RESEARCH COMMUNICATION

Multitasking in signal transduction by a promiscuous human Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase

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We describe a human cDNA encoding 1-kinase activity that inactivates Ins(3,4,5,6)P_4, an inhibitor of chloride-channel conductance that regulates epithelial salt and fluid secretion, as well as membrane excitability. Unexpectedly, we further discovered that this enzyme has alternative positional specificity (5/6-kinase activity) towards a different substrate, namely Ins(1,3,4)P_3. Kinetic data from a recombinant enzyme indicate that Ins(1,3,4)P_3 ($K_m = 0.3\ \mu M$; $V_{max} = 320$ pmol/min per μg) and Ins(3,4,5,6)P_4 ($K_m = 0.1\ \mu M$; $V_{max} = 780$ pmol/min per μg) actively compete for phosphorylation *in vivo*. This competition empowers the kinase with multitasking capability in several key aspects of inositol phosphate signalling.

Key words: chloride channel, inositol phosphates, signalling.

INTRODUCTION

Ins(3,4,5,6)P_4 is an established cellular signal that inhibits the conductance of Ca^{2+}-activated Cl^- channels in the plasma membrane, thereby making an important contribution to the complex homoeostatic control of salt and fluid secretion from epithelial cells [1–7]. Other physiological activities regulated by Cl^- channels include smooth-muscle contraction [8] and neurotransmission [9]. The general importance of Ins(3,4,5,6)P_4 necessitates that the cell exerts close control over its synthesis and metabolism. Our current understanding of this regulatory process is based on the paradigm [10–12] that the pathway leading to Ins(3,4,5,6)P_4 synthesis terminates within a metabolic cul-de-sac. In this scenario, Ins(3,4,5,6)P_4 levels are dynamically regulated by the competing activities of a receptor-regulated, Ins(1,3,4,5,6)P_5 1-phosphatase/Ins(3,4,5,6)P_4 1-kinase metabolic cycle [10–12]. In all signal-transduction pathways, it is important to identify the molecular nature of the participating proteins, especially when, as in this case, manipulation of their activities is potentially of therapeutic benefit [11]. There has been no previous molecular characterization of either the Ins(1,3,4,5,6)P_5 1-phosphatase or the Ins(3,4,5,6)P_4 1-kinase.

We have now approached the molecular characterization of the Ins(3,4,5,6)P_4 1-kinase from a novel perspective. The observation that Ins(1,3,4)P_3 inhibits the 1-kinase [11,12] appeared, to us, to be an intriguing coincidence in the light of earlier studies that showed Ins(3,4,5,6)P_4 itself to be a potent inhibitor of Ins(1,3,4)P_3 phosphorylation by a 5/6-kinase [13,14]. We therefore reasoned that there was a strong likelihood of there being active-site similarities between the 1-kinase and the 5/6-kinase. A cDNA encoding the Ins(1,3,4)P_3 5/6-kinase was cloned by Wilson and Majerus [15] several years ago. We have now used that published nucleotide sequence as a database query, in an effort to identify additional clones with sufficiently similar sequences that they could be candidate Ins(3,4,5,6)P_4 1-kinases. We now describe the success of this approach, not only in identifying a 1-kinase, but also in the determination that the same enzyme is also an Ins(1,3,4)P_3 5/6-kinase. These developments now rationalize some seemingly disparate observations in inositol phosphate metabolism. More importantly, our work establishes the molecular identity of a key 'on/off-switch' for an inositol phosphate signalling process, while also defining a new model for multitasking in signal transduction.

MATERIALS AND METHODS

Type α and type β Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase

The original Ins(1,3,4)P_3 5/6-kinase cDNA (GenBank® accession number U51336) cloned by Wilson and Majerus [15] is herein reclassified as 'type α' Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase. A human cDNA expressed sequence tag (EST) clone with IMAGE ID: 1606926 (GenBank® accession number AA991251; A.T.C.C., Manassas, VA, U.S.A.) was completely sequenced and deposited in the GenBank® database (accession number AF279372) as a cDNA encoding 'type β' Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase. The coding region of the type β cDNA was directly amplified from the plasmid prepared from this EST clone by PCR using two primers: 5′-CGCGGATCC-ATGCAGACCTTTCTGAAAGGGAAGAGAG-3′ and 5′-CGGGGTACCCTACTGGGAGGAGGCCTTGGTG-3′. The 1.4 kb PCR product obtained was then gel-purified and cloned into a PQE30 expression vector (Qiagen) between *Bam*HI and *Kpn*I restriction sites.

Expression in *Escherichia coli*, purification of the recombinant protein, and enzyme assay

The kinase cDNA construct was used to transform M15 competent *E. coli* cells. Overnight culture (100 ml) was used to inoculate 2 litres of Luria–Bertani medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin,which was then incubated until D_{600} reached 0.9–1.0. The expression was initiated by adding 1.5 mM isopropyl β-D-thiogalactoside, and continued for a further 5 h before the cells were harvested by centrifugation.

Abbreviations used: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; EST, expressed sequence tag; UTRs, untranslated regions.

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The cell pellet was resuspended in 100 ml of lysis buffer, containing 20 mM Tris (pH 8.0), 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mg/ml lysozyme, and was kept on ice for 1 h. Cells were then lysed by sonication following three freeze-and-thaw cycles. The cell lysate was centrifuged (15000 ***g*** for 15 min) and the supernatant was loaded (0.8 ml/min) on to a 5 ml Ni^{2+}-nitrilotriacetate affinity column. After washing with 40 ml of buffer A [20 mM Tris (pH 8.0) and 300 mM NaCl], bound protein was eluted with a 60 ml linear gradient of 0–100 mM imidazole in buffer A. Peak fractions were pooled and dialysed against 2×1.5 litres of 20 mM Tris (pH 8.0) for 4–6 h at 4 °C. Subsequently, 2 mM $MgCl_2$ was added to the dialysed protein extract, which was then loaded (0.8 ml/min) on to a UNO Q12 (15 mm $\times$ 68 mm) ion-exchange column. After a 40 ml wash with buffer B [20 mM Tris (pH 8.0), 1 μg/ml leupeptin and 2 mM $MgCl_2$] the kinase was eluted from the column with a 60 ml linear gradient of 0–350 mM NaCl in buffer B. Peak fractions were pooled and supplemented with 15% (v/v) glycerol. Kinase activity was assayed as described previously using radiolabelled Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 [11,16]. Non-radioactive inositol phosphates for kinetic assays were purchased from CellSignals Inc. (Lexington, KY, U.S.A.). Reaction products were separated either by gravity-fed anion-exchange columns [17] or by HPLC [16].

RESULTS AND DISCUSSION

Characterization of a new human cDNA encoding Ins(3,4,5,6)P_4 1-kinase activity, and expression of the recombinant enzyme in *E. coli*

This study began with the hypothesis that the Ins(1,3,4)P_3 5/6-kinase and Ins(3,4,5,6)P_4 1-kinase could be two closely related proteins (see the Introduction). Thus the nucleotide sequence of a cDNA clone of a human brain Ins(1,3,4)P_3 5/6-kinase (type α in Figure 1) [15] was used to screen nucleotide databases in the hope that a candidate 1-kinase could be distinguished. This approach identified a 3006 bp human breast EST-cDNA (GenBank® accession number AA991251) that we fully sequenced (type β in Figure 1). The latter's nucleotide sequence from nt -22 to 2949 is 97% identical to the corresponding region of the type α cDNA; some of the differences could be polymorphic. Database screening also identified a contiguous 2042 bp high-throughput genomic sequence (GenBank® accession number AL117192), assigned to chromosome 14, which is 96–99% identical to corresponding regions of the two cDNAs (Figure 1). There were five single-nucleotide differences between the two cDNAs within their coding regions, but only one of these led to an amino acid difference; asparagine-356 in the type α protein, described by Wilson and Majerus [15], is replaced by serine in our type β protein. The genomic sequence (AL117192), and an additional human brain EST-cDNA in the GenBank® database (accession number T09063), both encode the type β protein. The α and β cDNAs have different 5′-untranslated regions (UTRs), which we propose are derived by alternative splicing, based upon the high degree of similarity achieved in alignments with another chromosome 14 high-throughput genomic sequence (Figure 1).

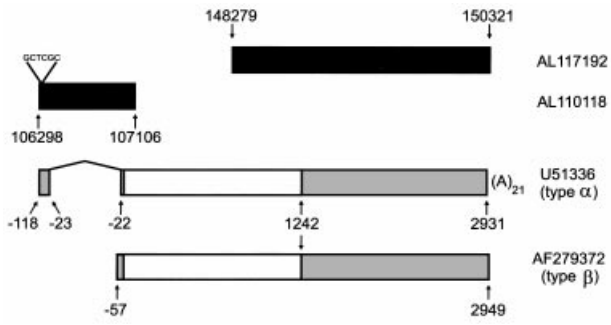

Figure 1 Alignment of the type α and type β cDNAs for the Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase with genomic sequences assigned to chromosome 14

The nucleotide sequence of the type β cDNA that we characterized in the present study (GenBank® accession number AF279372) was aligned with the type α cDNA that was described previously [15] (accession number U51336). The first nucleotide of the start codon is numbered +1. Coding regions are white and UTRs are shaded grey. The type α cDNA from nt -22 to 2931 is 97% identical to the corresponding region of the type β cDNA. The Figure includes two contiguous high-throughput genomic sequences (in black, AL110118.3 and AL117192.3; both phase 2); nt 106298 of AL110118 is aligned with nt -118 of the type α cDNA, and nt 107106 of AL110118 is aligned with nt 96 of both cDNAs. The sequence from nt -118 to -23 of the type α cDNA is 98% identical to the corresponding sequence of AL110118, excluding the indicated GCTCGC insert. The sequence from nt -57 to 96 of the type β cDNA is 100% identical to the corresponding sequence of AL110118. The genomic sequence of AL117192 is 96% and 99% identical to the corresponding regions of the type α and type β cDNAs respectively (nt 148279 and nt 150321 of AL117192 are aligned with nt 897 and nt 2939 of the type β cDNA).

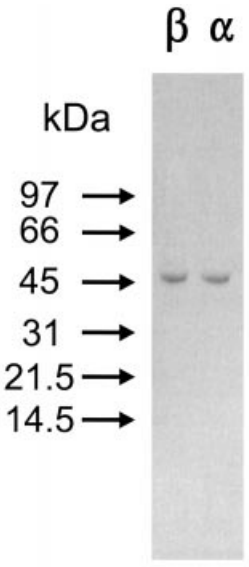

Figure 2 SDS/PAGE analysis of purified Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase and its $Ser^{356} \rightarrow$ Asn mutant

A 1.8 μg aliquot of purified recombinant type β kinase and its $Ser^{356} \rightarrow$ Asn mutant (i.e. type α) were each loaded on to a 4–12% (w/v) polyacrylamide/Bis-Tris gel and run with Mes-based running buffer (Novex). The protein bands were visualized using Coomassie Brilliant Blue stain.

We used *E. coli* as an expression system for an N-terminal His-tagged version of our type β protein (Figure 2). The apparent molecular mass of this enzyme (48 ± 0.4 kDa; $n = 4$) was close to the theoretical size (47 kDa). The purified enzyme showed time-dependent phosphorylation of Ins(3,4,5,6)P_4 to InsP_5 (Figure 3A). Ins(3,4,5,6)P_4 has two hydroxy groups at carbons 1 and 2 available for phosphorylation, so both Ins(1,3,4,5,6)P_5 and Ins(2,3,4,5,6)P_5 were potential products; these were resolved by Partisphere SAX HPLC, and Ins(1,3,4,5,6)P_5 was the only product formed (Figure 3). This represents the first molecular identification of the Ins(3,4,5,6)P_4 1-kinase.

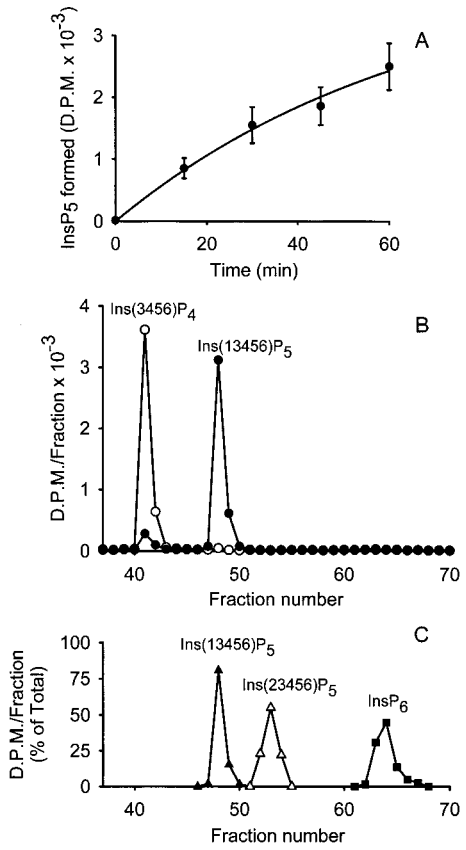

Figure 3 Characterization of recombinant Ins(3,4,5,6)P_4 1-kinase activity

Purified recombinant kinase (2 ng) was incubated for various times with approx. 4000 d.p.m. of [^{3}H]Ins(3,4,5,6)P_4, and the [^{3}H]InsP_5 formed was determined by gravity-fed anion-exchange chromatography (**A**). Data represent means ± S.E.M. from three experiments. In separate experiments (**B**), the kinase was incubated for either 0 min (open circles) or 30 min (solid circles) and samples were analysed by HPLC using a Partisphere SAX column. (**C**) The elution of standards of [^{3}H]Ins(1,3,4,5,6)P_5 (18000 d.p.m.; solid triangles), [^{3}H]Ins(2,3,4,5,6)P_5 (300 d.p.m.; open triangles) and [^{3}H]InsP_6 (4000 d.p.m.; solid squares), all of which were determined in a parallel HPLC run. Data are representative of three experiments.

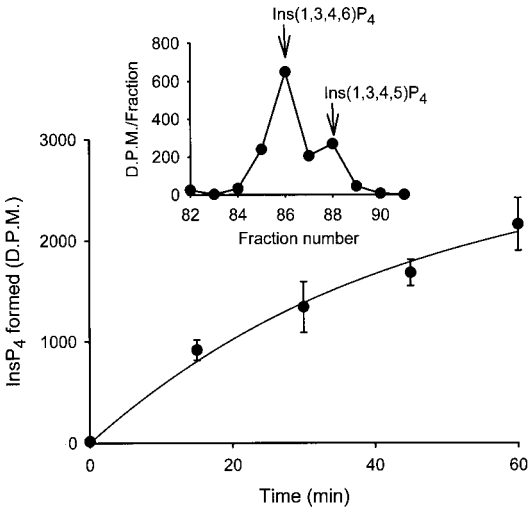

Figure 4 Recombinant Ins(3,4,5,6)P_4 1-kinase has Ins(1,3,4)P_3 5/6-kinase activity

Purified recombinant kinase (2 ng) was incubated for various times with approx. 4000 d.p.m. of [^{3}H]Ins(1,3,4)P_3, and the [^{3}H]InsP_4 formed was determined by gravity-fed anion-exchange chromatography (main panel). Data represent means ± S.E.M. from three experiments. The inset shows, in separate experiments, the products formed after phosphorylation of approx. 1500 d.p.m. of [^{3}H]Ins(1,3,4)P_3; the HPLC analysis was performed using a Synchropak Q100 column (see the Materials and methods section). The elution positions of standards of Ins(1,3,4,5)P_4 and Ins(1,3,4,6)P_4 are indicated by the arrows. No [^{3}H]InsP_5 was formed in these experiments. Data represent means ± S.E.M. from three experiments.

Recombinant Ins(3,4,5,6)P_4 1-kinase has Ins(1,3,4)P_3 5/6-kinase activity

Our new Ins(3,4,5,6)P_4 1-kinase has only one amino-acid difference from the protein previously shown to be a Ins(1,3,4)P_3 5/6-kinase [15]. This observation prompted us to examine if our enzyme could also phosphorylate Ins(1,3,4)P_3. This was indeed the case (see Figure 4). As in previous studies [13,15], HPLC analysis showed that phosphorylation of the 6-hydroxy group of Ins(1,3,4)P_3 was accompanied by the less productive phosphorylation of the 5-hydroxy group (inset to Figure 4). How can a single enzyme have the capacity to phosphorylate both the 5- and 6-hydroxy groups of Ins(1,3,4)P_3, as well as the 1-hydroxy group of Ins(3,4,5,6)P_4? In fact, Ins(1,3,4)P_3 can be oriented in such a way that its 1-phosphate, 3-phosphate, 4-phosphate and 6-hydroxy groups are, respectively, surrogates for the 6-phosphate, 4-phosphate, 3-phosphate and 1-hydroxy groups of Ins(3,4,5,6)P_4, while also maintaining the appropriate ring conformation (Figure 5A). Consistent with the idea that the latter four substituents help define substrate recognition, the enzyme cannot phosphorylate Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 or Ins(1,3,4,5,6)P_5 ([13,18,19], and see the legends to Figures 3 and 4). Further progress on this topic will be best addressed by determination of the structure of the active site.

The V_{max} value for Ins(3,4,5,6)P_4 phosphorylation by recombinant type β kinase (780 ± 69 pmol/min per μg; $n = 4$) was approximately twice the corresponding value for Ins(1,3,4)P_3 (320 ± 38 pmol/min per μg; $n = 4$). With Ins(3,4,5,6)P_4 as substrate, the K_m value was 0.1 ± 0.01 μM; similar to the K_m value of

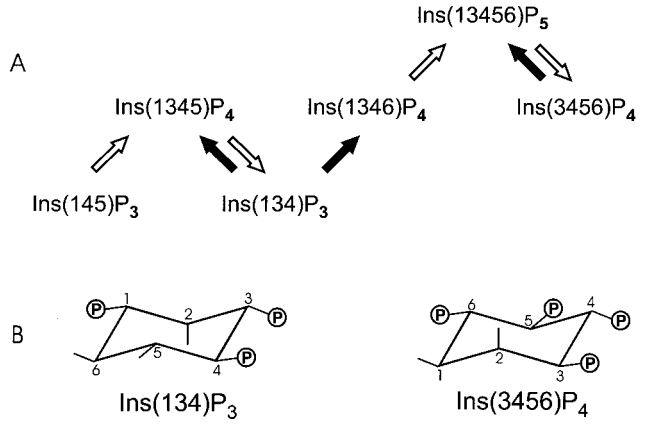

Figure 5 Metabolic and structural relationships between Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4

(**A**) The metabolism of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 are interconnected in higher eukaryotes. The filled arrows designate the reactions performed by the multifunctional Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase that is characterized in this study. (**B**) A comparison of the structures of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4.

0.1–0.2 μM for Ins(3,4,5,6)P_4 that we previously established for the native 1-kinase [11,12]. The K_m value for phosphorylation of Ins(1,3,4)P_3 by recombinant type β kinase was 0.3 ± 0.07 μM. When Wilson and Majerus [15] described their recombinant Ins(1,3,4)P_3 5/6-kinase, they did not report its kinetic parameters. However, others have shown that native rat and human Ins(1,3,4)P_3 kinase preparations have K_m values between 0.2 and 0.5 μM [18,20]. Overall, the kinetic properties of the recombinant enzyme are very similar to those of native enzyme preparations, and so we conclude from these kinetic data that Ins(3,4,5,6)P_4 and Ins(1,3,4)P_3 compete for phosphorylation *in vivo*.

We examined if the single amino-acid difference between the type α and type β kinases affected catalytic activity. Site-directed mutagenesis of the type β cDNA was used to change serine-356 to asparagine in the protein, thereby yielding type α kinase, which was expressed in *E. coli* (Figure 2). This type α enzyme phosphorylated both Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4, and the catalytic activities were indistinguishable from those of our wild-type enzyme (results not shown). Serine-356 introduces an Arg-Xaa-Xaa-Ser candidate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation site, but reconstitution of CaMKII (New England Biolabs) with either form of the kinase did not affect enzyme activity towards Ins(1,3,4)P_3 or Ins(3,4,5,6)P_4 (results not shown).

Conclusions

Our studies have led to an unexpected re-evaluation of the significance of an enzyme that was previously characterized solely as an Ins(1,3,4)P_3 5/6-kinase. The discovery that this enzyme has substrate-dependent positional specificity towards Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 (see Figure 5B) is of general enzymological significance, and our findings provide important new information that will ultimately be pertinent to characterizing the structure and evolution of this catalytic motif. This is an exceptional feature for inositol phosphate metabolism in higher eukaryotes, although there are a couple of precedents for a certain amount of catalytic promiscuity of inositol phosphate kinases in lower organisms. For example, the Arg82 protein from *Saccharomyces cerevisiae* shows both 3-kinase and 6-kinase activities towards Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 [16,21,22]. Another protein with Ins(1,4,5)P_3 3-kinase activity, this time from *Entamoeba histolytica*, also shows a weak ability to phosphorylate Ins(1,3,4)P_3 [23].

However, what makes our work with the mammalian Ins(3,4,5,6)P_4 1-kinase/Ins(1,3,4)P_3 5/6-kinase of such interest to the fields of signal transduction and ion transport physiology is the functional significance of the two substrates in higher organisms. The prevailing level of Ins(3,4,5,6)P_4 in the cell sets the degree of ionic conductance through calcium-activated Cl^- channels in the plasma membrane [1]. Thus the cloning of the Ins(3,4,5,6)P_4 1-kinase represents a significant development, since this finding establishes the molecular identity of an important signalling 'off-switch' that acts to reduce cellular Ins(3,4,5,6)P_4 levels. At the same time, our new data showing that Ins(3,4,5,6)P_4 and Ins(1,3,4)P_3 can actively compete for phosphorylation by a multifunctional kinase *in vivo* provides a molecular explanation for our previous observation that receptor-dependent increases in Ins(1,3,4)P_3 levels inhibit the 1-kinase and elevate Ins(3,4,5,6)P_4 levels [11]. We have therefore gained increased insight into the nature of a signalling 'on-switch'. This represents an unusual paradigm in signal transduction, whereby a single protein is charged with performing competing activities that both activate and deactivate a signalling pathway. These functions therefore add considerably to the significance of the enzyme previously characterized as solely an Ins(1,3,4)P_3 5/6-kinase, and which was already known to be responsible for supplying precursor material for Ins(1,3,4,5,6)P_5 [and therefore Ins(3,4,5,6)P_4; see Figure 5B], as well as for InsP_6 and the diphosphorylated inositol phosphates (which have their own roles in signal transduction; see [24]). This kinase also synthesizes Ins(1,3,4,5)P_4, thereby potentially prolonging the half-life of a cellular signal that mediates receptor-activated Ca^{2+} entry [25]. Since these different activities are concurrent, our new work has uncovered remarkable multitasking capability in an inositol phosphate kinase.

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