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GENETIC AND FUNCTIONAL STUDIES OF PHOSPHATIDYL-INOSITOL 4-KINASE TYPE III α

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Abstract

Phosphatidylinositol 4-kinase type III α (PI4KIII α) is one of four mammalian PI 4-kinases that catalyzes the first committed step in polyphosphoinositide synthesis. PI4KIII α has been linked to regulation of ER exit sites and to the synthesis of plasma membrane phosphoinositides and recent studies have also revealed its importance in replication of the Hepatitis C virus in liver. Two isoforms of the mammalian PI4KIII α have been described and annotated in GenBank: a larger, ~ 230 kDa (isoform 2) and a shorter splice variant containing only the ~97 kDa C-terminus that includes the catalytic domain (isoform 1). However, Northern analysis of human tissues and cancer cells showed only a single transcript of ~ 7.5 kb with the exception of the proerythroleukemia line K562, which contained significantly higher level of the 7.5 kb transcript along with smaller ones of 2.4, 3.5 and 4.2 kb size. Bioinformatic analysis also confirmed the high copy number of PI4KIII α transcript in K562 cells along with several genes located in the same region in Chr22, including two pseudogenes that cover most exons coding for isoform 1, consistent with chromosome amplification. A panel of polyclonal antibodies raised against peptides within the C-terminal half of PI4KIII α failed to detect the shorter isoform 1 either in COS-7 cells or K562 cells. Moreover, expression of a cDNA encoding isoform 1 yielded a protein of ~97 kDa that showed no catalytic activity and failed to rescue hepatitis C virus replication. These data draw attention to PI4KIII α as one of the genes found in Chr22q11, a region affected by chromosomal instability, but do not substantiate the existence of a functionally relevant short form of PI4KIII α .

Keywords

Phosphoinositide; PI 4-kinase; chromosome 22; K562 leukemia cells; Golgi; Endoplasmic reticulum

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1. INTRODUCTION

Phosphoinositides are membrane-bound regulatory lipids with remarkable importance in orchestrating a range of cellular functions including transmembrane signaling, lipid transport and vesicular trafficking [1]. These lipids are synthesized from phosphatidylinositol (PtdIns) by phosphorylation of various positions around the inositol ring. The most abundant and longest studied phosphoinositides are PtdIns4P and PtdIns(4,5)P₂, the products of phosphorylations by PI 4-kinase (PI4K) and PIP 5-kinase enzymes, respectively. PtdIns4P has long been viewed only as an intermediate to PtdIns(4,5)P₂ synthesis in the plasma membrane [2], but its presence in the Golgi [3, 4] and endocytic compartments [5] has firmly established its role as a regulatory lipid. PtdIns4P serves as a docking site for various clathrin adaptors [4], lipid transfer proteins [6, 7] and other regulatory proteins such as GOLPH3 [8] in these various locations. It is becoming more and more evident that PI4Ks have a major role in organelle dynamics and vesicular trafficking as evidenced by recent reports on the importance of PI4Ks in viral replication [9, 10]. However, progress in understanding the functions and regulation of these enzymes has been relatively slow.

In vertebrates, PtdIns4P is synthesized by four distinct PI4K enzymes that belong to either the type-II or type-III family, each having α - and β -forms [11]. The type-III PI4Ks are relatives of the PI 3-kinase family, while the smaller type-II enzymes form a separate family [12, 13]. The first cloned PI4Ks were the yeast orthologues of the type-III PI4Ks [14]. PIK1 was recognized as an essential gene important in Golgi to plasma membrane secretion [15, 16] whereas another yeast PI4K, STT4 was discovered as a gene whose mutations cause staurosporine hypersensitivity [17]. Stt4 is also essential in most yeast strains and plays a role in cell wall biogenesis and plasma membrane PtdIns(4,5)P₂ generation [18]. The mammalian type-III PI4K enzymes come in two sizes (210–230 and 110 kDa) and show sensitivity to PI3K inhibitors, while the type-II enzymes are completely resistant to this class of drugs [19]. The first mammalian PI4K cloned from human placenta was named PI4K α , contained 854 amino acid and had a predicted size of 97 kDa [20]. This protein showed high sequence similarity to the C-terminus of the larger (210–230 KDa) yeast Stt4p and was initially classified erroneously as a type-II PI4K [20]. Subsequent studies clarified that full-length PI4KIII α is a 210–230 kDa type-III PI4K that is an orthologue of the yeast Stt4 protein [21–23] and it was concluded that the original PI4K α was a short splice variant of the larger enzyme. The other type-III PI4K, PI4KIII β , which is the mammalian orthologue of the yeast Pik1p, was cloned from bovine rat and human [21, 24, 25] with a calculated size of 92 kDa but migrating as a 110 kDa protein on SDS gels.

Although the existence of the two splice variants of PI4KIII α has been accepted and is so annotated in GenBank, no systematic study has examined their presence and functions in mammalian cells. In the present study we scrutinized the genomic organization of PI4KIII α and investigated the presence of various transcripts as well as proteins in human and rat cells and tissues. These studies reveal several unique features of the PI4KIII α gene organization and question the existence of the short PI4K variant both at the mRNA and protein levels. These experiments also showed that the short form of the PI4KIII α expressed from a cDNA does not display PI4K activity and that a minimally active enzyme required additional extensions toward the N-terminal direction.

2. MATERIALS AND METHODS

2.1 Materials

[γ -³²P]-ATP was purchased from Perkin Elmer. All other materials were of the highest analytical grade. The monoclonal anti HA antibody was obtained from COVANCE and the

monoclonal anti FLAG antibody from Sigma. The polyclonal antibody against PI4KIII α was from Cell Signaling.

2.2 DNA constructs

The PI4KIII α isoform 1 was amplified with the following primer pair: 5'-ATATAAGCTTCGGGAGATGGCAGGGGC - 3' and 5' - ATATGAATTCTCAGTAGGGGATGTCATTCTGATAGTA - 3'. The PCR product was cut with HindIII and EcoRI and cloned into the HA-pcDNA 3.1(+). Amplification of the full-length isoform 2 in one piece was unsuccessful, therefore this clone was created from 3 fragments with the following strategy: the C-terminus was amplified with the following primer pair: Fw: 5'- ATATGATATCGACTACAAGTCTGGGACCCCGATGC-3' and Rev: 5'- ATATGCGGCCGCTCAGTAGGGGATGTCATTCTGATAGTACTGGATCATG-3' from human brain cDNA (Clontech) and ligated in to HA-pcDNA 3.1(+) between the EcoRV and NotI sites. To obtain the N-terminal pieces a nested PCR approach was used. The first round of amplification was done with the Fw: 5'- TGGCGGTGCAGAGACCAGCATC-3' and Rev: 5'- TGCTGCACTCATCCTCGGAGTCTGAG-3' primer pairs and the reaction product was used for the subsequent amplifications. For the N-terminal piece the primers were: Fw: 5'- ATATAAGCTTATGTGTCCAGTGGATTTCCATGGGATCTTC-3' and Rev: 5'- TTGGCCAGGGCATTAAATGACTGCCAG-3' and for the middle piece: Fw: 5'- AGTGATTAATGCCCTGGCCAACATCGC-3' and Rev: 5'- ATATGATATCCAGCACAATGGCCTCAGGGTTG-3'; both products were cloned into the TOPO cloning vector (Invitrogen). The two N-terminal fragments were then ligated together along the VspI restriction sites and this conjoined piece was then placed within the HA-pcDNA 3.1(+) plasmid already containing the C-terminal segment between the HindIII and EcoRV restriction sites. All pieces were fully sequenced and expression of the full-length protein was verified with Western analysis and its activity measured in *in vitro* kinase assays.

2.3 Quantitative RT-PCR

Total RNA was isolated from K562, COS7 and HEK293 cells using the RNeasy Mini Kit (Qiagen). 1 μ g of total RNA was used in the reverse transcriptase reaction using the ImProm II Reverse Transcription System (Promega), following the manufacturer's instructions. 1 μ l of the cDNA was analyzed by quantitative PCR with Light Cycler 1.5 (Roche) using the LightCycler FastStart DNA Master Plus SYBR Green I kit (Roche) following the manufacturer's instructions. To quantify transcripts from the PI4KA gene the following primer pair was used: 5' ATCGGCGACCTCCTGGATCA GTTG 3', 5' CTGCCGGCAGTCGTCTCCCACC 3'. To quantify transcripts from the pseudogenes the following primer pair was used: 5' ATCGGCGACCTCCGGGAGCAGTTA 3', 5' CTGCCGGCAGTCGTCTCCCAGT 3'. The PCR reaction was run 40 cycles with the amplification protocol as follows: 96C, 10 sec; 60C, 5 sec; 72C, 16 sec; 88C, 5 sec. The results were analyzed with the Light Cycler v1.5 software. Two ESTs were obtained to serve as controls during the amplification: BE407628 encoding the relevant region from the PI4KA gene and BG397684 for the pseudogenes. The reaction products were analyzed by agarose gels that confirmed their expected sizes. The cross-reactivity between the primers and the respective templates were tested using serial dilutions of the EST plasmids. This showed more than 6 log unit difference between amplification from the two templates for both primers.

2.4 Northern blot analysis

The commercially available membranes containing the human tissue panels and cancer cell lines have been purchased from Clontech. The probe and hybridization procedure has been described in detail in our previous publication [21].

2.5 Production of polyclonal antibody against PI4KIII α

The polyclonal antibody against PI4KIII α was produced in New Zealand Rabbits against the peptide: KYLTASQLVPPDNQDTRSC by New England Peptide (Gardner, MA) and it was affinity purified using the immobilized peptide.

2.6 Immunoprecipitation and Western Blot Analysis

COS-7 cells were cultured on 6 well culture plates and transfected with the LipofectAMINE 2000 reagent(Invitrogen) as described previously[5]. Transfected cells were rinsed three times with ice-cold PBS, and cell lysates were prepared by the addition 1.3 ml of ice-cold lysis buffer (20 mM Hepes (pH 7.5), 100 mM NaCl, 2.5 mM MgCl₂, 1mM EDTA, 40mM β -glycerophosphate, 1% Nonidet P-40, 0.5 mM Na₃VO₄, 1 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin) to each well. Samples were transferred to Eppendorf tubes, centrifuged at 20,000 $\times g$ for 20 min, and assayed for protein concentration by the BCA Protein Assay kit (Pierce). To an aliquot of the lysates containing equal amounts of protein, 1 μ g of anti-HA or anti-FLAG antibodies were added, and samples were incubated at 4 °C overnight. Immunoprecipitated proteins were collected by protein A and G-Sepharose. After washing five times with 0.5 ml of lysis buffer, samples were analyzed for PI kinase activity or processed for Western analysis. Western analysis was also performed on samples without immunoprecipitation in which case the cells were directly lysed in Laemmli buffer. For detection of the proteins, either the polyclonal PI4KIII α antibodies were used in (1:200 - 1:500) dilutions or the monoclonal anti-HA or anti-Flag antibodies in 1:500 - 1:1000 dilutions. For detection, the infrared fluorescent secondary antibodies and imaging from LI-COR (Lincoln, NE) was used.

2.7 RNAi

For RNA interference (RNAi)-mediated knockdown, K562 and COS7 cells (10⁵ cells in 2 ml) were cultured on 6-well plates. Two different duplexes were used for treatment: against sequences 1072–1092, and 4516–4536. Silencing RNAs were obtained from Qiagen (Valencia, CA). Cells were treated with 100 nM siRNA twice in two consecutive days using Oligofectamine (Invitrogen), and the effect of siRNA treatment on the PI4K expression levels was determined by Western blot analysis.

2.8 Assay of PI 4-kinase activity

The activity of PI-4 kinase was measured as incorporation of radioactivity from [γ -³²P]ATP into organic solvent-extractable material as described previously [26]. The standard reaction mixture for PI 4-kinase (50- μ l final volume) contained 50 mM Tris/HCl, pH 7.5, 20 mM MgCl₂, 1 mM EGTA, 1 mM PI, 0.4% Triton X-100, 0.5 mg/ml bovine serum albumin (lipid kinase buffer), 100 μ M [γ -³²P]ATP, and the enzyme. Reactions were started by the addition of [γ -³²P]ATP and terminated after 30 min by the addition of 3 ml of CHCl₃, CH₃OH, 37% HCl (200:100:0.75 (v/v/v)). The organic solvent phase was separated from [γ -³²P]ATP as described elsewhere [19], and after evaporation, its activity was determined in a liquid scintillation counter.

2.9 Viral replication studies

For the viral replication studies, 3XFLAG-tagged PI4KA constructs encoding isoforms 1 and 2 were subcloned into the pFB (Stratagene; La Jolla, CA) retroviral vector. VSV-G

pseudotyped retroviral particles were generated as described in [9] and transduced into OR6 cells expressing a full-length genotype 1b HCV replicon that encodes a luciferase reporter gene. Endogenous PI4KIII α was silenced using a lentiviral shRNA vector targeting the 3'UTR of the PI4KIII α transcript [9]. Luciferase activity was measured after four days of silencing.

3. RESULTS

3.1 Genomic organization of the PI4KA gene

The PI4KIII α gene (PI4KA) contains 55 exons (54 coding) and is located on human chromosome 22. Upon inspection of this locus we noted that in addition to the full gene, there were two additional pseudogenes containing most but not all exons encoding the C-terminal catalytic domain of the protein (Fig. 1A). The PI4KA gene and the two partial genes (PI4KAP1 and -2 located in centromeric and telomeric directions, respectively, on the same strand) are all found in locus 22q11.2, a region known for its genetic instability. Examination of other genomes showed that this gene multiplication is present in other primates (such as Chimpanzee) but not in any of the lower mammals, such as rat, mouse, pig or the cow. In humans, this locus has also been the site of monoallelic deletions, causing the "22q11.2 deletion syndromes" that manifest in developmental abnormalities of varying severity and include, among others, DiGeorge and velocardiofacial syndromes [27]. The size of deletion is variable but can affect as many as 50 genes located in this region (Fig. 1B) and it is not clear which gene(s) are responsible for the most severe symptoms. Interestingly, the 22q11.2 locus has also been linked to schizophrenia [28]. Because of the presence of the PI4KA gene in this locus we began to explore this region with more scrutiny.

3.2 Shorter transcripts for PI4K α can be present in cancer cells

To determine whether shorter transcripts are present in some tissues, we performed Northern analysis on human tissue RNA blots using the 3286–6200 fragment of the bovine PI4KIII α as a probe [21]. This probe detected only a 7.2 kb transcript in most tissues showing highest expression in the brain and hinted the possible presence of smaller transcripts in the placenta (Fig. 2). This was in good agreement with the findings of Wong and Cantley in describing PI4K α cloned from placenta [20]. However, hybridization with a panel of human tumors (Fig. 2A) showed prominent signals at ~2.4, 3.5 and 4.2 kb in addition to the 7.2 kb band in the promyelocytic leukemia cell line, K562. (These RNA blots were commercially available with documented GAPDH loading controls, therefore, we did not perform hybridization for such housekeeping genes).

Next we used quantitative RT-PCR analysis to determine the relative copy numbers of PI4KIII α transcripts in various cell lines. For this, we used a primer pair that specifically amplified a segment of the PI4KA transcript with more than 6 log units discrimination relative to the pseudogenes. For calibration, two ESTs were obtained that corresponded to the amplified segments of the PI4KA gene and one of the pseudogenes (see Methods). Serial dilutions of these plasmid templates were used for calibration and several dilutions of the cDNAs obtained from the cells were run to obtain values for the cells. These results confirmed that K562 cells had high copy numbers of the PI4KA transcripts (68.5 ± 9.3 copies/ng cDNA, SEM, $n=3$) compared to HEK293 cells (25.6 ± 7.6 copies/ng cDNA, Mean $\pm$ range, $n=2$) or COS-7 (0.2 ± 0.1 copies/ng cDNA, Mean $\pm$ range, $n=2$).

To detect transcripts derived from the pseudogenes, we designed primers that match the exonic regions in the PI4KAP2 that differ from the PI4KA sequence as a result of accumulating point mutations. Notably, we also found ESTs in GenBank that correspond to transcripts from the pseudogenes isolated from various cancers. This indicated that these

transcripts do exist with higher abundance in cancer cells. One of the ESTs (IMAGE: 4565073; BC020225) was used as a calibration control template in the qPCR analysis. It was confirmed that these primers showed good specificity and did not amplify from the EST plasmid containing the cDNA from the PI4KA gene in qPCR reactions. Using this analysis, we were unable to detect the presence of PI4KAP2 transcripts in cDNAs prepared from HEK293 or COS-7 cells using qPCR analysis, while they were clearly detectable in K562 cDNA. Accurate quantification of these transcripts was not attempted.

3.3 The pattern of PI4KIII α expression correlates with several other transcript patterns from the Chr22q11.2 region in a panel of tumor cells

The human tumor panel shown in Fig. 2, suggested that significant variations in the expression level of PI4KIII α exist between cell lines. This prompted us to perform a bioinformatic analysis querying array-based gene expression data that are publicly available for the NCI-60 cell lines [29]. In particular, we wished to identify genes whose expression patterns showed positive correlation with that of PI4KIII α . This analysis identified several genes, including CRKL, and adaptor protein for Fgf signaling; SNAP29, a synaptosome associated protein; THAP7, an inhibitor for histone deacetylation; PRKAB1, an AMP-activated protein kinase; and CDC45L, a CDC45-like protein (Table I). Intriguingly, with the exception of PRKAB1, genes of the top-scoring transcripts are located in the same chromosomal region, 22q11.2, in close proximity to the PI4KA gene (Fig. 1B). Quantitative RT-PCR analysis also confirmed a significantly higher level of PI4KA transcript in the K562 cell line and HEK293 cells than in COS-7 cells (not shown).

3.4 Is there a PI4KIII α protein corresponding to transcript variant 1?

Next we wanted to determine whether the shorter splice variant of PI4KIII α is detectable at the protein level. For this, we generated polyclonal antibodies against peptide sequences corresponding to the unique regions within the catalytic domain of the PI4KIII α protein (NEP-Ab), a region that is identical between the two isoforms. We also used the polyclonal PI4KIII α antibody from Cell Signaling that is also raised against the catalytic region of the protein. As shown in Fig. 3A, both of these antibodies detected both PI4KIII α isoforms when these were expressed in COS-7 cells as HA-tagged proteins. The antibodies also detected the full-length 210–230 kDa protein in rat tissues, COS-7, HEK293 and K562 cell lysates with the NEP-Ab being more sensitive (Fig. 3B). These results confirmed that the protein is expressed at highest levels in brain and cerebellum.

We noted that the NEP antibody detected a faint band at the position of the shorter (isoform1) PI4KIII α in non-transfected COS-7 cells (Fig.3A, right panel, first lane). Therefore, we wanted to test whether this signal could correspond to isoform 1 of the protein. This band (band2), which is better seen in Fig. 3C as shown in another Western analysis of lysates from untransfected COS-7 cell, was also more abundant in K562 cells. We performed siRNA-mediated knock down of PI4KIII α using two different RNA duplexes designed to target either only transcript 2 or both transcripts 1 and 2. These duplexes were used both in COS-7 cells and K562 cells. Western analysis showed that only the 210–230 kDa band showed a decrease in response to silencing with either siRNA, suggesting that these additional bands were unlikely to correspond to the shorter PI4KIII α variant (Fig. 3C).

3.5 The short splice variant of PI4KIII α is a catalytically inactive protein

Although Western analysis did not confirm the presence of the shorter PI4KIII α variant, we could not completely rule out that it might be present in a small amount undetectable with our antibodies. To investigate the possibility that this protein, if expressed, could contribute to the PI4K activity of cells, we compared the N-terminally HA-tagged splice variants of the human PI4KIII α enzyme for catalytic activity. These proteins were expressed in COS-7 cells

and their PI4K activity assayed in the anti-HA immunoprecipitates. As shown in Fig. 4, the short (isoform 1) lacked catalytic activity even though the two proteins were expressed at comparable levels as shown by the Western analysis of the same samples (Fig. 4A). In order to get a catalytically active fragment of the PI4KIII α enzyme, the cDNA sequence had to be extended in the N-terminal direction and a 130 kDa fragment was found to be catalytically active when expressed as a GST fusion protein in bacteria [30] or as an epitope-tagged enzyme in HEK293 cells (not shown). This was in agreement with earlier published reports on the minimally active fragment of the PI4KIII α enzyme [31].

To further investigate the possible biological activity of isoform1 of PI4KIII α , we performed rescue experiments to determine whether it was able to rescue the replication defect of the Hepatitis C virus in OR6 cells in which endogenous PI4KIII α was knocked down by shRNA targeting the non-coding region of PI4KIII α . The efficiency of targeting PI4KIII α with this procedure has been previously documented [9]. The OR6 cell line contains a full-length genotype 1b HCV replicon with a *Renilla* luciferase reporter gene. These cells were transduced with MMLV retroviral vectors encoding either GFP alone, full-length PI4KIII α (isoform 2), or the short PI4KIII α variant (isoform 1). The cells were then transduced 24 hr later with lentiviral vectors encoding either a nontargeting shRNA or a shRNA targeting the PI4KIII α 3'UTR. As shown in Fig. 4B, the full-length enzyme was able to substantially restore HCV replication while the short variant, isoform1 failed to do so. These data also suggested that isoform1 of PI4KIII α even if exists is not a functionally competent protein.

4. DISCUSSION

The present studies were designed to clarify important questions concerning major splice variants of the PI4KIII α enzyme. The orthologue of this enzyme, Stt4p is essential for yeast and increasing evidence suggests that it is also critical in maintaining the functional integrity of mammalian cells. There are several interesting features of this protein: first it is primarily located in the endoplasmic reticulum where it was shown to regulate ER exit sites [32, 33]. Curiously, in spite of its ER localization, PI4KIII α seems to be responsible for the maintenance of the plasma membrane phosphoinositide pools [34], similarly to the yeast Stt4p protein [18]. This might be related to the enrichment of the enzyme in signaling domains at ER-PM contact zones as shown in the yeast [35], a finding yet to be demonstrated in mammalian cells. Second, recent studies have demonstrated that PI4KIII α is essential for HCV viral replication in liver [9, 10] and possibly also for the replication of other small RNA viruses [36], thus gaining significant interest as a druggable target.

There are two splice variants of PI4KIII α listed in GenBank (GI:4505807= Isoform 1, GI:155030226= Isoform 2). The shorter isoform was the first PI4KIII α clone isolated from humans [20]. Since the smaller splice variant has never been shown to exist endogenously at the protein level, it was important to know whether it indeed is present and if so, whether it contributes to the PI4K activity of mammalian cells. Our analysis did not confirm the existence of the short splice variant of PI4KIII α , and also proved that even if it existed, this protein would be catalytically inactive. Consistent with this finding, unlike the full-length protein, this short splice variant was unable to restore HCV replication in OR6 cells with downregulated PI4KIII α . These data together make it quite unlikely that the short splice variant of the PI4KIII α has any major functional significance.

Quantitative analysis of the PI4KA mRNA copy numbers in several cell lines revealed a wide range, showing the most abundant message in K562 cells and HEK293 cells substantially higher than in COS-7 cells. In fact, earlier studies concluded that the enzyme was not expressed in COS-7 cells, which is consistent with the low level of the protein in these cells [37]. In the present study we were able to detect the low endogenous level of

PI4KIII α in COS-7 cells, although both K562 cells and HEK293 cells showed significantly higher levels of the protein. However, these differences in protein levels did not reflect the proportions found in the number of transcripts. This discrepancy can be caused by the non-linearity of the Western analysis, but most likely is due to differences in translational efficiency and protein stability between the cell lines. This aspect of regulation was beyond the scope of the current studies. The other important question that arises is the nature of the shorter transcripts detected in the K562 cells. These could represent shorter forms generated from the PI4KA gene or from the two pseudogenes. Since our Northern analysis cannot discriminate between these possibilities, we could not determine with certainty the contribution of these different sources to these smaller transcripts. However, our qPCR analysis suggested a contribution to these transcripts by the PI4KAP2 pseudogene, in agreement with the existence of a number of ESTs in the database corresponding to transcripts from the pseudogenes. Therefore, we concluded that the pseudogenes contribute to the transcript heterogeneity in K562 cells where chromosomal amplification likely yields abundant transcripts generated from this chromosomal region.

Our analysis of the genomic organization of the PI4KA gene in human Chr22 revealed important details with potential implications in human disease. First, the gene is located in the 22q11.2 locus that is known for its genetic instability. This is due to the presence of low copy sequence repeats (LCRs) that can result in recombination defects causing “22q11.2 deletion syndromes” [38]. Intriguingly, these repeats contain two partial copies of the PI4KA gene containing most (but not all) exons (exon 24 and from exon 33 upward) comprising part of what encodes the PI4KIII α catalytic domain. These observations are even more revealing in light of the high expression of PI4KA transcript (together with smaller transcript variants) in the K562 erythroleukemia cell line along with a number of other genes, all of which are located in the 22q11.2 locus. It is also notable that several genes that are found highly expressed in correlation with PI4KA are located in 22q11.2 and are involved in FGF signaling (see Table I). CRKL is a member of the Crk family of adaptor proteins that is a downstream target of Fgf receptors 1 and 2 and is a target of tyrosine phosphorylation by the BCR-Abl tyrosine kinase in CML cells [39, 40]. THAP7 is a histone tail-binding protein that represses transcription by recruiting the HDAC3 histone deacetylase to transcriptional complexes [41] and a close relationship between Fgf signaling and histone deacetylation has been documented [42, 43]. In this context it is notable that downregulation of PI4KIII α in zebrafish embryos results in defective Fgf signaling [44]. However, it will require further studies to determine whether PI4KA haploinsufficiency contributes to some of the symptoms associated with 22q11.2 deletion syndromes.

In summary, the present study shows that the functionally relevant PI4KIII α protein is the larger isoform 2 and that isoform 1, even if exists, is present at undetectable levels and encodes a catalytically inactive protein. These studies help to clarify the significance of the distinct isoforms listed in the GenBank and to draw attention to this enzyme as a potentially important candidate in several forms of human diseases associated with the human Chr22q11 region.

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ABBREVIATIONS

Phosphatidylinositol

PtdIns

phosphatidylinositol 4-phosphate	PtdIns4P
phosphatidylinositol 4,5-bisphosphate	PtdIns(4,5)P ₂
phosphatidylinositol 4 kinase type IIIα	PI4KIIIα

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Highlights

- PI4KA, the gene encoding phosphatidylinositol 4-kinase III α is surrounded by two partial pseudogenes on Chr22q11.
- Most human tissues and cancer cells do not express transcripts corresponding to isoform 1 of PI4KIII α .
- K562 proleukemia cells show high levels of full and partial PI4KIII α transcripts as well as several other genes in the vicinity of PI4KA suggesting chromosome amplification.
- The shorter, isoform 1 of PI4KIII α cannot be detected at the protein level.
- Expressed isoform 1 of PI4KIII α shows no catalytic activity and cannot rescue hepatitis C viral replication in cells depleted in endogenous PI4KIII α .

Figure 1. Genomic organization of the PI4KIIIα gene

The PI4KA gene is located on human chromosome 22, the black box indicates the q11.2 region. (A) The structural relationship between the two isoforms at the protein level is shown indicating the location of the functional domains. As indicated in the exon-intron organization of the PI4KA gene. In addition to the PI4KA gene, this chromosomal region contains two pseudogenes (PIKAP1 and -2), located centromeric and telomeric direction relative to PI4KA. The dark blue boxes represent exons. (B) The 22q11.2. locus, showing the relative positions of genes around PI4KA and the PIKAP pseudogenes. The green line indicates the site and extent of the most common deletion (typical deletion region, TDR) causing 22q11.2 deletion syndromes.

Figure 2. Expression of PI4KA transcripts in human tissues and cell lines

(A) Northern analysis of PI4KA transcripts in human tissues and selected cancer cell lines. Note the prominent appearance of shorter transcripts in K562 cells but not in normal tissues or most other cancer cells. The arrow shows the full-length transcript coding for isoform2 of PI4KIII α . It is not clear whether the shorter transcripts are transcribed from the pseudogenes or they also contain those coding for isoform1. This Northern blot was performed on the commercially available membrane for which equal loading has been documented by the manufacturer.

Figure 3. Expression and cellular localization of the PI4KIIIα protein

(A) Western analysis of total cell lysates from COS-7 cells expressing an HA-tagged version of isoform1 or -2 of PI4KIIIα. These samples were probed with an anti HA antibody and two different PI4KIIIα antibodies. (B) Detection of isoform 2 by the NEP antibody in various rat tissues (10 µg protein/lane). (C) siRNA-mediated-down of PI4KIIIα in COS-7 and K562 cells. Cells were treated with control siRNA or two targeting siRNA duplexes acting on different regions (numbers indicate nucleotides). Whole cell lysates were prepared from COS-7 and K562 cells and Western blot analysis was performed using the NEP polyclonal antibody. The diagram shows the quantification of band 1 corresponding to isoform 2 from two experiments one of which is shown on the Western-blot. Bands 2 and -3

were also quantified but they showed no change with either of the duplexes (not shown). In both cell lines only band 1, representing the full-length PI4KIII α enzyme shows siRNA specific decrease in expression.

Figure 4. Expression and catalytic activity of the two isoforms of PI4KIIIα

N-terminally HA-epitope tagged versions of the two isoforms of PI4KIIIα were expressed in COS7 cells. Whole cell lysates were immunoprecipitated with an anti HA-antibody and analyzed with Westernblotting with either anti-HA or anti-PI4KIIIα (NEP) antibodies. PI4K activity was measured on the anti-HA immunoprecipitates from the transfected COS-7 cells. Isoform 1 showed no detectable PI4K activity. (B) Inhibition of HCV replication by silencing of endogenous PI4KIIIα is rescued by expression of the full-length PI4KIIIα isoform but not by the short splice variant. The OR6 cell line, which contains a full-length genotype 1b HCV replicon with a *Renilla* luciferase reporter gene, was transduced with MMLV retroviral vectors encoding GFP, full-length PI4KIIIα (PI4K230), or the short PI4KIIIα variant (isoform1). The cells were then transduced 24 hr later with lentiviral vectors encoding a nontargeting shRNA (gray bar) or a shRNA targeting the PI4KIIIα 3'UTR (black bars). (A) Cells were assayed 96 hr after lentiviral transduction for *Renilla* luciferase activity. Values are means $\pm$ SD (n=3) (B) Immunoblotting of OR6 cells with anti-FLAG (upper panel) and actin (lower panel) antibodies demonstrates expression of the 3XFLAG-tagged isoform2 and isoform1 proteins. Two similar experiments were performed in triplicates with identical results.

Table 1
Correlation of gene expression with the PI4KIII α transcript based on 60 cancer cell lines.

Fragment Name	Known Gene Symbol	Accession number	Known Gene Name	Correlation coefficient	Chr. location
207081_s_at	PI4KA (PIK4CA)	NM_002650	phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	1	22q11.2
213408_s_at	PI4KA (PIK4CA)	AK024034	phosphatidylinositol 4-kinase, catalytic, alpha polypeptide	0.833508411	22q11.2
212180_at	CRKL	AK000311	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	0.747069964	22q11.2
218327_s_at	SNAP29	NM_004782	synaptosomal-associated protein, 29kD	0.699617306	22q11.2
218492_s_at	THAP7	NM_030573	inhibitor of histone acetylation	0.69880667	22q11.2
201834_at	PRKAB1	BC001007	protein kinase, AMP-activated, beta 1 non-catalytic subunit	0.653240376	12q24.1
204126_s_at	CDC45L	NM_003504	CDC45 cell division cycle 45-like (S. cerevisiae)	0.647949355	22q11.2