The Ins(1,3,4) P_3 5/6-kinase/Ins(3,4,5,6) P_4 1-kinase is not a protein kinase

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Among inositol phosphate kinases, Ins(3,4,5,6) P_4 1-kinase has been considered to be an outsider with disparate sequence, a proclaimed capacity to also phosphorylate proteins and apparent 1-phosphatase activity. Such multifunctionality, coupled with ignorance of its operational domains, complicates any mechanistic rationale behind literature reports that Ins(3,4,5,6) P_4 1-kinase regulates apoptosis, salt and fluid secretion, and transcription. We have expressed poly(His)-tagged human Ins(3,4,5,6) P_4 1-kinase in Sf9 insect cells and purified the enzyme using Ni-agarose chromatography. Protein kinase activity was eluted from the Ni-agarose column, but this did not co-elute with the Ins(3,4,5,6) P_4 1-kinase, indicating that the protein kinase and inositol kinase activities belong to separate proteins. To pursue this conclusion, we prepared catalytically inactive mutants of the Ins(3,4,5,6) P_4 1-kinase by identifying and targeting the ATP-binding site. Our strategy was based on sequence alignments suggesting homology of the Ins(3,4,5,6) P_4 1-kinase with ATP-

grasp metabolic enzymes. Individual mutation of four candidate MgATP-binding participants, Lys¹⁵⁷, Asp²⁸¹, Asp²⁹⁵ and Asn²⁹⁷, severely compromised Ins(3,4,5,6) P_4 1-kinase activity. Yet, these mutations did not affect the protein kinase activity. We conclude that the Ins(3,4,5,6) P_4 1-kinase is not a protein kinase, contrary to earlier reports [e.g. Wilson, Sun, Cao and Majerus (2001) *J. Biol. Chem.* **276**, 40998–41004]. Elimination of protein kinase activity from the enzyme's repertoire and recognition of its ATP-grasp homology together indicate that structural, functional and catalytic relationships between Ins(3,4,5,6) P_4 1-kinase and other inositol phosphate kinases are closer than previously thought [Gonzalez, Schell, Letcher, Veprintsev, Irvine and Williams (2004) *Mol. Cell* **15**, 689–701].

Key words: ATP-grasp, inositol phosphate, inositol 3,4,5,6-tetraphosphate 1-kinase, protein kinase.

INTRODUCTION

Countless biological processes are regulated by stimulus-dependent changes in cellular levels of inositol phosphates (for reviews, see [1,2]). In this context, the canonical roles of inositol phosphate kinases and phosphatases are to regulate synthesis and metabolism of these polyphosphates, thereby controlling their signalling strength. For example, the cellular levels of Ins(3,4,5,6) P_4 are regulated by a dynamic equilibrium between the competing Ins(3,4,5,6) P_4 1-kinase and Ins(1,3,4,5,6) P_5 1-phosphatase activities [3]. This has been thought to control the extent to which Ins(3,4,5,6) P_4 inhibits the conductance of calmodulin-dependent protein kinase II-activated Cl[−] channels [3,4]. The regulation by Ins(3,4,5,6) P_4 of Cl[−] channels in the plasma membrane controls epithelial salt and fluid secretion, and probably also neurotransmission and smooth muscle contraction [3]. Ins(3,4,5,6) P_4 also controls the conductance of Cl[−] channels in intracellular vesicles; this has been demonstrated directly to be biologically important for the regulation of insulin secretion from pancreatic cells, but probably extends to a wide range of endocytic and exocytic processes [5]. Clearly, control of Ins(3,4,5,6) P_4 levels by the 1-kinase/1-phosphatase is an important function that serves multiple biological processes.

Majerus and colleagues [6,7] have reported that the Ins(3,4,5,6) P_4 1-kinase has other important signalling functions which are apparently unrelated to its ability to metabolize inositol phosphates. This group used the baculovirus expression system

with insect cells to produce recombinant human 1-kinase (under its Ins(1,3,4) P_3 5/6-kinase pseudonym, a nomenclature that reflects its additional role in *de novo* Ins(1,3,4,5,6) P_5 synthesis). Their preparations of recombinant enzyme phosphorylated serine and threonine residues on several transcription factors, including ATF-2 (activating transcription factor-2) and IκBα (inhibitory κBα), at least *in vitro* [7]. This new idea that the Ins(3,4,5,6) P_4 1-kinase 'moonlights' [8] as a protein kinase makes it difficult to explain how changes in cellular 1-kinase expression can, for example, alter salt and fluid secretion [3] and regulate apoptosis [9]. Do these biological effects arise from the enzyme's reported protein kinase activity, or are they secondary consequences that arise from alterations in levels of inositol phosphates? These are urgent problems to resolve if we want to understand the biological significance of the Ins(3,4,5,6) P_4 1-kinase.

In a related development, recent crystallographic analyses have demonstrated that some other inositol phosphate kinases, namely, the Ins(1,4,5) P_3 3-kinases, possess vestigial elements of both protein kinases and also ATP-grasp loops [10,11]. The latter describe a protein's ability to grip ATP between two anti-parallel β-sheets [12,13]. The Ins(1,4,5) P_3 3-kinases are now considered to be structurally intermediate between the largely separate families of protein kinases and ATP-grasp metabolic proteins. This new information concerning the 3-kinases is being used as a template to understand their relationships with the other inositol phosphate kinases. Notably absent from this analysis is the 1-kinase. Its disparate sequence typically excludes it from multiple sequence

Abbreviations used: ATF-2, activating transcription factor-2; CV, column volumes; DTT, dithiothreitol; IκBα, inhibitory κBα; 3D-PSSM, three-dimensional position-specific scoring matrix.

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alignments of inositol phosphate kinases [10,11]. The 1-kinase has remained a mystifying outlier from the rest of the inositol phosphate kinase family.

However, protein structure is frequently more tightly conserved than is amino acid sequence [14]. There are structural algorithms, such as those used by the 3D-PSSM (three-dimensional position-specific scoring matrix), that can recognize structural and functional relationships that are beyond even state-of-the-art sequence alignments [15]. In the present study, we show that the threading of the 1-kinase sequence through the 3D-PSSM server (version 2.6.0; <http://www.sbg.bio.ic.ac.uk/~3dpssm>) provides high-confidence predictions that the structure of the 1-kinase is likely to have similarities with ATP-grasp family members. This analysis also identified a candidate ATP-binding domain within the 1-kinase, which we targeted by site-directed mutagenesis. We examined the effects of these mutations upon 1-kinase and protein kinase activities.

The results that were obtained indicate that the 1-kinase is not a protein kinase. Moreover, our data lead us to propose that the 1-kinase has a much closer relationship with the Ins(1,4,5) P_3 3-kinase family than has previously been suspected.

MATERIALS AND METHODS

Materials

[^3H]Ins(1,3,4,5,6) P_5 was prepared by phosphorylation of [^3H]Ins(1,3,4,5) P_4 using recombinant human inositol polyphosphate multikinase as described in [16]. [^3H]Ins(3,4,5,6) P_4 was prepared by 1-phosphatase dephosphorylation of [^3H]Ins(1,3,4,5,6) P_5 (see the Results section, and [3]). Non-radiolabelled inositol phosphates were purchased from Cell Signals (Columbus, OH, U.S.A.). [γ - ^{32}P]ATP was purchased from Amersham Biosciences. Rabbit polyclonal anti-[human Ins(3,4,5,6) P_4 kinase] antibodies (Sigma-Genosys) were generated against the C-terminal peptide QHCVASLTKASSQ, which was conjugated to keyhole-limpet haemocyanin via an engineered N-terminal cysteine residue. The peptide was used for immunoaffinity purification of the anti-kinase antibodies, which were used at a 1:500 final dilution.

Preparation of recombinant human Ins(3,4,5,6) P_4 1-kinase

A recombinant baculovirus genome harbouring the Ins(3,4,5,6) P_4 1-kinase open reading frame, separated from an N-terminal His $_6$ -epitope tag with a thrombin-cleavage site, was constructed using Gateway technology (Invitrogen) as follows: our original Ins(3,4,5,6) P_4 1-kinase cDNA [17] was subcloned into Invitrogen's pDONR 221 vector incorporating a thrombin-cleavage site on the N-terminus of the gene reading frame. The open reading frame was shuttled from pDONR 221 to pDEST10 (Invitrogen) using the LR recombination reaction following the manufacturer's protocol. The destination construct was transformed into DH10Bac. Bacteria harbouring recombinant bacmid DNA containing the 1-kinase gene were identified using blue/white and antibiotic selection as prescribed by Invitrogen. Recombinant bacmid DNA was isolated and transfected into Sf9 insect cells according to the manufacturer's instructions. After a viral stock was generated, Sf9 cells were infected for 72 h at 27°C. Cells were then harvested by centrifugation (2800 g for 20 min at 4°C) and the cell pellets were stored temporarily at -80°C.

Cell pellets were thawed and lysed by resuspension for 5 min on ice in CytoBuster Protein Extraction Buffer (Novagen) mixed with 1 × protease inhibitor cocktail (from BD Biosciences). The suspension then was centrifuged at 15 000 g for 20 min. The supernatant was loaded on to a 1 cm × 6.4 cm Ni-agarose column (Invitrogen), and protein was purified using an AKTA FPLC system

(Amersham Biosciences) at 4°C. The column was washed with 10 CV (column volumes) of buffer A (20 mM Tris/HCl, pH 8.0, 500 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol and 10 % glycerol) at 0.5 ml/min, followed by 2 CV of buffer B (20 mM Tris/HCl, pH 8.0, 1 M KCl, 10 mM 2-mercaptoethanol and 10 % glycerol), and again with 2 CV of buffer A. The final step was a gradient elution created by mixing buffer A with buffer C (20 mM Tris/HCl, pH 8.0, 100 mM KCl, 200 mM imidazole, 10 mM 2-mercaptoethanol and 10 % glycerol) using 0–100 % buffer C over 3 CV. Fractions of 0.5 ml were collected, and those containing Ins(3,4,5,6) P_4 kinase were stored in aliquots at -80°C. Once the aliquots had been thawed once, they were discarded. For some experiments, the fractions containing the 1-kinase after elution from the Ni-agarose column were pooled and loaded on to a HiPrep 16/10 Butyl FF column (Amersham Biosciences, #17-5096-01). A linear gradient of 0–100 % 50 mM sodium phosphate (pH 7.4) over 5 CV was used to eliminate salt from the column. The enzyme was then eluted with a linear gradient of 0–100 % 50 mM sodium phosphate (pH 7.4) containing 1 % Triton X-100 over 5 CV. Protein concentration was then determined using a bicinchoninic acid assay (Pierce). For some experiments, we took advantage of the engineered N-terminal thrombin-cleavage site to remove the poly(His) tag from the enzyme; this had no effect upon catalytic activity (results not shown).

Site-directed mutagenesis

Mutagenesis was performed by using the Stratagene QuikChange Site-Directed Mutagenesis kit according to the manufacturer's instructions, using as a template the wild-type Ins(3,4,5,6) P_4 1-kinase cDNA in the pDONR 221 vector. The primers that were used (reverse primers were exact complements) are as follows, with the mutagenic codons underlined: 5'-GCCATCAGAACCTGCTTGACGCTCCAAGTCCTATGAGC-3' (for R106A), 5'-CCCATTTCATTTGCGCAACCAGAGTGGCTCATGGC-3' (for K157A), 5'-GCAAAACCAGAGTGGCTCATGCCACCACTCTCACG-3' (for G163A), 5'-GCGTGTCACTCTTTGGCATCGCCATCATCATCAACAACC-3' (for D281A), 5'-GGGCA-GCAGCCGTCATTGCCATCAATGCCTTCCC-3' (for D295A), 5'-CCGTCATTGACATCGCTGCCTTCCCAGGCTACGAGGG-3' (for N297A) and 5'-CCGTCATTGACATCGATGCCTTCC-CAGGCTACGAGGG-3' (for N297D).

After PCR amplification/mutagenesis, the mutant gene was excised with DpnI and gel-purified. The purified product was then subcloned into pDONR 221 which had been cut with DpnI to generate a new pDONR 221 vector containing the mutant Ins(3,4,5,6) P_4 1-kinase. After ligation, the vector was transformed into XL1-Blue Supercompetent Cell. Colonies were selected, and the presence of the Ins(3,4,5,6) P_4 1-kinase gene was confirmed by PCR. Positive clones had their sequence verified. The DH10Bac cells were then transformed and the mutants were expressed as described for wild-type protein (see above).

MS

Gel bands were excised manually and digested with trypsin (Promega) for 8 h in an automated fashion with a Progest In-gel Digester from Genomics Solutions. Samples were freeze-dried to dryness, resuspended in 50:50 (v/v) 0.1 % methanoic (formic) acid/acetonitrile and 0.3 ml aliquots were spotted on to a 192-sample stainless steel MALDI (matrix-assisted laser-desorption ionization) plate accompanied by 0.3 ml of 33 % saturated α -cyano-4-hydroxycinnamic acid. Mass spectrometric analyses, MS and MS/MS, were then performed on an Applied Biosystems 4700 Proteomics Analyzer in the positive-ion and reflector modes.

The MS was internally calibrated using autolytic tryptic peptides and the MS/MS was calibrated externally using the fragment ions of the angiotensin I M^+ -H ion (m/z 1296.68). A focus mass of 2000 was used for the MS acquisition. For the MS/MS, 1000 V was used for the collision energy, and argon was used as the collision gas with a recharge threshold set at 1.0×10^{-7} . Protein identification was then performed using a GPS Workstation (Applied Biosystems, Foster City, CA, U.S.A.) which interrogated both the MS and the MS/MS using MASCOT (resident licence; Matrix Science, Boston, MA, U.S.A.) and the NCBI non-redundant database (resident copy). Search parameters included an allowance for two missed tryptic cleavages, a 0.06 Da mass tolerance for the MS data, a 0.1 Da mass tolerance for the MS/MS data, and an allowance for variable oxidation of methionine residues.

Enzyme assays

The first-order rate constant (k , in the equation $S_t/S_0 = e^{-kt}$, where t = time, and S_t and S_0 describe substrate concentration at zero time and at time t) for Ins(3,4,5,6) P_4 phosphorylation was determined in 25 μ l volume assays exactly as described by Majerus and colleagues [6,7]. All zero-order assays for Ins(3,4,5,6) P_4 1-kinase and Ins(1,3,4,5,6) P_5 1-phosphatase activities were performed in either 100 μ l or 200 μ l assays, as described previously [3], and analysed using either gravity-fed anion-exchange columns or HPLC, as described previously [18].

For the protein kinase assays, 1 μ g of purified Ins(3,4,5,6) P_4 1-kinase was incubated at 37°C for 20–30 min in 10–20 μ l of buffer containing 20 mM Tris (pH 7.6 with HCl), 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 10% (v/v) glycerol, 10 μ Ci [γ - ^{32}P]-ATP, plus 1 μ g of protein substrate. The reactions were quenched by addition of 4 $\times$ NuPAGE LDS loading buffer (Invitrogen), followed by boiling for 3 min. Samples were resolved on 4–12% NuPAGE pre-cast gels (Invitrogen), fixed with methanol/ethanoic (acetic) acid/water (5:1:4), and analysed with a phosphorimager.

Western blot analysis

Samples were resolved on 4–12% NuPAGE pre-cast gels in the presence of 50 mM DTT (dithiothreitol), transferred on to a nitrocellulose membrane (Schleicher & Schuell), and immuno-detected with a 1:500 dilution of immunoaffinity-purified anti-peptide antibodies to the C-terminus (see above). After incubation with secondary antibody (anti-rabbit, 1:5000), the immunoreactive bands were visualized with ECL[®] (enhanced chemiluminescence) reagents (Amersham Biosciences).

RESULTS AND DISCUSSION

The nature of the protein kinase activity in preparations of recombinant Ins(3,4,5,6) P_4 1-kinase

After expression of recombinant human Ins(3,4,5,6) P_4 1-kinase in insect cells, Majerus and colleagues [7] purified the enzyme using step ('batch') elution from an affinity column. However, a gradient elution employed in column chromatography typically enhances the purity of the final preparations. Thus, in the present study, we eluted recombinant Ins(3,4,5,6) P_4 1-kinase from a Ni-agarose affinity column using a linear imidazole gradient generated using FPLC (Figure 1A). Western blot analysis, using anti-peptide antibodies against the C-terminus, indicated that the 1-kinase migrated close to its predicted size of 50 kDa (Figure 1B). Our anti-1-kinase antibodies also recognized a faint band migrating at approx. 98 kDa, approximately twice the size of the major 50 kDa band (Figure 1B). The 98 kDa band was excised from gels and was analysed by MS (see the Materials and methods section),

and only the 1-kinase sequence was detected. This is consistent with a small proportion of 1-kinase forming a dimer under these conditions. The dimer could still be detected even if the gels were run with 250 mM DTT (results not shown).

The elution profile for 1-kinase activity (Figure 1A, $\circ$) was not significantly different from the immunoblot profile of the 1-kinase protein (Figures 1B and 1C; $\circ$). These data indicate that our preparations of 1-kinase are catalytically homogeneous. That is, there is no indication that a subfraction of the purified recombinant 1-kinase is somehow modified and has a different catalytic activity. The absence of covalent modification is also indicated by the 1-kinase band having the same apparent size across all of the fractions to elute from the Ni-agarose column (Figure 1B).

Using enzyme that eluted in fraction 9 (Figure 1), we determined the K_m and V_{max} for the Ins(3,4,5,6) P_4 1-kinase to be 0.05 μ M and 220 $nmol \cdot mg^{-1} \cdot min^{-1}$ respectively. We have reported previously that the enzyme can also act as an ADP-dependent Ins(1,3,4,5,6) P_5 1-phosphatase [3]; in the present study, we found the K_m and V_{max} for the phosphatase to be $0.03 \pm 0.001 \mu$ M and $16 \pm 0.5 nmol \cdot mg^{-1} \cdot min^{-1}$ respectively (kinetic parameters for the 1-phosphatase activity have not previously been published).

The Majerus group previously expressed the specific activity of the 1-kinase as a first-order rate constant: 500 000 $min^{-1} \cdot mg^{-1}$ [6]. They derived this value using the kinase's alternative substrate, Ins(1,3,4) P_3 [17]. Our earlier experiments have shown that the first-order rate constants are very similar for both Ins(3,4,5,6) P_4 and Ins(1,3,4) P_3 (Figures 3 and 4 in [17]). Therefore it is possible to directly compare the specific activity of our recombinant enzyme with that made in Majerus's laboratory, even though we have used Ins(3,4,5,6) P_4 as the substrate. The value of the specific activity obtained for our preparations (approx. 300 000 $min^{-1} \cdot mg^{-1}$; Figure 1E) is very close to that reported by Majerus and colleagues [6].

Majerus and colleagues have shown previously that as little as 5–8 ng of their preparations of recombinant human 1-kinase can phosphorylate full-length human IkB α [6]. We could not reproduce these results using such small amounts of protein (results not shown). When we used 125–200-fold larger amounts of protein (1 μ g) in our assays, we found that some of the early fractions that eluted from the Ni-agarose column did indeed phosphorylate IkB α (Figures 1C and 1F). Thus we observed a considerably lower level of protein kinase activity than did Majerus and colleagues [6], even though our inositol kinase activities are in good agreement with theirs. This experimental discrepancy would be completely understandable if the protein kinase was performed by contaminating protein(s). The latter idea is supported further by the observation that the peak of the elution profile for the protein kinase activity was clearly separated from the peak of 1-kinase activity (Figure 1). Indeed, fraction number 6 contains the peak of the protein kinase profile (Figures 1C and 1F), yet this fraction has a negligible proportion of total 1-kinase (Figures 1A and 1B). Finally, some preparations of 1-kinase were purified further using a hydrophobic affinity column (see the Materials and methods section); after SDS/PAGE, the only visible bands were attributable to the 50 kDa 1-kinase, and a small amount of the dimerized form of the protein (Figure 2A, lane B). These preparations did not display any protein kinase activity (Figure 2A, lane A).

Majerus and colleagues [7] were also able to phosphorylate the N-terminal activation domain of ATF-2 with their preparations of 1-kinase. We were able to confirm this observation using aliquots of impure 1-kinase (e.g. those in fraction 6, Figure 1) provided, as described above, that we used 125–200-fold larger amounts of protein (1 μ g) (Figure 2) than previously used in the Majerus

Figure 1 Chromatographic separation of protein kinase and Ins(3,4,5,6) P_4 1-kinase activities

(A) Recombinant Ins(3,4,5,6) P_4 1-kinase was expressed in Sf9 cells and bound to a Ni-agarose column, which was eluted with an imidazole gradient; 0.5 ml fractions were collected. ○, 1-kinase activity; ■, protein content of each fraction. (B) Western blot analysis was used to quantify the Ins(3,4,5,6) P_4 1-kinase protein in each of the fractions (identified along the top). Sizes are indicated in kDa. (C) The relative 1-kinase band intensities (○) were calculated using NIH Image software, and are computed from results shown in (B). Protein kinase band intensities (●) are also shown. (D) Aliquots (15 μ l) of the fractions (identified along the top) were boiled in 7 μ l of NuPAGE LDS buffer (Invitrogen) + 60 mM DTT, resolved by SDS/PAGE (4–25% NuPAGE pre-cast gels), and stained with 'SimplyBlue SafeStain' (Invitrogen). Sizes are indicated in kDa. (E) Approx. 2000 d.p.m. of [3 H]Ins(3,4,5,6) P_4 was incubated for 75 s in 25 μ l of assay buffer with 4.2 ng of 1-kinase from fraction 9. Samples were quenched, neutralized and resolved by HPLC. From the radioactivity in the two peaks [Ins(3,4,5,6) P_4 = 358 d.p.m. and Ins(1,3,4,5,6) P_5 = 1495 d.p.m.], we calculated the first-order rate constant for 1-kinase activity to be approx. 300 000 $\text{min}^{-1} \cdot \text{mg}^{-1}$. The closed circles show relative band intensities of phosphorylated I κ B α , in response to protein kinase activity in 5 μ l of each fraction using as a substrate 1 μ g of full-length human I κ B α (F) expressed as a 70 kDa GST (glutathione S-transferase)-fusion protein in *Escherichia coli* (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

laboratory [7]. Our preparations also phosphorylated histone (Figure 2). Negligible protein phosphorylation was observed in protein kinase assays supplemented with aliquots of relatively pure 1-kinase from fraction 9 (Figure 2).

Overall, our data (Figures 1 and 2) indicate that the protein kinase activity is due to a contaminant, and is not an inherent property of the 1-kinase. Indeed, the fractions that contained the protein kinase activity also contained proteins of various sizes (Figure 1D). We attempted to identify the protein kinase by excising bands from gel and analysing them by MS (see the Materials and methods section). No protein kinase sequence was detected, probably because it was not present at sufficiently high levels.

Selection of putative ATP-grasp targets for mutagenesis

The data described above indicate that preparations of recombinant 1-kinase expressed in Sf9 cells are contaminated with protein kinase activity. To verify this conclusion, we decided to identify amino acid residues that might comprise the MgATP-binding site, to target them by mutagenesis and study the consequences for both 1-kinase and protein kinase activity. We should note that there

are no previous mutagenic studies of this enzyme, so we first had to identify candidate residues. The sequence of the 1-kinase was threaded through the 3D-PSSM server, which predicted, with > 95% certainty, that there are substantial structural similarities between the 1-kinase and D-Ala-D-Ala ligase. The predicted similarities in secondary structure (α -helix and β -sheet) are especially notable across four subregions of the ATP-grasp domain (labelled a–d in Figure 3). The 'ATP-grasp' nomenclature describes the ability of a protein to grip ATP between two antiparallel β -sheets [12,13]. We examined the veracity of this prediction that the 1-kinase is also an ATP-grasp protein. We expanded the alignment output from the 3D-PSSM server by adding several other ATP-grasp proteins (Figure 3). Several residues in the 1-kinase align with residues shown previously to be catalytically important in ATP-grasp family members (these residues are underlined in Figure 3).

For example, Arg¹⁰⁶ and Lys¹⁵⁷ in the 1-kinase align with arginine/lysine residues in ATP-grasp domains a and b (Figure 3), which are known to interact electrostatically with the α - and β -phosphates of ATP [19–21]. Also in domain b, ATP-grasp proteins contain a conserved glycine residue (Figure 3). It is noteworthy

Figure 2 Characteristics of the protein kinase activity in preparations of recombinant Ins(3,4,5,6) P_4 1-kinase

For these experiments, we used aliquots of fractions 6 and 9 (see Figure 1) as indicated above each lane. Protein kinase assays were performed as described in the Materials and methods section. The following proteins (1 μ g) were used as substrates: (A) I κ B α (see Figure 1 legend); (B) residues 19–96 of human ATF-2 expressed as a 34 kDa GST (glutathione S-transferase)-fusion protein in *Escherichia coli* (Cell Signaling Technology, Waltham, MA, U.S.A.); (C) human histone H1 (Cell Signaling Technology). In addition, a preparation of recombinant 1-kinase was eluted from a Ni-agarose column and fractions containing the entire 1-kinase peak were pooled. This preparation was then purified further using a HiPrep 16/10 Butyl FF column as described in the Materials and methods section. The absence of protein kinase activity in the final preparation is shown in Lane A. Lane B shows the purity of 0.32 μ g of enzyme resolved by SDS/PAGE (4–12% bis-Tris gel) and silver-stained (SilverXpress, Invitrogen). Sizes are indicated in kDa.

that Gly¹⁶³ in the 1-kinase does not occur in the glycine-rich context (i.e. GXGXXG) normally associated with nucleotide-binding loops. Nevertheless, Cheek et al. [22] proposed that Gly¹⁶³ in the 1-kinase is equivalent to the conserved glycine residues (Figure 3) in domain b of ATP-grasp proteins. Next, Asp²⁷⁹ in the 1-kinase, aligns with aspartic/glutamic residues that co-ordinate to a Mg²⁺ ion [21,23]; glutamine also serves this function in the two subunits of carbamoyl phosphate synthetase [20]. Finally, in domain d (Figure 3), Asp²⁹⁵ and Asn²⁹⁷ in the 1-kinase align with ATP-grasp residues that co-ordinate to a second Mg²⁺ ion [19,20]. We examined the importance of these six residues in the 1-kinase (i.e. those underlined in Figure 3) by site-directed mutagenesis.

Effect of site-specific mutations upon Ins(3,4,5,6) P_4 1-kinase activity

Our mutagenic experiments began with Asn²⁹⁷ (see above). In view of the Mg²⁺-binding role of the equivalent asparagine residue in the ATP-grasp family, we made a conservative (N297D) mutation because there are examples of aspartic acid residues having a surrogate Mg²⁺-binding capacity [24]. We complemented this experiment with a non-conservative (N297A) mutation. The purity of these enzyme preparations was verified by Western blot analysis and by SDS/PAGE (Figures 4A and 4B). The conservative N297D mutation reduced 1-kinase activity by approx.

Figure 3 Proposed domain conservation between the Ins(3,4,5,6) P_4 1-kinase and representatives of the ATP-grasp family

Proposed regions of structural similarity between the 1-kinase and D-Ala-D-Ala ligase, within ATP-grasp microdomains (a–d), were obtained by threading the 1-kinase amino acid sequence through the 3D-PSSM server. The graphics above the ligase sequence represent regions of α -helix and β -sheet. The corresponding predicted secondary structures in the 1-kinase are indicated below its sequence. The amino acid sequence alignment was expanded to include other ATP-grasp proteins, as published previously by Galperin and Koonin [13], with the addition of the human Ins(3,4,5,6) P_4 1-kinase (1-K) as suggested by Cheek et al. [22]. Conserved hydrophobic clusters are shaded grey. Residues that are underlined in the 1-kinase sequence are those that were mutated in the present study. Residues in the other sequences that are underlined are those that have previously been indicated to be catalytically essential by mutagenesis or by structural analysis, as described for D-Ala-D-Ala ligase from *Escherichia coli* [19,23], the small subunit of *E. coli* carbamoyl phosphate synthetase (CPS sm.; [20]), biotin carboxylase from *E. coli* (BC; [21,28,29]), the large subunit of *E. coli* carbamoyl phosphate synthetase (CPS lg.; [30,31]). The number to the left of the first amino acid beginning each line of sequence identifies its position within the full protein. Numbers in square brackets depict residues in each sequence that are omitted.

60% (Figure 4C). The non-conservative N297A mutant had no detectable activity even when it was incubated at a > 50-fold higher concentration than the wild-type enzyme (Figure 4C). This demonstration of the functional significance of Asn²⁹⁷ is consistent with the idea (Figure 3) that the 1-kinase might utilize an ATP-grasp fold. We therefore next targeted five other residues that were implicated as being functionally significant by the sequence alignments (Figure 3).

Three of these additional five mutant proteins that we prepared had little (< 2%) or no detectable Ins(3,4,5,6) P_4 1-kinase activity: K157A, D281A and D295A (Table 1). The activity of R106A was also lower (by 33%) than that of the wild-type enzyme (Table 1). Since these residues are functionally-important in the ATP-grasp family, our data strongly suggest that the 1-kinase is very likely to be a new member of this group of proteins, although a definitive demonstration will require that structure of the protein is defined. The only one of the mutations described in the present paper that did not affect 1-kinase activity was the G163A modification (Table 1). As mentioned above, we had already noted that this glycine residue did not occur in the appropriate context normally associated with nucleotide binding.

A single active site for 1-kinase and 1-phosphatase activity?

The catalytically compromised mutants that we have prepared were also helpful in the pursuit of an earlier observation that the Ins(3,4,5,6) P_4 1-kinase can also act 'in reverse' as an ADP-dependent Ins(1,3,4,5,6) P_5 1-phosphatase [3]. We found that the effects

Figure 4 Impaired 1-kinase activity in N297D and N297A mutants

The purity of 1–2 μ g of either wild-type 1-kinase, or N297D and N297A mutants, was checked by Western blot analysis (A) or SDS/PAGE (B). (C) The 1-kinase activity was ascertained by HPLC as described in the Materials and methods section in incubations containing 0.2 μ M [3 H]Ins(3,4,5,6) P_4 and either 2.1 ng of wild-type enzyme (○), 2.1 ng of the N297D mutant (■) or 115 ng of the N297A mutant (●).

Table 1 Mutational analysis of the Ins(3,4,5,6) P_4 1-kinase/Ins(1,3,4,5,6)- P_5 1-phosphatase activities

Assays of Ins(3,4,5,6) P_4 1-kinase and Ins(1,3,4,5,6) P_5 1-phosphatase were performed as described in the Materials and methods section using 0.2 μ M of the appropriate substrate. Data are calculated relative to wild-type activity; means $\pm$ S.E.M. are shown ($n=3-4$). The absolute activities of the wild-type (WT) 1-kinase and 1-phosphatase were 205 ± 10 and 16 ± 0.5 nmol/mg of protein per min respectively.

	Activity (%)						
	WT	R106A	K157A	G163A	D281A	D295A	N297A
1-Kinase	100	67 $\pm$ 5	2 $\pm$ 0.5	104 $\pm$ 19	2 $\pm$ 1	0	0
1-Phosphatase	100	121 $\pm$ 15	11 $\pm$ 6	139 $\pm$ 23	0	0	0

of our mutations upon 1-phosphatase activity were very similar to their effects upon 1-kinase activity (Table 1). In particular, the four mutants that had the most impaired 1-kinase activity (K157A, D281A, D295A and N297A) also had little or no 1-phosphatase activity (Table 1); clearly, these residues are important to both reactions. These data indicate that the enzyme utilizes a single nucleotide-binding site for both the kinase and phosphatase reactions.

Protein kinase activity in preparations of recombinant catalytically compromised 1-kinase mutants

In experiments describe above, we demonstrated that, during the elution of recombinant 1-kinase from a Ni-agarose column, early fractions contain protein kinase activity. We concluded that the protein kinase activity was due to contaminants from the Sf9 expression system, rather than being an inherent property of the

Figure 5 Protein kinase activity in preparations of 1-kinase in which inositol kinase activity is compromised

Aliquots (5 μ l) of fractions 5 and 6 obtained following a Ni-agarose elution of preparations of three different catalytically compromised mutants of the 1-kinase (K157A, D281A and N297A) were assayed for protein kinase activity using 1 μ g of I κ B α as substrate (as in Figure 1). Aliquots of the same fractions were also subjected to Western blot analysis to assay for the 1-kinase protein (see Figure 1 legend). HPLC analysis of 1-kinase activity was determined as described in Figure 1 for the wild-type enzyme, except that the quantity of kinase that was assayed was increased 50-fold. The arrow marks the elution position of Ins(1,3,4,5,6) P_5 .

1-kinase. This conclusion was examined further using the mutant proteins that we have generated. Mutants for which the ATP-dependent 1-kinase activity is compromised should also lose other ATP-dependent catalytic activities, since the putative ATP-grasp domain encompasses half of its entire length (Figure 3). Despite losing 1-kinase activity (HPLC traces in Figure 5), aliquots of fractions 5 and 6 generated by elution from the Ni-agarose column still showed approximately the same protein kinase activity (Figure 5) that was observed in preparations of wild-type enzyme (Figure 1). The fact that catalytically compromised preparations of 1-kinase still contained protein kinase activity further confirms that the protein kinase is due to contaminating proteins.

Concluding comments

We have shown that the protein kinase activity previously attributed to the preparations of baculovirus-expressed, recombinant Ins(3,4,5,6) P_4 1-kinase [6,7,9] is in fact due to microcontamination from separate protein kinases. This is an important development, because it now allows us to focus future studies on the function of the 1-kinase purely in terms of its ability to regulate inositol phosphates. This will make it easier to determine how changes in 1-kinase activity influence apoptosis [9], salt and fluid secretion [3], and transcription [7]. Our studies also counter an earlier assertion [6] that the 1-kinase is a long-sought-after protein kinase that tightly associates with the COP9 multimeric signalosome, which regulates the activity of SCF (Skp1/Cullin/F-box protein)-type E3 ubiquitin ligases [25,26]. In any case, preparations of COP9 have been obtained which retained considerable protein kinase activity, yet no 1-kinase could be detected, either by using antibodies [27] or by direct assays of 1-kinase activity (W. Dubiel and S. B. Shears, unpublished work). Nevertheless, we should not necessarily neglect the observation that protein kinase activity persists during the purification of recombinant 1-kinase expressed in insect cells. This may signify strong protein-protein interactions that have some biological significance. In fact, protein kinase activity has also been detected in preparations of recombinant 1-kinase expressed and isolated from HEK-293 (human embryonic kidney) cells [6,7], although those preparations were recognized to be more contaminated than the baculovirus-expressed 1-kinase purified from insect cells [7].

Our mutagenic data strongly suggest that the 1-kinase should be considered a member of the ATP-grasp family of metabolic proteins. Of course, a definitive demonstration of the presence of this feature awaits a structural analysis of the 1-kinase, but to date the human enzyme has resisted sustained attempts at its crystallization (G. J. Miller, S. B. Shears and J. H. Hurley, unpublished work). Nevertheless, in our present study, we identified several residues in the 1-kinase as being conserved in a group of related ATP-grasp proteins, and mutation of these residues dramatically impaired 1-kinase activity. This is especially interesting in the light of a recent demonstration that the Ins(1,4,5) P_3 3-kinases possess vestigial ATP-grasp elements [10,11]. Thus we should consider the 1-kinase to be a closer relative of the 3-kinases than could previously have been considered based upon the lack of obvious sequence similarity. Considerable insight into catalytic mechanisms can be obtained by comparing structural features among different proteins. We are therefore now closer to determining the catalytic mechanism of the Ins(3,4,5,6) P_4 1-kinase.

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