Searching for Alternatively Spliced Variants of Phospholipase Domain-Containing 2 (Pnpla2), a Novel Gene in the Retina
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Abstract

Purpose: Ensembl and other expressed sequence tag (EST) databases reveal putative alternative splice variants in mouse and rat for Pnpla2, the gene encoding pigment epithelium-derived factor-receptor (PEDF-R). The purpose of this study was to obtain experimental evidence for Pnpla2 splice variants in mouse.

Materials and Methods: Cultures of a mouse cell line derived from photoreceptors (661W cells) and mouse eye, heart, adipose, kidney, and liver tissues were used. Messenger RNA (mRNA) was isolated from cells and tissues, and complementary DNA (cDNA) was synthesized. Polymerase chain reaction (PCR) primer pairs were designed to flank the putative splice sites. Exon exclusion real-time PCR was used to reduce amplification of the full-length Pnpla2 transcript and enhance amplification of low abundant splice variants. PCR products were resolved by agarose gel electrophoresis and detected with a UV transilluminator. Recombinant plasmids containing a human full-length Pnpla2 cDNA or a Pnpla2Δ5cDNA lacking exon 5b (E5b) were controls to validate the techniques. Total cell lysates from 661W cells were prepared. PEDF-R protein detection was performed using western blots.

Results: PCR products for Pnpla2 transcripts obtained from 661W cells or various mouse tissues resolved into a single band following amplification with multiple primer pairs. Simultaneous amplification of two Pnpla2 cDNAs at various molar ratios prevented the detection of lower abundant transcripts. However, even when the cDNA for the full-length Pnpla2 transcript was significantly excluded using the exon exclusion method, no bands corresponding to Pnpla2 splice variants were detectable. Nonetheless, western blots of total 661W cell lysates with two different antibodies revealed isoforms for the PEDF-R protein.

Conclusions: The data provide evidence for the existence of a single, full-length Pnpla2 transcript that could give rise to a single protein product that undergoes posttranslational processing.

Keywords: Pnpla2; Alternative splicing; PEDF; PEDF-R; Retina

Introduction

In recent years, a group of genes encoding proteins with a common domain termed patatin-like phospholipase (PNPLA domain) has been discovered in the human genome. There are nine members of the PNPLA family, all of which display lipase, phospholipase and transacylase enzymatic activities and have major roles in adipocyte differentiation, lipid metabolism and signaling [1,2]. One PNPLA gene, PNPLA2 codes for a protein that is present at high levels in adipose tissue as a triglyceride lipase involved in lipid turnover [3]. Interestingly, we have reported that the retina expresses PNPLA2 and its gene product PEDF-R throughout the retinal pigment epithelium, photoreceptors, and the ganglion cell layer [4-7]. PEDF-R acts as a cell-surface receptor for pigment epithelium-derived factor (PEDF) [8], a key factor for the neural and vascular retina [9-11]. It exhibits phospholipase activity that hydrolyzes the sn-2 acyl bond of phospholipid substrates to release lysophospholipid and fatty acids [4,5,8]. PEDF binding stimulates the PLA activity of PEDF-R [4,5,8], and in turn its fatty acid products can act as bioactive lipid second messengers to trigger downstream antiapoptotic signaling in retina cells [12]. The PEDF-R polypeptide sequence has a patatin-like phospholipase domain towards its amino end (10-179) and amino acids serine in position 47 (Ser47) and aspartic acid in position 166 (Asp166) form the catalytic dyad of the enzymatic active site [6-8]. Recently, we have mapped a functional PEDF binding region in PEDF-R (Threonine 210 (Thr210) to Leucine 232 (Leu232)) located on exon 5b of human PNPLA2 (Figure 5A) [8]. Moreover, the PNPLA2 gene plays a crucial role in human embryonic stem cell self-renewal [13], human melanoma metastasis inhibition [14], and human prostate cell growth inhibition [15], all of which depend on PEDF.

Alternative splicing is a common posttranscriptional process for protein diversification, with the majority of the human genes potentially giving rise to multiple variants and thus creating protein isoforms [16]. The human PNPLA2 gene has ten exons of which exons 2-10 are the coding exons and the mouse Pnpla2 gene contains nine exons, with a coding capacity of 504 and 486 amino acids, respectively. The PNPLA2 sequence is highly conserved among the mammalian species, with mouse and human species having 87% identity. Alternatively spliced Pnpla2 variants are predicted in mouse: one with partial E4 exon deletion (E4a) (Pnpla2Δ4a; base pairs 487-589), another with an exon 6 (E6) deletion (Pnpla2Δ6; base pairs 758-925) and a third with both the E4a and E6 deletions in the same transcript (Pnpla2Δ4aΔ6) (Figure 1A). The first alternative splice transcript would generate a protein without Asp166 of the catalytic dyad, implying an inactive PEDF-R that could have implications in disease progression where PEDF-R is unable to mediate the effects of PEDF. The second one encodes part of

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the extracellular domain of PEDF-R; however, it is unclear what the functional importance of E6 is to PEDF-R activity. While the E6 region of mice (E7 of human) is not necessary for the binding of PEDF, the possibility that this region plays an indirect role in interactions with PEDF remain to be explored.

Although databases predict alternative splicing for the Pnpla2 mRNA in mouse, it is not yet known whether multiple variants exist. It is of interest to obtain empirical evidence for alternative splice variants to understand the regulation of PEDF-R, which would have an impact on PEDF activity. In this study, we used RT-PCR-based methods to explore the predicted alternative splicing of Pnpla2. For this purpose, we used 661W cells, a mouse cone photoreceptor cell line that has been shown to respond to PEDF treatment in the event of light damage [17] and also mouse eye, heart, adipose, liver, and kidney tissues. With the tested samples, one main Pnpla2 transcript was detected.

Materials and Method

Cell culture

Photoreceptor-derived 661W cells (kind gift from Dr. Muayyad Al-Ubaidi, University of Oklahoma Health Sciences) were cultured in DMEM medium with 10% of fetal bovine serum (FBS) at 37°C with 5% CO2 and 95% humidity.

Expression vector/plasmid

Two PNPLA2-containing expression vectors were used: PEDF-R, containing the full-length human PNPLA2 open reading frame (ORF) of 1512 bp and PEDF-RAE5b, containing a human PNPLA2 cDNA missing the 90 base pairs of exon 5b (E5b; bp 607-696). PNPLA2 cDNA for PEDF-R and PEDF-RAE5b were constructed into pEXP1-DEST vectors with N-terminal epitope-tags (N-terminal Hist6/Xpress) under a T7 transcription promoter as previously described [4].

RNA extraction, cDNA synthesis and real-time PCR

For 661W cells, total RNA was purified using the RNeasy™ mini kit (Qiagen) according to the manufacturer’s instructions. For tissue samples (kind gift from Dr. Lars Von Buchholtz, NIDCR, National Institutes of Health), total RNA was isolated from fresh mouse tissues using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA concentrations were determined using the Beckman DU 640 Spectrophotometer. The mRNA (1-5 μg) was reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen) in a total volume of 20 μL.

Pnpla2 transcript was amplified in a total volume of 25 μL containing 400 nM forward primer, 400 nM reverse primer, 2X SYBR Green mix (Qiagen), and 2 μL cDNA in the Bio-Rad Chromo4 real-time system. All primers (Table 1) were custom synthesized by Invitrogen. The thermal cycling conditions were 95°C for 15 min, then 46 cycles of 95°C for 30 s to 1 min, 60°C for 30 s to 1 min, and 72°C for 30 s to 1 min.

Exon exclusion

The restriction endonucleases AcuI (New England BioLabs, R0641S), MfeI (New England BioLabs, R0589S), and BstEI (New England BioLabs, R0162S) were used. Deoxynucleotides (custom synthesized by Invitrogen) were designed with sequences complementary to regions within E4a (5’-TGAGGTGGAGGAGGGATGAGCTGAAGAAT-3’), E5 (5’-CTGGAGAGGAGGATGAGCTGAAGAAT-3’), E6 (5’-CTGGAGAGGAGGATGAGCTGAAGAAT-3’), or E5b (5’-CACGAGGTCGCTACAAACACCAGCTACCC-3’), included the cleavage recognition sites (shown in bold) and, to prevent PCR amplification, contained two unpaired nucleotides at the 3’ end (shown in lower case). As in Wang et al. [18], the deoxynucleotides (1 μM) were annealed by mixing with cDNA in a total volume of 20 μL and heating at 94°C for 2 min, 85°C for 15 min, 70°C for 15 min, 55°C for 15 min and 25°C for 15 min. Restriction enzymes (12.5 - 25 units) or equal volume of water (for “buffer” controls) were added with the provided digestion buffer and incubated at 37°C for 1 hour. CutSmartTM buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 μg/mL BSA, pH 7.9) was used for restriction digestion reactions. Following the restriction digest 2 μL of the product was used for RT-PCR amplification. While Pnpla2 has only one cleavage recognition site for MfeI, it has two for BstEI and AcuL. Amplification primer pairs (PPI or J) were chosen such that the additional site was excluded. Both primer pairs had the same forward primer (Figure 1B).

Agarose gel electrophoresis

PCR products were diluted 1:5 or 1:10 and a total of 20 μL was loaded into either 1.2% or 2% agarose E-gels® (Invitrogen). The gels, containing ethidium bromide, were run for 30-45 minutes using the E-Gel PowerBase™ v.4 (Invitrogen). Photographs were taken with a UV transilluminator. The TrackIt™ 100 bp DNA ladder (Invitrogen) or the 1Kb Plus DNA ladder (Invitrogen) was used to estimate molecular weights of DNA fragments.

Recombinant protein, cell lysis preparation and western blotting

Total cell lysate from 661W cells was prepared with RIPA buffer...
immuno-Blot

Total protein detection in nitrocellulose membranes was accomplished with Ponceau Red. Nitrocellulose membranes were incubated in blocking solution for 1 hour at room temperature and then in blocking solution with one of the following PEDF-R antibodies: 1:10,000 (Invitrogen, cat#R910-25), primary dilution was 1:10,000 in 1% BSA in TBS-T and secondary dilution was 1:200,000 of HRP-conjugated goat anti-mouse. Chemiluminescence detection was performed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) on X-Ray films.

Polyacrylamide Gel Electrophoresis

Protein samples were applied to NuPAGE 4-12% polyacrylamide gels in Bis-Tris buffer with NuPAGE MOPS SDS as running buffer (Invitrogen). After electrophoresis, proteins from the gel were transferred to nitrocellulose membranes using the iBlot Gel Transfer (Invitrogen). After electrophoresis, proteins from the gel were separated from the soluble by centrifugation (14,000 X g, 4°C, 5 min) and the pellet was resuspended in SDS-PAGE sample buffer.

Table 1: Primer pairs used for PCR amplification of Pnpla2/PNPLA2 transcripts.

| Primer Pair Name | Primer Sequences* | Template* | Expected PCR Product Size (bp)* |
|------------------|-------------------|-----------|---------------------------------|
| PPB              | 5'-tgtgcctcattcctctac-3' | PNPLA2    | 217                             |
|                  | 5'-tcgagaggtgagatgtg-3'  | PNPLA2Δ4a  | 115                             |
|                  | 5'-ctccagcatgtgcatgatc-3' | PNPLA2Δ4a  | 386                             |
|                  | 5'-aaacggatggtgaaggacaco-3' | PNPLA2Δ6   | 193                             |
| PPC              | 5'-tcgagaggtgagatgtg-3'  | PNPLA2Δ4a  | 608                             |
|                  | 5'-tcgagaggtgagatgtg-3'  | PNPLA2Δ4a  | 506                             |
|                  | 5'-tcgagaggtgagatgtg-3'  | PNPLA2Δ4a  | 440                             |
|                  | 5'-tcgagaggtgagatgtg-3'  | PNPLA2Δ4a  | 338                             |
| PPD              | 5'-tctccctcatactcactcag-3' | PNPLA2Δ6    | 923                             |
|                  | 5'-tctccctcatactcactcag-3' | PNPLA2Δ6    | 857                             |
|                  | 5'-tctccctcatactcactcag-3' | PNPLA2Δ6    | 755                             |
| PPH              | 5'-aacgccactacacactcag-3' | PNPLA2Δ4aΔ6 | 1025                            |
|                  | 5'-aacgccactacacactcag-3' | PNPLA2Δ4aΔ6 | 1025                            |
|                  | 5'-aacgccactacacactcag-3' | PNPLA2Δ4aΔ6 | 1025                            |
| PPI              | 5'-acggatatccgcatgtc-3'  | PNPLA2Δ6   | 1025                            |
|                  | 5'-acggatatccgcatgtc-3'  | PNPLA2Δ6   | 1025                            |
|                  | 5'-acggatatccgcatgtc-3'  | PNPLA2Δ6   | 1025                            |
| PPJ              | 5'-atctccctcatactcactcag-3' | PNPLA2Δ4a  | 888                             |
|                  | 5'-atctccctcatactcactcag-3' | PNPLA2Δ4a  | 888                             |
|                  | 5'-atctccctcatactcactcag-3' | PNPLA2Δ4a  | 888                             |

*Sequence of forward (top) and reverse (bottom) primers for each primer pair. (Note, PPB and D have the same forward primer, PPC and D have the same reverse primer, and PPH and PPI have the same reverse primer) Bolded and underlined letters indicate base pairs, which deviate in the human PNPLA2 sequence in PPH and I.

Results

Potential alternative splice transcripts for Pnpla2

Databases such as the Ensembl (http://useast.ensembl.org/index.html) and the UCSC genome browser (http://genome.ucsc.edu/) reveal two potential alternatively spliced Pnpla2 transcripts in the mouse genome [19,20]. These predictions are based on expressed sequence tags (ESTs), which are short (200-800 base pairs) single-pass sequence reads that contains partial coverage and thereby possible sequence errors [21]. For the mouse sequence, one potential transcript has an E4a deletion (Pnpla2Δ4a; bp 487 - 589), the other has an E6 deletion (Pnpla2Δ6; bp 758 - 925) and a third has both E4a and E6 deletion (Pnpla2Δ4aΔ6). Figure 1A shows a scheme of the predicted Pnpla2 mRNAs (Pnpla2Δ4a, Pnpla2Δ6 and Pnpla2Δ4aΔ6). The above observations led us to explore if the potential alternative splice transcripts for mouse Pnpla2 exist in vivo.

Pnpla2 transcript from 661W cells

To empirically determine the expression of Pnpla2 transcripts in mouse, we performed RT-PCR using cDNAs from mRNA of 661W cells. Figure 1B shows primer pairs designed to amplify Pnpla2 cDNAs by RT-PCR. Primer pairs (PP) B, C, and D were used to detect full...
length \textit{Pnpla2} as well as the putative spliced \textit{Pnpla2Δ4a}, \textit{PNPLA2Δ6}, and \textit{Pnpla2Δ4aΔ6} transcripts. PPD has the same forward primer as PPB and the same reverse primer as PPC. Amplification of cDNA fragments was expected to result in discrete and distinct lengths for each primer pair (Table 1). Figure 2 shows PCR products with the three primer pairs and 661W cDNA separated by gel electrophoresis, and reveals that each primer pair amplified a single band that migrated as a DNA fragment of the expected size for \textit{Pnpla2}. Our data show a single, full-length \textit{Pnpla2} transcript in 661W cells.

**\textit{Pnpla2} transcript from mouse tissues**

The UCSC genome browser suggests that \textit{Pnpla2} splice variants were detected in ESTs obtained from mouse mammary tumor (\textit{Pnpla2Δ4a}) or mouse kidney (\textit{Pnpla2Δ6}), and so we hypothesized that the expression of splice variants may be tissue-dependent [20].

To test this hypothesis, we employed the same method as above, using cDNA from mouse eye, heart, adipose, kidney, and liver tissues for amplification. Amplification with PPB, C, or D in mouse tissues was anticipated to result in the same sized PCR products as was expected for the 661W cells (Table 1). We found that amplification using any of the three primer pairs in any of the five tissues yielded a distinct single band of PCR product that migrated as DNA of the size expected for \textit{Pnpla2} (Figure 3). Again, our data show that a single \textit{Pnpla2} transcript was detected in mouse tissues as in 661W cells.

**Limitations of low abundant transcript detection**

As previously described, low abundant alternatively spliced transcripts may not be amplified at the same rate as a more abundant transcript, and would therefore be undetectable [18]. To determine if these limitations apply to our study, we designed an experiment in which cDNAs with deletions to emulate spliced regions were used at various ratios as templates in PCR reactions. Expression plasmids containing the full-length human \textit{PNPLA2} cDNA (PEDF-R) and a deletion of 90 base pairs of the 3' end region of human exon 5 (mouse exon 4) (PEDF-RAESb) were used as templates to validate our assay (Figure 4A). Two new primer pairs were designed (PPH and I) to be as homologous as possible to mouse and human sequences of \textit{Pnpla2}/\textit{PNPLA2}. The primer pairs have different forward primers and share the same reverse primer (Figure 4B). The sequences of the two forward primers and the reverse primer were identical to the mouse \textit{Pnpla2} sequence, but each primer diverged by a single base pair from the human \textit{PNPLA2} sequence. Nonetheless, both PPH and PPI amplified mouse and human \textit{Pnpla2}/\textit{PNPLA2} transcripts from expression plasmids. Plasmids PEDFRAESb and PEDF-R were mixed in various molar ratios: 1:1 (5 ng PEDF-R to 5 ng PEDF-RAESb), 1:5 (2 ng PEDF-RAESb, 8 ng PEDF-R), 1:10 (1 ng PEDF-RAESb, 9 ng PEDF-R), or 1:20 (0.5 ng PEDF-RAESb, 9.5 ng PEDF-R) molar ratios prior to PCR amplification with PPH (C) or PPI (D). Also shown is amplification with specified amounts (0.1 or 2 ng) of each plasmid alone with PPH or PPI. E) Amplification of \textit{Pnpla2} transcripts from 661W cells as well as mouse eye and liver tissues was tested with both PPH (E) and PPI (F). For C-E, PCR products were diluted 1:10, resolved by 1.2% agarose gel electrophoresis, and stained with ethidium bromide. Photographs of UV exposed gels are shown. Numbers indicate migration pattern of DNA ladder. See Table 1 for expected product sizes.

![Figure 2](image1.png)

**Figure 2:** RT-PCR of \textit{Pnpla2} transcript from 661W cells. Amplification of \textit{Pnpla2} cDNA from 661W cells using PPB, C, or D was performed as in methods. PCR products were diluted 1:10 and resolved by 2% agarose gel electrophoresis. DNA was stained with ethidium bromide. Photograph of the UV exposed gels is shown. Numbers on left indicate migration pattern of DNA ladder (L). Primer pairs used for each PCR product are indicated at the top. See Table 1 for expected product sizes.

![Figure 3](image2.png)

**Figure 3:** RT-PCR of \textit{Pnpla2} transcript from mouse tissues. Amplification of \textit{Pnpla2} cDNA from mouse eye, heart, adipose, kidney, and liver tissues using primer pair (PP) B, C, or D and analyzed as in Figure 2. Numbers indicate migration pattern of DNA ladder (L). See Table 1 for expected product sizes.

![Figure 4](image3.png)

**Figure 4:** Detection limits of low abundant transcripts. A) Schematic of the open reading frame of full-length human \textit{PNPLA2} cDNA (PEDF-R) or a \textit{PNPLA2} cDNA lacking E5b (PEDF-RAESb) expression plasmid. B) Location of primer pairs (PP) H and I on the full-length human \textit{Pnpla2} transcript. The location for the primer pairs on the mouse \textit{Pnpla2} transcript are in Figure 1B, 1C and 1D) The plasmids were mixed in 1:1 (5ng each), 1:5 (2ng PEDF-RAESb, 8 ng PEDF-R), 1:10 (1 ng PEDF-RAESb, 9 ng PEDF-R), or 1:20 (0.5 ng PEDF-RAESb, 9.5 ng PEDF-R) molar ratios prior to PCR amplification with PPH (C) or PPI (D). Also shown is amplification with specified amounts (0.1 or 2 ng) of each plasmid alone with PPH or PPI. E,F) Amplification of \textit{Pnpla2} transcripts from 661W cells as well as mouse eye and liver tissues was tested with both PPH (E) and PPI (F). For C-E, PCR products were diluted 1:10, resolved by 1.2% agarose gel electrophoresis, and stained with ethidium bromide. Photographs of UV exposed gels are shown. Numbers indicate migration pattern of DNA ladder. See Table 1 for expected product sizes.
(661W cells, eye tissue, and liver tissue) to detect Pnpla2 transcript. In all three samples of cDNA, with either PPH or PPI, a single DNA of the size expected for Pnpla2 (Table 1) was detected (Figure 4E–4F), implying a single transcript.

Exon exclusion for cleavage of full-length Pnpla2/PNPLA2 transcript

To reduce competition between the multiple Pnpla2/PNPLA2 transcripts, we used the exon exclusion method in which cDNA amplification of the highly abundant transcript is limited by endonuclease digestion at a chosen exon. It was expected that introduction of the restriction digestion step would significantly reduce competition and allow amplification of the low abundant transcripts that do not have the chosen exon [18]. We identified that the human E5b region of PNPLA2 has a BstEII restriction recognition site (Figure 5A), which is missing in PEDF-RAE5b plasmid. When a 1:5 molar ratio of PEDF-RAE5b to PEDF-R (4 ng to 1 ng) was amplified and resolved by gel electrophoresis, the PCR reaction yielded products of ~880 bp (size expected for PNPLA2) and ~790 bp (size expected for PNPLA2ΔE5b), with the top band appearing significantly more intense than the bottom band (Figure 5B). However, Figure 5B also shows that when the 1:5 molar ratio was treated with BstEII prior to amplification, the bottom band increased in intensity and the top band decreased in intensity. These findings indicate that we successfully decreased PNPLA2 amplification to allow for greater detection of the low abundant PNPLA2ΔE5b transcript.

Exon region E4a and E6 have cleavage recognition sites for AcuI and MfeI enzymes, respectively. When using the AcuI and/or MfeI restriction enzymes on 661W or mouse kidney cDNA, we found similar efficiency in reducing the amplification of the full-length Pnpla2 transcript. Controls with cDNA under the same condition as above in the absence of restriction enzyme (“Buffer” control) yielded a single band as expected for the high abundance full-length Pnpla2. We found that treatment with AcuI and/or MfeI reduced the intensity of the ~808 bp product (size expected for full-length Pnpla2) for 661W (Figure 5C) and for kidney cDNA (Figure 5D). Despite this, no additional band corresponding to DNA fragments of the size expected for Pnpla2Δ4a, Pnpla2Δ6, or Pnpla2Δ4aΔ6 was detected (Figures 5C and 5D), even when loading greater amounts of PCR product (data not shown). Thus, our data point to the presence of a single Pnpla2 transcript in mouse cells and tissues, even after significantly improving the sensitivity of our method for low abundant transcripts by excluding the cDNA of the large transcript.

**Figure 6:** PEDF-R protein in mouse 661W cells. Equal amounts of 661W total cell lysate were run on the same 4-12% polyacrylamide Bis-Tris gel. Nitrocellulose membranes were either stained with Ponceau Red total protein stain (Lane 1) and then probed with AF5365 anti-PDF-R (Lane 3) or stained with Ponceau Red (Lane 2) and then probed with SAB2500132 anti-PEDF-R (Lane 4). Recombinant human PEDF-R was also run on a 4-12% Bis-Tris gel and probed with anti-Xpress (Lane 5). Labels of Ppb (phosphorylase b), BSA (bovine serum albumin), Ova (ovalbumin), and CA (carbonic anhydrase) show the migration pattern of the prestained molecular weight markers.

**Discussion**

In this study, alternative splicing was examined in biological samples because mouse Pnpla2 has two potential alternatively spliced transcripts in silico. Despite the prediction of alternatively spliced PNPLA2 transcripts, we detected only a single Pnpla2 transcript in...
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