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A novel murine CTP:phosphoethanolamine cytidylyltransferase splice variant is a post-translational repressor and an indicator that both cytidylyltransferase domains are required for activity

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ABSTRACT

CTP:phosphoethanolamine cytidylyltransferase (Pcyt2) has an important regulatory function in biosynthesis of the membrane phospholipid phosphatidylethanolamine. We previously determined that the full-length Pcyt2α and its splice variant Pcyt2β are the main active isoforms of this enzyme. Here we report that mouse *Pcyt2* could be spliced at Introns 7 and 8 to produce a unique third isoform, Pcyt2γ, in which the second cytidylyltransferase domain at the C-terminus becomes deleted. *Pcyt2γ* is ubiquitously expressed in embryonic and adult mouse tissues, and is the most abundant in the kidney, skeletal muscle and testis. *Pcyt2γ* splicing mechanism dominates over *Pcyt2β* exon-skipping mechanism in most examined tissues. Although Pcyt2γ maintains the N-terminal cytidylyltransferase domain as most cytidylyltransferases, the lack of the C-terminal cytidylyltransferase domain causes a complete loss of catalytic activity. However, Pcyt2γ interacts with the active isoform, Pcyt2α, and significantly reduces Pcyt2α homodimerization and activity. The inactive N-domain (H35Y, H35A) and C-domain (H244Y, H244A) mutants of Pcyt2α also reduce Pcyt2α homodimerization and activity. This study revealed the importance of both cytidylyltransferase ^{35}HYGH and ^{244}HIGH motifs for the activity of murine Pcyt2α and established that the naturally occurring splice variant Pcyt2γ has a function to restrain the enzyme activity through the formation of unproductive enzyme complexes.

1. Introduction

CTP:phosphoethanolamine cytidylyltransferase (Pcyt2) plays an important regulatory role in de novo biosynthesis of phosphatidylethanolamine (PE) by the CDP-ethanolamine (Kennedy) pathway. The Kennedy pathway is initiated by ethanolamine kinase (EK) that phosphorylates ethanolamine to produce phosphoethanolamine (P-Etn). The subsequent reaction between P-Etn and CTP is mediated by Pcyt2 and results in the production of CDP-ethanolamine (CDP-Etn) and pyrophosphate. The final step of the pathway is carried out by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) to produce PE from CDP-Etn and diacylglycerol. PE is the most abundant lipid in the cytoplasmic leaflet of cellular membranes. It has important roles in cellular processes including protein folding and lipidation (Deleault et al., 2012; Girardi et al., 2011; Pereira et al., 2012), membrane fusion (Deeba et al., 2005), cell cycle (Emoto et al., 1996), autophagy (Ichimura et al., 2000; Pereira et al., 2012), and apoptosis (Emoto et al., 1997). We recently reviewed the most current state of knowledge on the regulatory role of Pcyt2 in PE homeostasis and pathologies including the metabolic syndrome and cancer (Pavlovic and Bakovic, 2013).

Alternative splicing is considered to be the most plausible explanation for the apparent paradox that evolutionary distinctive organisms resemble in the number of their gene products. There are four major types of alternative splicing: exon-skipping, alternative usage of 5′-donor site or 3′-acceptor site and intron-retention (Galante et al., 2004). The most frequent splicing mechanism is the exon-skipping while the intron-retention represents the least established mechanism (Galante et al., 2004). There are also many examples of alternative splicing in which two or more donor splice sites compete for a single acceptor site (Eperon et al., 2000). Intron-retention and usage of an alternative donor splice site can cause a frame-shift and the formation of a premature stop-codon; this will generate a truncated protein product that can interfere with normal protein function (Gervois et al., 1999; Kim et al., 2006; Lundell et al., 2007). There are numerous examples of the alternatively spliced products that impose a dominant negative effect through the formation of nonproductive complexes with their functional partners

Abbreviations: Pcyt2, CTP:phosphoethanolamine cytidylyltransferase; PE, phosphatidylethanolamine; EK, ethanolamine kinase; P-Etn, phosphoethanolamine; EPT, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; PfECT, *Plasmodium falciparum* Pcyt2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCT, CTP:glycerol-3-phosphate cytidylyltransferase; CCT1, CTP:phosphocholine cytidylyltransferase 1; HMGCS1, HMG-CoA synthase; LDLR, LDL receptor; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; PCSK9, proprotein convertase subtilisin/kexin type 9; ASM, acid sphingomyelinase.

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leading to misfolding and degradation of the complexes (Klemm et al., 1998; Li et al., 2012; Mezghrani et al., 2008). According to the GenBank database human and rodent Pcyt2 genes encode multiple transcripts, which can be recognized as combinations of the internal, 5′-end, and 3′-end splice site variants. The only fully characterized variants are the longest Pcyt2α that is produced by canonical splicing, and Pcyt2β that is produced by skipping of the centrally located Exon 7 in Pcyt2α (Poloumienko et al., 2004; Tie and Bakovic, 2007).

Both Pcyt2α and -β naturally associate as homo- (α/α and β/β) or heterodimers (α/β) (Tie and Bakovic, 2007) and dimerization is critical for catalysis by other cytidylyltransferases (Lee et al., 2009; Veitch et al., 1998; Weber et al., 1999). However, all cytidylyltransferases except Pcyt2 have one catalytic domain, containing the CTP binding motif HXGH. Unique feature of Pcyt2 is that it has two cytidylyltransferase domains each with its own HXGH motif. Similar to other cytidylyltransferases, the functional role of the N-terminal HXGH motif of Pcyt2 is assumed to be the CTP binding site (Veitch et al., 1998; Weber et al., 1999). The presence of the second putative catalytic motif has been known since the initial cloning of the yeast, human, rat and murine Pcyt2 (Bladergroen et al., 1999; Min-Seok et al., 1996; Nakashima et al., 1997; Poloumienko et al., 2004). Two Km values were measured in the kinetic studies of Pcyt2 partially purified from rat liver implicating that both catalytic motifs contribute to the enzyme activity (Vermeulen et al., 1994). On the other hand, redundancy of the second catalytic motif was recently reported in a study of *Plasmodium falciparum* Pcyt2 (PfECT) ^{419}HIGH motif (Maheshwari et al., 2013).

Here we identify a natural splice variant Pcyt2γ that is ubiquitously expressed in all adult mouse tissues and in embryonic tissues during development. We establish that Pcyt2γ is a product of a specific splicing mechanism different from the exon-skipping that leads to the production of Pcyt2β. Pcyt2γ is derived by an alternative usage of 5′ donor site in Introns 7 and 8 while maintaining the same 3′-acceptor site as in the case of Pcyt2α. Consequently, as a derivative of Pcyt2α, Pcyt2γ maintains the first cytidylyltransferase domain including the N-terminal ^{35}HYGH motif and parts of the central linker region. Pcyt2γ lacks the second cytidylyltransferase domain including the second CTP binding motif, ^{244}HIGH, and it is catalytically inactive. However, Pcyt2γ retains the ability to interact with Pcyt2α and to dramatically reduce its homodimerization and enzyme activity. The site-directed mutagenesis of Pcyt2α revealed the necessity of both ^{35}HYGH and ^{244}HIGH motifs for the catalytic function. Similarly to Pcyt2γ, Pcyt2α mutants were inactive and inhibited Pcyt2α activity and homodimerization. Altogether the study demonstrates the importance of both cytidylyltransferase domains for the enzyme function, the critical role of dimerization, and the role of the natural variant Pcyt2γ to restrain the activity of Pcyt2α through dominant-negative interactions.

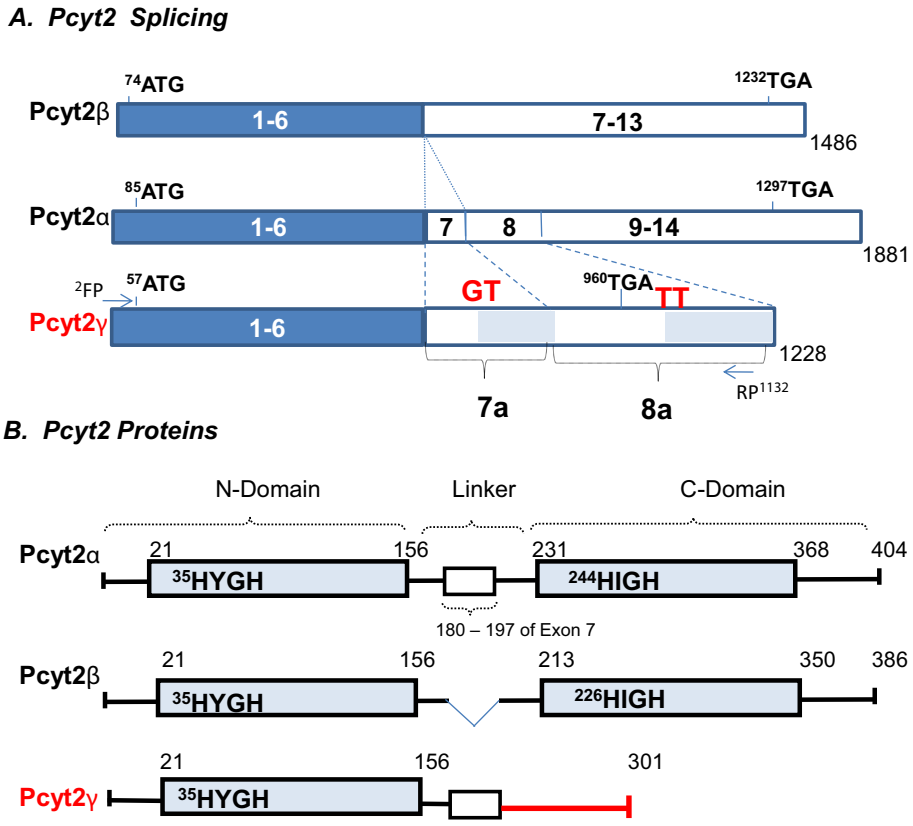

Fig. 1. Splicing mechanisms and the structure of the murine *Pcyt2γ*. (A) Schematic representation of *Pcyt2α* mRNA (1881 bp) and alternatively spliced variants *Pcyt2β* (1486 bp) and *Pcyt2γ* (1228 bp); *Pcyt2α* transcript is the longest and is composed of 14 Exons. *Pcyt2β* transcript is made by Exon 7 skipping mechanism and retains all other Exons present in *Pcyt2α*; *Pcyt2γ* transcript resembles *Pcyt2α* in sharing Exons 1–6 (blue) and retaining the Exon 7 and Exon 8 (white) sequence. Intron 7 and Intron 8 (not shown) are 428 and 379 bp, respectively. In *Pcyt2γ*, alternative 5′ donor sites are used in Intron 7 at position 185-GT (red) and Intron 8 at position 259-TT (red) instead of regular splice sites that generated Exon 7 and Exon 8 in *Pcyt2α*. This new type of splicing changed the 3′ boundary of the Exon 7 and Exon 8 making them longer. Consequently, Pcyt2γ has the specific Exon 7a of 238 bp and Exon 8a of 397 bp. Retention of the intron areas (light blue) also introduced a stop codon in Exon 8a at the position 960 and consequently *Pcyt2γ* does not maintain the rest of the *Pcyt2α* area including Exons 9–14. Indicated are the positions of the *Pcyt2γ*-specific forward primer (Exon 1) and reverse primer (Exon 8a). (B) A schematic representation of Pcyt2 proteins. Pcyt2α and Pcyt2β contain two cytidylyltransferase catalytic folds in N-terminal domain (21-156 aa; boxed) and C-terminal domain (α:231-368 aa; β:213-350 aa; boxed). Pcyt2α (404 aa) and Pcyt2β (386 aa) only differ in the length of the central linker segment where 18 amino acids (box) encoded by Exon 7 are absent due to exon skipping in Pcyt2β. Their cytidylyltransferase folds contain two CTP binding sites, $^{\alpha/\beta\text{-}35}$HYGH in N-domain and $^{\alpha\text{-}244/\beta\text{-}226}$HIGH in C-domain. The novel variant Pcyt2γ (301 aa) maintains the first cytidylyltransferase fold including the N-terminus ^{35}HYGH motif and parts of the central linker region including the Exon 7 of Pcyt2α. Pcyt2γ lacks the C-terminus cytidylyltransferase domain including the HIGH motif and forms a new C-terminus tail (red) of completely unique composition.