



Faculty of Resource Science and Technology

**Molecular Cloning and Expression Study of Proline Rich ENA/VASP
Ligand 2A (PREL-2A) Gene in Zebrafish Development**

Winnie Chuo

(22629)

**Bachelor of Science with Honours
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**Winnie Chuo
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This project is submitted in partial fulfilment of the requirement for the degree of
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DECLARATION

I hereby declare that all work submitted for assessment in this dissertation is my own work and that no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other institution of higher learning. Where other sources of information have been used, they have been acknowledged

Signature:

Date:

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LIST OF ABBREVIATIONS

BPS	Between Pleckstrin Homology and Src Homology 2
DEPC	Diethylpyrocarbonate
Dpf	Day post fertilization
EVH1	Ena/VASP Homology 1
EVH2	Ena/VASP Homology 2
EVL	Ena/VASP like
GM	Grb-Mig-10
Hpf	Hour post fertilization
IPTG	Isopropyl-b-d-thiogalactopyranoside
LPD	Lamellipodin
MRL	Mig10/RIAM/Lpd
PBST	Phosphate Buffered Saline Tween-20
PBS	Phosphate Buffered Saline
PH	Pleckstrin Homology
PREL-2	Proline rich Ena/VASP Ligand 2
RA	Ras-association
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SSC	Standard saline citrate
SH2	Sarcoma Homology 2 domain
SH3	Sarcoma Homology 3 domain
VASP	Vasodilator-stimulated phosphoprotein

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Molecular Cloning and Expression Study of Proline-rich Ena/VASP Ligand 2a (PREL-2a) Gene in Zebrafish Development

Winnie Chuo

Resource Biotechnology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

Proline rich Ena/VASP Ligand 2 (PREL 2) is designated as Lamellipodin (Lpd) in mammals. PREL-2 possesses the ability to colocalize with Ena/VASP proteins at the tips of lamellipodia. Mammalian PREL-2 was found to have significant functions on cell signalling, direct migration, promoting axon guidance, affecting adhesion and regulating lamellipodial dynamics. Zebrafish PREL-2 gene is duplicated into two copies namely, PREL-2a and PREL-2b during a genome duplication event. The expression data of PREL-2 family only available for selected adult organs in human and mice but still no detailed information of PREL-2a in the embryonic stages. In this study, the cloning and expression profile of zebrafish PREL-2a gene was described. Semiquantitative RT-PCR analysis revealed that zebrafish PREL-2a gene is expressed throughout the development. PREL-2a gene expression was first expressed at 1K cells stage (3hpf) and constantly expressed throughout zebrafish development up to 5dpf. The RT-PCR result indicated that PREL-2a gene show maternal and zygotic expression.

Key words: Zebrafish, PREL-2a gene, RT-PCR, gene expression

ABSTRAK

Proline rich Ena/VASP Ligand 2 (PREL 2) ditetapkan sebagai Lamellipodin (LPD) di mamalia. PREL-2 mempunyai keupayaan untuk colocalize dengan Ena / protein VASP di hujung lamellipodia. Mamalia PREL-2 dijumpai memiliki fungsi yang signifikan terhadap isyarat sel, penghijrahan langsung, mempromosikan bimbingan akson, mempengaruhi adhesi dan menetapkan dinamik lamellipodial. Zebrafish PREL-2 gen diulang menjadi dua salinan iaitu, PREL-2a dan PREL-2b selama acara duplikasi genom. Data ekspresi PREL-2 keluarga hanya untuk organ dewasa yang dipilih pada tikus dan manusia, namun masih tidak ada maklumat terperinci tentang PREL-2a dalam tahap embrio. Dalam kajian ini, profil pengklonan dan ekspresi gen PREL-2a zebrafish telah dijelaskan. Semiquantitative RT-PCR analisis menunjukkan bahawa gen PREL-2a zebrafish ekspres sepanjang pembangunan zebrafish. ekspresi gen PREL-2a pertama kali disajikan pada 1K sel (3hpf) dan terus-menerus ekspres sampai 5dpf PREL-2a gen menunjukkan ekspresi ibu dan zigotik.

Kata kunci: Zebrafish, gen PREL-2a, , RT-PCR, ekspresi gen

1.0 INTRODUCTION

Proline rich Ena/VASP Ligand 2 (PREL-2) gene is known as Lamellipodin in mammals. PREL-2/Lamellipodin is identified by Krause *et al.* (2004) as an Ena/VASP homology 1 (EVH 1) ligand of Ena/VASP proteins since both proteins can colocalize in migrating cells at tips of lamellipodia and filopodia. Besides, PREL-2 is also known as Ras-associated and pleckstrin homology domains-containing protein 1 (RAPH1).

PREL-2 has a very long proline-rich region at C-terminus. The C-terminus is harbour eight potential SH3 binding sites, three potential Profilin binding sites and four clusters containing a total of six putative EVH1 (Ena/VASP homology domain 1) binding sites (Krause *et al.*, 2003). Laflaute *et al.* (2004) has proposed it as a part of the 'MRL family' (*Caenorhabditis elegans* Mig-10/Rap1-GTP-interacting adaptor molecules (RIAM)/Lamellipodin (Lpd) family). MRL family proteins and Growth receptor bound 7 (Grb 7) family share a conserved Grb-Mig (GM) domain which consists of Pleckstrin Homology (PH) and Ras-Associating (RA) domain (Manser *et al.*, 1997).

According to Krause *et al.* (2004), PREL-2 can colocalize with Ena/VASP proteins at the distal tips of lamellipodia and filopodia and targets them to the leading edge which required for lamellipodia formation. Overexpression of PREL-2 leads to the faster protrusion of lamellipodial. Conversely, knockdown of PREL-2 expression results in impairs lamellipodia formation and reduce its protrusion velocity. In addition, Krause *et al.* (2004) also proposed that, mammalian PREL-2 have significant functions on cell signalling, direct migration, promoting axon guidance and regulating cell adhesion.

Two copies of PREL-2 gene are found in zebrafish genome, namely PREL-2a and PREL-2b (Lee, 2008) which consistent with the recent genome duplication in ray-finned fish occurred before the teleost radiation (Amores *et al.*, 1998; Postlethwait *et al.*, 1998).

PREL-2a is expressed broadly at midbrain-hindbrain region and ventral hindbrain. During early somitogenesis, PREL-2a was strongly expressed along segmentation and posterior tail. This suggested PREL-2a might play a role in regulating the subdivision of presomitic mesoderm before morphological distinctive somites are formed (Lee, 2008). Thus, it is important to understand the role of PREL-2a in somite development and may suggest a potential role in muscular dystrophy or congenital myopathy. Furthermore, the expression patterns of PREL-2a at early embryonic and adult developmental stages are still unknown.

The research objectives of this study are to clone Proline-rich Ena/VASP Ligand 2a (PREL-2a) gene from zebrafish then identify and study its expression pattern in developing zebrafish by performed RT-PCR analysis.

2.0 LITERATURE REVIEW

2.1 Proline-rich Ena/VASP ligand 2 (PREL-2) gene/ Lamellipodin

Proline rich Ena/VASP Ligand 2 (PREL-2) gene is designated as Lamellipodin in mammals. PREL 2/Lamellipodin is identified by Krause *et al.* (2004) as an Ena/VASP homology 1 (EVH 1) ligand of Ena/VASP proteins since both proteins can colocalize in migrating cells at tips of lamellipodia and filopodia. Besides, PREL-2 is also known as Ras-associated and pleckstrin homology domains-containing protein 1 (RAPH1).

PREL-2 protein possesses a very long proline stretch at C-terminus, which harbours eight potential SH3 binding sites, three potential Profilin binding sites and four clusters containing a total of six putative EVH1 binding sites (Krause *et al.*, 2004).

Lafluyente *et al.* (2004) proposed it as a part of the MRL (Mig-10/RIAM/Lpd) family. MRL family proteins and Grb 7 family share the conserved GM region that consists of Ras-associated (RA) and Pleckstrin Homology (PH) domains (Manser *et al.*, 1997). However, Grb7 family consensus motif; BPS and SH2 domains are absent in MRL family proteins (Figure 1).

According to Krause *et al.* (2004), the proline-dense motifs of PREL-2 could bind up to four Ena/VASP homology EVH1 molecules both *in vitro* and *in vivo*. All the six potential EVH1 binding sites in PREL-2 showed medium to strong binding to EVL. Both PREL-2 and Ena/VASP play a role as mediators of localized membrane signal and colocalize at the tips of filopodia and lamellipodia. Krause *et al.* (2004) reported that

overexpression of PREL-2 increases the lamellipodia protrusion velocity. Besides, PREL-2 was found to have significant functions on cell signalling and direct migration.

Northern blot analysis from Krause *et al*, (2004) study revealed that mammalian PREL-2 is highly expressed in the mouse's brain, ovary and developing embryos whereas the isoform is expressed in the mouse' embryo, ovary and liver.

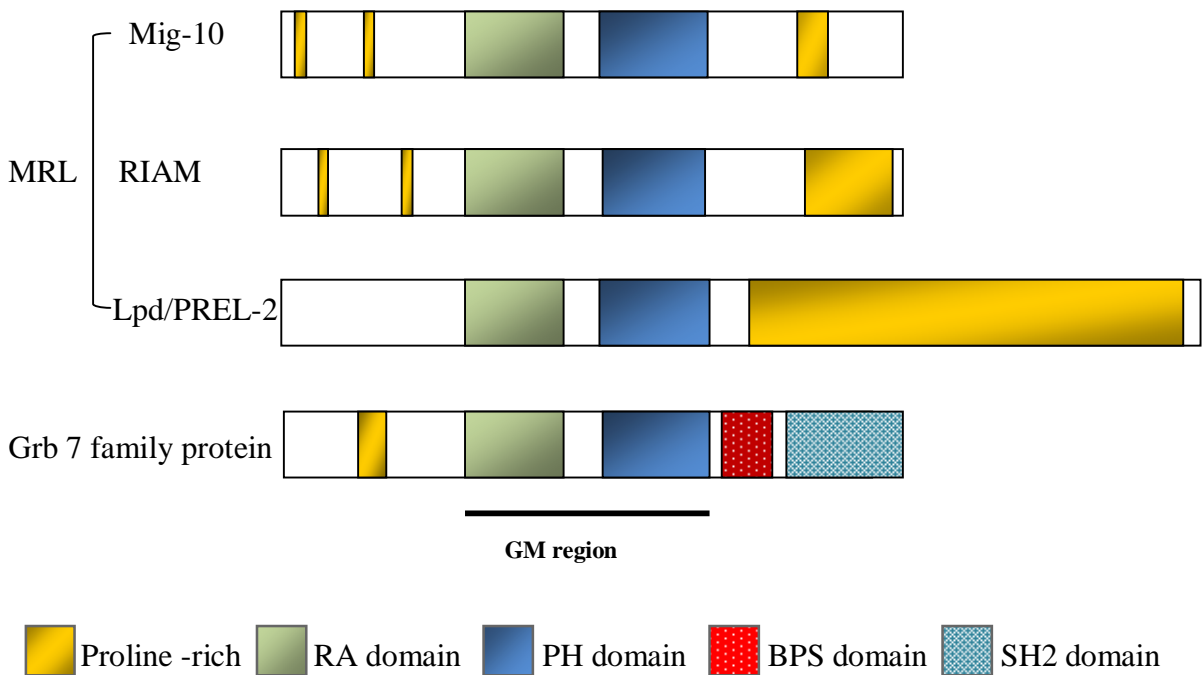


Figure 1.0 MRL protein family (Mig-10/RIAM/Lamellipodin) share a conserved domain structure with Grb 7 family proteins in GM region which containing RA and PH domains. Grb 7 family proteins possess two extra binding motifs; BPS and SH2 domain which absent in MRL family. Grb 7 family proteins only contain one proline-rich domain at N-terminus. In contrast, Mig-10 and RIAM have proline-rich domain at both N- and C- terminus compare to Lpd/PREL-2 which possess a very long proline-dense stretches at C-terminus. GM, Grb-Mig-10; RA, Ras-associated; PH, Pleckstrin Homology; BPS, between PH and SH2; SH2, Src Homology 2.

(Adopted and modified from Lee, 2008; Legg and Machesky, 2004)

2.2 Proline-rich Ena/VASP Ligand 2a (PREL-2a)

The PREL-2 in zebrafish is duplicated into PREL-2a and PREL-2b which is consistent with the recent genome duplication in ray-finned fish occurred before the teleost radiation (Amores *et al.*, 1998; Postlethwait *et al.*, 1998). Many studies indicated that genome duplication took place in teleost more than 100 million years ago (Amores *et al.*, 1998).

It was also noted that the two duplicated PREL-2 are highly identical to each other. PREL-2a has 1224 amino acids residues, possess longer amino acid sequences than PREL-2b which only has 1020 amino acids residues. Both of them show high homology in FPPPP proline-rich motifs, these raise interesting question regarding their functional relationship.

Previous studies have demonstrated that both zebrafish PREL-2a and PREL-2b display similar spatial-temporal and complementary expression patterns in somite development and brain region. This complementary expression pattern can be explained by duplication-complementary-degenerative (DDC) model (Lee, 2008).

In particular, PREL-2a is broadly expressed in midbrain-hindbrain region and ventral hindbrain. Besides, PREL-2a also found expressed during early segmentation and posterior tail. This suggests that it might play a role in regulating the proper subdivision of presomitic mesoderm before morphological distinctive somites are formed (Lee, 2008). This expression patterns are consistent with the role of this gene in regulating cell adhesion in cultured cells (Krause *et al.*, 2004, cited in Lee, 2008).

2.3 Zebrafish

2.3.1 Zebrafish as model organism

The zebrafish (*Danio rerio* and also known as *Brachydanio rerio*) is a small tropical freshwater fish. They are distributed throughout South Asia, north-eastern India, Bangladesh and Myanmar (Spence *et al.*, n.d; Wixon, 2000). The zebrafish belongs to the family of the Cyprinidae in the class of ray-finned fishes (Actinopterygii) (Dahm *et al.*, 2005).

Kimmel *et al.* (1995) defined that zebrafish has seven broad periods of embryogenesis which consists of zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. Zebrafish has very rapid early development with the entire body plan established by 24 hours post fertilization (hpf). This rapid development allows observation of development process and identification of gene expression within a few hours or few days.

Zebrafish has become widely used as model organism for studying molecular genetics development, cell biological process and gene functions (Altmann *et al.*, 2003). This mainly due to it possesses the advantages of optical transparency, easily to manipulate and the range of genetic and genomic tools to study it (Briggs, 2002).

Nowadays, more information and knowledge on zebrafish anatomy, cell biology, living behaviour and genetic strains can be organized and presented on the web via The Zebrafish Information Network (ZFIN). Within the future and present technologies and infrastructure, zebrafish will make important contributions to enhance our understanding of vertebrate development.

2.3.2 Stages of Embryonic Development of the Zebrafish

Kimmel *et al.* (1995) defined that zebrafish has seven broad periods of embryogenesis which consists of zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. The newly fertilized egg is in the zygote period until the first cleavage occurs about 40 minutes after fertilization. Embryos during the cleavage period are twisted about the animal-vegetal axis, roughly 45° from the face view (Kimmel and Law, 1985a). In blastula stage, the blastomere names are according to cell lineage and indicate cell positions. Blastodisc begins to look ball-like at the 128-cell stage and until the time of onset of gastrulation. An organ and tissue level fate map is available for the onset of gastrulation which broadly equivalent to fate maps of other chordates, notably amphibians (Keller, 1975; 1976; Dale and Slack, 1981, and also birds (Hatada and Stern, 1994) and mammals (Lawson *et al.*, 1991).

Segmentation period starts at 10 hpf, the somites start develop, the tail bud becomes more prominent and the embryo elongates. The first cells differentiate morphologically particularly along the AP axis and the first body movements appear. The forebrain, midbrain and hindbrain also can be visible in this stage. Ventrolaterally to the brain primordium and posteriorly to the eye, a primordium appears form the series of pharyngeal or visceral arches including the pharyngeal arches derive from all three germ layers, head neural crest contributing prominently to the arch mesenchyme (Schilling and Kimmel 1994). Besides, pigment cells extend almost to the end of the tail during the prim-25 stage which is 36 hpf.

During the hatching period, pharyngeal walls undergo both morphogenesis and differentiation dramatically in this stage. The development of pectoral fin to be useful

features for staging especially during the early part of the hatching period. The heart is prominent and beating strongly while the circulation in the pharyngeal arch region is becoming more complex with the advent of gill filament development. The hatched larva has completed most of its morphogenesis and continues to grow rapidly in 3dpf (Kimmel *et al.*, 1995).

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Fish

Zebrafish embryos were obtained from the fish facility in Animal Biotechnology Laboratory, University Malaysia Sarawak (UNIMAS). Eggs were collected from successfully mated fish then immersed in embryo medium. Fertilized eggs with good conditions were sorted while unfertilized eggs were discarded with Pasteur pipette.

3.1.2 Embryo medium

The concentration of embryo medium was prepared in 50X stock by dissolved all the chemicals listed in Table 3.1 in 1 liter of distilled water. Dilution was done to prepare 1 X stock embryo medium.

Materials	Molarity (mM)	Amounts (g)
Sodium chloride (NaCl)	250	14.610
Potassium chloride (KCl)	8.5	0.634
Calcium chloride dihydrate (CaCl ₂ .2H ₂ O)	16.5	2.426
Magnesium sulfate heptahydrate (MgSO ₄ .7H ₂ O)	16.5	4.067

Table 3.1 Molarity and amounts used in 50X embryo medium preparation

3.1.3 Ampicillin (50 mg/ml)

To prepare Ampicillin stock with concentration of 50 mg/ml, 0.1 g of Ampicillin was added to 2 ml of MiliQ water then filter-sterilized using 0.2 μ M syringe filter. This was aliquoted to 1 ml working stocks and stored at -20°C until further use.

3.1.4 IPTG (Isopropyl-b-d-thiogalactopyranoside)

To prepare IPTG stock solution with concentration of 24 mg/ml, 0.12 g of IPTG was dissolved in 5 ml of MiliQ water then filter-sterilized using 0.2 μ M syringe filter. It was stored at 4°C and should be stable for many months.

3.1.5 LAIX Plates (LB/Ampicillin/IPTG/X-GAL)

LB/Ampicillin plates was taken into laminar flow, 100 μ l of IPTG was spread onto plates and let the reagent be absorbed by placing them in an inverted position inside a 37°C incubator for 30 minutes. After this, 20 μ l of X-gal was spread onto each plate. The reagent was allowed to be absorbed then stored the plate at 4°C.

3.2 Methods

3.2.1 Eggs collection

For eggs collection, adult male and female zebrafish were placed in a mating tank at the evening before fertilized eggs were needed. Eggs were collected from successful mating fish on the following morning by the used of tea strainer. The good eggs were sorted and transferred to a fresh Petri dish using a Pasteur pipette, while the unfertilized eggs were discarded. Good and fertilized eggs were transferred to a Petri-dish which containing 1X embryo medium and labeled. All the embryos were raised in incubator at 28.5°C.

3.2.2 Staging

Microscopic observation was performed to learn about the developmental stage of living embryos of zebrafish. Zebrafish embryos were identified by morphological features then staged according to (Kimmel *et al.*, 1995). The developmental stage of the zebrafish embryos was examined with dissecting stereo-microscope. Staging was carried out to provide accuracy in developmental studies. A number of embryos at the following developmental stages 1K-cell (3hpf), 75% epiboly (8hpf), 1-somite (11hpf), 18-somite (18hpf), prim-5 (24hpf), prim-25 (36hpf), long-pec (48hpf), protruding mouth (72hpf), 4dpf and 5dpf were highlighted. Dechoriation was needed for the early stage of zebrafish embryos before proceed to RNA isolation. During the late pharyngula period, the embryos were removed from chorions and swims away in response to touch. This can be prevented by anaesthetise them with incubation in ais for 1 minute.

3.2.3 Primer design and sequences

The primers used in the project were designed by using two different programmes. Suitable primer pairs for PCR amplification were suggested by Primer3 (<http://waldo.wi.mit.edu/cgi-bin/primer3.cgi/primer3www.cgi>). All suitable primer pairs suggested by Primer3 were then analysed for hairpin, palindromes, dimmers and melting temperature with a second programme, NetPrimer (<http://www.premierbiosoft.com/netprimer/netprimer.html>). Primers with a rating of at least 85 (out of 100; Netprimer) were selected.

3.2.4 Preparation of samples

Approximately 130 embryos from each different stage (3hpf, 8hpf, 11hpf, 18hpf, 24hpf, 36hpf, 2dpf, 3dpf, 4dpf and 5dpf) were dechorionated using needles. The embryos were placed in a 2 ml eppendoff tube and removed the embryo medium before 1 ml of Tri Reagent was added. The embryos were homogenized using a gauge and syringe then stored at -20°C before RNA isolation.

3.2.5 Isolation of total RNA using Tri Reagent

The homogenate were centrifuged at 12,000 rpm for 10 minutes at 4°C. After centrifugation, 200 µl of chloroform was added. The samples were then shaken for 15 seconds and left at room temperature for approximately 5 minutes. The samples were then centrifuged at 12,000 rpm for 15 minutes and the upper phase was transferred to a new tube. 500 µl of total isopropanol was added to each sample then incubated at room temperature for 10 minutes to precipitate RNA. The samples were centrifuged again at

12,000 rpm for 10 minutes to pellet the RNA. The supernatant was discarded and washed the RNA pellet with 1ml of 70% ethanol. After that, the samples were centrifuged again at 7,500 rpm for 5 minutes. The supernatant was removed and the RNA pellet was air-dried for 3-5 minutes before addition of 10 μ l of Nuclease-Free Water. To visualize the RNA, 1 μ l of the solution was run on a 1% agarose gel at 120V for 30 minutes.

3.2.6 Agarose gel electrophoresis

A 1% agarose gel was prepared by adding 1.0 g of agarose powder into 100 ml 1 \times TAE buffer. The solution was heated and boiling in the microwave around 2 minutes to dissolve the agarose. The gel solution was then cooled to approximately 60°C and 2 μ l of ethidium bromide (10 mg/ml) was added and the gel was poured into a gel mould. 1 μ l of 6X loading dye and 4 μ l of distilled water were added for every 1 μ l of sample. The gels were run at 120V for 30 minutes in 1 \times TAE buffer then examined using an ultraviolet illuminator.

3.2.7 Quantification of RNA

For spectrophotometric measurement, 2 μ l of DNA aliquots was diluted with 68 μ l of distilled water in the cuvette and quantified using Ultrospec 1100pro. The absorbance values of 260 nm, 280 nm and 320 nm were recorded. The purity of the samples was determined by calculating the ratio of $A_{260-320}/A_{280-320}$ and the concentration of RNA was calculated as show below:

$$\text{RNA } (\mu\text{g}/\mu\text{l}) = [A_{260} \times (40 \mu\text{g RNA/ml}) / (1 A_{260} \text{ unit}) \times (\text{dilution factor})] / 100$$