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Cloning and characterization of a 92 kDa soluble phosphatidylinositol 4-kinase

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A phosphatidylinositol (PtdIns) 4-kinase cDNA cloned from a rat brain cDNA library encoded a protein of 816 amino acids with a calculated molecular mass of 91 654 Da. This molecule contained a lipid-kinase-unique domain and a presumed lipid/protein kinase homology domain that are found in other PtdIns 4-kinases and PtdIns 3-kinases. Furthermore, this kinase molecule had 43.3 % shared identity with the presumed catalytic domain of yeast PtdIns 4-kinase, PtdInsK1, and the two molecules had a region of similarity that is not conserved in other lipid kinases. By examining PtdIns kinase activity in transfected COS-7 cells using epitope tag immunoprecipitation as well as conventional methods, the product PtdIns phosphate was identified as phosphatidylinositol 4-phosphate (PtdIns4*P*), but not phosphatidylinositol 3-phosphate (PtdIns3*P*). The PtdIns 4-kinase activity was recovered predominantly from the soluble fraction and the activity was markedly enhanced in the presence of Triton X-100 and was relatively insensitive to inhibition by adenosine. In addition, the PtdIns 4-kinase activity was completely inhibited in the presence of 10 μM wortmannin. When examined by epitope tag immunocytochemistry, the immunoreactivity for the PtdIns 4-kinase molecule was dominantly aggregated in a cytoplasmic region juxtaposed to the nuclei and was faintly but widely dispersed in the cytoplasm. By *in situ* hybridization analysis, the mRNA for PtdIns 4-kinase was expressed ubiquitously and was detected in most neurons throughout the grey matter of the brain, with higher expression intensity found in fetal than in adult brain.

INTRODUCTION

Inositol phospholipids act as messengers in intracellular signal transduction which is initiated by stimulation of a variety of cell surface receptors [1,2]. The synthesis, degradation and subsequent resynthesis of inositol phospholipids constitute a metabolic cycle known as the inositol phospholipid cycle, and the phosphorylation of phosphatidylinositol (PtdIns) at the D4 position of the inositol ring by PtdIns 4-kinase is considered an important step in the cycle [3]. Multiple isoenzymes of PtdIns 4-kinase have been purified from various mammalian sources and from yeast, which suggests that the enzyme may have more than one function. In support of this suggestion, there is increasing evidence of non-signalling roles for PtdIns 4-kinase in intracellular vesicular traffic [4].

Previous studies have identified three cDNAs encoding distinct proteins which exhibit PtdIns 4-kinase activity in yeast: PtdInsK1, STT4 and TOR2 [5–8]. In mammals, a rat homologue to TOR (RAFT1) and a human homologue (PtdIns4Kα) to yeast PtdIns kinase have also been reported to possess PtdIns 4-kinase activity [9,10]. However, a recent study by us has established that the human homologue is an alternative or rather truncated form of another authentic 230 kDa PtdIns 4-kinase, which has more similarity with STT4 than PtdInsK1 [11].

While the 230 kDa PtdIns 4-kinase molecule and RAFT1 are membrane-associated, there is increasing evidence suggesting the existence of a soluble PtdIns 4-kinase molecule which may be involved in Ca^{2+}-activated priming for exocytosis [12–14]. The present study addressed this point and was undertaken to identify an isoform of rat PtdIns 4-kinase by gene cloning. Consequently, in contrast to the molecules mentioned above, the newly identified PtdIns 4-kinase was shown to be soluble and bore more similarity to yeast PtdInsK1 than to STT4.

EXPERIMENTAL

Isolation and characterization of cDNA clones

Extraction of total RNA from adult rat brain, isolation of poly(A) RNA and PCR-amplification procedures were carried out as described previously [11]. As primers for PCR, two degenerate oligonucleotides were prepared based on the amino acid sequences of conserved regions among the putative lipid kinase domains of rat 230 kDa PtdIns 4-kinase, STT4 and PtdInsK. The regions corresponded to the amino acid sequences (V/T)GDD(C/L)RQ (residues 1648–1654, 5′ primer) and HIDFGF(I/M) (residues 1770–1776, 3′ primer). The nucleotide numbers represent those of STT4. The sequences of the primers were designed according to the mammalian codon usage: CGGAATTCCGG(A/T/C)GA(T/C)GA(T/C)GA(T/C)T-(G/T)(T/C)CG(G/C)CA(G/A)GA for the 5′ primer and CGG-AATTCAT(G/A)AA(G/A/T)CC(G/A)AA(G/A)TC(G/A)-AT(G/A)TG for the 3′ primer. The 5′ ends of the 5′ and 3′ primers contained an *Eco*RI restriction sequence for subsequent cleavage of cloned cDNA fragments. Sequence analysis revealed that one of 12 clones (pNK1) showed identity with PtdInsK1, rat 230 kDa PtdIns 4-kinase and STT4, and this clone was used for further analysis.

Clones (3×10^6) derived from a rat brain cDNA library, which was constructed as described previously [15], were screened by

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns3*P*, phosphatidylinositol 3-phosphate; PtdIns4*P*, phosphatidylinositol 4-phosphate; PEP, priming in exocytosis protein; FLAG, marker peptide.

The EMBL, GenBank, DDBJ accession number for the phosphatidylinositol 4-kinase sequence reported in this paper is D84667.

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hybridization with a 387 bp cDNA fragment of pNK1. Hybridization was carried out at 42 °C for 16 h in a buffer containing 50 % (v/v) formamide, 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 1 × Denhardt's [0.02 % (w/v) Ficoll 400, 0.02 % (w/v) poly(vinyl pyrrolidone), 0.02 % (w/v) BSA], 50 mM sodium phosphate (pH 7.2) and 250 μg/ml of heat-denatured salmon sperm DNA. The membranes were washed twice at room temperature in 2 × SSC containing 0.1 % (w/v) SDS for 10 min, followed by two washes in 0.1 × SSC containing 0.1 % (w/v) SDS at 42 °C for 30 min and finally at 55 °C for 30 min. Among five hybridization-positive clones isolated, two clones containing large cDNA inserts were selected and subcloned into the Bluescript vector, SK^+ (Stratagene). The cDNA inserts of these clones showed an identical digestion pattern with the restriction enzymes except for some length difference in their extreme 5′ portions, and one clone containing the largest cDNA inserts (3.4 kb) was chosen for further sequence analysis of both strands by the dideoxy chain-termination method [16] with a 373A DNA sequencer (Applied Biosystems), used according to the supplier's instructions.

Northern blot analysis

Total RNA was extracted from several adult rat tissues as described previously [17]. Each of the total RNA samples (30 μg per lane) was denatured with formaldehyde and size-separated by agarose gel electrophoresis. The RNA was transferred and fixed to a nylon membrane (Nytran, Schleicher and Schuell) and hybridized with a probe corresponding to the sequence (nt 105–1843) labelled with [^{32}P]dCTP. Autoradiography was performed at −80 °C for 3 days.

Transfection and PtdIns kinase activity

The full length cDNA for the newly identified molecule was subcloned into the expression vector, pSRE (pcDL–SRa 296, [18]), as modified by Sakane et al. [19]. The vector alone or the constructs were transfected into COS-7 cells by a DEAE–dextran method [20]. After incubation for 3 days in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum, transfected COS-7 cells were harvested and lysed by sonication in lysis buffer containing 20 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 4 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml pepstatin, 50 mg/ml soybean trypsin inhibitor and 1 mM PMSF. Undisrupted cells were removed by centrifugation (550 ***g*** for 10 min) and the supernatant, hereafter referred to as the total lysate, was centrifuged at 105000 ***g*** for 30 min to separate soluble and particulate fractions. Protein concentrations were determined by the method of Lowry et al. using BSA as a standard [21].

PtdIns kinase activity was measured by the method of Kato et al. [22] with modifications. The reaction mixture (50 μl) contained 0.3 % (v/v) Triton, 50 mM Tris/HCl (pH 7.4), 20 mM $MgCl_2$, 1 mM EGTA, 5 mg/ml PtdIns (Sigma), and 1 mM [γ-^{32}P]ATP (5000 c.p.m./nmol; ICN). The reaction was continued for 5 min at 30 °C and stopped with 100 μl of 1 M HCl. The lipid was extracted with 250 μl of chloroform/methanol (1:1, v/v). The chloroform phase was extracted with 100 μl of methanol/1 M HCl (1:1 v/v) and evaporated to dryness. The dried lipids were resuspended and analysed by TLC. Silica gel plates (Merck) were pretreated with 40 % (v/v) methanol containing 1 % (w/v) potassium oxalate and 2 mM EDTA (pH 7.2), and were baked at 105 °C for 50 min. The lipids were separated by elution in chloroform/methanol/28 % (v/v) ammonia solution/distilled water (70:100:15:25, by vol.). The band of gel containing PtdIns*P*, detected by autoradiography, was removed from the TLC plate with a sharp spatula and the radioactivity was measured by liquid scintillation counting. For further separation of the PtdIns*P* product into PtdIns3*P* and PtdIns4*P*, thin layer plates precoated with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid were used as described by Walsh et al. [23]. The position of PtdIns3*P* was determined by using A431 cell lysate that had been reported to contain PtdIns 3-kinase activity as a standard [24]. To analyse the sensitivity of PtdIns 4-kinase activity to wortmannin, this reagent (Sigma) was added to the sample and incubated for 3 min at 4 °C before the start of the reaction.

Epitope tagging and immunoprecipitation

An epitope tag composed of eight amino acids (FLAG marker peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Kodak) was fused to the newly identified molecule by cloning the 24 bp FLAG coding sequence next to the initiation codon, ATG, of the novel cDNA. The FLAG epitope-tagged molecule was expressed in COS-7 cells using the expression vector, pSRE, by a DEAE–dextran method. Transfected COS-7 cells were harvested and lysed with 1 ml of 1 % (v/v) Nonidet P-40 in lysis buffer [20 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 4 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml pepstatin, 50 mg/ml soybean trypsin inhibitor and 1 mM PMSF]. Total lysates were incubated for 40 min at 4 °C with gentle rocking and centrifuged at 10000 ***g*** for 20 min at 4 °C. Equal quantities of soluble protein were immunoprecipitated for 1 h at 4 °C with a monoclonal antibody (anti-FLAG-M2, Kodak) specific for the FLAG marker peptide. Protein A–Sepharose beads precoated with 2 mg/ml BSA were added to the lysates and incubated for 1 h. The beads were washed twice with 1 % (v/v) Nonidet P-40 in lysis buffer and three times with lysis buffer alone. The beads were assayed for PtdIns kinase activity as described above.

Immunoblotting, immunohistochemistry and *in situ* hybridization histochemistry

For immunoblotting, the total lysates of the cells containing overexpressed protein were boiled for 4 min in Laemmli's sample buffer and subjected to SDS/7.5 %-PAGE [25]. The proteins were electrophoretically transferred to a nitrocellulose membrane (pore size, 0.45 μm). After blocking the non-specific binding sites with 5 % (w/v) skimmed milk in PBS, the membrane was incubated with the antibody to FLAG for 2 h at room temperature and was then treated with peroxidase-conjugated anti-(rabbit IgG) antibody for 1 h. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) kit (Amersham).

The transfected cells, fixed with 4 % (v/v) paraformaldehyde, were processed for immunohistochemistry, and freshly frozen brain from adult (postnatal day 49) male and fetal (prenatal day 15 or 18) rats were processed for *in situ* hybridization, as described in detail in our previous study [11].

RESULTS

The nucleotide and deduced amino acid sequences of the composite cDNA are presented in Figure 1. The putative initiation codon was preceded by in-frame stop codons at nucleotides −93, −216 and −351. The deduced amino acid sequence encoded a protein of 816 amino acids with a calculated molecular mass of 91654 Da. Comparison with sequences in protein and DNA data banks revealed that the protein encoded by this cDNA had more identity with PtdInsK1 than with any

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-366  TTACTATGGCATCGGTAGTGTCCTGACAGTTCGGAAAGCAGGAAAGCAAGCGCCGAGAAGAAAGGCTCGGGCCCAGCGGACTCTTACATCGCAACCCATCAAGGAATTGTTATTTTGGGT
-246  CCTGCAAGTGGGGTCCGGTCCAACTTGAGGTAGCGTGAAGGCCCCTTCAGGCGGCGCCGCGGGCAGCCCCGCAGCCGGGGCCTGGTGCAGCCGCCGCGGCCGCTGTCAGGGAAGCGCAGG
-126  CGGCCAATGGAACCCGGGAGCGGCCGCTGCTGCTGAGGCGGCGGTGTCGGCAGTCCGACCCCGACCGCCTGCACCCCCTCCGCGAGGGTCCCCCGGAGCTTGGAAGCTCGAAGTCTGGCT
-6    GTAGCCATGGGAGACATGGTAGTGGAGCCTGCCACCCTGAAGCCAACTTCTGAGCCTACTCCTAGCCCATCAGGGAATAACGGGGGCTCCCTACTAAGCGTCATCACGGAGGGGGTCGGG
1         M  G  D  M  V  V  E  P  A  T  L  K  P  T  S  E  P  T  P  S  P  S  G  N  N  G  G  S  L  L  S  V  I  T  E  G  V  G
115   GAACTGTCAGTGATTGACCCTGAGGTGGCCCAGAAGGCCTGCCAGGAGGTACTGGAGAAAGTCAAGCTTTTGCATGGAGGTGTAGCCATCTCTAGCAAAGGCAGCCCACTGGAGTTGGTT
39    E  L  S  V  I  D  P  E  V  A  Q  K  A  C  Q  E  V  L  E  K  V  K  L  L  H  G  G  V  A  I  S  S  K  G  S  P  L  E  L  V
235   AATGGGGATGGTGTGGACAATGAGATCCGTTGCCTAGATGATCCACCTACCGAGATCAGGGAGGAAGAAGATGAGATGGAGCCTGGTGTGGTCTCTGGCACAGCCAAAGGAACAAGAAGA
79    N  G  D  G  V  D  N  E  I  R  C  L  D  D  P  P  T  E  I  R  E  E  E  D  E  M  E  P  G  V  V  S  G  T  A  K  G  T  R  R
355   AGACGACAGAACAACTCAGCCAAACAGTCTTGGCTCCTGAGGCTGTTTGAATCAAAACTATTTGACATCTCTATGGCCATTTCATACTTGTATAACTCCAAGGAGCCGGGAGTGCAAGCC
119   R  R  Q  N  N  S  A  K  Q  S  W  L  L  R  L  F  E  S  K  L  F  D  I  S  M  A  I  S  Y  L  Y  N  S  K  E  P  G  V  Q  A
475   TACATTGGCAACCGGCTCTTCTGCTTTCGCAATGAGGATGTGGACTTCTATTTGCCCCAGTTGCTTAACATGTATATCCATATGGATGAGGATGTGGGTGATGCCATTAAACCCTACATA
159   Y  I  G  N  R  L  F  C  F  R  N  E  D  V  D  F  Y  L  P  Q  L  L  N  M  Y  I  H  M  D  E  D  V  G  D  A  I  K  P  Y  I
595   GTCCACCGTTGTCGCCAGAGCATCAACTTTTCCCTCCAGTGTGCCCTGTTGCTTGGGGCCTACTCTTCAGACATGCACATTTCCACTCAACGACACTCCCGAGGGACCAAGTTACGGAAG
199   V  H  R  C  R  Q  S  I  N  F  S  L  Q  C  A  L  L  L  G  A  Y  S  S  D  M  H  I  S  T  Q  R  H  S  R  G  T  K  L  R  K
715   CTAATCCTCTCAGATGAGCTGAAGCCAGCTCACCGAAAGAGGGAGCTGCCAACTTTAAGCCCAGCCCCTGACACAGGACTGTCTCCCTCTAAAAGGACTCACCAGCGCTCTAAGTCAGAT
239   L  I  L  S  D  E  L  K  P  A  H  R  K  R  E  L  P  T  L  S  P  A  P  D  T  G  L  S  P  S  K  R  T  H  Q  R  S  K  S  D
835   GCCACGGCCAGCATAAGTCTCAGCAGCAACCTGAAACGAACAGCCAGCAACCCTAAAGTGGAGAATGAGGATGAGGAGCTCTCCTCCAGCACCGAGAGTATTGATAATTCATTCAGTTCC
279   A  T  A  S  I  S  L  S  S  N  L  K  R  T  A  S  N  P  K  V  E  N  E  D  E  E  L  S  S  S  T  E  S  I  D  N  S  F  S  S
955   CCTGTCCGGCTGGCCCCTGAGCGAGAATTCATCAAGTCCTTGATGGCAATCGGCAAACGGCTGGCCACGCTCCCCACTAAAGAGCAAAAAACACAAAGGCTGATCTCAGAACTTTCCCTG
319   P  V  R  L  A  P  E  R  E  F  I  K  S  L  M  A  I  G  K  R  L  A  T  L  P  T  K  E  Q  K  T  Q  R  L  I  S  E  L  S  L
1075  CTCAACCATAAGCTCCCTGCCCGAGTCTGGCTGCCCACAGCTGGCTTTGACCACCACGTGGTCCGTGTGCCCCACACACAAGCTGTTGTCCTCAATTCCAAGGATAAGGCTCCCTACCTG
359   L  N  H  K  L  P  A  R  V  W  L  P  T  A  G  F  D  H  H  V  V  R  V  P  H  T  Q  A  V  V  L  N  S  K  D  K  A  P  Y  L
1195  ATCTATGTGGAAGTTCTTGAATGTGAAAACTTCGACACAACTAATGTTCCTGCCCGGATTCCTGAAAACCGAATTCGGAGTACGAGGTCTGTGGAGAACCTGCCGGAATGTGGTATCACT
399   I  Y  V  E  V  L  E  C  E  N  F  D  T  T  N  V  P  A  R  I  P  E  N  R  I  R  S  T  R  S  V  E  N  L  P  E  C  G  I  T
1315  CACGAGCAGCGGGCTGGCAGCTTCAGCACTGTGCCCAATTATGACAATGACGATGAAGCCTGGTCGGTGGATGACATAGGCGAGCTGCAGGTGGAGCTCCCAGAAGTGCACACCAACAGC
439   H  E  Q  R  A  G  S  F  S  T  V  P  N  Y  D  N  D  D  E  A  W  S  V  D  D  I  G  E  L  Q  V  E  L  P  E  V  H  T  N  S
1435  TGTGACAACATCTCTCAGTTCTCGGTGGACAGCATCACCAGCCAGGAGAGCAAGGAGCCTGTGTTCATTGCGGCAGGGGACATCAGACGGCGCCTTTCAGAACAGCTGGCTCACACTCCC
479   C  D  N  I  S  Q  F  S  V  D  S  I  T  S  Q  E  S  K  E  P  V  F  I  A  A  G  D  I  R  R  R  L  S  E  Q  L  A  H  T  P
1555  ACAGCCTTCAAACGAGACCCTGAAGACCCTTCTGCAGTCGCCCTCAAAGAGCCCTGGCAGGAGAAAGTGCGGAGGATCAGAGAAGGTTCCCCGTATGGCCATCTTCCCAATTGGCGACTC
519   T  A  F  K  R  D  P  E  D  P  S  A  V  A  L  K  E  P  W  Q  E  K  V  R  R  I  R  E  G  S  P  Y  G  H  L  P  N  W  R  L
1675  CTTTCAGTCATTGTCAAGTGTGGAGATGACCTTCGCCAGGAGCTGCTGGCTTTCCAGGTGTTGAAACAACTGCAGTCCATTTGGGAACAGGAGCGAGTGCCTCTTTGGATCAAGCCATAT
559   L  S  V  I  V  K  C  G  D  D  L  R  Q  E  L  L  A  F  Q  V  L  K  Q  L  Q  S  I  W  E  Q  E  R  V  P  L  W  I  K  P  Y
1795  AAGATTCTTGTGATTTCAGCTGACAGTGGCATGATTGAACCAGTAGTCAACGCTGTGTCCATCCACCAGGTGAAGAAACAGTCACAGCTCTCCTTGCTCGATTACTTCCTGCAGGAGCAT
599   K  I  L  V  I  S  A  D  S  G  M  I  E  P  V  V  N  A  V  S  I  H  Q  V  K  K  Q  S  Q  L  S  L  L  D  Y  F  L  Q  E  H
1915  GGCAGTTACACCACTGAGGCATTCCTCAGTGCCCAGCGCAATTTTGTGCAAAGTTGTGCTGGCTACTGCTTGGTCTGCTACCTATTACAAGTCAAGGACAGGCACAATGGGAACATCCTT
639   G  S  Y  T  T  E  A  F  L  S  A  Q  R  N  F  V  Q  S  C  A  G  Y  C  L  V  C  Y  L  L  Q  V  K  D  R  H  N  G  N  I  L
2035  CTGGACGCAGAAGGTCACATCATCCACATCGACTTTGGCTTCATCCTTTCCAGCTCACCCCGAAACCTGGGCTTCGAGACGTCAGCCTTTAAGCTGACCACAGAATTTGTGGATGTAATG
679   L  D  A  E  G  H  I  I  H  I  D  F  G  F  I  L  S  S  S  P  R  N  L  G  F  E  T  S  A  F  K  L  T  T  E  F  V  D  V  M
2155  GGCGGCCTGAACGGTGATATGTTCAACTACTATAAGATGCTCATGCTGCAAGGGCTGATCGCTGCTCGCAAACACATGGACAAGGTGGTACAGATTGTGGAGATCATGCAGCAAGGTTCT
719   G  G  L  N  G  D  M  F  N  Y  Y  K  M  L  M  L  Q  G  L  I  A  A  R  K  H  M  D  K  V  V  Q  I  V  E  I  M  Q  Q  G  S
2275  CAGCTTCCTTGCTTCCATGGCTCCAGCACCATTCGCAACCTCAAAGAGAGGTTCCACATGAGCATGACTGAGGAGCAGCTGCAGCTGCTGGTGGAGCAGATGGTGGACGGCAGCATGAGG
759   Q  L  P  C  F  H  G  S  S  T  I  R  N  L  K  E  R  F  H  M  S  M  T  E  E  Q  L  Q  L  L  V  E  Q  M  V  D  G  S  M  R
2395  TCCATCACCACCAAACTCTACGATGGCTTCCAGTACCTCACCAATGGCATCATGTGACACCACTTTCAGGCCCAGAAGTGGTGGGATTGAAGGCACCCTCCTTAGGAGGACCCTTGCCTA
799   S  I  T  T  K  L  Y  D  G  F  Q  Y  L  T  N  G  I  M
2515  AGAAAACCTAAATCACAAAATCCCACCTACCTAACCACCTACCCAAGGGAAATGGAAGGCAAAAAACACAAAGGATCATGTGGTAACCATGAGAGCTGGCCAAAGGGTAGGAGAGCCAAC
2635  CATGGGGTCCAGACTTGCTGGGGCTTCCCCACCTCTTGCTGTGTCAGTATTGCCACCTGACAGATCCCAGGACTCACTGCCCTCCAGAGAACAGAGATGATGAACATGAGGAGGACTGGA
2755  ACCTTCTTCCCCTCGGGGATCTCATCTTTTACTATCCCTGGGTCCCAGCAAGATGGGAGGAGTGGGTTCTTGGTACTTAGGACTT
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Figure 1 Nucleotide sequence and predicted amino acid sequence of the 92 kDa PtdIns 4-kinase cDNA

In-frame stop codons in the 5′ untranslated region are underlined.

other PtdIns 4-kinases and had also significant similarity with the PtdIns 3-kinase family (Figure 2B). In fact, this molecule contained the lipid-kinase-unique domain and the presumed lipid–protein kinase catalytic domain, showing 43.3% identity with PtdInsK1. These two domains are conserved in all other lipid kinases including the 230 kDa PtdIns 4-kinase isolated in our previous study [11]. However, unlike the 230 kDa PtdIns 4-kinase, no pleckstrin-homology domains, SH3 domain or proline-rich regions were present in this novel molecule, similar to the findings for PtdInsK1. Furthermore, downstream, next to the lipid-kinase-unique domain, this molecule and PtdInsK1 had a region in common with each other that was not found in any other lipid kinases (Figures 2A and 2C).

In a Northern blot analysis of adult rat tissues using the probe corresponding to the nucleotide sequence −105 to 1843 of the present composite cDNA (Figure 3), a single hybridization band of 3.5 kb in size, which was consistent with almost the full length of the present cDNA, was intense in the brain, kidney, lung, small intestine, uterus and adrenal gland, whereas it was faintly visible in the liver, heart, skeletal muscle, thymus and testis. No distinct hybridization band was detected in the spleen.

The lipid kinase activity of this novel molecule was measured by transfection into COS-7 cells of the full-length cDNA including the non-coding regions. As control, an expression vector, pSRE [18], was expressed in COS-7 cells. The PtdIns kinase activity of the total lysate from COS-7 cells transfected with the present cDNA was 13.2 nmol/min per mg of protein and was 6-fold higher than that in the control lysate. A similarly high kinase activity was detected also in another lysate from COS-7 cells transfected with the epitope-tagged cDNA (results not shown). After immunoprecipitation of total lysates from COS-7 cells overexpressing the epitope-tagged cDNA, using the antibody to the FLAG tag, the kinase activity in the immunoprecipitated lysates was at least 100-fold higher than that of the control (results not shown). When PtdIns*P* products were analysed further to separate PtdIns4*P* and PtdIns3*P* according to Walsh et al. [23], the products obtained with the present molecule were almost exclusively PtdIns4*P* (Figure 4A). In the immunoblot using the antibody to the FLAG tag, a single immunoreactive band with a molecular mass of approx. 100 kDa was detected in total lysates from the transfected cells (Figure 4B).

After separation of the total lysate into soluble and particulate

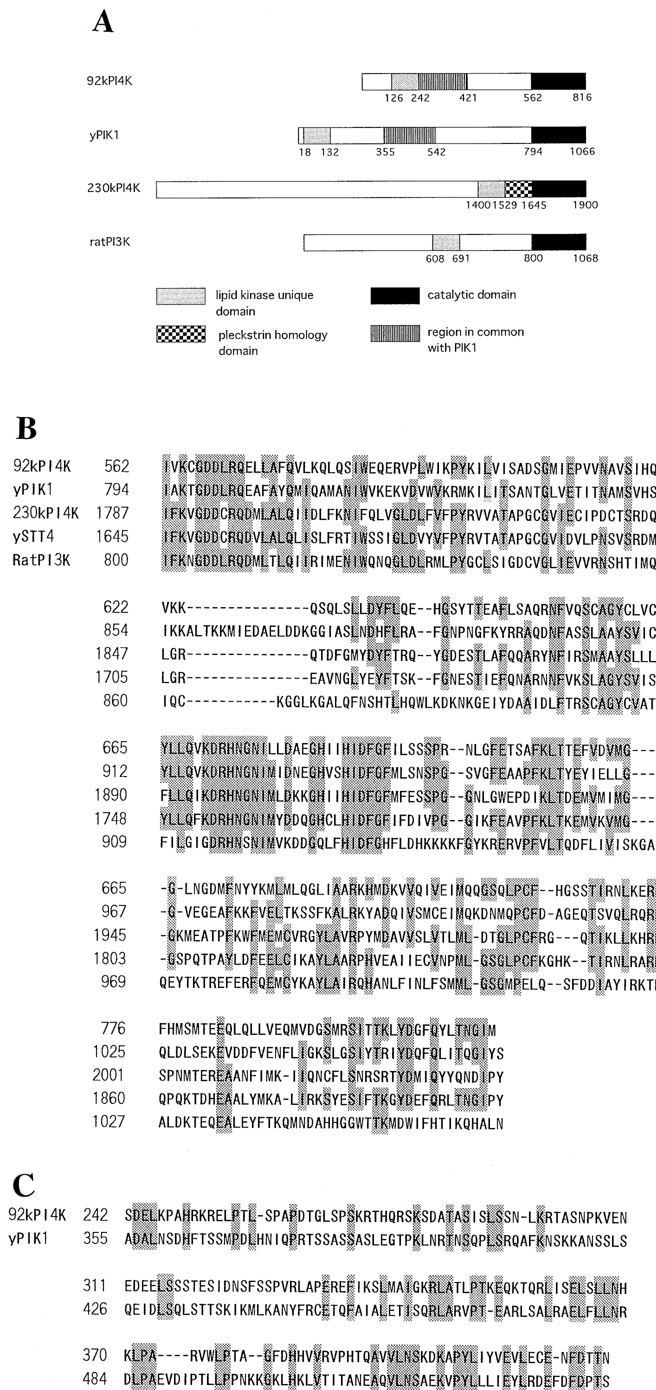

Figure 2 Comparison of the amino acid sequence of the 92 kDa PtdIns 4-kinase with other PtdIns kinases

(**A**) Schematic representation of 92 kDa PtdIns 4-kinase (92kPI4K), yeast PtdInsK1 (yPIK1), 230 kDa PtdIns 4-kinase (230kPI4K) and rat PtdIns 3-kinase (ratPI3K) p110α subunit. The lipid-kinase-unique domain, pleckstrin homology domain, catalytic domain and the conserved domain between the 92 kDa PtdIns 4-kinase and PtdInsK1 are shown. (**B**) Detailed comparison of the catalytic domains among the PtdIns kinases shown in (**A**). Conserved residues are shaded. (**C**) Comparison of amino acid sequence of the regions in common with the 92 kDa PtdIns4 kinase and PtdInsK1, downstream of the lipid-kinase-unique domain. Identical residues are shaded.

fractions, the kinase activity of the present molecule recovered from the soluble fraction was 22.5 nmol/min per mg of protein and was 17-fold higher than that of the particulate fraction. The

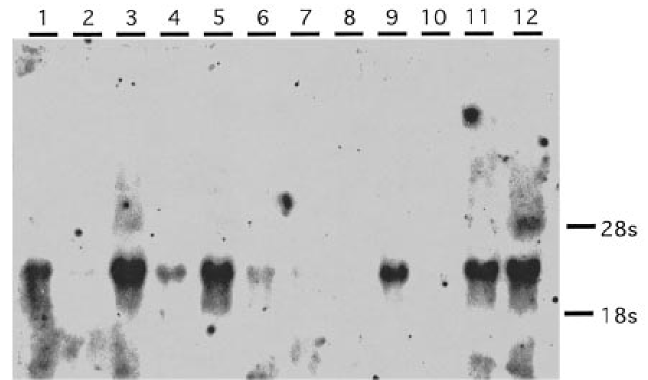

Figure 3 Northern blot analysis of the 92 kDa PtdIns 4-kinase mRNA in various rat tissues

Total RNAs (30 μg per lane) were electrophoresed and transferred to a nylon membrane. The blot was hybridized with a ^{32}P-labelled probe. Lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, lung; lane 6, skeletal muscle; lane 7, thymus; lane 8, spleen; lane 9, small intestine; lane 10, testis; lane 11, uterus; lane 12, adrenal gland. Size markers represent 28 S and 18 S rRNAs.

predominance of kinase activity in the soluble fraction was true also in the case of transfection with the epitope-tagged cDNA (results not shown). In the immunoblot with the antibody to the FLAG tag, a single immunoreactive band was predominant in the soluble fraction (Figure 4C). Equal amounts of lysates from COS-7 cells transfected with the FLAG-tagged cDNA for this molecule were immunoprecipitated with the anti-FLAG antibody and assayed with various concentrations of Triton X-100 and adenosine. The kinase activity of the molecule was stimulated markedly in the presence of increasing concentrations, from 0.1 to 0.4% (v/v), of Triton X-100, but was inhibited slightly as the concentration of Triton X-100 exceeded 0.8% (Figure 5A). The kinase activity of the molecule was relatively insensitive to increasing concentrations of adenosine, although the decrease in activity was slightly greater than that found for our previous 230 kDa PtdIns 4-kinase (Figure 5B). Furthermore, the activity of the 92 kDa molecule, as well as of the 230 kDa molecule, was almost completely inhibited by 10 μM wortmannnin (Figure 5C). The kinase activity was not changed in the presence or absence of calcium ions (results not shown).

When the full-length epitope-tagged molecule was transiently expressed in COS-7 cells, cells immunoreactive for FLAG accounted for approx. 2–5% of the total cell population and appeared to be randomly dispersed in each culture dish. The immunoreactive products showed a strong tendency to be aggregated in one pole of the cytoplasmic region, juxtaposed to the nuclei, although they were faintly dispersed as fine dots and granules throughout the cytoplasm (Figure 6a). No significant immunoreactivity was discerned in any other region, such as the cell margins or nuclei. Using immuno-electron microscopy, the immunoreaction products were localized densely in the narrow cytoplasmic space among many Golgi vesicles and vacuoles (Figure 6b). No immunoreactivity was detected in any cells when the transfection was made using cDNA without the tag (results not shown).

By *in situ* hybridization histochemistry of entire fetuses on prenatal days 15 and 18, positive expression signals for this novel molecule were detected ubiquitously in most tissues including the brain (Figures 7a and 7b). The expression was detected widely throughout the mantle zone of the fore-, mid- and hind-brain. In

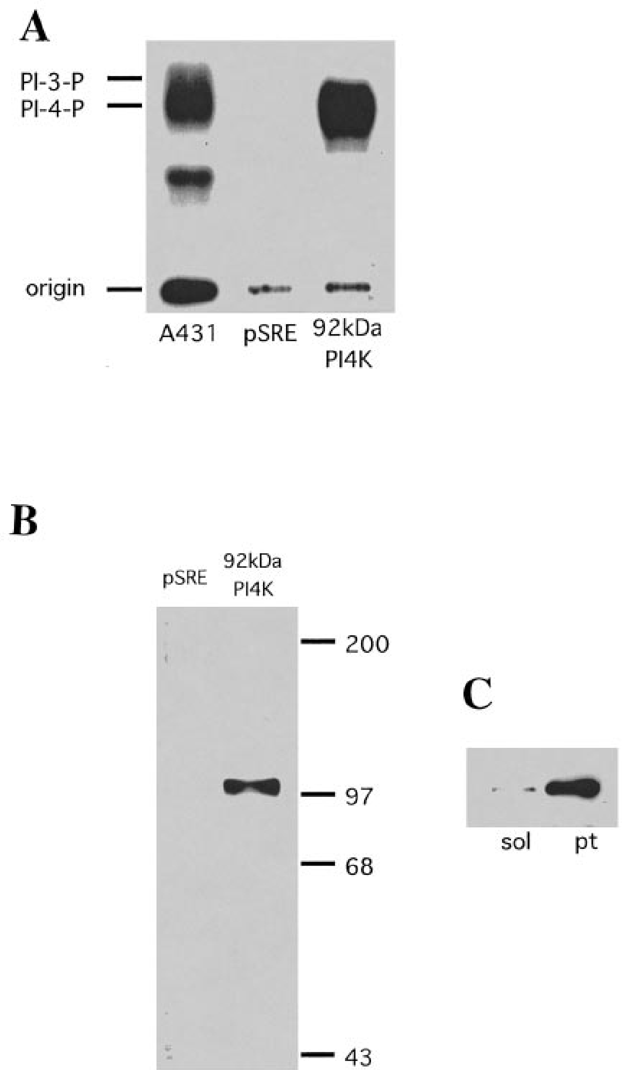

Figure 4 Thin layer chromatography and immunoblot of PtdIns*P* products

(**A**) Equal quantities of the lysates were immunoprecipitated with anti-FLAG antibody and assayed. PtdIns4*P* (PI-4-P) and PtdIns3*P* (PI-3-P) were separated as described in the Experimental section. PI4K, PtdIns 4-kinase. The position of PtdIns3*P* was determined by using A431 cell lysate as a standard. (**B**) Immunoblot of cells transfected with the epitope-tagged new cDNA using anti-FLAG antibody. Numbers are molecular mass in kDa. pSRE, control. (**C**) Immunoblot of soluble (sol) and particulate (pt) fractions of the transfected cells isolated in (**B**) using anti-FLAG antibody.

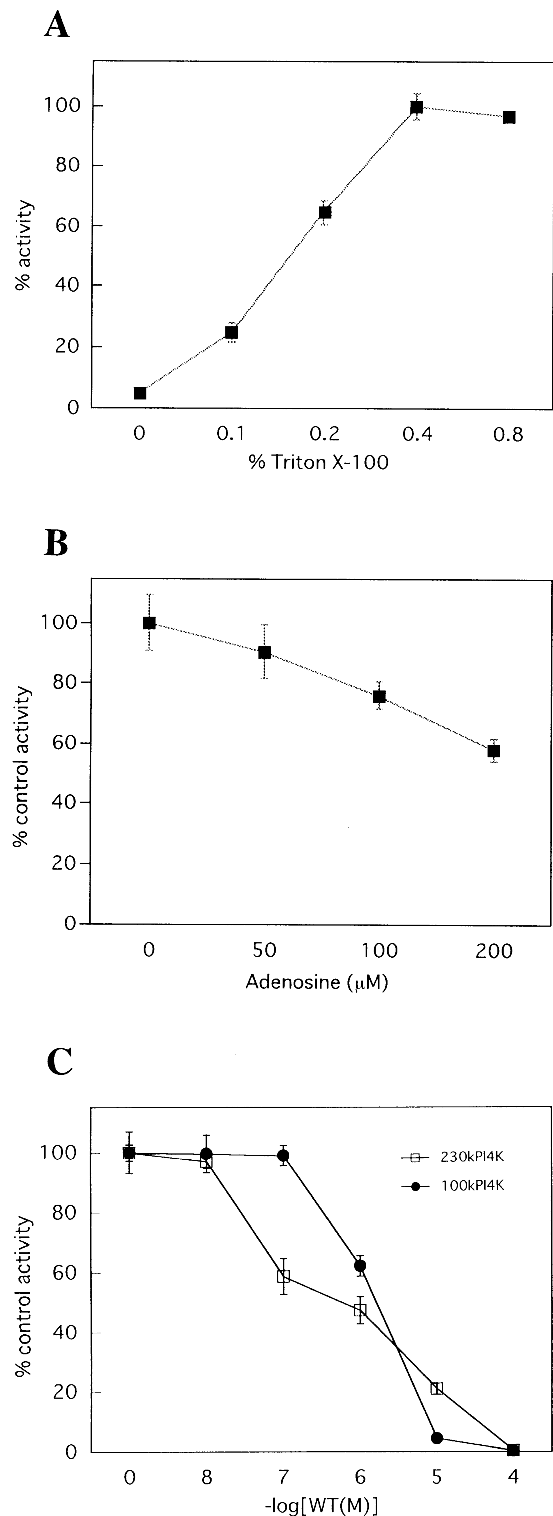

Figure 5 Effect of Triton X-100, adenosine and wortmannin on the activity of 92 kDa PtdIns 4-kinase

COS-7 cells were transfected with 92 kDa PtdIns 4-kinase plus FLAG, the lysates were precipitated with anti-FLAG antibody and assayed for kinase activity in the presence of various concentrations of (**A**) Triton X-100, (**B**) adenosine and (**C**) wortmannin (WT). Values shown are means ± S.D. ($n = 6$). PI4K, PtdIns 4-kinase.

the cerebrum, positive expression was observed in the cortical plate and ventricular zone and no expression was seen in the intermediate zone (Figure 7b). On postnatal day 49, weak expression of this molecule was detected more or less throughout the grey matter of the entire brain, and expression was evident in the hippocampal pyramidal cells, dentate granule cells and the cerebellar granule cells (Figure 7c). These findings indicate expression of this molecule in almost all neurons, but not in the glia. When the expression signals in sections from fetal and adult brains were compared by simultaneous exposure of both sections to the same Hyperfilm-β max, the expression appeared, in general, to be much greater in the fetal brain than in the adult brain. When fetal and adult brain sections were hybridized with the control probe, a cDNA fragment of about 800 bp (*Pst*I–*Hin*dIII) from the pBR322 plasmid vector without any insert cDNA, no significant hybridization signals were detected (results not shown).

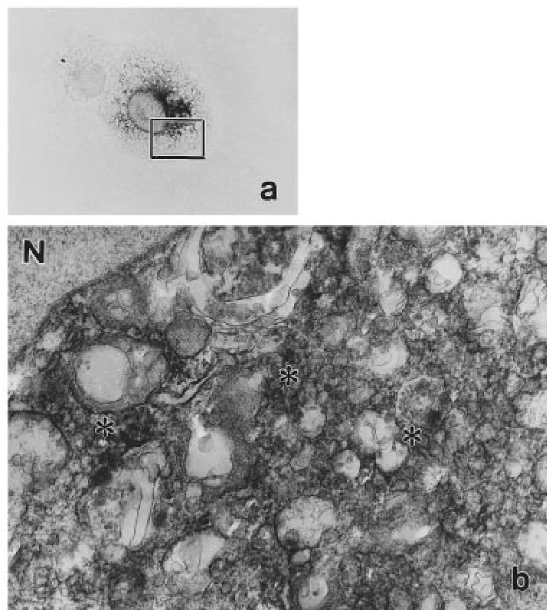

Figure 6 Immuno-light (a) and -electron (b) micrographs of COS-7 cells transfected with 92 kDa PtdIns 4-kinase and detected with anti-FLAG antibody

(**b**) Immunoelectron micrograph of the juxtanuclear cytoplasmic region [corresponding to the region marked by a rectangle in (**a**)] of a COS-7 cell transfected with the cDNA for 92 kDa PtdIns 4-kinase. Immunoreaction products (*) are dense in the cytoplasmic spaces among many Golgi vesicles and vacuoles. N, nucleus. Magnification in (**b**) $\times 15000$.

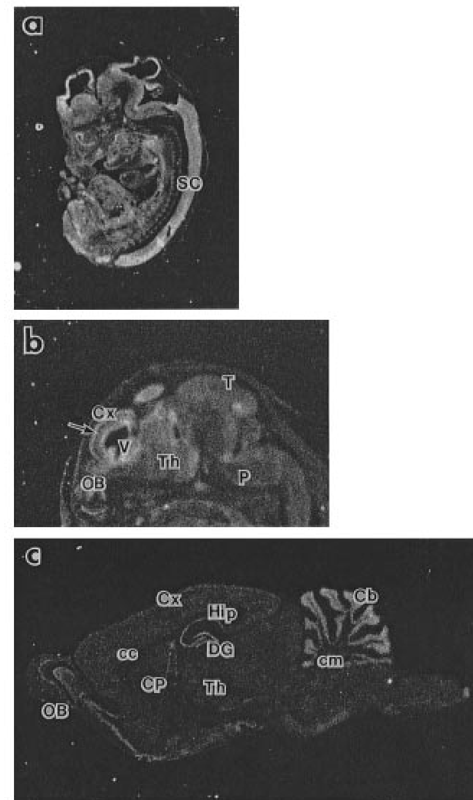

Figure 7 *In situ* hybridization of 92 kDa PtdIns 4-kinase mRNA in fetal and adult rat brain

(**a**) Whole body on prenatal day 15; (**b**) brain on prenatal day 18; (**c**) brain on postnatal day 49. Autoradiographic image of parasagittal section. Note positive expression signals throughout the mantle zone of the entire brain and spinal cord (SC) on prenatal days 15 and 18. The ventricular zone (V) also expresses mRNA positively and no significant expression is seen in the intermediate zone (arrow). Also note the positive expression throughout the grey matter of the entire brain, with varying intensity in different loci on postnatal day 49. No expression signals were seen in the white matter, such as the corpus callosum (cc) and the cerebellar medulla (cm). Abbreviations: CP, caudate putamen; Cb, cerebellar cortex; Cx, cerebral cortex; DG, dentate granular cell layer; Hip, hippocampal pyramidal cell layer; OB, olfactory bulb; P, pons; T, tectum; Th, thalamus.

DISCUSSION

It is well known that PtdIns 4-kinase is predominantly a membrane-bound protein [26–30] although the activity of PtdIns 4-kinase has recently been reported to occur in the soluble fraction of bovine adrenal cortical cells [31]. The present study clarifies for the first time, by demonstration of its molecular structure and characteristics, the presence of a mammalian soluble PtdIns 4-kinase as a distinct entity. Although the conventional criteria for the differentiation of PtdIns 4-kinase into type II and type III, based on the sensitivity to non-ionic detergents and adenosine, was originally applied to the membrane-associated activity [32], the sensitivity of the cytosolic PtdIns 4-kinase indicates that the present molecule does not represent either a conventional type II or type III PtdIns 4-kinase. Sensitivity of the present PtdIns 4-kinase molecule to inhibition by wortmannin was also noted in this study. The sensitivity to inhibition was approx. 2–3 orders of magnitude less than that of PtdIns 3-kinases [33], which was also the case for our previous 230 kDa PtdIns 4-kinase molecule, where activity was associated with the particulate fraction [11]. It has been reported recently that the kinase activity of the soluble PtdIns 4-kinase from bovine adrenal tissue is also wortmannin-sensitive [31]. The enzyme constituted a small proportion of the kinase activity in the adrenal cells and the inhibition by wortmannin was of the same order as that observed for the present PtdIns 4-kinase. The bovine adrenal enzyme had an apparent molecular mass of 125 kDa [31], which is roughly comparable with the molecular mass of 92 kDa calculated for the present molecule and that of 100 kDa estimated from SDS/PAGE of the epitope-tagged translation molecule.

The molecular structure of this novel rat PtdIns 4-kinase closely resembles that of a previously cloned yeast PtdIns 4-kinase, PtdInsK1. There has been dissension regarding the subcellular localization of PtdInsK1 activity, the cytosolic versus nuclear-associated [5,6]. Because no immunocytochemical examination was performed for PtdInsK1, the apparent difference could be attributed to the different methods of cellular fractionation employed by the two groups. In this regard, the immunocytochemical findings of the present study should be noted. The characteristic features common to this molecule and to the membrane-bound 230 kDa PtdIns 4-kinase recently cloned by us [11] were the cytoplasmic localization of the immunoreactivity to the epitope-tagged molecule for the novel PtdIns 4-kinase and the tendency of the immunoreactive products of the epitope-tagged molecule to aggregate in one cytoplasmic pole juxtaposed to the nucleus, in what was presumed to be the Golgi apparatus of COS-7 cells. The close spatial association of the PtdIns 4-kinase molecules with the intracellular membranes of vesicles and vacuoles was clearly shown in mammalian cells by our present and previous immuno-electron microscopy. However, no significant immunoreactivity for either molecule was

detected in the cell surface membrane. These features suggest that the two rat PtdIns 4-kinase molecules are more actively implicated in vesicular trafficking rather than in ligand-stimulated, receptor-mediated signal transduction via PtdIns turnover in the plasma membrane, as suggested by Liscovitch et al. [34]. The involvement of PtdIns 4-kinase, as well as PtdIns 3-kinase, in vesicular trafficking is already known in plants. It has been shown that the treatment with wortmannin inhibits the activity of the two kinases and the synthesis of phospholipids, and that the mis-sorting of vacuolar proteins caused by wortmannin shows a dose dependency that is similar to the dose dependency for the inhibition of synthesis of PtdIns4*P* in tobacco cells [35].

With regard to the vesicular traffic in mammalian cells *in vitro*, recent studies have shown that three rat brain cytosolic proteins termed PEP (priming in exocytosis protein) 1, 2 and 3 are required for ATP-dependent steps in Ca^{2+}-activated secretion from PC12 cells, and PEPs 1, 2 and 3 have been shown to have molecular masses of 500, 120 and 21 kDa respectively. PEP 1 has further been identified as PtdIns4*P* 5-kinase and PEP 3 as PtdIns-transfer protein [12–14]. Taking into account that PtdIns-transfer protein (PEP 3) and PtdIns4*P* 5-kinase (PEP 1) are components of a sequential pathway of inositol phospholipid synthesis, the present soluble PtdIns 4-kinase of approx. 100 kDa may catalyse the second step in this pathway. This remains to be verified.

The functional implications for this kinase in mammalian cells *in situ* may be deduced from the present hybridization findings. The expression of this novel kinase molecule in almost all neurons, but not in glia, throughout the fetal brain, and the marked decrease in the neuronal expression in the adult brain, suggest that this PtdIns 4-kinase may be intimately involved in neuronal differentiation and maturation. Neuronal differentiation includes neurite prolongation and bifurcation, synapse formation and establishment of the axoplasmic flow, all of which require active intracellular vesicle traffic. It is thus inferred that PtdIns 4-kinase and its product, PtdIns4*P*, are closely involved in the regulation of intracellular membrane transport and sorting in relation to neuronal differentiation in the brain.

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