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Identification of Four Novel Human Phosphoinositide 3-Kinases Defines a Multi-isoform Subfamily

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Phosphoinositide (PI) 3-kinases have critical roles in diverse cellular signalling processes and in protein trafficking. This suggests that like other intracellular signalling molecules, e.g., phospholipase C and protein kinase C, there might be a large family of PI 3-kinase isoforms with the individual members having discrete signalling roles. Reverse transcription-polymerase chain reaction methods, using degenerate oligonucleotide primers against the lipid kinase consensus region, revealed eight sequences from human cDNA containing a high degree of identity to the family of PI 3-kinases. The sequences obtained included the previously described p110α, p110β, and p110γ isoforms and HsVps34. Additionally, we have identified four novel sequences which are related to PI 3-kinases. Three of the novel sequences would appear to form a distinct sub-family of PI 3-kinases. We report the expression of these novel PI 3-kinases in human tissues and in cells derived from normal breast.

Phosphoinositide (PI) kinase activity has been found to be associated with activated receptors and oncogene products and to be an activity of major importance involved in diverse cellular processes including cell migration, cell proliferation, oncogenic transformation, cell survival and intracellular trafficking of proteins [reviewed in 1, 2, 3]. PI 3-kinase was originally identified as a heterodimeric complex consisting of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit (termed p85 and p110) [4, 5, 6]. The p110 catalytic subunit is capable of generating the 3-phosphorylated forms of phosphatidylinositol (PtdIns), PtdIns4P and PtdIns(4,5)P_2 as the potential second messengers PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively. Receptor activation results in stimulation of PI 3-kinase activity, leading to an increase in cellular levels of the D-3 phosphorylated lipid PtdIns(3,4,5)P_3. Increased levels of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 have been detected during diverse cellular processes, including oncogenic transformation, mitogenesis and insulin triggered changes in glucose transport [reviewed in 1, 2].

Since the original identification of the p85-p110 complex a larger family of proteins related to the p110 catalytic subunit has begun to emerge. Analysis of the sequence homology between the different family members, their substrate specificities and structural characteristics has enabled the different PI 3-kinase isoforms to be assigned to three classes [7]. Class IA PI 3-kinases include p110α [6, 8] and p110β [9] which bind p85 adaptor proteins and associate with receptor protein-tyrosine kinases. The p85 adaptors add to this diversity with a least three distinct isoforms and several splice variants in addition [4, 10, 11, 12, 13]. These p85 proteins link associated PI 3-kinase catalytic subunits to regulation by protein-tyrosine kinases [2]. Class IB is represented by the p110γ isoform which does not bind p85 adaptor subunits and is regulated by α and $\beta\gamma$ subunits of heterotrimeric G-proteins [14, 15]. Members of the Class I p110 sub-family have also been reported in *Drosophila* [16] and *Caenorhabditis elegans* [17]. Class I PI 3-kinases are able to phosphorylate PtdIns, PtdIns4P and PtdIns(4,5)P_2 to PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively *in vitro* and their major *in vivo* product is thought to be PtdIns(3,4,5)P_3 [1, 18]. Class II PI 3-kinases are defined by the presence of a carboxy-terminal C2 domain. This sub-family includes PI3K_68D/cpk [19, 20] from *Drosophila* and cpk-m/p170 from mouse [20, 21]. Members of this sub-family have been suggested to have a dis-

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Abbreviations used: PCR, polymerase chain reaction; PI, phosphoinositide; PtdIns, phosphatidylinositol; PTK, protein-tyrosine kinase.

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tinct *in vitro* substrate specificity phosphorylating PtdIns and PtdIns4P, but not PtdIns(4,5)P_2, at the 3 position of the inositol ring. The phospholipid products generated by this PI 3-kinase family *in vivo* remains to be determined. Class III is composed of PtdIns-specific PI 3-kinases which are only capable of phosphorylating PtdIns to PtdIns3P both *in vitro* and *in vivo*. The prototype of this sub-family is Vps34p which was originally identified as a protein in *Saccharomyces cerevisae* which was involved in vesicular trafficking and vacuolar protein sorting [22]. Vps34 homologues have been described in *Schizosaccharomyces pombe* [23], *Dictyostelium discoidium* [24], Soybean (*Glycine max*) [25], *Arabidopsis thaliana* [26], *Drosophila* [27] and in humans [28, 29]. Further more distantly related proteins to the above PI 3-kinases include the PI 4-kinase family [30, 31], the TOR/FRAP family [32, 33, 34] and the large protein kinases related to DNA-dependent protein kinase [reviewed in 35].

Research on PI 3-kinases has reached the stage at which in order to fully understand the role of PI 3-kinase activity in specific cell functions we need a clearer picture of the total spectrum of isoforms present within a particular cell type or tissue, how they are individually regulated and their sensitivities to commonly used PI 3-kinase inhibitors such as wortmannin [36] and LY294002 [37]. The polymerase chain reaction (PCR) has been extensively used to identify new members of the protein-tyrosine kinase (PTK) family and to survey the repertoire of PTKs expressed in specific cell types [38, 39, 40]. This approach has recently been applied to the analysis of PI 3-kinases in *Dictyostelium discoidium* [24] and *Drosophila* [19] utilising redundant oligonucleotide primers based on conserved sequence motifs within the PI 3-kinase domain. We have carried out a molecular analysis of PI 3-kinase isoforms by a RT-PCR approach in order to gain a better understanding of the role of this family of enzymes in signalling processes in human tissues. Here we describe the identification of four novel and four known PI 3-kinases by PCR and show that these kinases exhibit differential tissue and cell type expression. One of the novel isoforms is likely to be an additional member of Class I PI 3-kinases. The other three of these novel isoforms form a discrete subfamily of PI 3-kinases which possess C2 domains at their carboxy-termini.

MATERIALS AND METHODS

Tumour Material and Breast Cell Preparation

Organoids and purified human breast cell populations were prepared essentially as described previously [41]. Briefly, normal breast tissue obtained from reduction mammoplasty was finely chopped and then digested overnight with collagenase (5 mg/ml, Sigma) in Dulbecco's Modified Eagle Medium (GibcoBRL) supplemented with 5% foetal calf serum (TCS Biologicals), fungizone (2.5 μg/ml), kanamycin (100 μg/ml), penicillin (100 μg/ml) and streptomycin (100 μg/ml). Organoids were collected by filtration and plated out in RPMI 1640 supplemented with 10% foetal calf serum, insulin (5 μg/ml), hydrocortisone (5 μg/ml) and cholera toxin (100 ng/ml). Luminal and myoepithelial cells were then isolated by immunomagnetic separation utilising antibodies directed against the epithelial membrane antigen (EMA) found exclusively on luminal epithelial cells and CALLA (CD10) on myoepithelial cells.

RT-PCR

Total RNA was prepared from non-involved and involved axillary lymph nodes removed from a patient with metastatic breast carcinoma, from total breast tissue obtained from reduction mammoplasty, from breast-derived organoids and from isolated primary cells [42]. 5 μg of total RNA was DNase-treated and reverse transcribed using random hexamers (Pharmacia) and SuperScript II RNase H- reverse transcriptase (GibcoBRL). The resultant cDNA was amplified by PCR using a mixture of degenerate oligonucleotides. The sense and antisense primers used to generate the mini library of PI 3-kinase related sequences (and their corresponding amino acid sequences) were as follows.

Sense PI 3-kinase family primers

PI 3-kinase GDDLRQD
GG[A/C/G/T]GA[T/C]GA[T/C][T/C]T[A/G]CG[A/C/G/T]CA[A/G]GA

PI 3/4-kinase GDD[L/C]RQ[D/E]
GGQGA[T/C]GA[T/C][T/C][T/G][A/C/G/T][C/A]G[A/C/G/T]CA[A/G]GA

TOR/FRAP HED[L/I]RQD
CA[C/T]GA[A/G]GA[T/C][A/C/T]T[A/C/G/T][C/A]G[A/C/G/T]CA[A/G]GA

Antisense PI 3-kinase family primers

PI 3-kinase FHIDFGHF
[A/G]AA[A/G]TGQCC[A/G]AA[A/G]TC[A/G/T]AT[A/G]TG[A/G]AA

TOR/FRAP HIDFGDC
CA[A/G]TCQCC[A/G]AA[A/G]TC[A/G/T]AT[A/G]TG

PCR was carried out in a buffer containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTP mixture and 1 U Taq Polymerase (GibcoBRL). The PCR reaction went through 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min. PCR products were subcloned into pCR II (Invitrogen). Three hundred insert containing plasmids were grown up and gridded out onto Hybond-N (Amersham) membranes in duplicate. Randomly picked clones from the PI 3-kinase plasmid library were sequenced and the products identified. [^{32}P]-labelled probes were prepared using random primers and used as hybridisation probes to eliminate identical clones from the remaining gridded PCR PI 3-kinase mini library. Several hybridising clones were sequenced to confirm their identity to the probe sequence. Further non-hybridising clones were then sequenced and used as probes as outlined above until all the clones on the filter had been identified. Inserts were sequenced on both strands by the chain termination method using a Sequenase kit, version 2 (USB) or by

cycle sequencing with dye terminators and analysed on an ABI473 automated sequencer.

Northern Blot Analysis

Two Northern blots containing 2 μg of poly(A)$^+$ RNA isolated from human tissues (MTNI and MTNII, Clontech) were hybridized overnight at 42°C using conditions described by the supplier, with [^{32}P]-labelled probes prepared from the PI 3-kinase PCR products. A final high stringency wash was performed at 65°C in 0.1×SSC, 0.1% SDS and the blots were subjected to autoradiography. The blots were normalised by probing with a β-actin probe provided by the manufacturer.

RESULTS

PCR Cloning of PI 3-Kinases from Metastatic Lymph Node

It has recently become clear that there are more members of the family of PI 3-kinases than previously expected and that they have a region of weak sequence homology to the kinase domain of protein kinases [43] which has been termed the lipid kinase domain [6]. Alignments of the amino acid sequences of known PI kinase family members at that time (p110α, p110β, PI 4-kinase, ScVps34 and TOR/RAFT) were made using UWGCG package [44] and several fully degenerate primers were designed around two highly conserved sequences, (G/H)(D/E)D(L/I/C)RQ(D/E) and (F)HIDFG(D/H)(C/F), found within the lipid kinase domain. The primers used were more degenerate than those used previously to isolate PI 3-kinases from *Dictyostelium* and *Drosophila* [19, 24], the aim being to isolate further more distantly related PI 3-kinase family members if any existed. The full sequence of the primers used in this study is given in the materials and methods. PCR amplification of reverse transcribed RNA from an involved axillary node from a patient with metastatic breast carcinoma yielded the expected 400 bp fragment. In addition a 500 bp fragment was observed which is the predicted size for the amplification of members of the TOR/FRAP family due to the presence of additional sequences between the two chosen primers. This 500 bp fragment proved difficult to subclone and has not been followed further in this study. The 400 bp product was subcloned to create a plasmid mini library of PI 3-kinase cDNAs. Three hundred insert containing colonies were grown up and spotted onto Nylon membranes in duplicate. Radiolabelled probes prepared from PI 3-kinase fragments amplified from both the involved and non-involved node RNAs were used to screen these filters as described previously [45]. This approach has been used successfully to identify PTKs which display elevated expression in breast cancer [46]. However, no strong preferential hybridization was observed with the probe prepared from the PI 3-kinase cDNA fragment pool amplified from the involved node RNA. We therefore decided to investigate the complexity of the PI 3-kinase mini library derived from the involved axillary node by a hybridization and DNA sequencing approach. Of the 300 clones analysed 285 encoded PI 3-kinase related sequences. Only 15 clones lacked PI 3-kinase related sequences between the primer regions. In total, eight distinct sequences were identified which displayed sequence similarity with known PI 3-kinases. An alignment of the amino acid sequences derived from these eight cDNA clones is shown in Figure 1A with absolutely conserved amino acids being marked by an asterisk. The relative frequencies of the different PI 3-kinases in the PCR library and their closest known family members are documented in Table 1.

Current sequence databases were searched using the BLAST and FASTA programs [44]. Of the eight PI 3-kinase sequences identified, four were either known family members or have subsequently been reported. These include the human homologues of p110α [6, 8], p110β [9], p110γ [15] and HsVps34 [29]. The other four sequences (T14, T96, T105 and T119) represent novel PI 3-kinase isoforms. The kinase domain fragments were aligned and a tree showing their relative sequence similarity was derived (see Figure 1B). Both our database searches and the kinase domain alignment suggest that novel clone T119 is a fourth member of the Class I p110 family showing greatest sequence similarity to p110β. The other three novel sequences also show considerable sequence similarity with the Class I p110 family members, but are more closely related to each other and to members of the recently described C2 domain containing family of PI 3-kinases which is made up of the *Drosophila* PI 3-kinase called PI3K_68D or cpk [19, 20] and the murine PI 3-kinase cpk-m/p170 [20, 21] (see Figure 1A and 1B). Based on amino acid sequence identity of $>$99% over the kinase domain PCR product clone T96 is identified as the human homologue of the recently described cpk-m/p170 [20, 21]. Clones T14 and T105 appear to be novel genes and exhibit a lower degree of sequence identity to cpk-m over the PCR-derived kinase domain fragment at 60% and 81% respectively. We have isolated the full cDNA for T105 and find it is indeed a novel member of the C2 domain family of PI 3-kinases. This clone will be described in detail elsewhere [47]. Clone T14 shows a similar degree of sequence identity to clone T105 (61%) as it does to clone T96/cpk-m.

Tissue Expression Profile of the Novel PI 3-Kinase mRNAs

We have characterised the expression pattern of the four novel PI 3-kinase clones by examining the relative levels of mRNA present in sixteen adult human tissues. Radiolabelled probes for each of the novel clones and a β-actin probe were prepared and were sequentially hybridised to the same two multiple tissue Northern

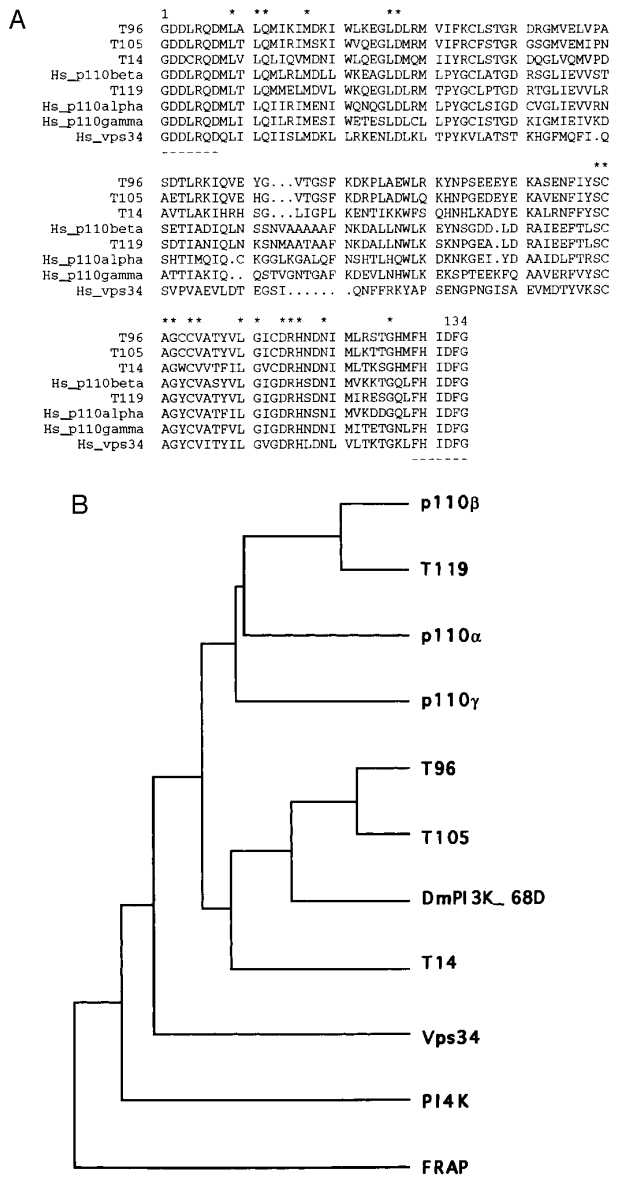

FIG. 1. (A) PI 3-Kinase domain amino acid sequence alignment. Alignment of PI 3-kinase kinase domain fragments of the known and novel PI 3-Kinases. The sequence alignment was made using the PILEUP program and the consensus sequence was displayed using the PRETTY program. Absolutely conserved residues are indicated by asterisks. The primer regions are indicated by a dashed underline. (B) Kinase domain family tree. Dendrogram of PI 3-kinase kinase domains showing their degree of relatedness. FRAP [33], PI4K [30] and DmPI3K_68D [19] are included for comparative purposes. Sequences were aligned using the PILEUP program and the dendrogram was displayed using FIGURE.

blots. From our original screening of the PI 3-kinase domain mini library we are confident that under high stringency conditions these probes are highly specific for their respective PI 3-kinase. In each case a single major transcript was observed (Figure 2).

All four novel PI 3-kinases exhibited distinct expression patterns. Clone T119, the putative Class I p110 family member, exhibited highest levels of a 6 kb transcript in spleen, thymus and peripheral blood leukocytes (PBL) suggesting a possible haematopoeitic origin. Lower levels of the same transcript were observed in heart, liver, skeletal muscle, prostate, testis, ovary and small intestine. No transcripts were detected in brain, placenta, lung or colon.

All three of the putative C2 domain containing family PI 3-kinases had distinct tissue expression patterns. Clone T14 showed the most limited expression pattern reported for a PI 3-kinase to date with a 6.5 kb transcript being readily detectable in liver and prostate samples. A smaller transcript of 2.6 kb was observed only in testis which is probably the result of alternate splicing. Longer exposures revealed lower levels of T14 transcript in small intestine, pancreas and kidney. Clone T96 was detected as a transcript of approximately 9.5 kb. Highest levels were seen in testis with expression also detected in heart, brain, placenta, skeletal muscle, pancreas, spleen, thymus, prostate, ovary, small intestine and colon. Clone T105 hybridised to a major transcript of 8.5 kb which was detected in all tissues analysed. Highest levels were in placenta and spleen and lowest levels were in peripheral blood leukocytes, skeletal muscle and kidney. A larger transcript $>$9.5 kb was detected at a lower level and paralleled expression of the smaller transcript and thus may represent an alternative spliced form of the mRNA.

Expression of PI 3-Kinases in the Breast

The parenchyma of the adult human breast consist of two main cell types; luminal epithelial cells which secrete milk during lactation and are the source of most breast malignancies and basally situated myoepithelial cells which are contractile in nature. We have also used PCR to examine the expression of all

TABLE 1

Incidence of Different PI 3-Kinase Isoforms in Involved Lymph Node Tissue PCR Mini Library

Clone	Frequency	Closest relative
T1	49	Identical to Hs p110α
T83	40	Identical to Hs p110β
T105	77	Novel PIK, C2 family member, closest relative cpk-m
T200	51	Identical to Hs p110γ
T96	45	Novel PIK, C2 family member, cpk-m homologue
T2	14	Identical to HsVPS34
T119	7	Novel PIK, closest relative p110β
T14	2	Novel PIK, C2 family member, closest relative cpk-m
	15	No sequence similarity.
Total	300	

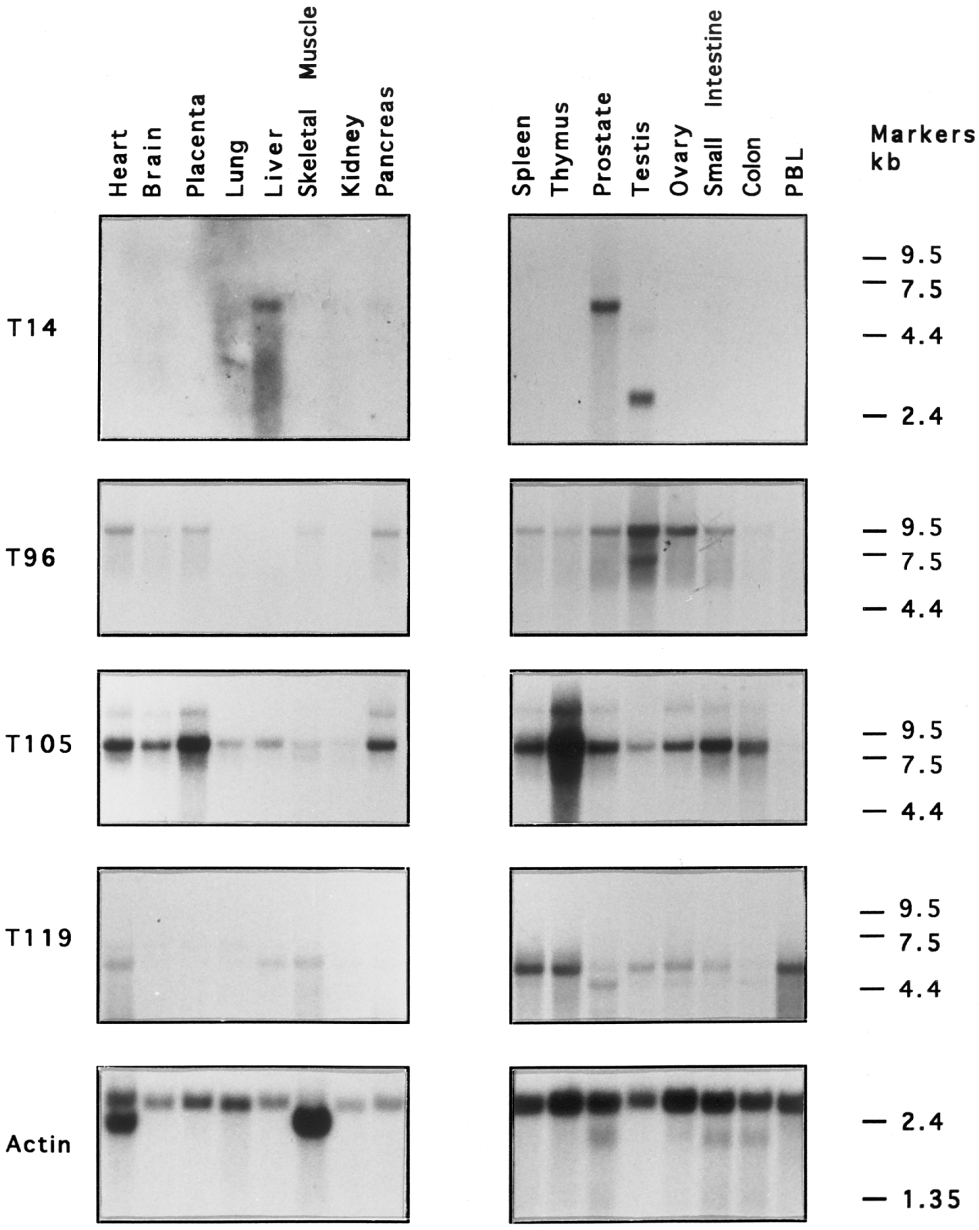

FIG. 2. Expression of novel PI 3-kinases in human tissues. Multiple tissue Northern blots (MTNI and MTNII, Clontech) were sequentially probed with ^{32}P-labelled fragments from T14, T96, T105, T119 and a β-actin probe supplied by the manufacturer. The mobilities of the RNA markers are indicated in kb. The blots were not stripped between probing, but the bound probe was allowed to decay below the level of detection before applying the next probe. Exposure times for autoradiographs shown were: T14, 3 days; T96, 18 days; T105, 24 hours; T119, 7 days; β-actin, 3 hours.

eight PI 3-kinases in normal breast tissue, breast derived organoids (which are the epithelial elements, i.e., ducts and lobulo-alveolar fragments, remaining after digestion and removal of collagen and fat) and in the three major primary cell types (luminal epithelial cells, myoepithelial cells and mammary fibroblasts) derived from this tissue. We adopted this approach due to the problems of obtaining sufficient poly(A)$^+$ mRNA from the sorted primary cells to allow Northern blot analysis (we have been unable to detect PI 3-kinase transcripts on Northern blots of total RNA preparations). Oligonucleotide primers specific for each PI 3-kinase were employed and are shown in Table 2. The results of this analysis are shown in Table 3. Representative PCR products obtained were directly sequenced to confirm their identity. The results were similar to those seen in the tissue Northerns. All eight PI 3-kinases were found in total breast tissue. Three of the Class I kinases (p110α, p110β and T119) were found in all samples tested as were

TABLE 2

Primers Used in PCR Expression Analysis of PI 3-Kinase Isoforms

Clone	Orientation	Primer sequence	Annealing temp.
110α	Sense	GACTTATTGAGGTGGTG	56°C
110α	Antisense	GGCATGCTGTCGAATAG	
110β	Sense	GCTAATGTGTCAAGTCG	56°C
110β	Antisense	CCGATTACCAAGTGCTC	
110γ	Sense	CCTGCAGAATTCTCAAC	56°C
110γ	Antisense	CACAATCTCGATCATTC	
T14	Sense	CTGCCAAATCTACTTCACAG	45°C
T14	Antisense	TCCTCCTCAGAACTTCACT	
T96	Sense	CAAATGTCTCTCAACTG	52°C
T96	Antisense	CTTCGAAGCATTATATTG	
T105	Sense	GGATCCCTGCCCCGGGATGAGAAGGCTATGGG	60°C
T105	Antisense	CTCGAGGCTGGGCTCACAAGGTGCCATGACTTC	
T119	Sense	GTACTCCGTTCAGACACC	60°C
T119	Antisense	CATGATGTTGTCGCTGTG	
Vps34	Sense	CTTACGAATCAAGATAAAGCC	60°C
Vps34	Antisense	GATTTTCGCCATCTGGAACTTC	

Note. PCR was carried out in a buffer containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.75 mM $MgCl_2$, 1 mM dNTP mixture and 2.5 U Taq Polymerase (GibcoBRL). The PCR went through 35 cycles of 94°C for 1 min, Annealing temperature (see table above) for 1 min, and 72°C for 1 min. 2.5 U Pfu, rather than Taq, was used for the amplification of T14.

Vps34 and T96. The G-protein regulated p110γ was not found in any of the three breast cell types tested. It is likely that its expression in the whole tissue is due to the presence of lymphoid cell types. We readily detected p110γ expression in U937, Jurkat and HL60 cells (data not shown). T105 was found at very low levels myoepithelial cells and mammary fibroblasts, but was not detectable in luminal epithelial cells.

TABLE 3

Expression of PI 3-Kinases in Breast Cells by PCR

Kinase	Whole breast	Organoid	Lum	Myo	Fibro
Class I					
110α	+	+	+	+	+
110β	+	+	+	+	+
T119	+	+	+	+	+
110γ	+	−	−	−	−
Class II					
T14	+	+	−	+	−
T96	+	+	+	+	+
T105	+	+	−	−/+	−/+
Class III					
Vps34	+	+	+	+	+

Note. Lum, luminal epithelial cells; Myo, myoepithelial cells; Fibro, mammary fibroblasts. −, not detected; −/+, weak signal; + readily detected.

Finally T14 was found in the myoepithelial cells, but not in the luminal epithelial or fibroblasts.

DISCUSSION

The identification of four new PI 3-kinase clones T14, T96, T105 and T119 significantly extends the repertoire of human PI 3-kinases. Although we have only deduced the amino acid sequence for a small portion of these proteins, the presence of highly conserved features of the lipid kinases domain, e.g., the motif SCAxCV which is diagnostic for the PI 3-kinase family, suggests that all the novel isoforms are likely to encode PI 3-kinases.

The four new PI 3-kinase clones described here fall into two classes. Clone T119 is a fourth member of the Class I p110 subfamily and is most closely related to p110β within the deduced PI 3-kinase domain sequence. It is not possible to predict from our sequence whether clone T119 will be a p85 binding isoform or not (although the strong homology with p110β would seem to favour the former possibility). The expression of the p110β message has previously been investigated in mouse rather than human tissues where it was found to be ubiquitous, although mRNA levels did vary considerably between tissues examined [9]. Highest p110β mRNA tissue expression was observed in kidney, whereas T119 is absent from this tissue. Conversely T119 showed high levels in spleen, a tissue

where p110β expression was observed to be very low. The significance of this observation is not clear and will require more detailed examination of the expression of the different PI 3-kinase catalytic subunits at the protein level. Unfortunately, good specific antisera against all the known PI 3-kinase isoforms are not currently available. The expression pattern of p110α mRNA has not been described, but the protein has been described in a number of diverse tissue and cell types [6, 48]. By the PCR approach we have detected p110α in all cell and tissue types tested to date (LKFH and MJF, unpublished observations). The p110γ isoform mRNA also appears to be fairly widely expressed being detected in all tissues tested except brain [15]. Human Vps34 has also been described as having a ubiquitous expression pattern [29].

The other three novel sequences are most closely related to members of the recently described C2 domain containing family of PI 3-kinases (Class II PI 3-kinases). To date this includes a single *Drosophila* family member called PI3K_68D [19] or cpk [20] and a single murine representative termed p170 or cpk-m [20, 21]. Our T96 clone is the human homologue of cpk-m. Clones T105 and T14 are new members of this family. We have isolated a full length cDNA for Clone T105 and it shows all the features of this new family [47]. The expression pattern of the C2 family members have not previously been described. Several lines of evidence suggest that T14 is a more divergent third member of the C2 domain family of PI 3-kinases. Both kinase domain alignment and family tree clearly place T14 in this group. T96, T105 and T14 all possess a three amino acid deletion in their kinase domain relative to the Class I p110 members including T119 (see Figure 1A). Additionally we have identified EST sequences from human tissue sources in databases with sequence identity to clone T14. Further sequencing of these ESTs reveal sequence similarity to C2 domains carboxy terminal to the kinase domain homology (MR and MJF, unpublished observations).

The expression patterns of the PI 3-kinase genes suggests the possibility of distinct roles for these putative PI 3-kinases. The broad expression pattern of T96 and T105 suggests that these kinases may have a more general role in signalling. T119 has a somewhat more restricted expression pattern, but is still found in the majority of tissues examined. In contrast, clone T14 has a highly restricted expression pattern being expressed in breast, prostate and liver (with very low levels in kidney, pancreas and small intestine) of the tissues thus far examined. This restricted expression is also found at the level of which cells in a tissue (e.g., the breast) that T14 is found in. We are currently isolating a full-length cDNA for T14 and raising antisera against a portion of its protein product in order to allow us to examine its sensitivity to PI 3-kinase inhibitors and its possible functions in more detail.

What is required now is functional analysis of these PI 3-kinases to determine whether they have specific or redundant roles in signalling pathways. Might Class I p110 family members regulate the proliferative functions of PI 3-kinases [48] and Vps34 intracellular trafficking events [22], whilst the C2 domain PI 3-kinase family might regulate cell survival through the Akt serine/threonine kinase [49, 50] ? To date a major approach to analyse PI 3-kinase function has been the use of inhibitors such as wortmannin and LY294002 [36, 37]. However these compounds have shown little specificity for the different PI 3-kinase isoforms tested to date and members of related enzyme families such as some PI 4-kinases and members of the TOR/DNA-dependent protein kinase families may also be sensitive to inhibition by these compounds [31, 51, 52, Rushika Sumathipala and MJF, unpublished observations]. It is likely that all the PI 3-kinases described here will be inhibited by these compounds to some extent. Unravelling the roles of the individual PI 3-kinases isoforms is thus likely to require the use of knockout mutations, specific dominant interfering mutants or constitutively active mutants of the individual PI 3-kinases present in a given system. The identification of all of the players is the first step in this analysis.

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