A novel neuronal-specific splice variant of Type I phosphatidylinositol 4-phosphate 5-kinase isoform γ

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Type I PIPkins (phosphatidylinositol 4-phosphate 5-kinases) are the enzymes that catalyse the major cellular route of synthesis of PtdIns(4,5)P_2, and three isoforms (α, β and γ) with several splice variants have been found to date. In the present paper, we describe the discovery of a novel splice variant of the γ isoform, which we call PIPkin Iγc, and which is characterized by the inclusion of a 26-amino-acid insert near the C-terminus. Its transcript appears to be selectively expressed in brain, where it locates in the neurons of restricted regions, such as cerebellum, hippocampus, cortex and olfactory bulb, as indicated by *in situ* hybridization studies. Overexpression of two different catalytically inactive constructs of PIPkin Iγc in rat cerebellar granule cells causes a progressive loss of their neuronal processes, whereas equivalent kinase-dead versions of PIPkin Iγa did not induce any such effect, suggesting the possible existence of a specific PtdIns(4,5)P_2 pool synthesized by PIPkin Iγc, which is involved in the maintenance of some neuronal cellular processes.

Key words: cerebellar granule cell, neuronal process, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], splice variant, Type I phosphatidylinositol 4-phosphate 5-kinase γ.

INTRODUCTION

PtdIns(4,5)P_2 is a remarkably versatile signal mediator which regulates multiple intracellular processes. Its functions take two basic forms: first, it is the precursor for a number of second messengers such as InsP_3, diacylglycerol and PtdIns(3,4,5)P_3 [1,2]. Secondly, PtdIns(4,5)P_2 itself regulates many cellular events, such as the control of ion channels and nuclear events, remodelling of the actin cytoskeleton, cell motility, process formation, and membrane trafficking [3,4]. Most of these processes are mediated through a direct binding with an extensive repertoire of proteins that contain PtdIns(4,5)P_2-binding domains [5]. As a result of the contact with PtdIns(4,5)P_2, the target proteins change their intracellular location and/or enzymic activity, and thus their functions are modulated.

As shown by imaging studies using a GFP (green fluorescent protein)-conjugated PtdIns(4,5)P_2-binding domain [6], PtdIns(4,5)P_2 is located mostly at the plasma membrane, but extensive biochemical and immunocytochemical evidence also indicates the presence of PtdIns(4,5)P_2 in the Golgi apparatus [7–9], the endoplasmic reticulum [7,10] and the nucleus [7,11]. The presence of both a series of PtdIns(4,5)P_2-binding proteins that perform distinct tasks and several PtdIns(4,5)P_2 pools suggests the need for multiple mechanisms for the spatial and temporal regulation of PtdIns(4,5)P_2 synthesis.

PtdIns(4,5)P_2 is synthesized via two independent reactions [12]. It is the product of Type I PIPkins (phosphatidylinositol 4-phosphate 5-kinases) which phosphorylate PtdIns4*P* on the 5-position of the inositol ring, and it is also produced by Type II PIPkins, which catalyse the phosphorylation of PtdIns5*P* on the 4-position of the inositol ring. Radiolabelling data suggest that the major route of synthesis in intact cells [13] or isolated nuclei [14] is by the Type I PIPkin route.

Little detail is known about the regulation of PtdIns(4,5)P_2 synthesis. One way to control its location and turnover in a membrane would be to regulate the targeting of a synthesizing enzyme at a subcellular site. The existence of three isoforms (α, β and γ) and of alternative splice variants [15–17] prompts the question of whether the differences among them are required to perform specific tasks at specific cellular locations and, if so, which ones. Recent experiments have revealed important findings, although a comprehensive picture is not yet completely clear. A few studies show differences in localizations that reflect a distinct function among isoforms. For example, an extracellular stimulus such as PDGF (platelet-derived growth factor) induces membrane ruffling in MG-63 osteosarcoma cells and a concomitant translocation to the ruffles of endogenous human PIPkin Iα from cytosol, whereas the location of human Iβ in perinuclear vesicles is not altered [18]. The process of ruffle formation depends on PtdIns(4,5)P_2, which, the authors propose, is generated exclusively by PIPkin Iα [18]. Also, constitutive endocytosis in CV-1 and HeLa cells requires mouse PIPkin Iβ specifically [19].

Type Iγ PIPkin is particularly highly expressed in neurons, and has been shown to have an important role in synaptic function [20]. A putative role of a PIPkin I in neurosecretion [21] has been recently confirmed by the discovery that PIPkin Iγ generates a PtdIns(4,5)P_2 pool at the synapse [20]. This pool is implicated in clathrin-mediated endocytosis. Importantly, there are differences between the two known splice variants of this enzyme. Thus, by interacting with talin, an integrin-binding protein, the splice variant Iγ 661 (called PIPkin Iγa in the present paper) targets to focal adhesions and regulates the focal assembly through PtdIns(4,5)P_2 production [22,23]. The splice variant Iγ 635 (PIPkin Iγb) also locates at the plasma membrane, but not in these specialized domains, and, consequently, its activity is not required for their regulation [22,23].

Splice variation is becoming increasingly central to our understanding of the complexities of cell function, especially in the context of the emerging realization that mammals may have fewer genes than had originally been supposed. In the present paper, we report the discovery of a novel splice variant of PIPkin Iγ, which we call PIPkin Iγc. We demonstrate its apparent specific

Abbreviations used: GFP, green fluorescent protein; PIPkin, phosphatidylinositol 4-phosphate 5-kinase; RT, reverse transcriptase.

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rPIPKin Iγa    DTSEQPRYRRRTQSSGQDGRPQEEPHAEDLQKITVQVEPVCGVG-VVPKEEGAGVEVPPC 599
rPIPKin Iγb    DTSEQPRYRRRTQSSGQDGRPQEEPHAEDLQKITVQVEPVCGVG-VVPKEEGAGVEVPPC 599
mPIPKin Iγc    DTSEQPRYRRRTQSSGQDGRPQEELHAEDLQKITVQVEPVCGVGVVVPKEQGAGVEVPPS 600
mPIPKin Iγa    DTSEQPRYRRRTQSSGQDGRPQEELHAEDLQKITVQVEPVCGVGVVVPKEQGAGVEVPPS 600
                                    *                   *     *        *

rPIPKin Iγa    GASAAASVEIDAASQASEPASQASDEEDAPSTDIYF---------------------- 636
rPIPKin Iγb    GASAAASVEIDAASQASEPASQASDEEDAPSTDIYF---------------------- 635
mPIPKin Iγc    GASAAATVEVDAASQASEPASQASDEEDAPSTDIYFFAHGRYWLFSPRRRRLRAVTPSHT 660
mPIPKin Iγa    GASAAATVEVDAASQASEPASQASDEEDAPSTDIYF---------------------- 636
                     *  *                        ************************

rPIPKin Iγa    --PTDERSWVYSPLHYSARPASDGESDT 661
rPIPKin Iγb    ---------------------------
mPIPKin Iγc    GAPTDGRSWVYSPLHYSARPASDGESDT 688
mPIPKin Iγa    --PTDGRSWVYSPLHYSARPASDGESDT 662
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Figure 1 Comparison of the splice variants of the mouse and rat type I PIPkin Iγ isoform

The alignment of the C-terminal amino acid sequences of the splice variants is shown. Dashes indicate that the amino acid is not present. Asterisks indicate residues that are not common to all splice variants of PIPkin isoform Iγ. mPIPkin, mouse PIPkin; rPIPkin, rat PIPkin.

expression in brain, with particularly high levels of expression in the cortex, hippocampus, cerebellum and olfactory bulb of mouse brain. We also present evidence that it may play a specific role in the maintenance of neuronal processes.

MATERIALS AND METHODS

Molecular isolation of PIPkin Iγ cDNA

Full-length cDNA of rat PIPkin Iγ was amplified from a cDNA library derived from rat hippocampal cultured neurons [titre 9×10^9 plaque-forming units/ml; a gift from Dr H. Bading, Laboratory of Molecular Biology (LMB), Cambridge, U.K.] with 5′-CCGGAATTCATGGAGCTAGAGGTGCCGGAC-3′ [sense primer containing an *Eco*RI site (underlined) and corresponding to nucleotides 1–21 of mouse PIPkin Iγ] and with 5′-CCCGCTCGAGTTATGTGTCGCTCTCGCCGT [antisense primer containing a *Xho*I site (underlined) and corresponding to nucleotides 1967–1986 of the mouse splice variant a of PIPkin Iγ]. PCR was performed in a 50 μl volume containing 1 μl of cDNA library, 4 mM dNTPs, 4 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) and 25 pmol of sense and antisense primers in the buffer (pH 8.8) supplied with the enzyme for 35 cycles (1 min at 96 °C, 1 min at 55 °C and 3 min at 72 °C), preceded by a hot start (5 min at 96 °C, 1 min at 55 °C and 3 min at 72 °C).

The amplified cDNA was inserted into the bacterial expression vector, pGEX-4T2 (Amersham Biosciences, Little Chalfont, Bucks., U.K.) and was then subcloned into a mammalian expression vector, pCMV-2B (Stratagene, Cambridge, U.K.) containing a FLAG epitope tag. Recombinant plasmid DNAs were purified with a Miniprep or an endotoxin-free Maxiprep plasmid isolation kit (Qiagen, Crawley, U.K.).

Site-directed mutagenesis

Asp^{316} and Lys^{188} of the PIPkin Iγa and Iγc were mutated into lysine and alanine respectively by using the QuickChange™ Site-Directed Mutagenesis kit (Stratagene). Mutations were generated in the constructs cloned into the pCMV-2B vector.

RT (reverse transcriptase)-PCR

Total RNA (0.5 μg) derived from various tissues of Swiss Webster mice (Ambion, Austin, TX, U.S.A.) were reverse transcribed with the sense primer 5′-AAGGAGGAGGGTGCAGGAGTGGAGGTC-3′ and the antisense primer 5′-TCCGTCAGTGGGGAGGGAGAGAACAAG-3′ using the Access RT-PCR System (Promega, Madison, WI, U.S.A.). Approx. 20 % of each total reaction mixture was analysed by agarose (1.7 %) gel electrophoresis.

Probes and probe labelling

Oligonucleotide DNA probes complementary to bases 1909–1945 and bases 1950–1986 of the mouse PIPkin Iγc (Figure 1) were synthesized: antisense 1, 5′-ACGGGGAGAGAAAAGCCAGTATCTCCCGTGGGCGAA-3′; antisense 2, 5′-AGTGCCTGTGTGGTTTGGTGTCACGGCCCGCAGTTG-3′.

The probes (concentration 0.1 pmol/μl) were 3′-tail-labelled with [^{35}S]dATP (NEN, Hounslow, U.K.) by a 1 h incubation at 37 °C with 50 units of terminal deoxynucleotide transferase (Roche, Mannheim, Germany) and 1 pmol/μl [^{35}S]dATP in 1× tailing buffer containing 3.3 mM $CoCl_2$. The labelled probes were purified using a QIAquick Nucleotide Removal kit (Qiagen).

In situ hybridization

Mouse tissue sections were taken directly from the −80 °C freezer and warmed to room temperature (20 °C) before being immersion-fixed for 10 min in 4 % (w/v) formaldehyde in 0.1 M phosphate buffer pH 7.4. Sections were then rinsed twice briefly in 0.1 M PBS and were then dehydrated through graded alcohols. Hybridization was carried out at 42 °C overnight with labelled probe (specific radioactivity $> 1 \times 10^7$ d.p.m./μg) diluted in hybridization buffer containing 4× SSC (standard saline citrate; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate), 50 % formamide, salmon testis DNA (200 μg/ml), 10 % dextran sulphate, 1× Denhardt's solution (0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA) and 100 mM dithiothreitol. Following hybridization, the sections were washed in 1× SSC containing 0.2 % sodium thiosulphate first for 1 h at room temperature and then three times for 30 min each at 55 °C. Excess salt was removed by a brief rinse in 1× SSC and 0.1× SSC/0.2 % sodium thiosulphate, dehydrated in graded alcohols and air-dried before exposure to Film Biomax MR (Kodak) for 5 weeks to produce film autoradiographs. Subsequently sections were coated with K5 nuclear emulsion (Ilford, Mobberley, Cheshire, U.K.) and exposed in the dark for 12 weeks before development.

Sections were counterstained with Methylene Blue and mounted on coverslips with Ralmount (BDH, Poole, Dorset, U.K.).

Cell culture and transfection

Primary cerebellar granule cells were prepared by trypsin digestion from postnatal day 6–8 rat cerebella, grown for 2 days in Neurobasal Medium with B27 supplement (Life Technologies, Paisley, U.K.) and 10 % (v/v) horse serum and thereafter in serum-free medium for up to 5 weeks. The KCl concentration in the medium was 20 mM and cytosine arabinoside (Sigma, St. Louis, MO, U.S.A.) was present between 24 and 72 h after plating. Transfection of neurons between 1 and 4 weeks was achieved with a calcium-phosphate technique modified from [24]. Cells were incubated for 2 h with calcium phosphate/DNA precipitate in Neurobasal Medium with 10 mM $MgCl_2$/10 mM Hepes (pH 7.2), briefly washed and left for 30 min in the same medium then returned to the growth medium.

Immunofluorescence

Primary cerebellar granule cells seeded on glass coverslips pre-coated with poly-lysine and transfected with FLAG constructs were washed once with PBS/0.9 mM $CaCl_2$/0.52 mM $MgCl_2$/ 0.16 mM $MgSO_4$ and fixed with 4 % (w/v) formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min. The cells were then permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 5 min, washed with PBS and blocked with 2 % fish gelatin (Sigma) 2 % goat serum (Sigma) for 1 h. The cells were incubated for 1 h with the monoclonal M2 anti-FLAG antibody (4 μg/ml) (Stratagene), washed in PBS and re-blocked with 2 % goat serum. Detection was performed by incubation for 30 min with anti-mouse Cy3- or TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.). All steps were performed at room temperature. Stained coverslips were mounted in Prolong anti-fade reagent (Molecular Probes, Leiden, The Netherlands). Images were acquired with a Zeiss confocal microscope.

Lipid kinase assay

The lipid kinase assay was performed on immunoprecipitates obtained from COS-7 cells transiently transfected with either wild-type or mutant FLAG–PIPkins. Cells were maintained in 5 % CO_2 in Dulbecco's modified Eagle's medium plus 10 % (v/v) foetal calf serum, and seeded on to 10-cm-diameter dishes approx. 24 h before transfection. They were transfected by the calcium-phosphate technique by exposing them to the DNA precipitate overnight with a 2 min glycerol shock at the end. The lysates were made after 24 h by freezing and thawing the cells scraped from the dishes in 1 % (v/v) Triton X-100, 5 mM EDTA, 5 mM EGTA plus 50 μg/ml leupeptin, 1 mM PMSF in PBS. The homogenates were then centrifuged at 14000 ***g*** for 10 min at 4 °C. The supernatants were stored at − 80 °C.

The FLAG-tagged wild-type and mutant Iγ were immunoprecipitated by incubation of the cell lysates with the monoclonal M2 anti-FLAG antibody (4 μg/ml) for 1 h at 4 °C and subsequent incubation with Protein G–Sepharose (Amersham Biosciences) beads for 2–3 h at 4 °C. The immunoprecipitates were first washed in TBS and then in the lipid kinase assay buffer at 4 °C. The lipid kinase activity was assayed in the final volume of 100 μl at 30 °C in 80 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 50 mM Tris/HCl, pH 7.4, by using 10 μM ATP (containing [γ-^{32}P]ATP 50–100 μCi/ml) and 10 μM PtdIns4*P* (Echelon

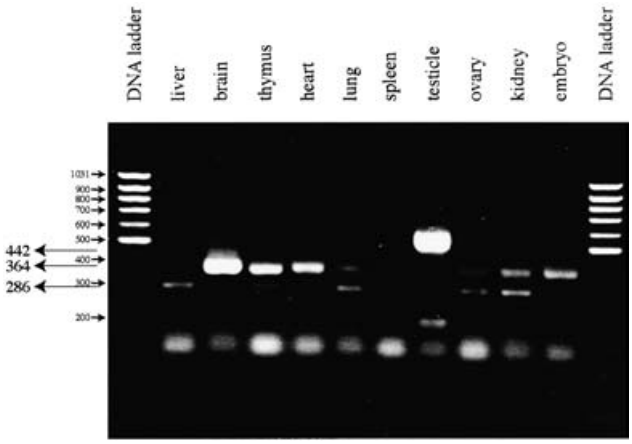

Figure 2 Detection of mRNA of Iγa, b and c in various mouse tissues by RT-PCR

Total mRNA from different mouse tissues was subjected to RT-PCR. This analysis was performed by using primers that amplify the bands of 442, 364 and 286 bp corresponding to splices c, a and b respectively (see the Materials and methods section for details). Sequence analysis of the broad band of around 530 bp identified in testicles has established that it does not correspond to any PIPkin I. Sizes (in bp) are indicated on the left-hand side.

Research Laboratories, Salt Lake City, UT, U.S.A.) as substrates. The assay mixture was always supplemented with 10 μM phosphatidic acid. The reaction was stopped by adding 500 μl of chloroform/ methanol (1:1, v/v) and subsequently 125 μl of 2.4 M HCl was added. After removal of the upper phase, the lower phase was washed once with 490 μl of chloroform/methanol/1 M HCl (2:48:47, by vol.) and then dried under nitrogen. Lipids were separated by TLC on silica-gel plates (Merck, Darmstadt, Germany) impregnated with 1 % potassium oxalate and containing 4 mM EGTA, and pre-conditioned at 110 °C for 1 h. The lipids extracted from each sample were dissolved in chloroform, loaded on to plates and separated in methanol/chloroform/water/ saturated ammonium hydroxide solution (40:28:10:6, by vol.). The plates were dried and exposed to X-ray film (Kodak) at − 80 °C. PtdIns(4,5)P_2 was identified by comparison with a radioactive standard run on the same plates.

The relative activity of the kinase-dead mutants compared with the wild-type PIPkins was measured on immunoprecipitates containing similar amounts of recombinant enzyme. This was carefully determined by analysis of the immunoprecipitates with SDS/10 % (w/v) PAGE and Western blotting on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blots were blocked with 5 % (w/v) non-fat dried milk and 0.05 % (v/v) Tween 20 in TBS and then probed with monoclonal M2 anti-FLAG antibody. Horseradish-peroxidase-linked anti-mouse secondary antibodies (Amersham Biosciences) were visualized by SuperSignal® West Dura (Pierce, Tattenhall, Cheshire, U.K.). The linearity of the lipid kinase assay for each recombinant PIPkin was also determined.

Quantification of neuronal processes

Images of fields of neurons were examined, and, for each cell in the field, the first, second and third branch points of its processes were counted manually to give a measure of process complexity. To give an estimate of cell survival, the number of transfected cell bodies was counted in a larger number of randomly selected fields of view.

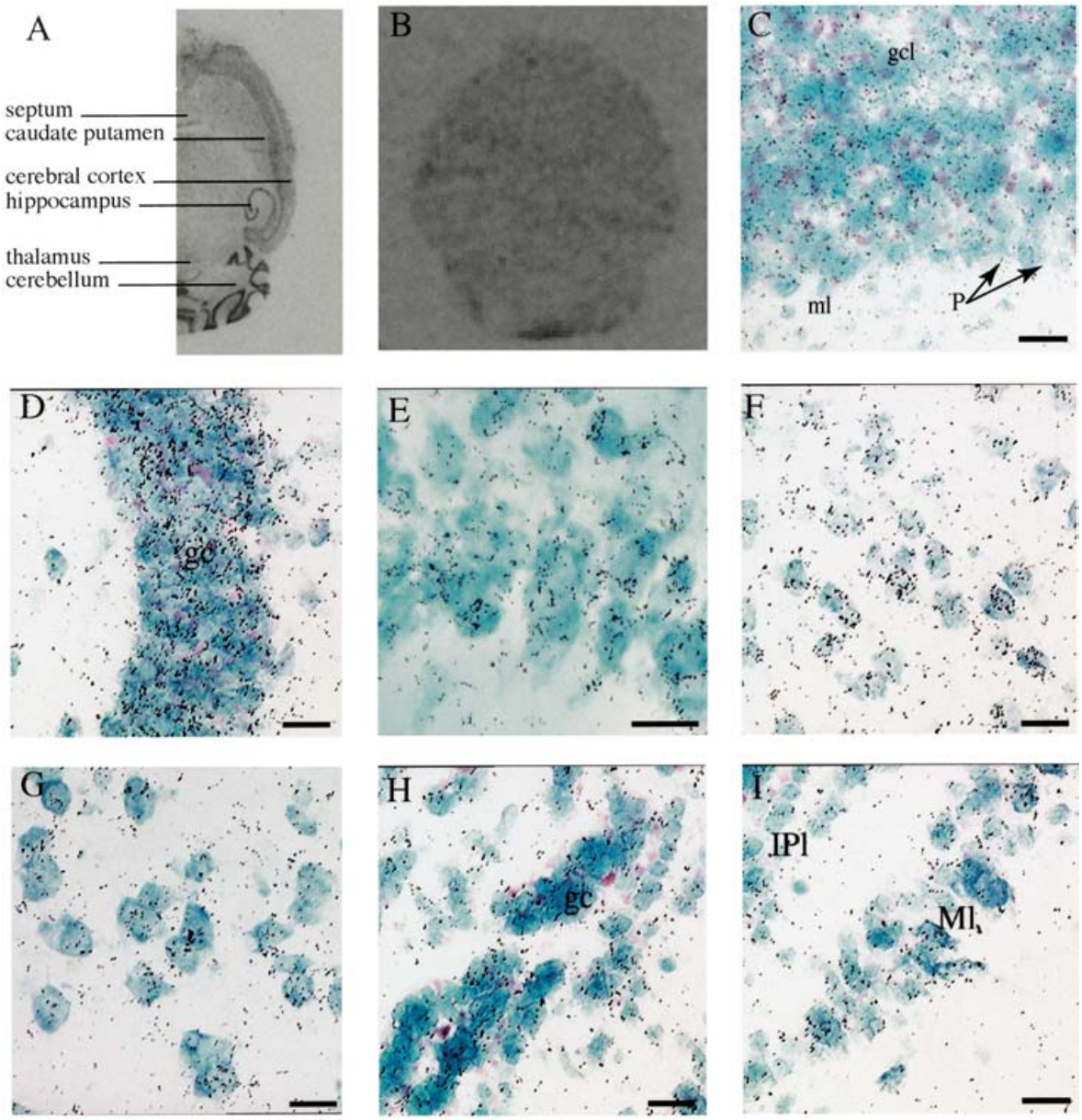

Figure 3 ***In situ*** **hybridization for PIPkin Iγ c in the adult mouse brain**

Autoradiographs of parasagittal section of the adult mouse brain hybridized with ^{35}S-labelled antisense oligonucleotides specific for Iγc mRNA. (**A**) Lower power brain section. Note the strong Iγc mRNA expression in the cerebral cortex, hippocampus and cerebellum, with some weaker expression in the septum, the caudate putamen and the thalamus. (**B**) Control hybridization with excess unlabelled oligonucleotide showed only weak background labelling. Higher magnification of the brain sections shows labelling of: (**C**) cerebellar granule cells (gcl, granule cell layer), low signal is detected in the molecular layer (ml) and in Purkinje cells (P); (**D**) granule cells (gc) of the dentate gyrus; (**E**) CA1 pyramidal cells of hippocampus; (**F**) pyramidal cells of cerebral cortical layers II–III; (**G**) pyramidal-like cells in layers V–VI of the cerebral cortex; (**H**) granule cells (gc) of the olfactory bulb; (**I**) inner plexiform layer (IPl) and mitral cell layer (Ml) of the olfactory bulb. Scale bars, 25 μm.

RESULTS AND DISCUSSION

Isolation of a novel splice variant of PIPkin Iγ

A cDNA encoding a novel splice variant of the PIPkin Iγ isoform was cloned from a rat hippocampal library. The new cDNA was 2067 nt in length and encodes a protein of 688 amino acids with a predicted molecular mass of 75.6 kDa.

It has been previously shown that mouse PIPkin Iγ is alternatively spliced depending on the inclusion (splice variant a) or the exclusion (splice variant b) of a 26-amino-acid tail at the C-terminus [17]. The novel isoform identified here (splice variant c) presents a further 26 additional amino acids after Phe^{636} encoded by a 78 bp insert, presumably resulting from alternative splicing (Figure 1).

We searched the nucleotide database and found an EST (expressed sequence tag; ID 9550937) from mouse eye retina. The corresponding I.M.A.G.E. clone (ID No. 5360818) contains a fragment that is 95 % identical with the C-terminal sequence of rat PIPkin Iγc and includes the spliced insert. The nucleotide sequence of the PIPkin Iγc insert was then compared with the High Throughput Genomic Sequence database which identified the mouse clone RP24-535C14 (AC102226.2). Sequence alignment

of the latter with the full-coding rat PIPkin Iγc cDNA showed that the gene consists of 18 exons and revealed the existence of the additional splice sites that led to the insertion of the 78 bp at the 3′-end of exon 16.

We also searched the Ensembl version of the human genome database. Results of a BLAST search using the rat Iγc insert gave a sequence that was 77 % identical, which is located on chromosome 19 (bp 3588534–3588611). The identified sequence belongs to the gene ENSG00000185111 that codes for human Iγ and this therefore suggests that the novel splice variant Iγc might be also expressed in human tissues.

mRNA of PIPkin Iγc is expressed specifically in mouse brain

Isoforms α and β of PIPkin I [15] are widely distributed among tissues and are expressed with different relative levels, which implies that they have a function applicable to many cell types. The γ isoform, on the other hand, is preferentially expressed in kidney, lung and, in higher amounts, in brain [17,20], as expected for an enzyme that is shown to play a major role at the synapse [20].

We determined the expression of mouse PIPkin Iγ splice variants by RT-PCR on commercially available mouse total RNA derived from various tissues. We carried out the RT-PCR with probes generated from a region in the coding sequence common to the splice variants and from the 3′-untranslated region of the mRNA. These would amplify three products from the transcripts of the PIPkin Iγ splice variants: a fragment of 364 bp of the a variant, one of 286 bp of the b and a 442 bp fragment of the c variant. This was indeed observed (Figure 2), suggesting that all three transcripts are expressed in mouse tissues. However, the relative occurrence of the three fragments was different in each tissue. The 364 bp band representing the a variant is widely expressed and present in larger amounts in brain. The PIPkin Iγb transcript is less broadly distributed and is hardly detectable in brain and testis. Interestingly, the PIPkin Iγc transcript is detected exclusively in brain.

Northern blot and Western blot analysis of PIPkin Iγ in brain [20,25] have identified a doublet of bands at approx. 90 kDa. Since, as shown in the present paper, the b splice variant is scarcely present in brain, it is possible that the two PIPkin Iγs are in fact the a and the c splice variants. Moreover, an antibody against the 26-amino-acid tail of the Iγa (which is also present in the Iγc, but not in the Iγb) immunoprecipitates two bands from a brain extract [22,23]. These could represent PIPkin Iγa in different phosphorylation states, as suggested by these authors or, alternatively, might correspond to the PIPkin Iγa and the new brain-specific variant c. It is noteworthy that talin, an integrin-binding protein located in focal adhesions, interacts with just one of the two PIPkin Iγs. This may indicate that talin binds PIPkin Iγa only when in a phosphorylated/dephosphorylated state [22,23], but an alternative explanation suggested by our current data is that it specifically recognizes only the a splice variant.

In situ hybridization

In situ hybridization with ^{35}S-labelled oligonucleotides specific for the PIPkin Iγc variant, which would not detect the Iγa or b variants, revealed a strong expression of the Iγc in the forebrain, particularly the cerebellum, the hippocampus and the cerebral cortex (Figures 3A and 3B). The signal was low or undetectable in the thalamus, hypothalamus and pons medulla, and moderate in the basal ganglia. Strong Iγc mRNA expression was found in the granule cells of the cerebellum (Figure 3C) and in the dentate gyrus of the hippocampus (Figure 3D). Hybridization signals were localized to neurons mainly in the pyramidal cells of the hippocampus (Figure 3E) and of the cerebral cortex (Figures 3F and 3G). The Iγc transcript was also identified in the granule cells of the olfactory bulb, specifically the mitral and the inner plexiform layers (Figures 3H and 3I). The signal distribution suggests that Iγc localizes in major excitatory output neurons especially in the forebrain.

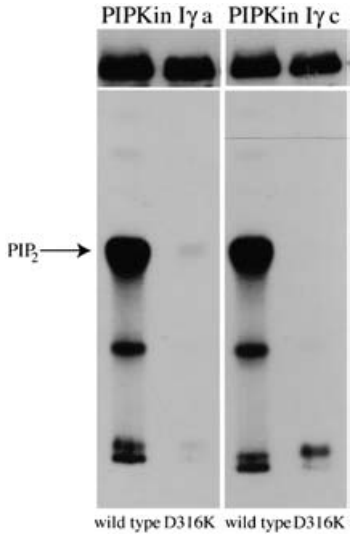

Figure 4 Reduced activity of D316K mutants of the a and c spice variants of PIP Iγ

Wild-type or D3166K mutants of PIPkin Iγa (left) or Iγc (right) were immunoprecipitated from COS-7-transfected cells, and assayed for PIPkin activity as described in the Materials and methods section. Above the measurements of activity are shown the respective quantification of enzyme present in each assay by Western blotting of a parallel aliquot (see the Materials and methods section).

Possible function of PIPkin Iγc

Kinase-dead PIPkin Type Iα constructs have been successfully used as dominant-negative mutants by van Horck et al. [26] and Yamazaki et al. [27] to show an involvement of this isoform in neurite retraction. To explore a possible function for PIPkin Iγc, we made some kinase-dead constructs and followed a similar strategy. For most of the experiments we used a kinase-negative mutant of Iγc in which Asp^{316}, which has a role in general base catalysis, was changed into a lysine residue (Iγc-D316K). *In vitro* lipid kinase activity of recombinant FLAG–Iγc-D316K immunoprecipitated from COS-7 cells was less then 1 % of that of the wild-type (Figure 4). As a control, we used the equivalent catalytically compromised PIPkin Iγa (Figure 4). We also confirmed the effects of the kinase-dead PIPkin Iγc with an independent mutant that has a point mutation in the ATP-binding site [Lys^{188} → Ala (K188A)] and retains less then 1 % of the wild-type activity (results not shown). This construct and its PIPkin Iγa equivalent were indistinguishable in their effects (described below) on cerebellar granule cells from the corresponding D316K mutants (results not shown).

The overexpression of the four kinase-dead mutants (two each of PIPkin Iγa and PIPkin Iγc) gave distinct results in cerebellar granule cells. The kinase-dead Iγa mutants did not induce any obvious change in the neurons compared with GFP-transfected controls in our experiments (Figure 5B compared with Figure 5C at 24 h, and Figure 5E compared with Figure 5F at 65 h), though

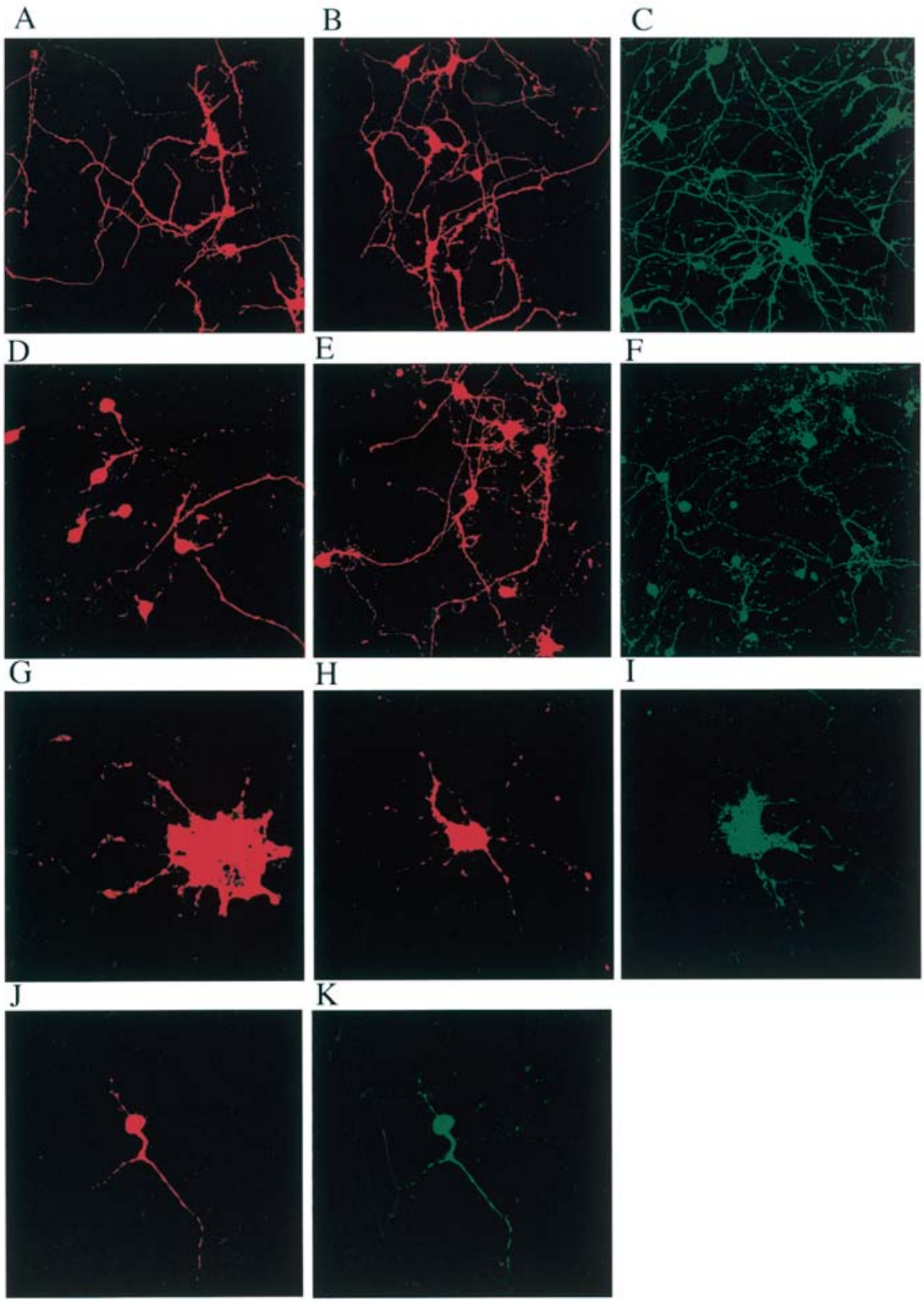

Figure 5 PIPkin Iγc-D316K induces process remodelling in cerebellum granule neurons

Granule cells isolated from mouse cerebellum after 7–21 days in culture were transfected with plasmids for FLAG–Iγc-D316K (**A**, **D**), FLAG–Iγa-D316K (**B**, **E**) and enhanced GFP (**C**, **F**). They were cultured for the next 14 h (**A**, **B**, **C**) or 60–70 h (**D**, **E**, **F**), then fixed and stained as described under the Materials and methods section. (**G**, **H** and **I**) show glial-like cells expressing either the kinase-dead Iγc (**G**) or the kinase-dead Iγa splice variant (**H**) or enhanced GFP (**I**), which were identified amidst the primary granule cells. All images are single sections obtained at a confocal microscope. (**J** and **K**) show granule cells co-transfected with kinase-dead Iγc and GFP for 65 h; (**J**) is stained with anti-FLAG antibody, and (**K**) shows GFP fluorescence.

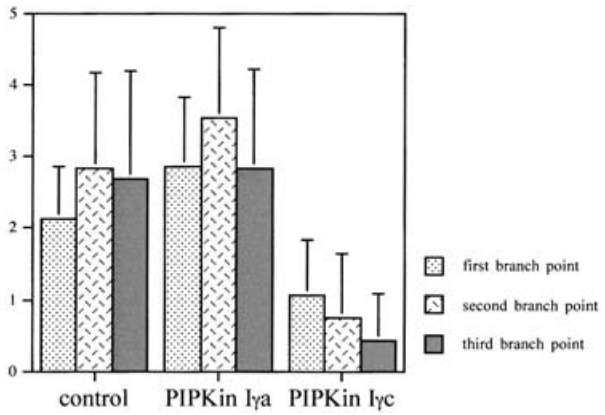

Figure 6 Quantification of neuronal processes

Results represent the first, second and third branch points of axons and dendrites for each cell. Results were derived from 60 (for both GFP-transfected granule cells and PIPkin Iγa kinase-dead-transfected cells) and 56 (PIPkin Iγc kinase-dead) cerebellar granule cells, all transfected for 66 h. The total number of branch points (i.e. the sum of first, second and third) was 7.7 ± 2.9, 9.2 ± 3.0* and 2.2 ± 2.0** (means $\pm$ S.D.) for cells transfected with GFP, PIPkin Iγa and PIPkin Iγc (both kinase-dead) respectively. *$P < 0.05$ and **$P < 0.001$, compared with the control, as determined by Student's t test. The decrease in branch points in PIPkin Iγc kinase-dead-transfected cells was accompanied by a clear decrease in cell number. At 24 h after transfection, there was no significant difference in the number of cells transfected per cover slip for any of the three constructs, yet, for example, in 17 randomly selected fields of cells 66 h after transfection, for GFP-transfected cells there were 807 cells visible and for PIPkin Iγa-transfected there were 805, whereas for the PIPkin Iγc kinase-dead-transfected cells there were 148 cells (a decrease of approx. 80 %).

there may be a small increase in the number of branch points per neuron (Figure 6) that requires further investigation. Transfection of granule cells with the kinase-negative version of PIPkin Iγc, however, produced a striking effect on their morphology. There was little obvious effect within 24 h of expression (Figure 5A compared with Figures 5B and 5C). However, over a longer period, and becoming fully evident within 60–70 h of expression, neurons containing the kinase-dead Type Iγc constructs had processes greatly reduced in length and complexity (Figure 5D), which was accompanied by an extensive loss of neurons (Figure 6).

That the processes were truly shorter and less complex, rather than this being due to PIPkin Iγc taking on a more restricted localization by 65 h, was confirmed by co-transfecting the cells with GFP (Figures 5J and 5K). Note that control (GFP or PIPkin Iγa kinase-dead-transfected) cells grew long multi-branching processes during the 60–70 h, whereas cells transfected with inactive PIPkin Iγc ended up with processes even shorter and simpler than they had when they were transfected at zero time [Figures 5 and 6; note that the transfection took place after 1–3 weeks in culture (see the Materials and methods section)]. This implies that an actual decrease in processes, rather than a simple failure of them to grow, has occurred. The long time that this effect took to develop fully suggests that this phenomenon is unlikely to be due to neurite retraction, which is usually a much faster process [26,28], and it may instead involve some slower mechanism such as degeneration (e.g. see [29]).

The ideal control for this effect of kinase-dead PIPkin Iγc constructs would be to use wild-type PIPkin Iγc to titrate out the effect of the kinase-dead mutants. Unfortunately, we found that in control experiments, expression of high levels of both PIPkin Iγa and PIPkin Iγc induced changes in the appearance of cerebellar neurons within 24 h, particularly some beading along their processes (results not shown). We have not explored this effect further in the present study. Transfection of CV-1 cells with PIPkin Iγa does not increase total cellular PtdIns(4,5)P_2 [19], but as the two splice variants in our experiments caused a similar effect in cerebellar granule cells, it might nevertheless be a consequence of more synthesis of PtdIns(4,5)P_2. The relevance of this phenomenon in the present context is that it would make any attempt to reverse effects of the kinase-dead PIPkin Iγc with kinase-live constructs very difficult to interpret.

However, evidence for the likely specific nature of this phenomenon can be found in three observations. First, it appears to be unique to neurons, the cells that express PIPkin Iγc naturally (Figure 3), at least insofar as glial-like cells in the same cultures that became transfected with Iγc-D316K (Figure 5G) showed no morphological difference from PIPkin Iγa-D316K- or enhanced GFP-transfected cells (Figures 5H and 5I). Secondly, as described above, kinase-dead PIPkin Iγa controls, which differ from their PIPkin Iγc equivalents only by 26 amino acids, caused no obvious visible effect (Figure 5), consistent with the idea that this is unlikely to be a non-specific artifact of transfecting the neurons with a kinase-dead PIPkin. Thirdly, two independent kinase-dead constructs of PIPkin Iγa and c (K188A) had effects identical with the D316K mutants (results not shown), effectively ruling out a non-specific protein misfolding artifact.

The most likely reason for the change seen is the simple one that neurons transfected with inactive PIPkin Iγc are unable to maintain their processes so that these degenerate, leading ultimately to cell death, which in turn suggests that a PtdIns(4,5)P_2 pool synthesized by PIPkin Type Iγc may be involved specifically in their growth and maintenance. For example, such a PtdIns(4,5)P_2 pool might be involved in the transport events that are essential to such growth and maintenance [30,31], and it is interesting that De Vos et al. [32] have recently shown that PtdIns(4,5)P_2 regulates the direction of the axonal transport of mitochondria.

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