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Neuroscience Research 56 (2006) 80–90
Neuroscience Research
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Proliferin enhances microvilli formation and cell growth of neuroblastoma cells

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Received 6 December 2005; accepted 29 May 2006
Available online 28 July 2006

Abstract

Proliferins (also termed mitogen-regulated proteins; MRP/PLFs) belong to the prolactin gene family. *Mrp*/*Plfs* are involved in angiogenesis of the uterus and placenta and maximally expressed during midgestation and decline through the remainder of the gestation period in mouse placenta. The tissue expressions of *Mrp*/*Plfs* are mainly documented in placenta, hair follicles of skin and in wound healing. In this report, we demonstrate that *Plf1*, *Plf1* minus exon3, *Plf2* and *Mrp3* but not *Mrp4* are expressed in mouse whole brain by diagnostic RT-PCR and Western blotting. The expression levels of *Mrp*/*Plf* mRNAs in mouse brains were low during the neonatal period, but higher in embryonic and adult stages, indicating *Mrp*/*Plfs* expression profiles are different in mouse brain and placenta. Interestingly, endogenous *Mrp*/*Plfs* were detected using immunostaining both in mouse brain sections and the neuroblastoma cell line, Neuro-2a cells. The function of PLF1 was explored by expressing exogenous PLF1 in Neuro-2a cells. This resulted in increased microvilli. Neuro-2a cells with stable expression of PLF1 had increased proliferation compared with normal and stable expressing EGFP cells when cell reached saturation density. Together these data, strongly suggest that MRP/PLFs mediate microvilli formation and contribute to cell proliferation of neuroblastoma cells.

Keywords: Proliferin; Mitogen-regulated protein; Proliferation; Microvilli; Neuroblastoma; IGF-II/M6P

1. Introduction

Mitogen-regulated protein/proliferin (*Mrp*/*Plf*) was originally cloned from serum inducing quiescent BABL/c 3T3 cells (Nilsen-Hamilton et al., 1980; Linzer and Nathans, 1984). Expression of MRP/PLF increases when immortalized mesenchymal cells derived from mouse embryos are stimulated with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Nilsen-Hamilton et al., 1980). MRP/PLF's amino acid sequence most closely resembles prolactin-like protein A (PLP-A) (Duckworth et al., 1986) and MRP/PLFs have been classified as members of the prolactin/growth hormone superfamily (Wiemers et al., 2003).

To date, four complementary *Mrp*/*Plf* genes (*Plf1*, *Plf2*, *Mrp3* and *Mrp4*) have been cloned and found to share 91% amino acid sequence identity (Connor et al., 1989; Fassett et al., 2000; Fassett and Nilsen-Hamilton, 2001). Each of the MRP/PLF proteins was mainly detected in trophoblast giant cells of the mouse placenta at midgestation (Linzer et al., 1985). *Plf1* was proved to stimulate angiogenesis both in developing mouse placenta (Jackson et al., 1994) and a progressing fibrosarcoma tumor (Toft et al., 2001). *Plf2* was obtained from a BABL/c mouse placental cDNA library (Wilder and Linzer, 1986). *Mrp3* was originally cloned from a Swiss mouse embryonic fibroblast cDNA library (Connor et al., 1989) and its expression was highly induced in keratinocytes at the edge of wounds during cutaneous wound healing and in hair follicles (Fassett and Nilsen-Hamilton, 2001). *Mrp4* cDNA was obtained from CF-1 mouse placenta at day 13 of gestation and was detected in hair follicles of the tail and ear (Fassett et al., 2000). All of these studies suggest that *Mrp*/*Plfs* are engaged in conveying growth signals between the uterus and placenta by enhancing angiogenic activity (Jackson et al., 1994), migration and proliferation of keratinocytes for repairing cutaneous wounds

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0168-0102/$ – see front matter
doi:10.1016/j.neures.2006.05.011

and in the hair follicle cycle (Fassett et al., 2000; Fassett and Nilsen-Hamilton, 2001).

MRP/PLF proteins bind insulin-like growth factor II (IGF-II)/mannose 6-phosphate (M6P) receptor located on the trans-Golgi reticulum, then traffic to lysosomes or receptors on the cell membrane in the mouse fetus (Jackson and Linzer, 1997). IGF-II/M6P receptor has been detected as being widely but selectively distributed in adult rat brain. Double-labeling studies have indicated that a subset of IGF-II/M6P receptor localizes with cholinergic neuronal cells in the central nervous system (Hawkes and Kar, 2003). Furthermore, *Mrp*/*Plfs* expression is increased after basic fibroblast growth factor (bFGF) stimulation in Swiss 3T3 cells and bFGF is highly expressed in mouse brain (Ford-Perriss et al., 2001). Because MRP/PLF receptor and bFGF are expressed in mouse brain, we hypothesize that *Mrp*/*Plfs* is expressed in mouse brain.

In this study, expression of *Mrp*/*Plf* mRNAs and proteins was demonstrated in adult mouse brain and also in a neuroblastoma cell line, Neuro-2a. Using diagnostic restriction endonuclease digestion RT-PCR, we showed that all of the *Mrp*/*Plf* gene family were expressed in Neuro-2a cells, whereas *Plf1*, *Plf2* and *Mrp3* were the major species expressed in mouse brain. A *Plf1* alternative splicing *Plf1* minus exon3 was specifically detected in mouse brain. The *Mrp*/*Plf* mRNAs were detected in mouse brain from embryonic day 14 to adults. In order to characterize MRP/PLF functions in Neuro-2a cells, Myc-tagged PLF1 was transiently or stably expressed in Neuro-2a cells. The formation of microvilli on the dorsal region of the Neuro-2a cell membrane was increased by transiently expressed exogenous PLF1 and cellular proliferation was enhanced in cells with stable expression of PLF1. Together these results suggest that *Mrp*/*Plf* genes may require for cell adapting external signals in the central nervous system.

2. Materials and methods

2.1. Cloning and construction of mouse brain Mrp/Plf cDNAs

We purchased ICR and C57Bl/6 mice from the National laboratory animal center (Taipei, Taiwan) at different stages of development (indicated in text). Mice were killed to obtain their whole brains; the animal protocols conformed to those approved by the National Taiwan University Animal Care and Use Committee. *Plf1* and *Plf1* (−exon3) complementary DNAs (cDNAs) were obtained from whole brain of 8-week-old female C57Bl/6 mice. Total RNA isolation and reverse transcription were performed as described below. The primer sequences used for semi-quantitative *Mrp*/*Plf* cDNAs were as follows: forward, CTCTGCAGAGATGCTCCCTTC; backward, CATGATATTTCAGAAGCACAGCAC. The double stranded cDNAs were then subcloned into pGEM-T Easy vector by T–A cloning (Promega, Madison, WI). These constructed plasmids were termed pGEM-T Easy Plf1 and pGEM-T Easy Plf1 (−exon3). All of the inserted cDNAs were sequenced by the Applied Biosystems 3730 DNA analyzer. In order to subclone the full open reading frame of *Plf1* cDNA into pcDNA4/Myc-HisA (Roche, Alcaloid, Mannheim), a *Plf1* cDNA fragment was amplified by PCR from pGEM-T Easy Plf1 plasmid using the following primers: T7, TAATACGACTCACTATAGGG; mPB-XbaI-His, AATGGTGATGGTGATGATGTCTAGAGCAGTTATCTATTTTTA. The following PCR conditions were used: denaturation at 94 °C for 3 min followed by 40 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min, then a final elongation step at 72 °C for 10 min. The PCR product was then simultaneously digested with *EcoR* I and *Xba* I restriction endonucleases (New England Biolabs, Beverly, MA). The resulting PCR product was ligated into pcDNA4/Myc-HisA that had been digested with the same restriction endonucleases. The sequence of pcDNA4/Myc-HisA containing Plf1 was confirmed and termed pcDNA4-Plf1. The pcDNA4-EGFP construct was made by digesting both pEGFP-N1 (Clonetch, Palo Alto, CA) and pcDNA4/Myc-HisA with *Pst* I and *Not* I restriction enzymes (New England Biolabs, Beverly, MA). The EGFP cDNA fragment was then recovered and purified by a Gel Extraction system (Marligen Bioscience, Ijamsville, MD) and cloned into pcDNA4//Myc-HisA. This plasmid was designated pcDNA4-EGFP.

2.2. Reverse transcription-PCR

Total RNA from C57Bl/6 mouse brain at different developmental stages and Neuro-2a cells was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (0.8 μg) was reverse transcribed in a reaction volume of 20 μl, containing 2 μl of 10× reverse transcription buffer, 4 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 1 μl of 50 μM oligo$(dT)_{20}$ primer, 1 μl of 40 U RNaseOUT (Invitrogen, Carlsbad, CA), and 1 μl of 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Synthesis of cDNA was carried out for 50 min at 50 °C. A mock control without reverse transcriptase was performed for each RT-PCR in order to exclude genomic DNA contamination. PCR amplifications were carried out in 50 μl reaction volumes that contained 2.5 μl of cDNA, 5 μl of 10× PCR buffer, 0.5 μl of 10 mM dNTP, 20 pmol sense and antisense primers, and 0.5 U of DNA Taq polymerase (Takara, Otsu, Shiga). The primer sequences used for amplification and cloning *Mrp*/*Plf* and CI-MPR cDNAs were as follows: *Mrp*/*Plf* forward primer: AGAGATGCTCCCTTCTTTG; *Mrp*/*Plf* backward primer, GAAGCAGAGCACATGAAAG; CI-MPR forward primer, CTTCTTCC ACTGTGACCCTC; CI-MPR backward primer, GGATCTCTTCCATCAGCC. G3PDH was used as an internal control; its pair of primer sequences was as follows: forward primer, GCAAATTCAACGGCACAGTC; backward primer, TCTTCTGGGTGGCAGTG ATG. The thermal cycling conditions were as follows: 94 °C for 3 min, then 40 cycles at 95 °C for 30 s, 60 °C for 20 s, 72 °C for 30 s, with a final 10 min at 72 °C. The PCR products were resolved by electrophoresis through a 1.5% agarose gel and examined by ethidium bromide staining.

2.3. Diagnostic restriction endonuclease digestion RT-PCR

The diagnostic restriction endonuclease digestion RT-PCR assay was used to distinguish between expression of different *Mrp*/*Plf* cDNAs in mouse brain and Neuro-2a cells. Its principle is based on the minor differences found in the sequences of different *Mrp*/*Plf* cDNAs that leads to distinct mapping after restriction enzyme digestion as indicated in Fig. 3A. For restriction enzyme digestion, one-fifth of the products from the RT-PCR reactions were subjected to either individual *Bst*X I or *Fnu4*H I digestions (New England Biolabs, Beverly, MA) or a combination of both digestions. The digested products were then resolved by electrophoresis through an 8% non-denaturing polyacrylamide gel. The fragmented cDNAs in polyacrylamide gel were detected by silver staining (Nesterenko et al., 1994).

2.4. Cell culture and transfection

Mouse Neuro-2a cells (ATCC CCL-131) were obtained from the American Type Cell Collection (ATCC) and were propagated in DMEM medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin. Neuro-2a cells were either transfected with pcDNA4-Plf1 or cotransfected with pcDNA4-EGFP or pEYFP-ER (Clontech, Palo Alto, CA). Transfections were conducted using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, the day before transfection, 3×10^5 cells were plated on a six-well cell culture grade Petri dish (Falcon, Franklin Lakes, NJ). A 6 μl Plus™ reagent were added to 96 μl of serum-free medium containing 1 μg DNA then mixed with 100 μl serum-free medium containing 4 μl lipofectamine and incubated for 15 min at room temperature. The DNA-Plus™-lipofectamine reagent complexes were added to each well containing cells in fresh serum-free medium and incubated at 37 °C in 5% CO_2 for 3 h, then replaced with fresh complete medium. Immunocytochemical analysis was performed

48 h after transfection. For screening stable expressing PLF1 or EGFP Neuro-2a cells, 700 μg/ml of G418 and Zeocin were used to screen the stable clones respectively. Proliferation of Neuro-2a cells stable expressing PLF1 and EGFP was determined by counting cell number every day after plating cells at 5×10^4 cells/ml density.

2.5. Immunohistochemical staining

Twelve micrometers thick sagittal brain cryosections were obtained from 4% paraformaldehyde perfused adult female ICR mouse. Sections were permeabilized by incubating with 0.2% Triton X-100 in PBS for 10 min. The endogenous peroxidase activity was quenched by incubating the sections in 0.3% H_2O_2 for 30 min. After three washes, the sections were blocked with 10% normal goat serum, then were immersed in antigen affinity purified MRP/PLF polyclonal antibody overnight at 4 °C. After three washes, the sections were incubated with diluted anti-rabbit IgG secondary antibody for 30 min at room temperature. After three washes, the sections were incubated with peroxidase-linked VECTASTAIN ABC reagent (Vector Laboratories, Inc. Burlingame, CA) for 30 min. After three washes, the peroxidase activity was developed with 1.39 mM diaminobenzidine, 0.01% H_2O_2 in 50 mM Tris–HCl, pH 7.3 and the sections were mounted on glass slides.

2.6. Immunocytofluorescent staining

Neuro-2a cells were transfected with pcDNA4-Plf1 or pcDNA4-EGFP for 48 h then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized by incubation in PBS containing 0.5% Triton X-100 at room temperature for 10 min. Cells were then immersed in blocking solution containing 10% normal goat serum in PBS for 1 h. Cells were incubated with anti-Myc primary antibody (9E10) (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After three washes with PBS, cells were incubated with the Texas Red-conjugated donkey anti-mouse IgG secondary antibody (1:300 in PBS; Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h at room temperature. After three washes with PBS, cells were incubated with Hoechst 33342 dye (10 ng/ml; Sigma–Aldrich Fine Chemical, Inc.) for 10 min at room temperature and finally washed three times with PBS. Slides were mounted with Mowiol 4-88 (Calbiochem, La Jolla, CA). For detecting endogenous MRP/PLFs protein in Neuro-2a cells, an anti-PLF1 peptide polyclonal antiserum was obtained by immunonizing a rabbit with synthesized PLF1 peptide (NGDEEKKNPAWFLQSDNED). Immunocytofluorescent staining was performed as described above by using the anti-PLF1 peptide polyclonal antiserum (1:300 in PBS) as the primary and FITC conjugated donkey anti-rabbit IgG (1:300 in PBS, Jackson Immunoresearch Laboratories, West Grove, PA) as the secondary antibodies. Cells were visualized by confocal laser scanning microscopy (Zeiss, LSM 510) and digitally photographed. The series of differential interference contrast (DIC) photographs shown in Fig. 7 was then taken and composed by the Auto-Montage program (Synoptics com., Frederick, MD).

2.7. Western blotting

Placentas obtained from pregnant day 14 mice and brain from 8-week-old female mice was freshly dissected and proteins were extracted with RIPA buffer (50 mM Tris–HCl, pH 7.0, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptine, 1 μg/ml peptasin A, 1 mM Na_3VO_4, 1 mM NaF, 10 mM $Na_4P_2O_7$). Cellular extracts were then centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants collected. A 50 μg of each sample was denatured for 5 min at 95 °C in Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris–HCl, pH 6.8, 0.01% bromophenol blue). Proteins were separated by SDS-PAGE in a 12% polyacrylamide. Proteins were transferred to a PVDF membrane and protein bands were visualized with amido-black to verify the quantity of the samples loaded. Blots were washed with TBST buffer (120 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) then placed in TBST buffer supplemented with 5% skimmed milk and blocked for 1 h at room temperature. Blots were then incubated with rabbit polyclonal anti-PLF1 peptide antiserum (1:3000 in TBST) for 24 h at 4 °C. After washing the blots three times with TBST, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:3000, Amersham Biosciences, Arlington Heights, IL) for 2 h and washed again as described previously. Membrane-bound secondary antibodies were detected using the ECL procedure developed by Amersham Biosciences.

3. Results

3.1. Mrp/Plf mRNAs express in the whole brain and are developmental regulated

After bFGF (Nilsen-Hamilton et al., 1980), 17β-estradiol (Connor et al., 1989) or cyclic adenosine 3′,5′-monophosphate (Yamaguchi et al., 1995) stimulation, expression of *Mrp/Plf* is observed in Swiss 3T3 cells, liver BLN cell line and placenta. The receptors for bFGF, 17β-estradiol and cAMP are expressed abundantly in mouse brain (Dono, 2003; Mordacq and Linzer, 1989). We speculated therefore that *Mrp/Plf* genes might be expressed in mouse brain. Therefore, adult mice whole brain extracts were subjected to RT-PCR with conserved primers for all of *Mrp/Plf* mRNAs. Both male and female adult mice whole brains contained *Mrp/Plf* mRNAs (Fig. 1A, lanes 3 and 5). Total RNA from the placenta of 14 days pregnant mice was used as a positive RT-PCR control. No genomic DNA contamination was detected when total RNA was directly subjected to PCR (Fig. 1A, lanes 2, 4 and 6). This result demonstrated that *Mrp/Plf* is expressed in mouse brain.

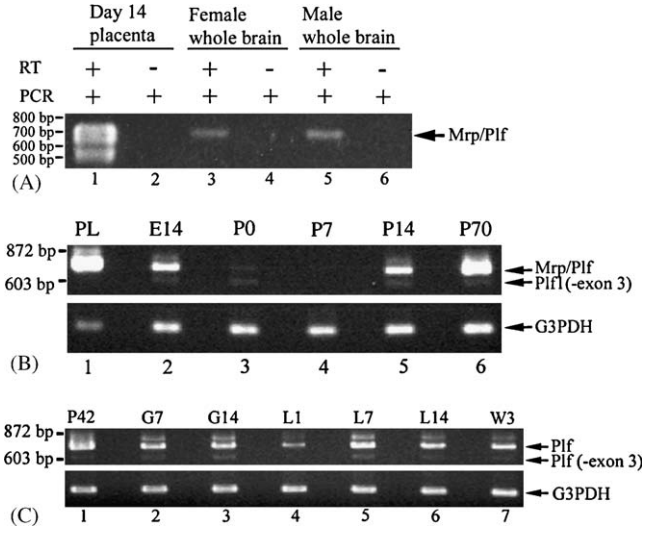

Fig. 1. (A) *Mrp/Plf* mRNAs expressed in the brain of both adult male and female mice. RT-PCRs were performed by using total RNAs from whole brain tissues of both adult female (lane 3) and male (lane 5) mice. The 721 bp of the amplified-*Mrp/Plf* cDNA product is 721 bp as expected. Male (lane 6) and female (lane 4) mice total RNA were used in PCR directly as controls for genomic DNA contamination. (B) The expression of *Mrp/Plf* mRNAs in neonatal mouse brain is developmentally regulated. Total RNAs were extracted from mouse brain tissues at different developmental stages as indicated and subjected to RT-PCR. One-tenth of the volume of total RNA from mid-gestation placenta was used as a positive control for RT-PCR in panels B (PL). (C) The expression pattern of *Mrp/Plf* mRNAs in the brains of adult female mice. Total RNAs were extracted from different developmental stages of whole mouse brain tissues as indicated and subjected to RT-PCR. The *Mrp/Plf mRNA* expression is shown with G3PDH employed as an internal control to normalize the amount of total RNA that was used for RT-PCR analyses. Abbreviations: embryonic (E); postnatal (P); gestation (G); lactation (L) and weaning (W).

In mouse placenta, *Mrp/Plf* mRNAs expression peaks at mid-gestation (Fang et al., 1999). To understand whether the regulatory mechanism of *Mrp/Plf* expression is identical in mouse brain and placenta, we investigated whether the temporal expression pattern of *Mrp/Plf* mRNAs at different stages of development of adult and neonatal mouse brains is the same as in the placenta. We performed semi-quantitative RT-PCR on developing whole brain to explore this question. There was considerably more expression of *Mrp/Plf* mRNAs in mouse placenta than in developing brain (Fig. 1B). *Mrp/Plf* mRNAs were detected in embryonic mouse brain at pregnancy day 14. However, *Mrp/Plf* mRNAs in neonatal mouse brain decreased markedly by parturition and postnatal day 7 (Fig. 1B, lanes 3 and 4) and expression increased after postnatal day 14 (Fig. 1B, lane 5). The highest level of *Mrp/Plf* mRNA expression in mouse brain was detected on postnatal day 70 (Fig. 1B, lane 6). These data suggest that the expression of *Mrp/Plf* mRNAs in mouse brain is under developmental regulation. We next determined if the expression pattern of *Mrp/Plf* mRNAs in adult mouse brain was identical to that in the placenta during gestation. A constant level of *Mrp/Plf* mRNAs was detected in adult mouse brain during gestation, lactation and weaning (Fig. 1C), but apparently decreased at parturition (Fig. 1C, lane 4). The level of *Mrp/Plf* mRNAs in placenta peaked between days 8 and 10 of gestation in mice (Linzer et al., 1985). Here we showed *Mrp/Plf* mRNAs had a constant expression level in pregnant adult mouse brains during gestation. These results suggest that *Mrp/Plf* mRNAs expression is regulated in a different manner in mouse placenta and brain.

3.2. *Plf1 minus exon3 [Plf1 (−exon3)], a novel Plf1 splicing form exists in mouse brain*

The expected 721 bp of *Mrp/Plf* mRNA was obtained after performed RT-PCR on mouse brain and placenta total RNA (Fig. 1A). A faint band around 600 bp also was detected after

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Plf1          ATGCTCCCTTCTTTGATTCAACCATGCTCCTGGATACTGCTCCTACTACTGGTGAACAGC
Plf1(-exon3)  ATGCTCCCTTCTTTGATTCAACCATGCTCCTGGATACTGCTCCTACTACTGGTGAACAGC
          1   M  L  P  S  L  I  Q  P  C  S  W  I  L  L  L  L  L  V  N  S

Plf1          TCGTTATTGTGGAAGAATGTTGCCTCATTTCCCATGTGTGCAATGAGGAATGGTCGTTGC
Plf1(-exon3)  TCGTTATTGTGGAAGAATGTTGCCTCATTTCCCATGTGTGCAATGAGGAATGGTCGTTGC
         21   S  L  L  W  K  N  V  A  S  F  P  M  C  A  M  R  N  G  R  C

Plf1          TTTATGTCCTTTGAAGACACATTTGAATTAGCCGGCAGTTTGTCTCATAATATCAGTATA
Plf1(-exon3)  TTTATGTCCTTTGAAGACACATTTGAATTAGCCGGCAGTTTGTCTCATAATATCAGTATA
         41   F  M  S  F  E  D  T  F  E  L  A  G  S  L  S  H  N  I  S  I

Plf1          GAAGTTTCAGAACTGTTCACTGAATTTGAAAAACATTATTCTAACGTGTCTGGGCTCAGA
Plf1(-exon3)  GAAGTTTCAGAACTGTTCACTGAATT..............................
         61   E  V  S  E  L  F  T  E  F  E  K  H  Y  S  N  V  S  G  L  R

Plf1          GACAAAAGCCCCATGAGATGCAATACTTCTTTCCTTCCAACTCCAGAAAACAAGGAACAA
Plf1(-exon3)  ..........................................................
         81   D  K  S  P  M  F  C  N  T  S  F  L  P  T  P  E  N  K  E  Q

Plf1          GCCAGGCTCACACACTATTCAGCTCTTCTGAAATCAGGAGCCATGATTTTGGATGCCTGG
Plf1(-exon3)  ..............TTATTCAGCTCTTCTGAAATCAGGAGCCATGATTTTGGATGCCTGG
        101   A  R  L  T  H  Y  S  A  L  L  K  S  G  A  M  I  L  D  A  W

Plf1          GAAAGCCCTCTGGACGATCTAGTGAGTGAATTATCTACCATAAAAAATGTCCCTGATATA
Plf1(-exon3)  GAAAGCCCTCTGGACGATCTAGTGAGTGAATTATCTACCATAAAAAATGTCCCTGATATA
        121   E  S  P  L  D  D  L  V  S  E  L  S  T  I  K  N  V  P  D  I

Plf1          ATCATCTCCAAAGCCACAGACATAAAGAAAAAGATCAACGCAGTCCGGAACGGGGTTAAT
Plf1(-exon3)  ATCATCTCCAAAGCCACAGACATAAAGAAAAAGATCAACGCAGTCCGGAACGGGGTTAAT
        141   I  I  S  K  A  T  D  I  K  K  K  I  N  A  V  R  N  G  V  N

Plf1          GCCCTCATGAGCACCATGCTTCAGAATGGAGATGAAGAAAAGAAGAACCCTGCCTGGTTC
Plf1(-exon3)  GCCCTCATGAGCACCATGCTTCAGAATGGAGATGAAGAAAAGAAGAACCCTGCCTGGTTC
        161   A  L  M  S  T  M  L  Q  N  G  D  E  E  K  K  N  P  A  W  F

Plf1          TTGCAATCTGACAATGAAGATGCTCGCATTCATTCTTTATATGGCATGATCAGCTGCCTA
Plf1(-exon3)  TTGCAATCTGACAATGAAGATGCTCGCATTCATTCTTTATATGGCATGATCAGCTGCCTA
        181   L  Q  S  D  N  E  D  A  R  I  H  S  L  Y  G  M  I  S  C  L

Plf1          GACAATGACTTTAAGAAGGTTGATATTTATCTCAACGTCCTGAAGTGTTACATGTTAAAA
Plf1(-exon3)  GACAATGACTTTAAGAAGGTTGATATTTATCTCAACGTCCTGAAGTGTTACATGTTAAAA
        201   D  N  D  F  K  K  V  D  I  Y  L  N  V  L  K  C  Y  M  L  K

Plf1          ATAGATAACTGCTGA
Plf1(-exon3)  ATAGATAACTGCTGA
        221   I  D  N  C  *
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Fig. 2. cDNA and amino acid sequence comparisons between *Plf1* and *Plf1* (−exon3). The leader signal sequence is underlined. The predicted *N*-glycosylation sites are boxed. The numbers to the left of the protein sequences show the position of the first amino acids in each row of the PLF1 protein sequence. PLF1 (−exon3) lacks one cysteine residue (circled by ring) and two *N*-link glycosylation sites (rectangle boxed) in comparison to PLF1.

RT-PCR was performed to amplify *Mrp*/*Plf* cDNAs from developing mouse brain (Fig. 1B and C). To determine whether this signal was due to a differentially spliced form of *Mrp*/*Plf* mRNAs, we cloned this fragment into a pGEM-T Easy vector and sequenced it. The fragment's sequence was identical to *Plf1* (accession no. NM_031191) apart from lacking its exon3 (Fig. 2). This indicated that a differentially spliced form of *Plf1* existed in mouse brain.

3.3. *Plf1, Plf2 and Mrp3 are the major Mrp/Plf family members expressed both in mouse whole brain tissue and Neuro-2a cells*

We now examined which *Mrp*/*Plf* genes expressed in mouse brains at different developing stages including embryonic day 18, postnatal day 25 pre-mature and day 55 mature female mice. Since members of this gene family have more than 91% DNA sequence identity, diagnostic restriction endonuclease digestion RT-PCR was used to verify which genes were expressed in developing mouse brains. The *Mrp*/*Plf* cDNAs in developing mouse brain were amplified by RT-PCR, the resulting products were then subjected to *Bst*X *I* and *Fnu*4H I restriction endonuclease digestion and digested fragments were then used to map the type of *Mrp*/*Plfs*. The predicted length of restriction fragments after *Bst*X I and *Fnu*4H I digestion are shown in Fig. 3 (panel A). No 565-bp fragment was detected after *Bst*X I and *Fnu*4H I double digestion of *Mrp*/*Plf* cDNAs from developing brain, indicating that *Mrp4* was absent in developing brain (Fig. 3B, lanes 6, 9 and 12). A 682 bp fragment was present in cDNAs from all of stages of developing mouse brain after *Bst*X I digestion indicating that *Plf2* was expressed in developing mouse brain. A 604 bp fragment was detected after *Fnu*4H I digestion which revealed that *Plf1* existed in developing brain (Fig. 3B, lanes 5, 8 and 11). A 274 and a 246 bp intense fragment were present after double digestion with *Bst*X I and *Fun*4H I indicating that *Plf1*, *Plf2* and *Mrp3* mRNAs were expressed in brain (Fig. 3B, lanes 6, 9 and 12). We had cloned and sequenced *Plf3* cDNA from adult mouse brain by TA-cloning (data not shown). After *Bst*X I digestion, an extra 212 bp fragment corresponding to *Plf1* minus exon3 was measured only in developing mouse brain (Fig. 3B and C). This experiment demonstrated that *Plf1*, *Plf1* minus exon3, *Plf2* and *Mrp3* are expressed in mouse brain from embryo to adult (Fig. 3B).

Mrp/*Plf* genes are expressed in certain immortalized cell lines (Malyankar et al., 1994) and a fibrosarcoma tumor cell line (Toft et al., 2001). Here we had proved it was also expressed in mouse

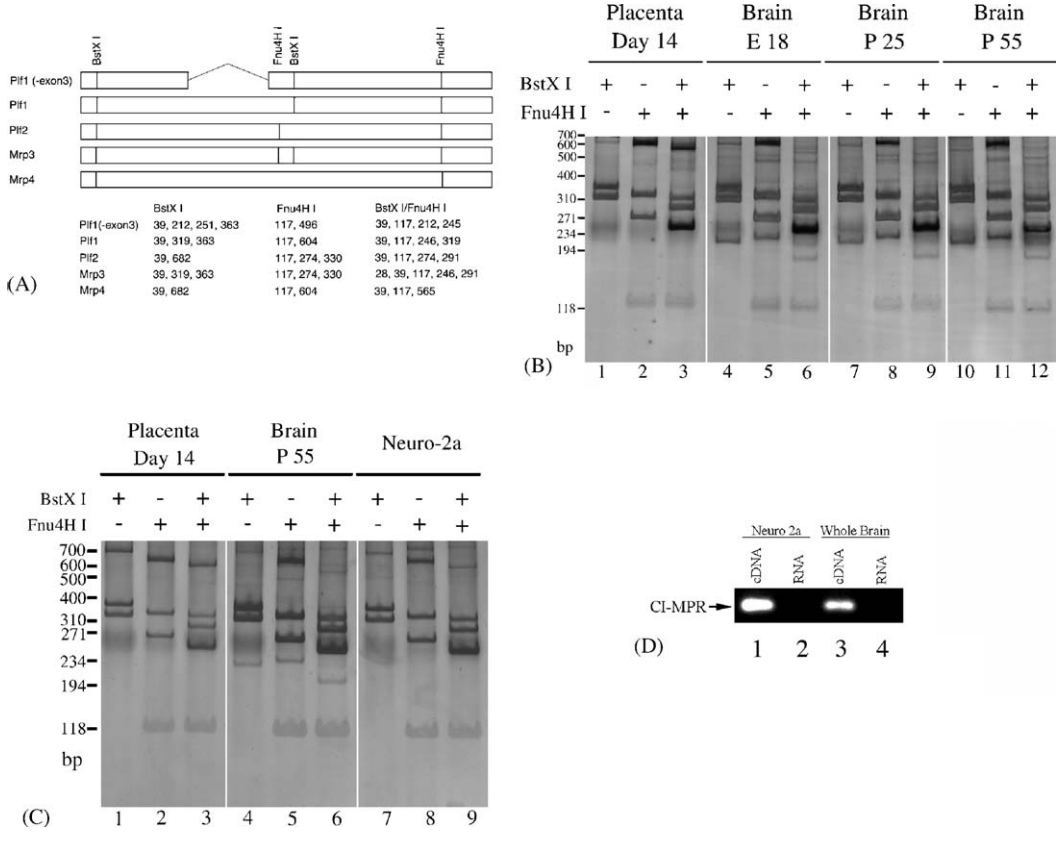


Fig. 3. *Plf1*, *Plf1* (−exon3), *Plf2* and *Mrp3* are expressed in mouse whole brain tissue. (A) The predicted length of *Plf1*, *Plf1* (−exon3), *Plf2*, *Mrp3* and *Mrp4* double strain cDNAs digested by *Bst*X I, *Fnu*4H I or simultaneously digested by both restriction endonucleases are shown in panel A. Panel B shows the a diagnostic restriction digestion RT-PCR of developing mouse brain. Expression of the *Mrp*/*Plf* genes family in mouse brain at embryonic day 18 (lanes 4–6), postnatal day 25 (lanes 7–9) and postnatal day 55 (lanes 10–12) is similar. (C) *Plf1*, *Plf2*, *Mrp3* and *Mrp4* are expressed in Neuro-2a cells. Total RNA extracted from mouse placenta at gestation day 14 (B and C) and adult female mouse whole brain (C) was used as experimental positive controls. (D) CI-MPR mRNA are detected in Neuro-2a cells. RT-PCR was performed on total RNA from Neuro-2a cells and adult mouse brain using CI-MPR primers. No genomic contamination was detected in either control experiments using total RNA directly for PCR (lanes 2 and 4). Total RNA from whole brain was used as an experimental positive control for RT-PCR (lane 3).

brain. Hence, for further functional studies, we tested whether *Mrp*/*Plf* genes were expressed in the mouse neuroblastoma cell line, Neuro-2a cell. Total RNA was extracted from Neuro-2a cells then diagnostic RT-PCR was employed. A 565 bp fragment, resulting from *Bst*X I and *Fnu*4H I double digestion of Neuro-2a *Mrp*/*Plf* cDNAs, indicated *Mrp4* mRNA existed in Neuro-2a cells (Fig. 3C, lane 9). A 604 bp intense band detected after *Fnu*4H I digestion of Neuro-2a *Mrp*/*Plf* cDNAs, revealing *Plf1* expression in Neuro-2a cells (Fig. 3C, lane 8). The restriction patterns from *Bst*X I and *Fnu*4H I double digestions were similar to the restriction patterns from postnatal day 55 mouse brain and indicated all of the known *Mrp*/*Plf* genes were expressed in Neuro-2a cells.

IGF II/M6P receptor is a MRP/PLF receptor located on the *trans*-Golgi reticulum membrane of rodent liver, placenta and brain (Lee and Nathans, 1988; Couce et al., 1992). To examine whether IGF II/M6P receptor was expressed in Neuro-2a cells, RT-PCR was used to analyze the expression of IGF II/M6P receptor. Total RNA from adult mouse whole brain was used as a RT-PCR positive control. A band of 383 bp corresponding to the expected size of IGF II/M6P receptor was detected after RT-PCR (Fig. 3D).

3.4. MRP/PLF proteins are expressed both in mouse brain and Neuro-2a cells

Our results thus far indicated that *Mrp*/*Plf* mRNAs were present in mouse brain and Neuro-2a cells. A polyclonal peptide antibody that specifically recognized a MRP/PLF peptide was now generated to determine whether MRP/PLF protein was expressed in mouse brain and Neuro-2a cells. Protein was extracted from mid-gestation placenta and used as the positive control for Western blotting by the polyclonal anti-MRP/PLF peptide antibody. The predicted 36–38 kDa glycosylated PLF1, PLF2 and MRP3 were present in the placenta as previously noted (Fassett et al., 2000; Fang et al., 1999). MRP/PLF proteins were also successfully detected in whole brain and the cerebellum of mice using the same anti-MRP/PLF peptide antibody (Fig. 4A). A 27 kDa band, previously identified as MRP4, was detected only in the placenta but not in whole brain or the cerebellum (Fig. 4A, lane 4), consistent with our mRNA results. We next determined the MRP/PLF expression pattern in mouse whole brain by immunohistochemical staining. This data demonstrated that MRP/PLF proteins are expressed in whole brain especially in cerebral cortex and cerebellum (Fig. 4B). The MRP/PLFs are expressed in more abundance in the granule layer of the cerebellum (Fig. 4C).

Based on the *Mrp*/*Plf* mRNAs detected in Neuro-2a cells, immunofluorescent staining was now used to examine the expression and distribution of endogenous MRP/PLFs in Neuro-2a cells. We found MRP/PLFs were expressed in a punctate pattern near the perinuclear region in the cytoplasm of Neuro-2a cells, suggesting MRP/PLFs presence might in the ER-Golgi protein secretory pathway (Fig. 5A and C). Cells expressing more MRP/PLF protein also presented a greater number of microvilli on their cellular dorsal region compared to

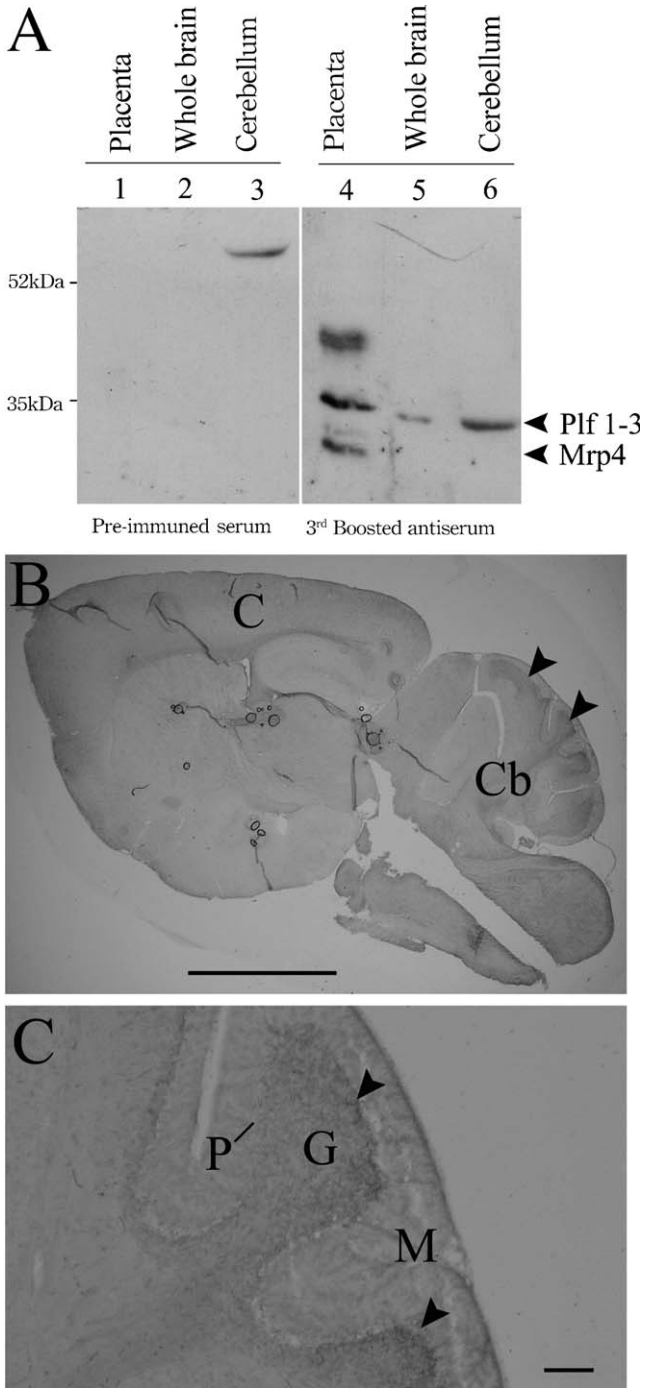


Fig. 4. MRP/PLF proteins expressed in adult mouse whole brain. (A) Proteins were extracted from the placenta (lanes 1 and 4), adult mouse whole brain (lanes 2 and 5) and the cerebellum (lanes 3 and 6) by RIPA buffer and used for Western blotting. Right panel, a third boosted rabbit polyclonal anti-MRP/PLF antibody was used to detect endogenous MRP/PLF proteins from the tissues mentioned above. Left panel, a rabbit pre-immune serum was used as a background control for Western blotting. Placental protein was used as a Western blotting positive control. MRP/PLF 1–3 and MRP4 (Mrp4) are shown as indicated according to Fassett et al. (2000). Protein standard markers are noted on the left of the figure. (B) Immunohistochemical staining of a mouse sagittal brain section with affinity purified polyclonal PLF peptide antibody. Scale bar = 3 mm. (C) The cerebellar photograph was magnified from B. Scale bar = 100 μm. The brain regions indicated are cerebral cortex (C), cerebellum (Cb), molecular cell layer (M), Purkinje cell layer (P) and granule cell layer (G). Arrows indicate the region expressed MRP/PLF.

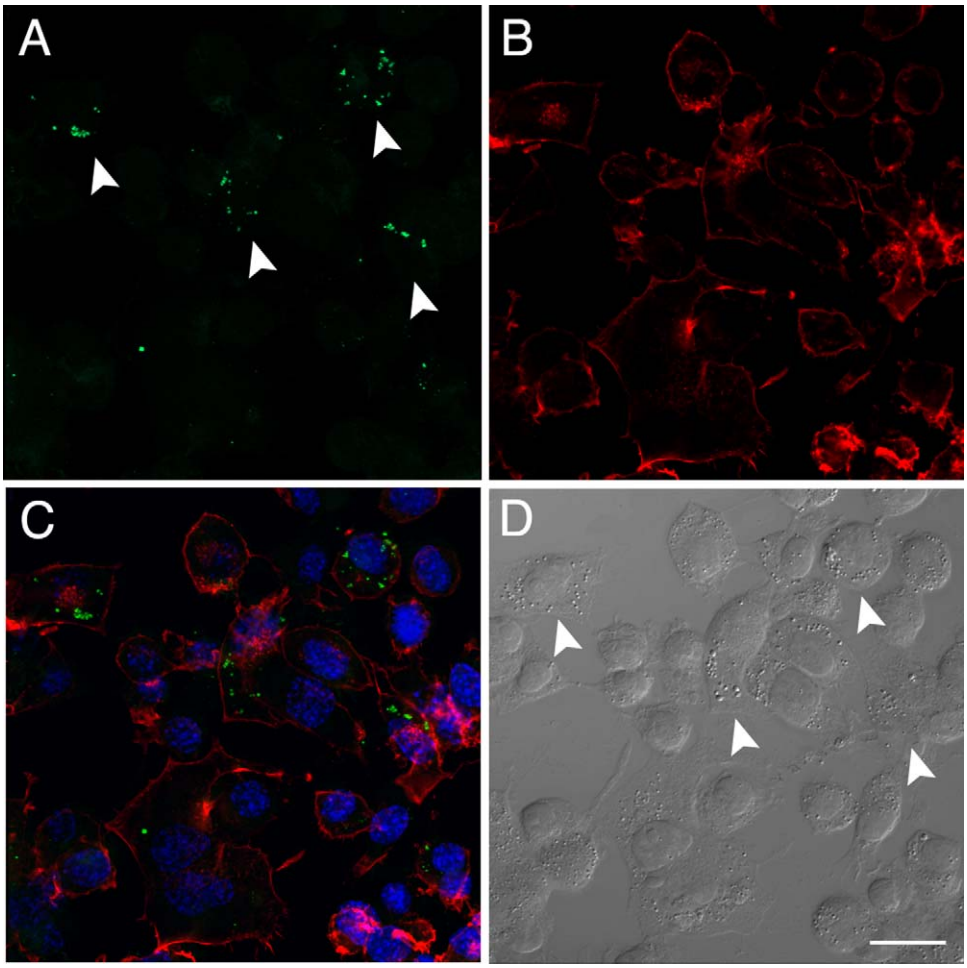


Fig. 5. Endogenous MRP/PLF punctate pattern inside the cytoplasm of Neuro-2a cells. Neuro-2a cells were fixed and subjected to immunocytofluorescent staining. (A) Endogenous MRP/PLFs were detected with rabbit polyclonal anti-MRP/PLFs antibody, then visualized by a FITC-labeled secondary antibody that recognized rabbit IgG. Texas-red conjugated-phalloidin (B) and Hoechst 33342 (C) were used to label filamentous actin and the nucleus, respectively. (C) Merged photographic images from (A) and (B). (D) Cellular microvilli were examined with a differential interference contrast (DIC) lens by confocal laser scanning microscopy. Arrows point to cells expressing MRP/PLF proteins. Scale bar is 20 μm.

cells in which MRP/PLF protein expression was less pronounced (Fig. 5D).

3.5. PLF1 protein enhances the formation of microvilli structure on the dorsal surface of Neuro-2a cells

Microvilli originating from the ER are packaged in exocytic secretory vesicles. The exocytic vesicles are then stabilized by a coating of proteoglycans with subsequent budding from the cell surface. Finally, nucleated F-actin bundle growth at the cytoplasmic surface of the membrane sites causes the newly budding membrane patch to grow out from the cell surface (Lange, 1999). Hence, the secretory vesicle has been proposed as a progenitor of microvilli (Lange, 2000). MRP/PLF secreted from Swiss 3T3 cells has been shown to follow the ER-Golgi secretory pathway (Nilsen-Hamilton et al., 1980) and to act as a ligand for the IGF-II/M6P receptor on the Golgi (Lee and Nathans, 1988). To address whether the localization of PLF1 followed the secretory pathway in Neuro-2a cells, we co-expressed Myc-tagged PLF1 and EYFP-ER encoding the yellow fluorescent protein fused to the endoplasmic reticulum targeting sequence of calreticulin and ER retrieval sequence KDEL into Neuro-2a cells. Cells were then immunocytofluorescently stained Myc-tagged PLF1 showed a punctate pattern in the cytoplasm of cells (Fig. 6C). Most of the Myc-tagged PLF1 protein co-localized with EYFP-ER, but was absent from the nucleus of cells (Fig. 6G). In the control experiment, EGFP did not co-localize with EYFP-ER and was randomly distributed in cells including the nucleus (Fig. 6H). We next asked whether transiently expressed PLF1 could enhance microvilli formation directly in Neuro-2a cells. To address this, any cell morphological changes resulting from transiently expressed recombinant PLF1 or EGFP protein were observed by confocal microscopy. We found that the 86% of the cells transiently expressing PLF1 had a greater dorsal display of microvilli-like structures than either cells expressing EGFP or normal control cells (Fig. 7A, e and f). These results suggested that MRP/PLF enhanced formation of microvilli might be through interaction with secretory vesicles. The increase in microvilli on the cellular membrane is required for adapting to environmental changes in growth confluent and mature cells (Lange, 2000). To study the role of PLF1 in Neuro-2a cells, we plated the same

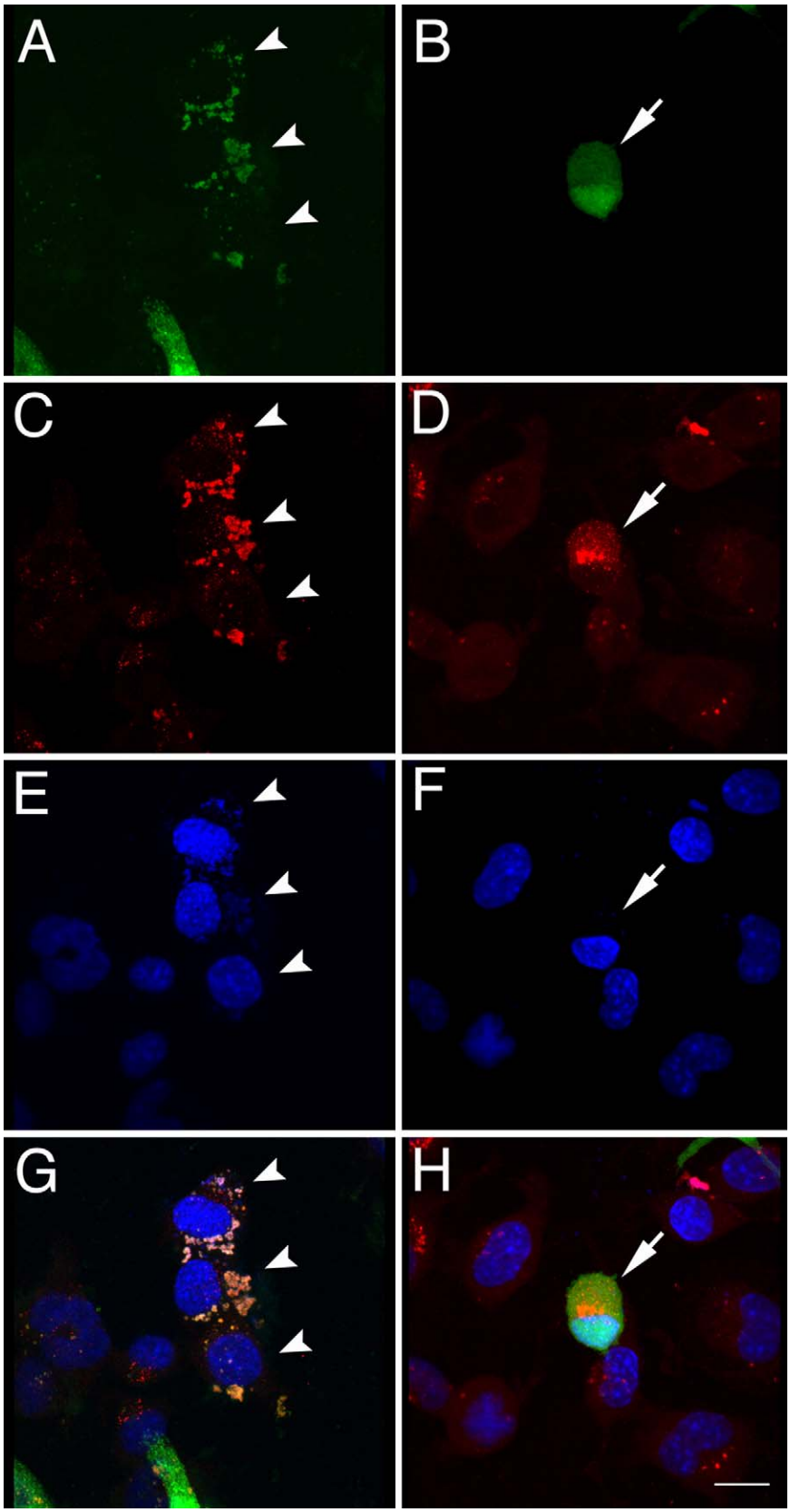
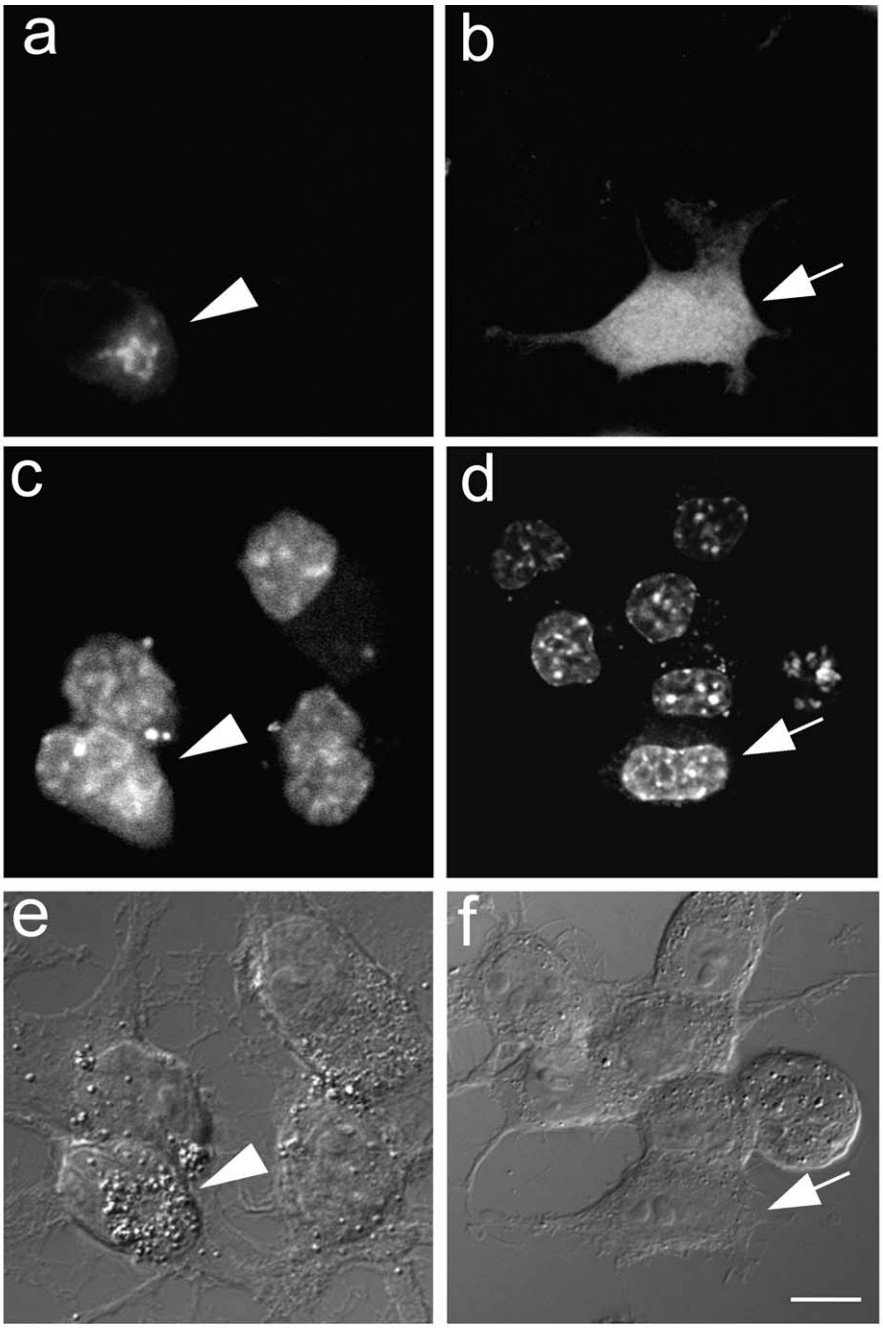


Fig. 6. Exogenous PLF1 distributed in the endoplasmic reticulum of Neuro-2a cells. pcDNA4-Plf1 (Myc-tagged) was cotransfected either with EYFP-ER (A, C, E and G) or pEGFP (B, D, F and H) into Neuro-2a cells. Cells were immunostained with mouse anti-Myc antibody then incubated with Texas-red conjugated anti-mouse IgG antibody (C and D) in order to observe the localization of PLF1 48 h after transfection. Hoechst 33,342 dye was used to stain nuclei (E and F). Panel G containing images merged from A, C and E; panel H contains merged images of B, D and F. Arrowheads mark Myc-tagged PLF1 expressing cells. Arrows point to cells expressing EGFP protein. Scale bar is 15 μm.


a
b
c
d
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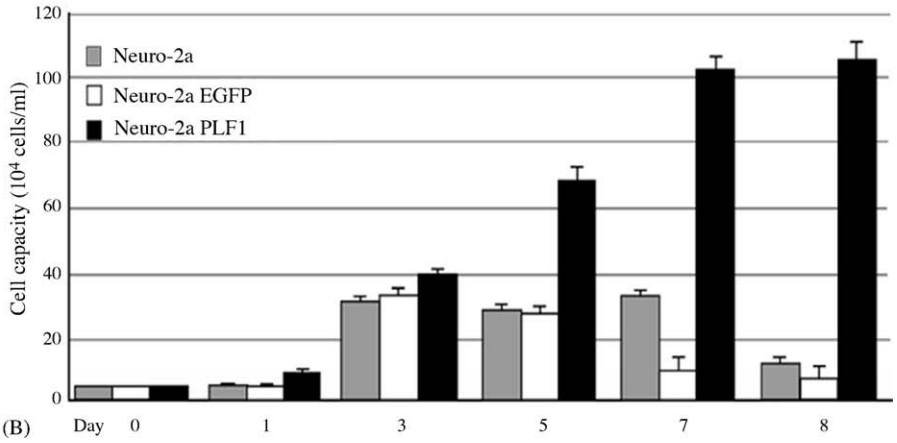
(A)


Neuro-2a
Neuro-2a EGFP
Neuro-2a PLF1
Cell capacity (10⁴ cells/ml)
120
100
80
60
40
20
0
Day 0 1 3 5 7 8

(B)

number of Neuro-2a cells stably expressing PLF1 or EGFP. Cellular proliferation was also determined. A significant increase of proliferation, ~8.3-fold of cell number was found at day 8 after plating ($P < 0.05$) in constitutive PLF1 expressing cells compared with EGFP expressing and normal cells (Fig. 7B). The proliferation declined both in normal and EGFP expressing cells after cells passed the plateau phase on day 7 after plating. Proliferation still increased in cells with stable expression of PLF1. This result suggests PLF1 is implicated in proliferation of mature cells.

4. Discussion

In this paper, we demonstrated that Mrp/Plfs are expressed both in mouse brain and also in Neuro-2a cells. Furthermore, the expression of *Mrp*/*Plf*s in mouse brain is developmentally regulated. In the developing fetal mouse brain, the expression level of *Mrp*/*Plf* mRNAs is lower at the neonatal stage during postnatal days 1 and 14 than that at the embryonic and mature stages. In the adult female mouse brain, the expression of *Mrp*/*Plf* mRNAs is constant throughout pregnancy and the weaning cycle, but slightly decreases during parturition. The amount of MRP/PLFs in the placenta appears at gestation day 8 and peak during days 10 and 13, then drops off rapidly. The time period and protein expression level of MRP/PLFs in placenta relates to the growth of the uterus and their production is proportional to the amount of fetal tissue. This suggests the number of fetuses may through MRP/PLFs pass a signal to uterus for proportional growth. In late gestation, the expression of MRP/PLFs quickly drops in placenta perhaps due to a negative feedback control loop that inhibits advance of uterine growth (Fang et al., 1999). Our findings clearly indicate that the regulating mechanism of expression of *Mrp*/*Plf* mRNAs in developing mouse brain is different from that in the placenta.

Mrp/*Plf* expression is mediated by intrinsic and extrinsic signaling. *Mrp*/*Plf* promoters contain an activator protein 1 (AP1) binding site and GATA2/3 consensus binding sites (Ng et al., 1994). The AP1 binding site has been shown to be responsible for inhibition of *Mrp*/*Plf* promoter activity after glucocorticoid treatment of mouse 3T3 cells (Mordacq and Linzer, 1989; Malkoski and Dorin, 1999). Because glucocorticoid is abundantly secreted into plasma from gonads at the onset of parturition in mammals (Whittle et al., 2001), we hypothesize that lower *Mrp*/*Plf* mRNAs in mouse brain during parturition and the neonatal stage of development may due to glucocorticoid suppression. Our data has shown that *Mrp*/*Plf* mRNAs are expressed in mouse fetal brains. The zinc finger transcription factors GATA2/3 are expressed and required for fetal nervous system development (Pandolfi et al., 1995; Nardelli et al., 1999) and induce MRP/PLFs expression in trophoblast giant cells of mouse placenta (Ma et al., 1997). Based on the existence of GATA2/3 response elements in *Mrp*/*Plf* promoter regions and the timing of *Mrp*/*Plf* fetal expression, we speculate that the expression of *Mrp*/*Plfs* at the embryonic stage in fetal brain may be regulated by GATA2/3.

In this study we found that the major *Mrp*/*Plf* genes expressing in mouse brain were *Plf1*, *Plf1* (−exon3), *Plf2* and *Mrp3*. *Plf1* (−exon3), a *Plf1* alternative splicing form had been found previously in cultured ehrlich carcinoma cells (Gil-Torregrosa et al., 1994). Our study reveals that *Plf1* (−exon3) not only exists in carcinoma cells but also in normal brain tissue. *Plf1* (−exon3) lacks two glycosylation sites and one cysteine residue needed for secretion and protein folding, respectively, suggesting that *Plf1* (−exon3) may not be secreted outside of cells and may have a different function to *Plf1*. In this study, for the first time *Mrp3* was demonstrated outside of muscle to be expressed in brain and Neuro-2a cells.

The microvillus is a new cell surface organelle. It originates from the endoplasmic reticulum, converts into exocytic membrane vesicles, then fuses and buds from the plasma membrane, where it is stabilized by F-actin bundle. The microvilli remain on the cell surface (Lange, 1999, 2000). Here we have demonstrated the recombinant PLF1 located in the ER-Golgi secretory pathway along with enhanced microvilli formation. We also have proved that IGF II/M6P receptor mRNA is expressed in Neuro-2a cells, and that the MRP/PLF receptor is located on Golgi membranes. The implication is that MRP/PLFs act for formation of microvilli through binding to IGF II/M6P receptors. We did not observe any exogenous PLF1 located at the cell membrane or co-localized with F-actin in microvilli, suggesting that PLF1 indirectly regulated the formation of the microvilli. We demonstrated that most of the MRP/PLFs were retained in the cytoplasm of cell stably expressing PLF1 (data not shown). Thus we hypothesized that PLF1 might not act through autocrine or paracrine mechanisms to achieve its function in Neuro-2a cells. Microvilli are enriched with glucose transporters and ion channels, hence are proposed to regulate glucose transport, ion fluxes, modulation of membrane potential and Ca^{2+} signaling. Microvilli also are the membrane structures to adapt environmental stimuli in many chemosensory and mechanosensory cells (Lange, 2000; Sekerková et al., 2004). The actin bundle in the tips of microvilli form the microvillar diffusion barrier, that can seal the cell surface to prevent unrestricted influx of metabolic substrates and ions in mature cells maintaining viability (Boman et al., 1983; Lange, 2000). We have shown that PLF1 increases cellular proliferation at the plateau growth phase of Neuro-2a cells, suggesting PLF1 is involved in maintaining survivability of mature neuronal cells in brain or is required for proliferation of neuroblastoma cells. We observed that *Mrp*/*Plf* mRNAs had increased expression in older mouse brain, when

Fig. 7. Expression of PLF1 enhances cellular proliferation and formation of microvilli structure on the dorsal surface of Neuro-2a cells. (A) Myc-tagged PLF1 or EGFP was transiently transfected into Neuro-2a cells as described. Cells expressing PLF1 were detected by immunofluorescent staining with anti-Myc monoclonal antibody and FITC conjugated secondary antibody. PLF1 transiently expressing cells are shown by arrowheads (a, c and e); EGFP transiently expressing cells are indicated by arrows (b, d and f). Hoechst 33,342 dye was used to stain nuclei (c and d). Scale bar is 15 μm. (B) PLF1 increases cellular proliferation in plateau growth phase of Neuro-2a cells. In the beginning, normal and stable expressing PLF1 or EGFP cells were seeded at same density of 4×10^5 cells/ml, cell number was counted as indicated. Three replicate experiments ($n = 3$) of each representative experiment were performed and analyzed by SAS software.

neurons were undergoing terminal differentiation. Based on the observation with Neuro-2a cells we speculated that PLF1 might be important for avoiding cell death at the mature stage. In conclusion, our data suggest the MRP/PLFs may play a role during brain development or neuroblastoma maturation.

Acknowledgements

This work was supported by a grant from the National Science Council of Taiwan, Republic of China (NSC 92-2313-B-002-145 and NSC 94-2317-B-002-020) and Council of Agriculture of Taiwan, Republic of China [93AD-4.1.3-AS-U1 (4)]. We are grateful to Dr. Kenrick Deen for help and advice in reviewing this manuscript.

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