Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C

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Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] 3-kinase catalyses the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4. cDNAs encoding two isoenzymes of Ins(1,4,5)P_3 3-kinase (3-kinases A and B) have been described previously. In the present study, we report the cloning of a full-length 2052 bp cDNA encoding a third human isoenzyme of the Ins(1,4,5)P_3 3-kinase family, referred to as isoform C. This novel enzyme has a calculated molecular mass of 75.207 kDa and a K_m for Ins(1,4,5)P_3 of 6 μM. Northern-blot analysis showed the presence of a transcript of approx. 3.9 kb in various human tissues. Inositol trisphosphate 3-kinase C demonstrates enzymic activity when expressed in DH5αF′ bacteria or COS-7 cells. Calcium alone decreases the Ins(1,4,5)P_3 3-kinase activity of the 3-kinase C isoenzyme in transfected COS-7 cells. This inhibitory effect is reversed in the presence of calmodulin. The recombinant bacterial 3-kinase C can be adsorbed on calmodulin–Sepharose in the presence of calcium. The present data show that Ins(1,4,5)P_3 3-kinase C: (i) shares a conserved catalytic domain of about 275 amino acids with the two other mammalian isoforms, (ii) could be purified on a calmodulin–Sepharose column and (iii) could be distinguished from the A and B isoenzymes by the effects of calcium and of calmodulin.

Key words: calcium, phosphatidylinositol metabolism, signal transduction.

INTRODUCTION

Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] 3-kinases are enzymes which catalyse the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4, both potential modulators of calcium homoeostasis [1,2]. Data presented by Irvine and colleagues [3,4] indicated that Ins(1,3,4,5)P_4 binds to a protein of the GTPase-activating protein-1 ('GAP1') family and thereby modulates Ca^{2+} mobilization by Ins(1,4,5)P_3. Another indication of a key regulatory role for the Ins(1,4,5)P_3 3-kinase(s) comes from recent data indicating that Ins(1,3,4,5)P_4 controls the frequency of calcium oscillations in HeLa cells [5]. cDNAs encoding two inositol trisphosphate 3-kinase isoenzymes have been reported to be present in rat and in human [referred to as Ins(1,4,5)P_3 3-kinases A and B] and these have been expressed in bacteria and in COS-7 cells [6–10]. Ins(1,4,5)P_3 3-kinase A was shown to be expressed at high levels in hippocampal CA1 pyramidal and in Purkinje cells [11]. Recent data obtained in our laboratory [12] have shown that Ins(1,4,5)P_3 3-kinase B is expressed in astrocytes and is phosphorylated in response to purinergic agonists.

In general, Ca^{2+} in a complex with calmodulin stimulates mammalian Ins(1,4,5)P_3 3-kinase activity, despite the presence of proteolytic fragments often present during extraction and purification of the enzyme [13,14]. This stimulating effect was attributed to the presence of a calmodulin-binding domain at the N-terminal end of the protein [15]. However, there is considerable cell-to-cell variability in the sensitivity of Ins(1,4,5)P_3 3-kinase activity to Ca^{2+}–calmodulin activation. This could result from the existence of different isoforms and/or splice variants of a single gene. This difference in sensitivity to Ca^{2+} was also shown for the two previously reported Ins(1,4,5)P_3 3-kinase A and B isoforms when expressed in *Escherichia coli*: the A isoform was stimulated 2- to 3-fold by Ca^{2+}–calmodulin, whereas the B isoform was stimulated up to 10-fold [8,9,16]. The specificity of Ins(1,4,5)P_3 phosphorylation by recombinant Ins(1,4,5)P_3 3-kinases A and B at the 3-position of the inositol ring has been resolved by HPLC [17–19].

Recently, a new family of inositol polyphosphate kinases has been reported and several cDNAs encoding these enzymes have been cloned; three of them (KIAA0263, PiUS and KCS1) are inositol hexakisphosphate (InsP_6) kinases [20,21], and ArgRIII (also known as Arg82) was shown to be a multifunctional kinase (IPMK) which phosphorylates Ins(1,4,5)P_3 and Ins(1,4,5,6)P_4 in *Saccharomyces cerevisiae* [22]. *In vitro*, ArgRIII catalyses the phosphorylation of Ins(1,4,5)P_3 at both the 3- and 6-position of the inositol ring, forming Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 [23]. A role of this multikinase in nuclear mRNA export was recently described [23,24]. In *S. pombe*, InsP_6 is also synthesized from Ins(1,4,5)P_3 and plays a role in osmotic regulation [25]. None of these enzymes appears to specifically produce Ins(1,3,4,5)P_4, as is shown for the A and B forms of mammalian Ins(1,4,5)P_3 3-kinases. In mammalian systems, no phosphorylation at the 6-position [which would form Ins(1,4,5,6)P_4] has ever been observed.

There is speculation in the literature that the family of Ins(1,4,5)P_3 3-kinases may be more diverse. In human platelets, rat thymus and thyroid cells, the Ins(1,4,5)P_3 3-kinase appears to run on SDS/polyacrylamide gels as higher-molecular-mass protein(s) when compared with Ins(1,4,5)P_3 3-kinases A and B [26–28]. In the present paper, we report the sequence and expression of a third mammalian Ins(1,4,5)P_3 3-kinase (3-kinase C), which demonstrates enzymic activity when expressed in *E. coli* or COS-7 cells. We have shown, for the first time, inhibition of activity by calcium when measured in transfected COS-7 cells, suggesting a different mode of Ins(1,3,4,5)P_4 production in intact cells when compared with cells expressing the A and B isoforms.

Abbreviation used: EST, expressed sequence tag.

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MATERIALS AND METHODS

Materials

DNA polymerase, restriction enzymes, calpain inhibitors I and II, Ins(1,4,5)P_3 and FuGENE™ 6 transfection reagent were obtained from Boehringer Mannheim. pcDNA3-His and pTrc-His vectors were from Invitrogen. [^{3}H]Ins(1,4,5)P_3 (15–30 Ci/mmol) was from DuPont–NEN. Dowex 1-X8 (formate form) was from Bio-Rad. Anti-His$_6$ monoclonal antibody was from Clontech. Haemocyanin from keyhole limpet and Freund's complete and incomplete adjuvants were from Calbiochem. Hyperfilm-MP, enhanced chemoluminescence (ECL®) Western-blotting reagents and calmodulin–Sepharose resin were from Amersham. Protan BA79 cellulose nitrate membranes were from Schleicher & Schuell. The human expressed sequence tag (EST) from clone DKFZp434B134 (accession number AL044962) was provided by the RessourcenZentrum/primär-Datenbank (Berlin-Charlottenburg, Germany).

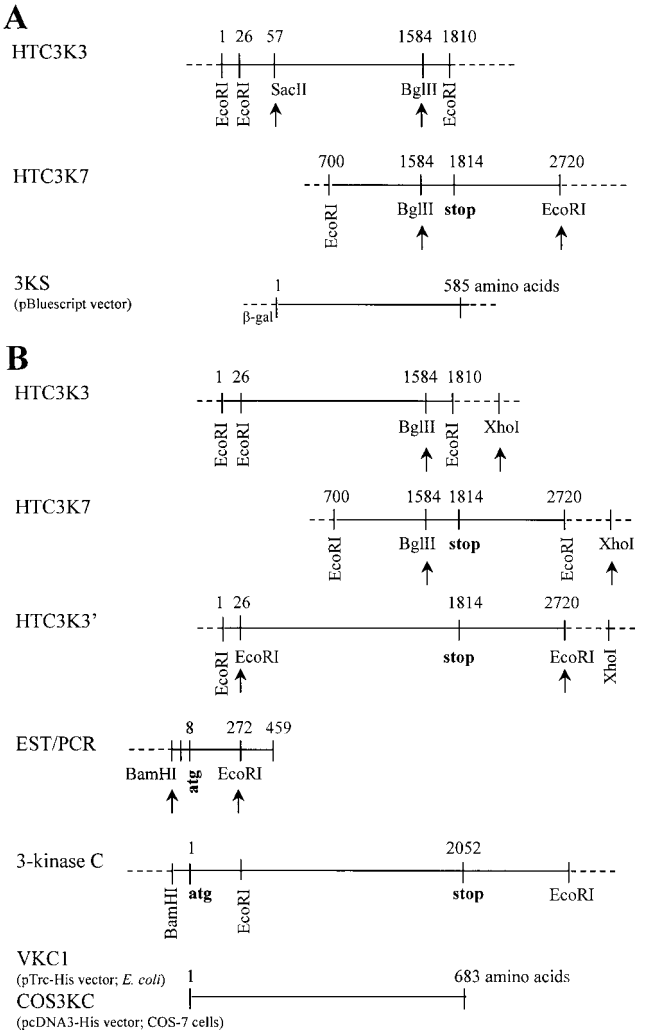

Figure 1 cDNA clones encoding human Ins(1,4,5)P_3 3-kinase C

(**A**) Restriction maps of the two clones HTC3K3 and HTC3K7 obtained following the screening of a human thyroid cDNA library. The 3KS plasmid of 585 amino acids was expressed in a pBluescript vector. (**B**) The EST sequence (accession number AL044962.1) contained the 5′-end of human Ins(1,4,5)P_3 3-kinase C. The coding region of 3-kinase C (accession number AJ290975) was subcloned into pTrcHis and pcDNA3-His. This resulted in the two constructs VKC1 and COS3KC. Solid lines indicate Ins(1,4,5)P_3 3-kinase C, broken lines indicate vector and arrows show restriction sites.

Molecular cloning of human Ins(1,4,5)P_3 3-kinase C

A human thyroid cDNA library in λgt11 vector (Clontech) was screened using a mixture of two probes encoding human Ins(1,4,5)P_3 3-kinase A and Ins(1,4,5)P_3 3-kinase B [9]. Approx. 5×10^5 clones were screened. The nitrocellulose filters were washed four times in 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1 % (w/v) SDS for 10 min at room temperature and twice in 0.2 × SSC/0.1 % SDS for 15 min at 60 °C. This resulted in the isolation of one clone (HTC3K3), which was used in a second screening to isolate HTC3K7. HTC3K3 was digested with *Sac*II and *Bgl*II and HTC3K7 with *Bgl*II and *Eco*RI. The two fragments obtained were subcloned into the pBluescript vector. The β-galactosidase in-frame fusion product encoded a partial sequence of human Ins(1,4,5)P_3 3-kinase C, i.e. 585 amino acids of the coding region, which was designated 3KS (Figure 1A).

The cDNA of clone HTC3K7 was also digested with the restriction enzymes *Bgl*II and *Xho*I. This fragment was used to replace a *Bgl*II–*Xho*I fragment of clone HTC3K3 to yield clone HTC3K3′. The EST sequence (AL044962) encoding the 5′-end of the cDNA of Ins(1,4,5)P_3 3-kinase C (459 bp) was amplified with a 5′-primer containing a *Bam*HI restriction site (underlined): 5′-CGGGATCCGGCATGAGGCGCTGCC-3′, and a 3′-primer: 5′-ATCAGTCCAAAGGCCATC-3′. The DNA of clone HTC3K3′ was digested with *Eco*RI and the EST/PCR product with *Bam*HI and *Eco*RI. The fragments obtained were subcloned into two expression vectors: pTrc-His A and pcDNA3-His C. The new His-tagged constructs were referred to as VKC1, when expressed in *E. coli* (DH5αF′), and COS3KC when expressed in COS-7 cells (Figure 1B).

Generation of a C-terminal peptide antibody against human Ins(1,4,5)P_3 3-kinase C

An immune serum was raised against the synthetic peptide of the C-terminal sequence of human Ins(1,4,5)P_3 3-kinase C (GNREDGYLWGLDNMI) derived from amino acids 660–674 (see Figure 2). The peptide was conjugated to keyhole-limpet haemocyanin (approx. 10 mg of peptide/carrier) in the presence of glutaraldehyde [29], diluted 2-fold (v/v) in Freund's complete adjuvant and injected into New Zealand rabbits. A booster dose of 0.5 mg of peptide/carrier in Freund's incomplete adjuvant (v/v) was given at 10 days and again at 20 days. Immunodetection of the enzyme expressed in DH5αF′ bacteria and COS-7 cells with both the peptide antibody (dilution 1:100) and the anti-His$_6$ monoclonal antibody (dilution 1:5000) was performed using the ECL® method.

Expression of recombinant Ins(1,4,5)P_3 3-kinase C in DH5αF′ bacteria

The full-length 683-amino-acid Ins(1,4,5)P_3 3-kinase C and the truncated construct of 585 amino acids were subcloned into pTrc-His A (VKC1) and pBluescript (3KS) expression vectors respectively. Both constructs were used to transform DH5αF′ bacteria. An overnight culture (5 ml) was used to inoculate 500 ml of Luria–Bertani medium containing 50 μg/ml ampicillin. After 6 h incubation at 37 °C, the medium was diluted to a D_{600} of 1, isopropyl β-thiogalactopyranoside was added to a final concentration of 1 mM and the incubation was continued for 3 h at 30 °C. The cells were harvested by centrifugation at 2500 ***g*** for

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  1  MRRCPCRGSLNEAEAGALPAAARMGLEAPRGGRRRQPGQQRPGPGAGAPA
 51  GRPEGGGPWARTEGSSLHSEPERAGLGPAPGTESPQAEFWTDGQTEPAAA
101  GLGVETERPKQKTEPDRSSLRTHLEWSWSELETTCLWTETGTDGLWTDPH
151  RSDLQFQPEEASPWTQPGVHGPWTELETHGSQTQPERVKSWADNLWTHQN
201  SSSLQTHPEGACPSKEPSADGSWKELYTDGSRTQQDIEGPWTEPYTDGSQ
251  KKQDTEAARKQPGTGGFQIQQDTDGSWTQPSTDGSQTAPGTDCLLGEPED
301  GPLEEPEPGELLTHLYSHLKCSPLCPVPRLIITPETPEPEAQPVGPPSRV
351  EGGSGGFSSASSFDESEDDVVAGGGGASDPEDRSGSKPWKKLKTVLKYSP
401  FVVSFRKHYPWVQLSGHAGNFQAGEDGRILKRFCQCEQRSLEQLMKDPLR
451  PFVPAYYGMVLQDGQTFNQMEDLLADFEGPSIMDCKMGSRTYLEEELVKA
501  RERPRPRKDMYEKMVAVDPGAPTPEEHAQGAVTKPRYMQWRETMSSTSTL
551  GFRIEGIKKADGTCNTNFKKTQALEQVTKVLEDFVDGDHVILQKYVACLE
601  ELREALEISPFFKTHEVVGSSLLFVHDHTGLAKVWMIDFGKTVALPDHQT
651  LSHRLPWAEGNREDGYLWGLDNMICLLQGLAQS*
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Figure 2 Deduced amino acid sequence of human Ins(1,4,5)P_3 3-kinase C

The 683-amino-acid protein has a calculated molecular mass of 75 kDa. MDCK, part of the typical Ins(1,4,5)P_3 inositol phosphate binding motif that is conserved in the various inositol phosphate kinases in mammals and yeast, is underlined. Putative phosphorylation sites for protein kinase C are doubly underlined. Critical residues for binding of ATP are in bold. The putative binding site for calmodulin is boxed.

15 min and were resuspended in 19 ml of lysis buffer [50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 10 % (w/v) sucrose, 12 mM 2-mercaptoethanol, 5 μM leupeptin and 50 μg/ml Pefabloc]. The cells, on ice, were briefly sonicated, using a microtip, three times for 5 s. Triton X-100 was added to a final concentration of 1 %, and the mixture was incubated for 10 min at 4 °C. The lysed cells were harvested by centrifugation at 10000 ***g*** for 10 min and 18 ml of the lysate was applied on to an 8 ml calmodulin–Sepharose column which had been equilibrated with 20 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 0.2 mM $CaCl_2$, 0.1 % (v/v) Triton X-100, 12 mM 2-mercaptoethanol, 5 μM leupeptin and 50 μg/ml Pefabloc. The lysate was diluted in 36 ml of the same buffer containing 2 mM $CaCl_2$. The column was washed with buffer A [20 mM Tris/HCl (pH 7.5), 0.2 mM $CaCl_2$, 0.4 M NaCl, 0.5 % (v/v) Triton X-100, 12 mM 2-mercaptoethanol, 5 μM leupeptin and 50 μg/ml Pefabloc] and then with the same buffer without Triton X-100. The adsorbed proteins were eluted with buffer B [20 mM Tris/HCl (pH 7.5), 2 mM EGTA, 0.4 M NaCl, 12 mM 2-mercaptoethanol, 5 μM leupeptin and 50 μg/ml Pefabloc] and then with the same buffer containing 0.2 % (w/v) SDS. Peak fractions of the last elution were identified by Western blotting and Ins(1,4,5)P_3 3-kinase assay [13].

Expression of Ins(1,4,5)P_3 3-kinase C in COS-7 cells

The full-length coding region of Ins(1,4,5)P_3 3-kinase C was subcloned into the pcDNA3-His C expression vector. Ins(1,4,5)P_3 3-kinase C was transfected into COS-7 cells (COS3KC) using the FuGENE method of transfection. The cells were grown in a humidified atmosphere of 5 % CO_2 at 37 °C and maintained in Dulbecco's modified Eagle's medium supplemented with 1 % (w/v) sodium pyruvate, 2 % penicillin/streptomycin and 10 % (v/v) fetal-calf serum. After 48 h, transfected cells were harvested, pelleted by centrifugation and resuspended in 300 μl of lysis buffer [20 mM Tris/HCl (pH 8.0), 150 mM KCl, 1 % (v/v) Triton X-100, 10 % (w/v) sucrose, 12 mM 2-mercaptoethanol, 5 μM leupeptin, 50 μg/ml Pefabloc and 10 μg/ml calpain inhibitors I and II). The cells were homogenized by passage five times through a 26-gauge needle and immediately used for Western blotting analysis and enzymic assay. For the subcellular distribution of enzyme activity, the cell lysates were centrifuged at 80000 ***g*** for 30 min at 4 °C and the pellet was resuspended to the same volume as that of the original homogenate.

Northern-blot analysis

A blot containing poly(A)$^+$-rich RNA from a variety of human tissues was purchased from Clontech. The membrane was probed with a 430 bp PCR product corresponding to the last 143 amino acids of human Ins(1,4,5)P_3 3-kinase C. Prehybridization was performed in 50 % formamide, 5 × SSPE (1 × SSPE = 0.15M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA), 5 × Denhardt's solution, 0.3 % SDS, 0.25 μg/ml herring sperm DNA and 200 μg/ml BSA at 42 °C. The hybridation procedure was similar, except that 10 % dextran sulphate was added to the hybridization solution. The blot was washed three times for 10 min in 2 × SSC/0.1 % SDS at room temperature and then three times for 30 min in 0.1 × SSC/0.1 % SDS at 60 °C.

HPLC analysis

The purified, recombinant enzyme (VKC1) was incubated at 37 °C with trace amounts (10000 d.p.m.) of [^{3}H]Ins(1,4,5)P_3 in 25 μl assay mixtures containing 20 mM Hepes (pH 7), 12 mM $MgSO_4$, 10 mM Na_2ATP, 20 mM phosphocreatine, 1 mM dithiothreitol, 1 mM EDTA, 360 units of phosphocreatine kinase (Calbiochem) and 0.5 mg/ml BSA. The reaction was stopped with 2 M perchloric acid and the mixture was neutralized with an appropriate volume of 1 M KCO_3/5 mM EDTA and analysed by HPLC using a SynChropak Q100 SAX column. Elution of the products was performed with a gradient generated by mixing buffer A (1 mM Na_2EDTA) with buffer B [buffer A containing 2 M $(NH_4)H_2PO_4$ and adjusted to pH 3.35 with H_3PO_4] as follows: 0–5 min, 0 % buffer B; 5–120 min, 0–65 % buffer B. Inositol phosphates were identified by their co-elution with the following standards: [^{14}C]Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, D/L-Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 isolated from [^{14}C]inositol-labelled parotid acinar glands [30].

RESULTS AND DISCUSSION

Cloning of cDNAs encoding human Ins(1,4,5)P_3 3-kinase C

The presence of a 100 kDa Ins(1,4,5)P_3 3-kinase has been reported in FRTL-5 cells. In these thyroid cells, activity was stimulated approx. 2-fold after activation by thyroid stimulating hormone [28]. This high-molecular-mass enzyme, which was poorly stimulated by Ca^{2+}, prompted us to screen a human thyroid cDNA library in order to search for new isoforms. Two cDNA clones encoding a novel Ins(1,4,5)P_3 3-kinase isoenzyme were isolated following this screening. These clones, referred to as HTC3K3 and HTC3K7 (GenBank accession number D38169, Figure 1), encoded a new partial sequence, referred to as 3-kinase C, which was different from the A and B isoforms described in human and rat [6–10]. A BLAST search in the human EST database identified a sequence available at the RZPD (Berlin, Germany) that covered the 5′-end of the cDNA (accession number AL044962). These three overlapping clones encoded a 683-amino-acid protein [Ins(1,4,5)P_3 3-kinase C; accession number AJ290975] with a calculated molecular mass of 75.207 kDa (Figure 1B and Figure 2). It contained the Ins(1,4,5)P_3 binding site (Figure 2, underlined), which is similar to that of the rat Ins(1,4,5)P_3 3-kinase A

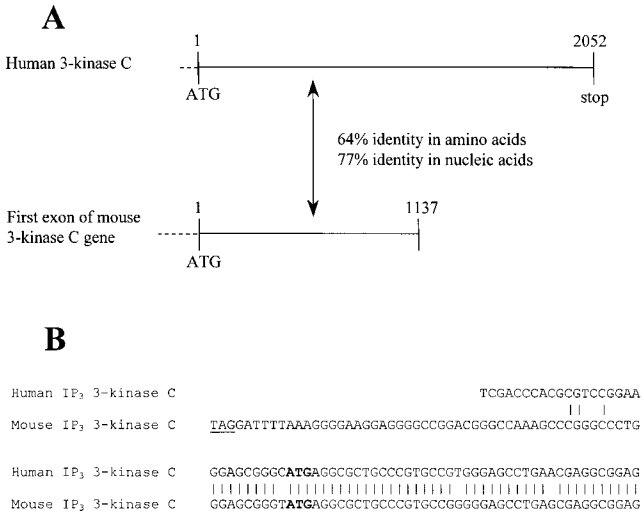

Figure 3 Alignment of human and mouse nucleotide sequence of Ins (1,4,5)P_3 3-kinase C on the starting methionine residue

(**A**) Alignment of the human nucleotide sequence of 3-kinase C and the first exon of the mouse Ins(1,4,5)P_3 3-kinase C gene. (**B**) The starting methionine residue is indicated in bold. The in-frame stop codon (TAG) upstream of the starting methionine in the mouse sequence is underlined.

isoform (LDCK^{262}). When the lysine residue (Lys^{262}) in 3-kinase A was substituted with alanine, no Ins(1,4,5)P_3 3-kinase activity was detected [31]. This very conserved and catalytically essential lysine residue (Lys^{486} in Figure 2), also present in the human 3-kinase A and B isoforms, was conserved in the different InsP_6 kinases [21,22] and in the yeast protein ArgRIII [24]. The sequence of Ins(1,4,5)P_3 3-kinase C also contained part of the ATP binding site described in 3-kinases A and B (Figure 2, DFG motif in bold type) [32]. Six sites, which were potential sites of phosphorylation by protein kinase C, were found at amino acids 106–108, 119–121, 222–224, 249–251, 404–406 and 652–654 (Figure 2, double-underlined residues). No calmodulin kinase II phosphorylation site was seen in the sequence, in contrast with that found in the A and B isoforms [12,33]. Potential residues essential for calmodulin binding were identified after an alignment with Ins(1,4,5)P_3 3-kinase A (i.e. PWKKLKTVLKYS^{399}; Figure 2, boxed residues) [15].

Identification of a potential initiator codon

Part of the sequence of Ins(1,4,5)P_3 3-kinase C was also found in a screening of a mouse genomic library. The sequences of the first three exons of the mouse Ins(1,4,5)P_3 3-kinase C gene were obtained (results not shown). An alignment of the first exon of the mouse sequence (1137 bp) with the human sequence showed 64% identity in terms of amino acids and 77% in terms of nucleic acids (Figure 3A). In the human sequence, we have assigned the initiation codon to the ATG at position 29 (Figure 3B, in bold) as it is flanked by a purine at position −3 and fits Kozak's criteria [34]. A PCR reaction on mouse uterus cDNA, using primers designed to hybridize within the first and third exons, confirmed that the first ATG is conserved and is preceded by an in-frame stop codon 61 bases upstream in the mouse sequence (Figure 3B, underlined). The putative starting methionine in the mouse cDNA is also flanked by a purine at position −3 and aligns with the human starting codon (Figure 3B).

A BLAST search in the protein domain database ProDom showed that the Ins(1,4,5)P_3 3-kinase C sequence contains a putative catalytic domain (domain PD010031), which is typical for Ins(1,4,5)P_3 3-kinase in mammalian and in *Caenorhabditis elegans*. The catalytic domain of Ins(1,4,5)P_3 3-kinase A was previously localized at the C-terminus and was restricted to a maximum of 275 amino acids [15]. An alignment of the corresponding catalytic domain of Ins(1,4,5)P_3 3-kinase C showed 73.2% identity with 3-kinase A and 73.8% identity with 3-kinase B. Mammalian Ins(1,4,5)P_3 3-kinases show four conserved motifs within the catalytic domain, which are 100% conserved between the A, B and C isoforms, relatively well conserved in *C. elegans* but not with the InsP_6 kinases or the yeast protein ArgRIII (Figure 4).

Amino acid residues showing similarity to a segment of the Ins(1,4,5)P_3 receptor [35] were also seen in the sequence of Ins(1,4,5)P_3 3-kinase C (i.e. LVKARERPRPR^{507}). When compared with the sequences of the human genome database, we found a considerable number of 100% identical partial clones which are localized on human chromosome 19. Northern-blot analysis of human Ins(1,4,5)P_3 3-kinase C expression showed the presence of a single transcript of approx. 3.9 kb in pancreas,

	Motif 1	Motif 2	Motif 3	Motif 4
Human InsP_3 3-kinase A (P23677)	**RTYLEEEL**^{275}	**AVTKPRYMQWRE**^{320}	**TLGFRIEGIKK**^{337}	**EGNREDGYL**^{445}
Human InsP_3 3-kinase B (Y18024)	**RTYLEEEL**^{657}	**AVTKPRYMQWRE**^{702}	**TLGFRIEGIKK**^{719}	**EGNREDGYL**^{827}
Human InsP_3 3-kinase C (AJ290975)	**RTYLEEEL**^{497}	**AVTKPRYMQWRE**^{542}	**TLGFRIEGIKK**^{559}	**EGNREDGYL**^{667}
C. elegans InsP_3 3-kinase (P91166)	**RT**F**LE**S**E**V^{179}	**A**I**TK**L**RYMQ**F**RE**^{224}	Q**LGFRIE**AA**K**R^{241}	P**GN**N**EDGYL**^{350}
Human InsP_6 kinase (Q92551)	**R**QHGDDAS^{237}	LD**T**GHYLCRNKY^{278}	IE**GFR**NALYQY^{296}	K**GFRDD**PTV^{414}
Yeast ArgIII (NP-010458)	TL**Y**DSKAS^{144}	S**V**LNQLSLEYY**E**^{188}	YI.**F**....**I**N**K**^{200}	DD**N**DD**D**...^{297}

Figure 4 Conserved residues between mammalian Ins(1,4,5)P_3 3-kinases

Four totally conserved motifs were found in the catalytic domain of all mammalian Ins(1,4,5)P_3 3-kinase isoforms (accession numbers P23677, Y18024 and AJ290975 for 3-kinases A, B and C respectively). These were also conserved in *C. elegans* (accession number P91166) but not in the InsP_6 kinases (accession number Q92551) or in the yeast protein ArgRIII (accession number NP-010458). Conserved residues are shown in bold. The dots are gaps introduced by the BESTFIT GCG program.

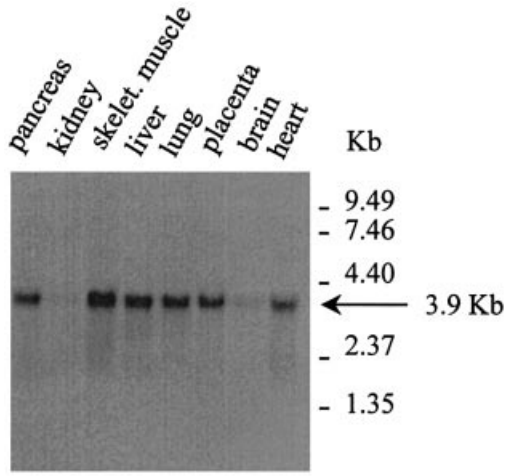

Figure 5 Tissue distribution of human Ins(1,4,5)P_3 3-kinase C

Northern-blot analysis of 2 μg of poly(A)$^+$ mRNA from various human tissues was hybridized with a catalytic probe of Ins(1,4,5)P_3 3-kinase C as described in the Materials and methods section. A single transcript of 3.9 kb is indicated by an arrow.

Table 1 Ins(1,4,5)P_3 3-kinase C activity in *E. coli* (DH5αF′) and COS-7 cells

Ins(1,4,5)P_3 3-kinase activity was determined in bacteria and in transfected COS-7 cells under basal conditions, i.e. in the presence of 1 mM EGTA or in the presence of 10 μM free Ca^{2+} and 2 μM calmodulin (Ca^{2+}–calmodulin complex). The stimulation of Ins(1,4,5)P_3 3-kinase C activity under these conditions varied between 1.2- and 1.5-fold. Activities are shown as means ± S.D. n.d., not detectable.

	Ins(1,4,5)P_3 3-kinase activity (nmol/min per ml)	
Added reagents ...	1 mM EGTA	Ca^{2+}–calmodulin
Bacterial crude lysate		
Non-recombinant	n.d.	n.d.
3KS 3-kinase C	141.4 ± 12.4	224.6 ± 5.1
VKC1 3-kinase C	23.8 ± 2.4	35.1 ± 1.5
COS-7 transfected cell crude lysate		
Vector	n.d.	n.d.
COS3KC 3-kinase C	303.4 ± 2.3	343.1 ± 1.1

skeletal muscle, liver, lung, placenta and with a lower expression in kidney and brain (Figure 5). The expression of the C isoform is therefore much more broadly expressed when compared with the A isoform, which is restricted to brain and testis [10]. On the other hand, this large distribution is comparable with that of the B isoform, which is expressed in rat lung, thymus, testis, brain and heart [10].

Expression of Ins(1,4,5)P_3 3-kinase activity in DH5αF′ bacteria

Ins(1,4,5)P_3 3-kinase C was subcloned into two different expression vectors (described in the Materials and methods section). The resulting plasmids were designated 3KS, which encoded a partial 3-kinase C sequence from amino acids 98–683 in the pBluescript vector, and VKC1, encoding the 683-amino-acid full-length sequence His-tagged in pTrc-His. Both constructs demonstrated Ins(1,4,5)P_3 3-kinase activity in bacteria, although crude lysates of the pBluescript construct had about 20-fold

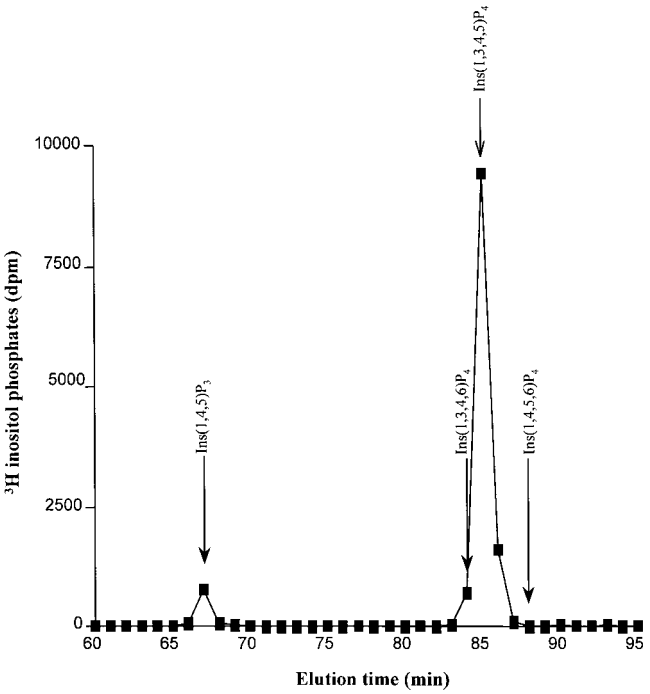

Figure 6 HPLC profile of the product of Ins(1,4,5)P_3 3-kinase C

[^{3}H]Ins(1,4,5)P_3 was incubated at 37 °C in the presence of purified Ins(1,4,5)P_3 3-kinase C (VKC1). The sample, containing ^{14}C-labelled standards of InsP_4, was separated by HPLC; the elution was performed as described in the Materials and methods section. Eluted ^{3}H-labelled InsP_4 peaks are indicated by arrows.

more activity (Table 1). When [^{3}H]Ins(1,4,5)P_3 was added to a bacterial lysate of VKC1, all of the phosphorylated product was found in a peak that co-migrated exactly with an internal standard of [^{14}C]Ins(1,3,4,5)P_4 (Figure 6). Standards for [^{14}C]-Ins(1,4,5,6)P_4 and [^{14}C]Ins(1,3,4,6)P_4 were also included but neither of these InsP_4 isomers was phosphorylated. Ins(1,4,5)P_3 3-kinase C did not phosphorylate Ins(1,4,5)P_3 to Ins(1,3,4,5,6)P_5, which is in contrast with the yeast enzyme ArgRIII when tested under similar incubation conditions [23].

Both of the recombinant enzymes (3KS and VKC1) expressed in DH5αF′ bacteria were stimulated by Ca^{2+} in the presence of calmodulin (2 μM). This degree of stimulation was much lower when compared with Ins(1,4,5)P_3 3-kinases A and B, and activity was only 1.5-fold greater than the basal activity when measured in the absence of the Ca^{2+}–calmodulin complex (Table 1), whereas recombinant 3-kinase A was typically stimulated 2- to 3-fold from basal levels [16]. The B isoform was stimulated more than 10-fold in the presence of Ca^{2+} and calmodulin [9].

Adsorption of recombinant Ins(1,4,5)P_3 3-kinase C on a calmodulin–Sepharose column

Mammalian Ins(1,4,5)P_3 3-kinases A and B could be purified by calmodulin–Sepharose affinity chromatography [16], providing direct evidence of a calmodulin binding site. Since Ins(1,4,5)P_3 3-kinase C was poorly activated by Ca^{2+}–calmodulin, we investigated if Ins(1,4,5)P_3 3-kinase C could be adsorbed on to the calmodulin–Sepharose matrix. We showed that the two bacterial Ins(1,4,5)P_3 3-kinase C constructs could be adsorbed on to this resin. An Ins(1,4,5)P_3 3-kinase antibody directed against a C-

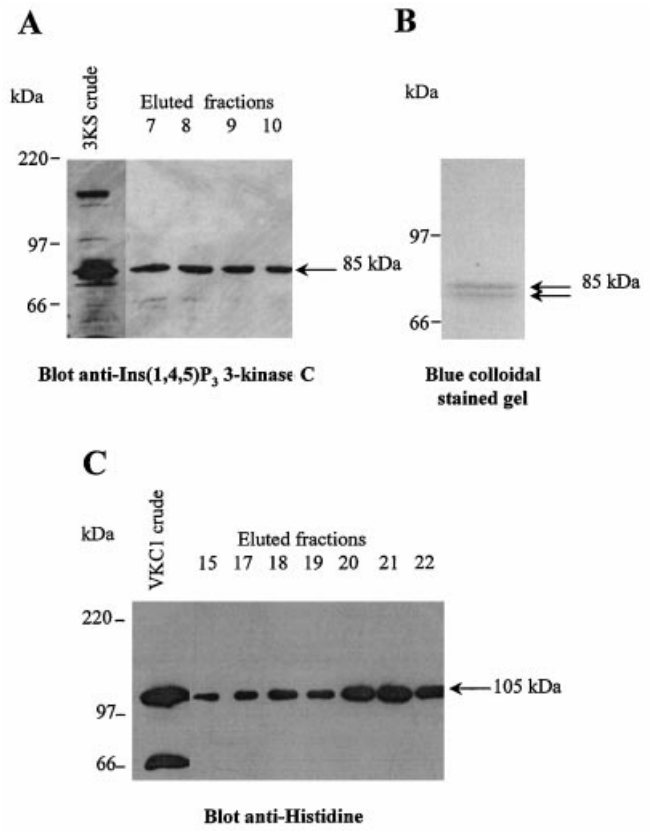

Figure 7 Purification of the two recombinant Ins(1,4,5)P_3 3-kinase C constructs 3KS and VKC1 using calmodulin–Sepharose

A crude lysate of each construct was applied on to a calmodulin–Sepharose column. A sample of the crude extract and the fractions eluted with EGTA were analysed by Western blotting. (**A**) An Ins(1,4,5)P_3 3-kinase C antibody (dilution 1:100) was used for the 3KS construct and a single band at approx. 85 kDa was detected. (**B**) Purified material was separated by SDS/PAGE and the products were stained with blue colloid. Two major bands were found having a similar molecular mass (approx. 80–85 kDa). (**C**) A His_6-tagged antibody (dilution 1:5000) for the VKC1 construct was used. A band at 105 kDa (indicated by an arrow) was revealed.

terminal peptide sequence of 3-kinase C recognized 3KS on a Western blot with an molecular mass of approx. 85 kDa (Figure 7A). No band was detected with a non-recombinant lysate (results not shown). A doublet with the same molecular mass was found on a blue-colloidal-stained gel (Figure 7B). MS analysis of the peptide fragments generated by trypsin treatment of these two bands showed that both corresponded to Ins(1,4,5)P_3 3-kinase C (D. Communi and V. Dewaste, unpublished work).

Western-blot analysis of the full-length active 3-kinase C (VKC1), purified by calmodulin–Sepharose chromatography, revealed a protein at 105 kDa after immunodetection with an anti-His_6 antibody (Figure 7C), confirming the adsorption of 3-kinase C on calmodulin–Sepharose. We were aware of the large difference between the predicted and the apparent size of the protein on SDS gels, and the reason for this is not understood but it did not result from sequencing errors. This difference in size was also observed with the 3KS construct (65.0 kDa for the predicted size compared with 85 kDa for the apparent size on gels).

The apparent K_m for Ins(1,4,5)P_3 and V_{max} values of 3KS were 6.4 ± 0.9 μM and 2.1 ± 0.1 μmol/min per mg of protein respectively (results not shown), which is comparable with the values obtained for Ins(1,4,5)P_3 3-kinases A and B when expressed in bacteria [9,16,32].

Inhibition by calcium of Ins(1,4,5)P_3 3-kinase C activity expressed in COS-7 cells

The coding region of Ins(1,4,5)P_3 3-kinase C was subcloned into pcDNA3-His vector (described in the Materials and methods section) and expressed in COS-7 cells (COS3KC, Figure 1B). As in DH5αF′ bacteria, the activation of Ins(1,4,5)P_3 3-kinase C by Ca^{2+}–calmodulin was rather weak and was only 20 % above the basal level (i.e. without Ca^{2+}–calmodulin; Table 1). In these transfected cells, Ca^{2+} (10 μM) alone was shown to inhibit Ins(1,4,5)P_3 3-kinase C activity when compared with Ca^{2+}-free solutions (Figure 8A, upper panel). This is in contrast with Ins(1,4,5)P_3 3-kinase A (Figure 8A, lower panel) or 3-kinase B, where Ca^{2+} alone had no effect on the kinase activity. This inhibitory effect on Ins(1,4,5)P_3 3-kinase C activity was also observed at lower calcium concentrations (0.1 and 1 μM; Figure 8B). This inhibition was reversed in the presence of calmodulin (2–20 μM; Figure 8A, upper panel). An inhibitory effect of Ca^{2+} at high concentrations (10^{-6} to 10^{-3} M free Ca^{2+}) has been reported in bovine brain inositol trisphosphate kinase after partial purification of the enzyme [14]. This could be related to the expression of Ins(1,4,5)P_3 3-kinase C in bovine brain.

Western-blot analysis followed by immunodetection with an anti-His_6 antibody of COS-7 cells transfected with Ins(1,4,5)P_3 3-kinase C also revealed a protein at 105 kDa (Figure 9A, lane 2). No activity could be detected in vector-transfected cells (Table 1), and no signal in a Western blot was detected with this control (Figure 9A, lane 1). Activity determinations (Figure 9C) and Western-blot analysis (Figure 9B) revealed that 90 % of total Ins(1,4,5)P_3 3-kinase C activity was in the soluble fraction of transfected COS-7 cell homogenate.

Conclusions

We have isolated the cDNA of a novel Ins(1,4,5)P_3 3-kinase (isoform C). The unique feature of this enzyme was its relatively weak activation by the Ca^{2+}–calmodulin complex, in contrast with the A and B isoforms, which are much more sensitive to the Ca^{2+}–calmodulin complex [9,16]. We have shown that Ins(1,4,5)P_3 is phosphorylated specifically at the 3-position and thus the enzyme is also distinct from the yeast enzyme ArgRIII, which was shown to phosphorylate Ins(1,4,5)P_3 at both the 3- and 6-carbons [22,23]. This was related to key amino acid residues within the catalytic domain, which are present only in the mammalian Ins(1,4,5)P_3 3-kinase isoforms and the *C. elegans* isoforms. Indeed, an alignment of the amino acid sequences of the various 3-kinase isoforms shows the presence of four conserved motifs, and these are the potential sites which account for the reported specificity of the Ins(1,4,5)P_3 3-kinase reaction in mammalian cells (Figure 4). The Ins(1,4,5)P_3 3-kinases are also unrelated to the different mammalian InsP_6 kinases, despite the presence of conserved residues in their catalytic domain, particularly in the Ins(1,4,5)P_3 inositol polyphosphate binding site {as identified by site-directed mutagenesis of the Ins(1,4,5)P_3 3-kinase A isoform [31]}. The four conserved motifs were not seen in the sequence of InsP_6 kinases (Figure 4).

In yeast, the Ins(1,4,5)P_3 kinase activity of ArgRIII has been reported to empower a transcriptional complex with the ability to co-regulate a group of genes that control the utilization of

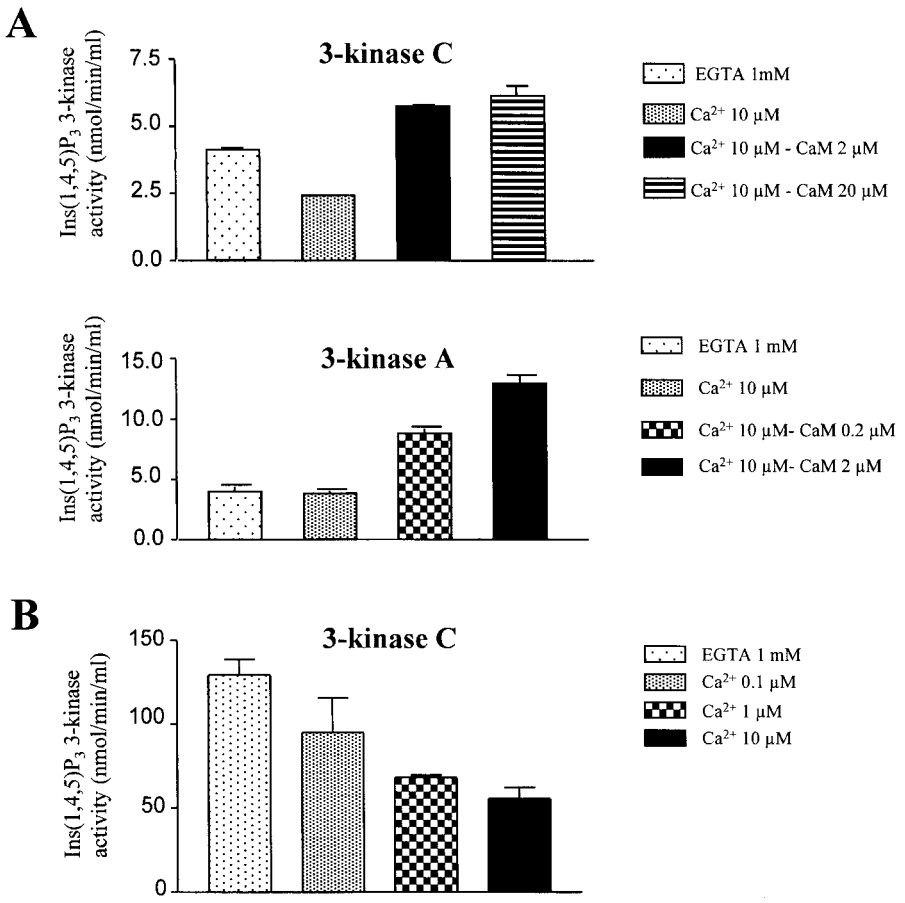

Figure 8 Inhibition of Ins(1,4,5)P_3 3-kinase C activity in transfected cells by calcium

Ins(1,4,5)P_3 3-kinase activity was determined in COS-7 cells transfected with: (**A**, upper panel) Ins(1,4,5)P_3 3-kinase C or (**A**, lower panel) human Ins(1,4,5)P_3 3-kinase A. The conditions used for the assays are shown in the key to the right of the panels [i.e. without calcium and calmodulin (1 mM EGTA), 10 μM calcium alone or with 10 μM calcium and 0.2 μM or 20 μM calmodulin (CaM)]. (**B**) Inhibitory effect of different calcium concentrations (0.1 μM, 1 μM or 10 μM free calcium) on Ins(1,4,5)P_3 3-kinase C activity.

arginine as a nitrogen source [24]. The kinase is also compartmentalized in the nucleus of yeasts [24,36] and *Dictyostelium* [37]. These findings naturally lead us to consider if transcriptional regulation may also be a function of mammalian 3-kinases. There are no indications in our studies that this might be a function for Ins(1,4,5)P_3 3-kinase C, which we identified to be a predominantly cytosolic enzyme with no defined nuclear targeting domains.

Theoretical studies have emphasized the potential importance of the activation of Ins(1,4,5)P_3 3-kinase activity by Ca^{2+} in the control of calcium oscillations [38]. This model proposes that oscillations of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are highly dependent upon the stimulation of Ins(1,4,5)P_3 3-kinase activity by Ca^{2+}. The new data shown in the present study make it clear that Ins(1,4,5)P_3 3-kinase C is unlikely to play a major role in this process. In the 3-kinase C isoform, which was poorly activated by Ca^{2+} and calmodulin, the ability to interact with a calmodulin–Sepharose matrix was nevertheless conserved, and this is therefore a common property of mammalian Ins(1,4,5)P_3 3-kinases.

However, one other possible role for the type C isoform of 3-kinase would be well served by its relative insensitivity to regulation by calcium and the apparent absence of consensus sites for phosphorylation by calmodulin kinase II. Recent studies have shown that one of the downstream metabolites of Ins(1,4,5)P_3, namely Ins(1,3,4)P_3, plays an important physiological role. Ins(1,3,4)P_3 links the degree and duration of receptor-dependent phospholipase C activation to the generation of Ins(3,4,5,6)P_4, a cellular signal that regulates the ionic conductance of Cl^- channels [39–41]. The degree of precision with which these two signalling activities are coupled together depends upon how closely the levels of Ins(1,3,4)P_3 mirror the rate of Ins(1,4,5)P_3 synthesis by phospholipase C. This could be most efficiently achieved by a species of 3-kinase in which the activity was largely dependent upon substrate supply, rather than being sensitive to cross-talk from other signalling pathways. In the event that different isoforms of 3-kinase do turn out to play subtly different physiological roles, we might consider functional specificity to be further augmented by their regulated assembly into multimeric signalling complexes.

The cloning of Ins(1,4,5)P_3 3-kinase C adds to the repertoire of molecular tools by which the impact of changes in inositol trisphosphate 3-kinase activities upon cellular function can be studied. These tools will be important in the study of the regulation of expression of the corresponding gene in future studies *in vivo*.

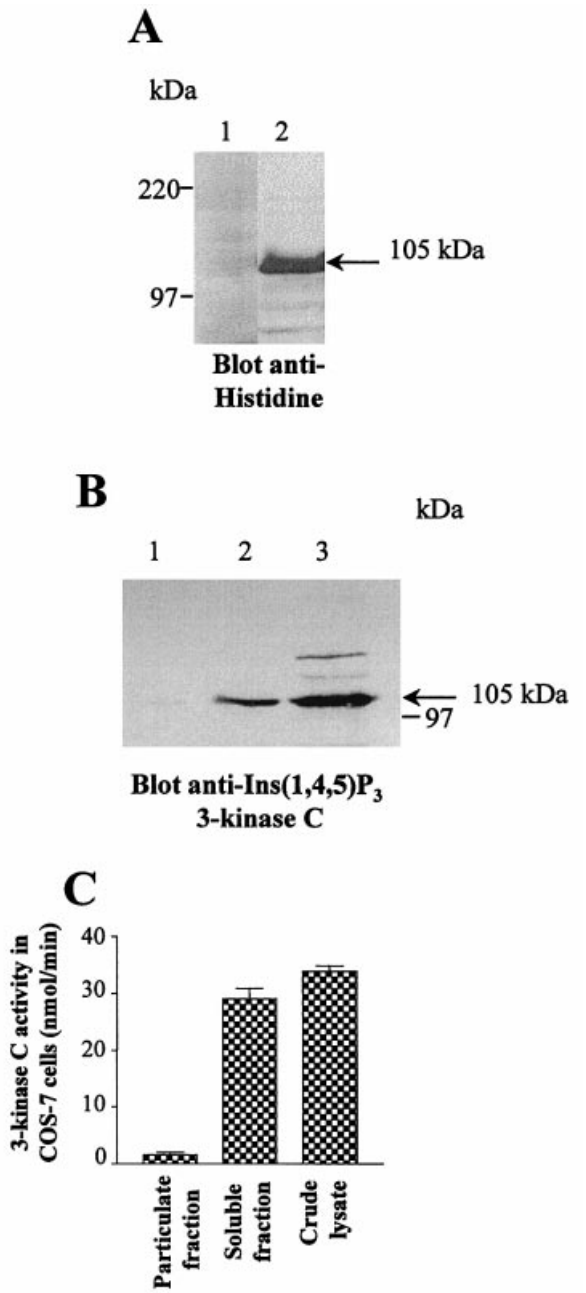

Figure 9 Western-blot analysis of Ins(1,4,5)P_3 3-kinase C expressed in COS-7 cells

(**A**) Crude homogenates of COS-7 cells transfected with vector alone (lane 1) or with the Ins(1,4,5)P_3 3-kinase C full-length construct (lane 2) were separated by SDS/PAGE. Immunodetection was performed with a His_6-tagged antibody (dilution 1:5000) and a band at 105 kDa was revealed for the transfected cells with Ins(1,4,5)P_3 3-kinase C. (**B**) An aliquot of the particulate fraction (lane 1), soluble fraction (lane 2) and crude homogenate (lane 3) (see the Materials and methods section) was analysed by Western blotting with an Ins(1,4,5)P_3 3-kinase C antibody (dilution 1:100). The 105 kDa band is indicated with an arrow. (**C**) Ins(1,4,5)P_3 3-kinase C activity was determined in the particulate and soluble fractions and crude lysate of COS-7 cells transfected with Ins(1,4,5)P_3 3-kinase C.

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