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Cloning, tissue distribution and sub-cellular localisation of phospholipase C X-domain containing protein (PLCXD) isoforms

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ABSTRACT

Phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes comprise a small family of receptor-regulated phosphodiesterases that control many cellular processes by the regulation of cytosolic calcium and/or the activity of several protein kinases. To date, six distinct classes of PI-PLC are known to exist in mammals. Here we characterise a seventh class of PI-PLC, which contains only the catalytic X domain in its structure, termed phospholipase C X-domain containing protein (PLCXD). At least three tissue-specific PLCXD isoforms exist in humans, comprising hPLCXD-1, hPLCXD-2 and hPLCXD-3, with hPLCXD-2 exhibiting three C-terminal spliceforms (2.1, 2.2 and 2.3). Specific amino acids known to be essential for the catalytic function of PI-PLCs were found to be conserved in all three human PLCXDs and over-expression of hPLCXD-1, 2.1 and 3 in the HeLa cell line increased endogenous PI-PLC activity. Human PLCXD isoforms exhibited tissue-specific expression profiles in mice and humans and immunocytochemistry revealed distinct sub-cellular localisations when over-expressed in human cultured cell lines. These novel proteins may therefore possess fundamental, and as yet uncharacterised roles in cell physiology.

1. Introduction

Inositol phospholipids comprise a group of up to eight membrane lipids with different arrangements of phosphate attached to the polar inositol head-group. Although together they only represent a small fraction of the total cellular phospholipid pool, regulation of the amounts of the different components by the enzymes involved in inositol phospholipid metabolism is fundamental to eukaryotic signal transduction [1]. An enzyme central to this is known as phosphatidylinositol-specific phospholipase C (PI-PLC). Through the generation of the calcium-releasing inositol-1,4,5-trisphosphate (IP_3) and the protein kinase C activator diacylglycerol (DAG), PI-PLC is able to mediate the physiological actions of various hormones, growth factors and neurotransmitters [2].

In mammals, six distinct classes of PI-PLCs have been identified (β, γ, δ, ε, ζ, η) and their molecular structures, functions and regulation have been extensively reviewed [2,3]. Each isozyme contains a well-conserved catalytic domain (40–60% amino acid identity), consisting of separate X- and Y-box domains, which fold into a characteristic α/β barrel secondary structure [2]. With the exception of PLC-ζ, all isozymes contain a pleckstrin homology (PH) domain, known to mediate interactions with plasma membrane phosphoinositides. PI-PLCs also contain a C2 domain which is generally known to be a calcium-binding motif important for additional membrane interactions, however, in PLC-β and PLC-γ the C2 domain has been shown to lack key amino acid residues involved in calcium binding and therefore functions in a calcium-independent manner [4]. Additionally, PLC isozymes contain subtype-specific domains. The PLC-γ isozymes have two Src homology domains (SH-2 and SH-3) between the X and Y domains, which are important for protein–protein interactions associated with a number of intracellular communication pathways including mitogenic signalling [5]. The PLC-β isozymes contain a C-terminal region (~400 residues) which has been reported to contribute to tethering of the enzyme to the membrane, given that truncation of this region blocked membrane association and enzymatic activation by heterotrimeric G-proteins of PLC-β1 [6]. PLC-ε contains an N-terminal RasGEF domain and two C-terminal Ras-association domains that are implicated in Ras-mediated signalling pathways [7]. The presence of distinct regulatory domains in PLC isozymes renders them susceptible to different modes of activation. All PI-PLC isozymes possess distinct tissue and subcellular distribution patterns and are known to play unique roles in regulating many aspects of cell metabolism, growth and division.

Abbreviations: PLC, phospholipase C; XD, X-domain containing protein; PI, phosphatidylinositol; BLAST, basic local alignment search tool; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Pk/V5, amino acid tag GKPIPNPLLGLDST; Rpl-P0, ribosomal large protein-P0; pac, puromycin N-acyl transferase; ORF, open reading frame; IMPA1, inositol monophosphatase.

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The present study was initiated following a microarray screen, which identified a novel PI-PLC-like gene that exhibited differential expression during the development of the European eel (unpublished). Subsequent BLAST analyses identified homologues in a wide range of species from bacteria to man. These homologues are members of a novel PI-PLC subtype which contain only the characteristic X-domain of the other multi-domained members of this enzyme family and are hence known as phospholipase C X-domain containing proteins (PLCXDs). To date, BLAST analyses has revealed the presence of at least three isoforms in a number of mammals, including man. Each PLCXD isoform shows a unique tissue distribution and cellular localisation, highly suggestive of distinct physiological functions. Preliminary results from experiments over-expressing PLCXDs in cultured cells indicate that these proteins may function as active phosphodiesterases as they are able to increase the turnover of inositol phosphate from some unknown phospholipid pool.

2. Materials and Methods

2.1. Quantitative and semi-quantitative RT-PCR

RNA was either extracted from various mouse tissues using Triazol reagent (Invitrogen), according to manufacturer's protocol or obtained commercially from human tissues (Clontech). RNA samples (2 μg) were incubated with DNase (New England Biolabs) to remove any contaminating genomic DNA and converted to cDNA using 200 units of MMLV reverse transcriptase according to manufacturers instructions (Promega, UK). RT-PCR was performed in a total reaction cocktail of 20 μl comprising 1 μl of a diluted sample of reverse transcribed RNA (40 ng), 300 nM each of gene-specific sense and antisense primers (Supplementary Table 1), 200 nM dNTPs, 1.25 units Taq DNA polymerase in 1×Taq DNA polymerase buffer (Biogene). The following PCR cycle was repeated for up to 40 cycles: 94 °C for 10 s, 60 °C for 30 s and 72 °C for 1 min. Products from the PCR reactions were analysed by electrophoresis in 1% agarose gels (Sigma–Aldrich) containing 0.1 μg/ml ethidium bromide and 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0 (1×TAE) as running buffer. Real-time quantitative PCR was performed as previously described with ribosomal large protein-P0 (Rpl-P0) used for data normalization [8].

2.2. Cloning and Lenti-viral expression of PLCXD isoforms in HeLa cells

Full-length cDNAs of all three human PLCXD isoforms were amplified from HeLa cell RNA as detailed above, but using the Platinum *Pfx* (Invitrogen) proof-reading DNA polymerase. cDNAs were cloned into pCR4-TOPO using a TA cloning kit (Invitrogen), and sequenced from both ends using the Big Dye di-deoxy chain termination method according to manufacturer's protocol (Applied Bio-Systems). Clones for all three genes, hPLCXD-1, 2.1 and 3, exhibiting identical sequences to that published in existing databases were selected and sub-cloned into pd NotI′MCS′R′Pk which contained the pac (puromycin N-acyl transferase) gene (provided by R.E. Randall, St Andrews University, UK) for generation of disabled lenti-virus particles, and subsequently used to generate puromycin-resistant HeLa cell lines stably expressing the hPLCXD genes [9]. Over-expression of each human PLCXD was confirmed by both quantitative PCR and Western Blotting with anti-hPLCXD (data not shown) and anti-Pk/V5 antibodies (Supplemental Fig. 1).

2.3. Bioinformatics

Amino acid identity and similarity analyses were calculated using Mat-Gat2.01 software. Amino acid sequences were obtained from NCBI and aligned using ClustalW. Phylogenetic trees were constructed and visualised using the p-distance matrix of the neighbour-joining (N-J) method [10]. The statistical reliability of the individual nodes for sequence similarity of the newly constructed tree was assessed by bootstrap analyses (1000 replicates).

2.4. In vivo PLC assay

Virally transfected cells over-expressing PLCXD-1, 2.1 and 3 constructs were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum, and 100 units/ml penicillin:100 μg/ml streptomycin at 37 °C in an atmosphere of 95% air/5% CO_2. The inositol phospholipid pools in control, non-transfected, and hPLCXD1, 2.1 and 3-transfected HeLa cells were radio-labelled by incubation for 24 h in inositol-free DMEM (MP Biomedicals, IIIkrich, France) containing 1 μCi/ml myo-D-[^{3}H]-inositol (GE Healthcare, Buckinghamshire, UK) before extensive washing in complete media. The turnover of inositol from the phospholipid pool was determined by measuring the accumulation of [^{3}H]-inositol phosphate in the cell cytosol during a subsequent 2 h incubation at 37 °C. During the incubations, 10 mM LiCl was added to the growth medium to inhibit endogenous inositol monophosphatase (IMPA1) activity and therefore block any re-cycling of inositol phosphate back into the phospholipid pool. Incubations were also conducted in the presence and absence of 1 μM of the calcium ionophore A23187. The reaction was stopped after 2 h by aspiration of the medium and the addition of 10 mM ice-cold formic acid. [^{3}H]-Inositol phosphates were isolated from the formic acid extract using ion exchange with Dowex AG 1-X8 ion exchange resin (BioRad, Hemel Hempstead, UK), after washing the resin with 60 mM ammonium formate/5 mM sodium tetraborate and following elution with 1 M ammonium formate/0.1 M formic acid and quantified by liquid scintillation counting. All experiments were conducted in triplicate.

2.5. Sub-cellular distribution

HeLa cells exhibiting stable transfection of Pk/V5-tagged hPLCXD-1, 2.1 and 3 were grown (as above) to sub-confluence for fluorescence immunocytochemistry, as described previously [11]. PLCXD expression was detected using a mouse monoclonal anti-Pk/V5 antibody (Invitrogen; 1/200 dilution) and a secondary labelled Alexa Fluor 633-labelled donkey anti-mouse polyclonal antibody (Invitrogen; 1:1000 dilution). Cover slips were mounted in ProLong gold antifade reagent (Sigma–Aldrich) and examined with a Zeiss Axioplan fluorescent microscope system. The specificity of the primary antibody toward Pk/V5-tagged proteins in HeLa whole cell extracts was confirmed by Western Blotting (Supplementary Fig. 1).

2.6. Statistical analysis

Data are represented as means ± SEM and where appropriate compared using a Student's *T*-test. Differences were considered significant with $P < 0.05$

3. Results and Discussion

3.1. Molecular cloning and sequence comparisons of human PLCXD-1, 2 and 3

Cloning allowed the verification of the in silico-sequence of three human orthologues of a novel sub-group of phosphatidylinositol-specific phospholipase Cs, known as hPLCXD-1, 2.1 and 3. The cloning of full-length reading frames for hPLCXD-1, 2.1 and 3

revealed 971 bp, 914 bp and 965 bp cDNAs with ORFs generating proteins of 323, 305 and 321 amino acid residues respectively, which are much smaller than all other known mammalian PLCs. Two additional C-terminal spliceforms of hPLCXD-2 (variants 2.2 and 2.3) were also detected in cultured cell lines (results not shown). In humans, the PLCXD-1 gene is unusual, in as much as it is one of only 29 pseudo-autosomal genes that are present on both X (Xp22.33) and Y (Yp11.32) chromosomes. Like most other pseudo-autosomal genes, this highlights PLCXD-1 as having possible roles in neonatal development and, as all pseudo-autosomal genes escape X-inactivation there is the potential for PLCXD-1 being linked to the variety and physical and mental disorders associated with sex chromosome aneuploidy [12], such as found in Triple X syndrome. In humans the PLCXD-2 gene is found on chromosome 3 (3q13.2) and PLCXD-3 is found on chromosome 5 (5p13.1).

Sequence analysis indicated that all three hPLCXDs contain only the X-domain of the normal X and Y catalytic domains found in all known mammalian PLC family members and are also devoid of all other regulatory domains. The sequences of the hPLCXDs are more closely related to bacterial X-domain containing homologues such as the PI-PLC enzyme from *Bacillus cereus*, where PLCXDs 1, 2.1 and 3 share ~35%, 32% and 33% amino acid identity, respectively. The PI-PLC from *Bacillus cereus* is known to hydrolyse both phosphatidylinositol (PI) and also cleave glycosylphosphatidylinositol (GPI)-anchored membrane proteins [13]. As a result of various biochemical and structural studies, the invariant His32 and His82 amino acids (*Bacillus cereus* sequence, Supplementary Fig. 1) have been shown to act together in the enzymatic hydrolysis of certain phosphodiesters and to be essential for the catalytic activity of the bacterial enzymes [14]. An alignment of the amino acid sequences corresponding to all three human PLCXDs and PI-PLC from *Bacillus cereus* is shown in Supplementary Fig. 2. Although the amino acid identities between the human and bacterial proteins are low, the two key catalytic histidines are conserved in all three human PLCXDs, suggestive of the retention of similar catalytic properties in the human proteins.

3.2. Phylogenetic analysis of PLCXD proteins

The amino acid sequences of all three human PLCXD proteins were compared with sequences from other species including mammals, amphibians, reptiles, birds, fish, and insects. Some organisms, such as mouse, zebrafish and humans have at least 3 isoforms whereas in invertebrates such as the nematodes, sequence information for only one homologue is available in databases. Collectively, these sequences allowed for the construction of a phylogenetic tree of PLCXD isoforms from different species (Fig. 1). Phylogenetic analysis indicates that known PLCXD-1, 2 and 3 sequences cluster into three separate groups with each isoform being highly conserved across species, suggestive of conserved functions.

3.3. In vivo PLC assays

A potential role of hPLCXDs acting as functional PLCs was investigated using a cell-based phosphatidylinositol turnover assay. The relative [^{3}H]-inositol phosphate (IP) content of non-transfected (control) HeLa cells and cells transfected with hPLCXD-1, 2.1 and 3 was determined following a 2 h incubation in the presence of the IMPA1 inhibitor LiCl, either in the absence or presence of the calcium ionophore, A23187. As shown in Fig. 2, HeLa cells transfec-

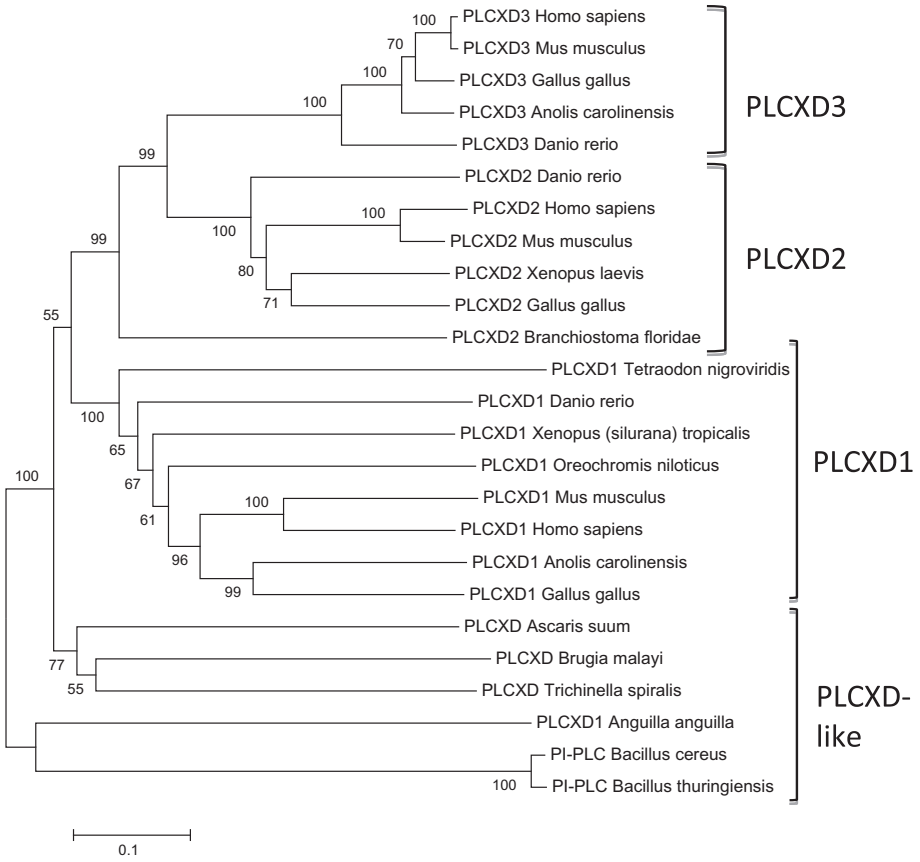

Fig. 1. Phylogenetic analysis of PLCXDs. The tree was constructed using the neighbour-joining (N-J) method. Bootstrap percentages are shown if >50. Bar represents the p distance of the N-J method. NCBI accession numbers are shown in Supplementary Table 2.

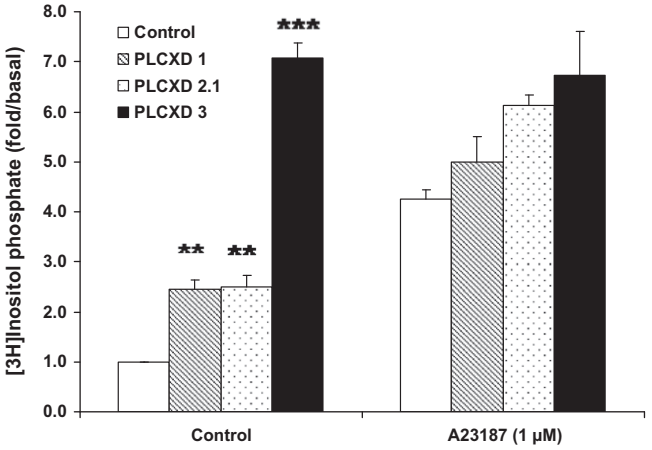

Fig. 2. Relative [^{3}H]-IP content of non-transfected (control) HeLa cells and cells transfected with hPLCXD-1, hPLCXD-2.1, hPLCXD-3, following a 2 h incubation in the presence of 10 mM LiCl plus or minus 1 μM calcium ionophore A23187. Bars indicate the fold difference over non-transfected, control cells. Error bars indicate SEM for $n = 3$ replicates. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

ted with hPLCXD constructs all exhibited significant (2.5- to 7-fold) increases in basal turnover of the phosphatidylinositol pool compared to the non-transfected control cells. Interestingly, compared with both PLCXD-1 and PLCXD-2.1 transfected cells, the turnover of PI-phospholipids was significantly higher in cells transfected with PLCXD-3, even although the levels of expression of this construct were much lower in the HeLa cells (results not shown). The calcium ionophore A23187 increased PI turnover to the same extent in control and PLCXD-1 and PLCXD-2.1 transfected cells but failed to enhance the already high turnover in cells over-expressing hPLCXD-3. These data suggest that over-expression of all three hPLCXD isoforms increased endogenous phospholipase C activity in cultured HeLa cells in a calcium independent manner. Although it is possible that the expressed PLCXDs mediate increases in expression and/or activity of the endogenous HeLa cell PLCs, given the sequence similarities with the bacterial PI-PLCs, it is likely that the proteins have their own enzyme activity. The increased activity in all cells seen with the ionophore could be accounted for by the enhanced activity of endogenous PLCs, most of which are stimulated by increasing calcium concentrations [2]. All existing mammalian PI-PLCs utilise a catalytic calcium ion to preferentially cleave phosphatidylinositol-4,5 bisphosphate (PIP_2) whereas bacterial PI-PLCs have no metal ion dependence and do not cleave the multi-phosphorylated forms of PI [15]. Strangely, the fold increase in phospholipase C activity in cells over-expressing PLCXD1 and, especially PLCXD3 was lower in the presence of the ionophore (Fig. 2), indicating a possible attenuation of PLCXD-1 and -3 actions in the presence of excess calcium. With respect to the apparent lack of stimulation by calcium in vivo and the absence of putative calcium co-ordinating amino acids [17] within their sequence, the human PLCXDs may be functionally more similar to bacterial PI-PLCs than other mammalian PI-PLCs. Although the transfected PLCXDs appear not to be activated by intracellular calcium, their endogenous substrates and/or binding partners are still unknown. The ability of the different human PLCXD isoforms to directly hydrolyse various multi-phosphorylated derivatives of PI is currently under investigation.

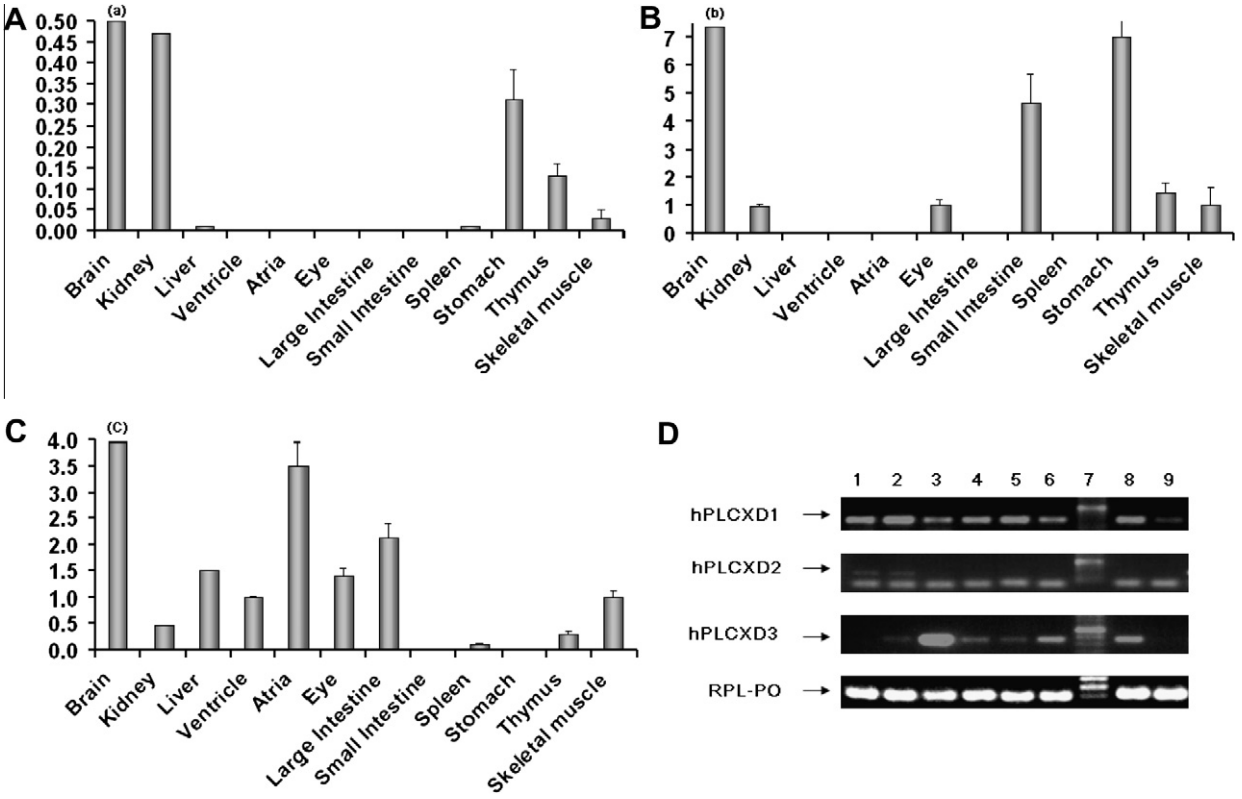

Fig. 3. Relative expression of mPLCXD-1, 2.1 and 3 mRNAs in selected mouse tissues (A-C). Expression of PLCXD mRNAs was determined from Ct values obtained and normalized to Rpl-P0 expression in each tissue as detailed in the materials and methods. Samples were run in triplicate; error bars indicate SEM. Mean values for brain samples (a), (b) and (c) are 1.0, 100 and 20, respectively. Values on Y-axis indicate expression relative to Rpl-P0 ($\times 10^{-3}$). (D) Semi-quantitative expression levels of hPLCXD-1, -2.1 and -3 mRNAs by RT-PCR analysis from a restricted number of human tissues (1 – leukocytes, 2 – thymus, 3 – heart, 4 – small intestine, 5 – colon, 6 – kidney, 7 – molecular weight markers, 8 – lung and 9 – skeletal muscle).

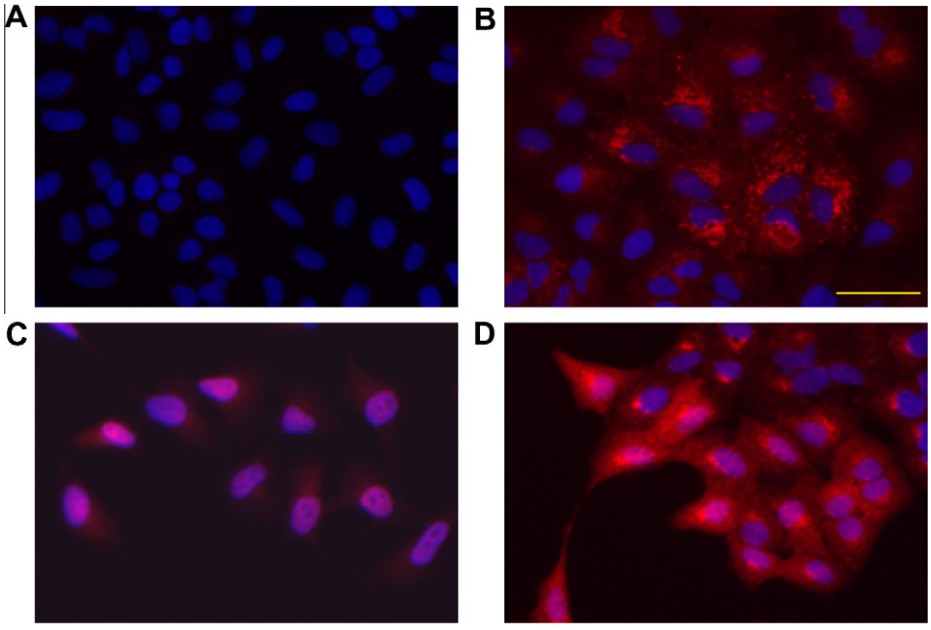

Fig. 4. Sub-cellular localisation of hPLCXD isoforms (red) in HeLa cell lines stably transfected with Pk-tagged hPLCXD-1 (B), 2.1 (C) or 3 (D), respectively. Control cell lines (A) were transfected with an empty vector. Hoescht stained nucleus is shown in blue. Photomicrographs were captured using Zeiss Axiocam light microscope. Scale bar indicates 50 μm.

3.4. Tissue distribution and sub-cellular localisation of hPLCXD-1, 2 and 3

Quantitative RT-PCR was used to determine the distribution and relative levels of expression of all three PLCXD mRNAs in both mouse and a restricted number of human tissues (Fig. 3A-C). PCR analyses clearly indicated that PLCXD-1, 2 (probe for all splice-forms) and 3 mRNAs show distinct tissue expression patterns. For all PLCXD isoforms, the highest mRNA expression levels were observed in the brain (Fig. 3A). PLCXD-3 mRNA was observed in virtually all tissues tested indicating a potential fundamental role in cell function.

The localisation of all three hPLCXDs was investigated by the expression of Pk/V5-tagged forms of the proteins transfected into HeLa cells using a lenti-viral expression system. When HeLa cells over-expressing either PLCXD-1 or PLCXD-3 were probed with an anti-Pk/V5 antibody the most prominent signals were present in uncharacterised cytoplasmic and perinuclear vesicles suggestive of endoplasmic reticulum and/ or Golgi apparatus (Fig. 4B and D). This indicates that both proteins may be involved in unknown intracellular functions rather than existing as part of extracellular signalling processes occurring at the plasma membrane. In contrast, transfected PLCXD-2.1 protein was found to be entirely nuclear (Fig. 4C), indicative of a putative transcriptional role. Similar sub-cellular distributions were found when over-expressing PLCXD isoform constructs in A549 cells (results not shown). The functional significance of these sub-cellular localisations are, however, currently unknown. Interestingly, the nucleus has been shown to contain a variety of phosphoinositides along with a number of enzymes able to metabolise them [16], and therefore it is hypothesised that PLCXD 2.1 may be an integral component of a nuclear phosphoinositide signalling pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.079.

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