online data

MARCKS-like protein, a membrane protein identified for its expression in developing neural retina, plays a role in regulating retinal cell proliferation

Jing ZHAO*, Tomonori IZUMI†[1], Kazuto NUNOMURA†[2], Shinya SATOH* and Sumiko WATANABE*[3]

*Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639, Tokyo, Japan, and †Department of Functional Proteomics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, 108-8639 Tokyo, Japan

Membrane proteins are expressed in a specific manner in developing tissues, and characterization of these proteins is valuable because it allows them to be used as cell surface markers. Furthermore, they are potentially important for the regulation of organogenesis because some may participate in signal transduction. In the present study, we used proteomics to examine the comprehensive protein expression profile of the membrane fraction in the embryonic and adult mouse retina. We purified the retinal membrane fraction by sucrose-density-gradient centrifugation and analysed total proteins using shotgun analysis on a nanoflow LC–MS/MS (liquid chromatography tandem MS) system. Approximately half of the 326 proteins from the adult retina and a quarter of the 310 proteins from the embryonic retina (day 17) appeared to be membrane-associated proteins. Among these, MLP [MARCKS (myristoylated alanine-rich C-kinase substrate)-like protein], which shares approx. 50 % amino acid identity with MARCKS, was selected for further characterization. The mRNA and surface protein expression of MLP decreased as retinal development progressed. Overexpression of MLP by retrovirus-mediated gene transfer enhanced the proliferation of retinal progenitor cells without affecting differentiation or cell migration in a retinal explant culture system. In contrast, MLP overexpression did not promote proliferation in fibroblasts (NIH 3T3 cells). Mutation analysis of MLP demonstrated that myristoylation was necessary to promote proliferation and that phosphorylation inhibited proliferation, indicating the functional importance of membrane localization.

Key words: cell proliferation, membrane protein, myristoylated alanine-rich C-kinase substrate (MARCKS), neural retina, protein kinase C (PKC), proteomics.

INTRODUCTION

The mature neural retina is organized into a three-layered structure consisting of six types of neurons and one type of glial cell. These seven types of cell are assumed to differentiate in a precise histogenic order from a single population of multipotent retinal precursors [1]. Various molecules, such as transcription factors and neurotrophic factors, have been reported to play important roles in retinal cell differentiation [2]. However, the intrinsic properties of retinal cells at different developmental stages are still vague. This is in part due to the lack of markers that can identify distinct stages of retinal progenitor cells. Although combinatorial expression of transcriptional factors and cell cycle regulators may represent intrinsic properties of the cells, these molecules are intracellular, limiting their usefulness as cell markers. In our previous studies, we have tried to identify markers of retinal progenitor cells using flow cytometry/cell sorting. We screened mouse retinal cells at various developmental stages for reactivity with a panel of antibodies against cell surface antigens. This technique revealed unique expression patterns for more than 30 antigens in the developing retina. Among these, SSEA-1 (stage-specific embryonic antigen-1) and c-kit were identified as retinal progenitor cell markers of early and late immature stages respectively [3,4].

However, since this approach identifies only known molecules, we used proteomics to examine the comprehensive expression profile of total membrane proteins from embryonic and adult mouse retina. Information about membrane proteins, which are expressed in a specific manner in the developing retina, may not only serve as tools for purifying retinal subfractions by cell sorting, but also for analysing the regulation of retinal development by receptor-signalling and cell surface molecules. To establish such a database, we used shotgun analysis and a nanoflow LC–MS/MS (liquid chromatography tandem MS) system to examine total protein expression in purified membrane fractions. Among these proteins, we focused on MLP [MARCKS (myristoylated alanine-rich C-kinase substrate)-like protein], which appeared to be expressed in the embryonic retina but not in the adult retina.

PKC (protein kinase C) is a family of diacylglycerol-activated, calcium-dependent protein kinases that have been implicated in a variety of biological processes, including embryogenesis, morphogenesis, growth and differentiation. MARCKS is a specific, widely distributed PKC substrate and its phosphorylation has been used as a marker of PKC activation *in vivo* [5]. MLP is homologous with MARCKS [6]; both proteins are myristoylated and contain three evolutionarily conserved motifs in the N-terminus, the intron splice site and the phosphorylation site. The expression patterns of MARCKS and MLP are very similar; mRNAs for both proteins are expressed in the developing neural tube and brain, and then in the spinal cord during later development [7]. They are also detected in the lung, adrenal gland, gut and kidney of adult mice. In neonatal mice brain, *in situ* hybridization has revealed that both MARCKS and MLP are very highly expressed in the retina [7].

Abbreviations used: BrdU, bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; E0, embryonic day 0; ED, effector domain; FCS, foetal calf serum; GFP, green fluorescent protein; EGFP, enhanced GFP; IRES, internal ribosome entry site; LC–MS/MS, liquid chromatography tandem MS; MARCKS, myristoylated alanine-rich C-kinase substrate; MLP, MARCKS-like protein; P0, postnatal day 0; PFA, paraformaldehyde; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; RT, reverse transcriptase.

[1] Present address: Department of Stress and Bio-response Medicine, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi, Japan.
[2] Present address: Link Genomics, Inc., Ube Research Institute, Ube-shi, Yamaguchi, Japan.
[3] To whom correspondence should be addressed (email sumiko@ims.u-tokyo.ac.jp).

Although MARCKS and MLP have similar expression patterns, knockout mice of each gene showed distinct phenotypes, suggesting that the biological significance of these genes are distinct and non-redundant. Targeted disruption of the gene encoding MARCKS resulted in universal perinatal death and defects in retinal lamination, in addition to decreased brain size and increased ventricular volume [8]. In homozygous knockouts of MLP, approx. 60 % of the embryos developed neural tube defects, including exencephaly and spina bifida, which resulted in high prenatal lethality [9]. Some MLP-deficient mice survived, but they displayed brain abnormalities. Furthermore, thinner, compressed retinas have been reported [9]; however, detailed analysis of this abnormality and the function and mechanisms of MLP in retinal development have not been investigated.

In the present study, we employed proteomics to obtain comprehensive protein expression profiles from membrane fractions purified from embryonic and adult neural retina. This analysis demonstrated that MLP expression decreases dramatically as the retinal development proceeds. By using an *in vitro* retinal explant culture system, we found that overexpression of MLP by retrovirus-mediated gene transfer enhanced the proliferation of the retinal precursor cells without affecting cell migration or cell fate. Mutation analysis of the conserved domains of MLP revealed that myristoylation is necessary for MLP-induced proliferation of retinal progenitors. In contrast, a mutation that mimics constitutive phosphorylation blocked MLP-enhanced proliferation, indicating that the protein must remain dephosphorylated for optimal function.

MATERIALS AND METHODS

Mice

ICR mice were obtained from Japan SLC Co. and Japan Clea Co. The day on which a vaginal plug was observed was considered to be E0 (embryonic day 0), and the day of birth was marked as P0 (postnatal day 0). All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Preparation of retinal membrane fractions

Eyes ($n = 210$) from E17 mice and 16 eyes from adult mice were used for protein purification. Isolated retinas were rinsed twice in ice-cold PBS supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+) and then suspended in 10 mM Hepes/NaOH (pH 7.5) supplemented with 0.25 M sucrose [8.5 % (w/v)] and protease inhibitor cocktail (Roche). The cells were lysed by nitrogen cavitation {at 800 lbf/in^2 (1 lbf/in^2 = 6.9 kPa) on ice for 20 min [10]} and then centrifuged at 3000 ***g*** for 10 min to remove large cell debris and nuclei. The supernatant was layered on a discontinuous sucrose density gradient [15, 30, 45 and 60 % (w/v) sucrose in 10 mM Hepes/NaOH, pH 7.5], and then centrifuged at 25 000 rev./min for 17 h [11]. The cell lysates were divided into eight fractions according to their density. Each fraction was harvested from the bottom of the centrifugation tubes and subjected to the following steps. Fractions were analysed by SDS/PAGE followed by Western blotting as described previously [12]. To identify the fraction enriched for plasma membrane proteins, we used anti-mouse gp130 [IL-6R (interleukin-6 receptor) subunit] antibody (1:1000; MBL Co.) and an appropriate secondary antibody. Western blots were visualized by LumiLight (Roche) according to the manufacturer's instructions.

Tryptic digestion

The protein fractions that contained membrane proteins were combined and diluted 4-fold with distilled water and then centrifuged at 26 000 rev./min for 2 h to obtain membrane-rich pellets. The membrane fractions purified by sucrose-density-gradient centrifugation were delipidated twice with unlabelled acetone, dried and solubilized with 8 M urea in 400 mM NH_4HCO_3 (pH 8.5). Protein samples were reduced with 2 mM dithiothreitol at room temperature (25 °C) for 30 min, alkylated with 2 mM iodoacetamide for 30 min and diluted 4-fold with distilled water. Protein samples were then digested with Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane; 'TPCK')-treated porcine trypsin (Promega, Madison, WI, U.S.A.) at an enzyme/substrate ratio of 1:100 (w/w) at 37 °C for 16 h.

Automated multidimensional LC–MS/MS analysis

The peptide mixtures were analysed on an automated nanoflow LC–MS/MS system using reverse-phase chromatography as described previously [13]. Briefly, the peptide mixture was captured on a trap column (Mightysil C18, 0.5 mm internal diameter × 1 mm long, 3 μm particles; Kanto Chemicals, Tokyo, Japan) for desalting and then separated on a Mightysil C18 column (0.15 mm internal diameter × 40 mm long, 3 μm particles; Kanto Chemicals) using a three-step linear gradient (0–30 % acetonitrile in 0.1 % formic acid for 120 min, 30–70 % acetonitrile in 0.1 % formic acid for 40 min and 70 % acetonitrile in 0.1 % formic acid for an additional 10 min) at a flow rate of 50 nl/min. The eluted peptides were sprayed directly into a high-resolution quadrupole time-of-flight hybrid mass spectrometer (Q-Tof-2; Micromass, Manchester, U.K.).

Protein identification by database search

The MS/MS signals were acquired by MassLynx (Micromass) and converted into text files by ProteinLynx software (Micromass). The database search was performed in triplicate by MASCOT (Matrix Science, London, U.K.) against the Refseq mouse, human and rat sequence databases with the following parameters: fixed modification, carbamoylmethylation (cysteine); variable modification, oxidation (methionine); maximum missed cleavages = 3; peptide mass tolerance = 150 p.p.m.; and MS/MS tolerance = 0.5 Da. For peptide and protein identification, the search results were processed as follows. (i) The candidate peptide sequences were screened with the probability-based MOWSE (molecular weight search) scores that exceeded their thresholds ($P < 0.05$) and with MS/MS signals for *y*- or *b*-ions 3. (ii) Redundant peptide sequences were removed. (iii) Each peptide sequence was assigned to a protein that gave the maximal number of peptide assignments among the candidates. (iv) The mouse, human and rat datasets were combined. (v) Interspecies redundancy of proteins was removed. Methods of protein identification and data processing are described in greater detail in a previous study [14]. A proteomics table containing all the identified proteins is provided as Supplementary Table 1 (http://www.BiochemJ.org/bj/408/bj4080051add.htm).

RT (reverse transcriptase)–PCR

Total RNA was purified from different stages of mouse retina, brain and liver cells using TRIzol® reagent (Gibco BRL), and cDNA was synthesized using Superscript II (Gibco BRL). All primer sets were tested for several different cycling numbers (25–35 cycles) using ExTaq or rTaq (Takara), and the optimal semi-quantitative cycle number was determined for each primer set. Bands were visualized with ethidium bromide.

Plasmid construction and production of retrovirus

The mouse MLP cDNA was cloned by RT–PCR from pooled mouse cDNA from P1 retina. The primers were designed based on the sequences available in the database. A full-length fragment of MLP was cloned into pMXc-IRES-EGFP [internal ribosome entry site-enhanced GFP (green fluorescent protein)] retrovirus vector at the NotI site. The vector was kindly provided by Dr T. Kitamura (Institute of Medical Science, Tokyo University, Tokyo, Japan). Production of the retrovirus was performed using the PLAT-E packaging cell line [15] as described previously [16].

Retinal explant culture, retrovirus infection and re-aggregation culture

Retinal explant cultures were prepared as described previously [16]. Briefly, the neural retina was isolated on a Millicell chamber filter and placed with the ganglion cell layer facing upwards. The filters were inserted into 6-well plates and cultured in 1 ml of explant culture medium [50 % (v/v) minimum essential medium containing Hepes buffer (Gibco), 25 % (v/v) Hanks balanced salt solution (Gibco) and 25 % (v/v) heat-inactivated horse serum (JRH Biosciences) supplemented with 200 μM L-glutamine, 5.75 mg/ml glucose, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco)] [16]. Explants were cultured at 34 °C in 5 % CO_2, and the medium was changed every other day. In some cases, the explants were infected with retrovirus and cultured for several more days. Cells were then fixed with 4 % (w/v) PFA (paraformaldehyde) and frozen-sectioned.

Re-aggregation cultures were prepared as described previously [17] with minor modifications [4]. After overnight retrovirus infection, the cells were dissociated and re-aggregated. The ratio of the host to donor cells was 1000-fold for clonal analysis.

In situ hybridization, immunohistochemistry and antibodies

In situ hybridization was performed using a DIG (digoxigenin)-labelled RNA probe according to a standard protocol [3]. Hybridized DIG-labelled RNA probes were detected with an alkaline phosphatase-conjugated anti-DIG antibody and visualized using a BCIP (5-bromo-4-chloroindol-3-yl phosphate)/NBT (Nitro Blue Tetrazolium) solution kit (Nacalai Tesque).

Immunohistochemistry of retinal explants was carried out as described previously [4,16]. The primary antibodies used were anti-GFP polyclonal antibody (ClonTech Laboratories), anti-Rho4D2 (kindly provided by Dr R. S. Molday, Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, BC, Canada), anti-(glutamine synthetase) (Chemicon), anti-HuC/D (1:1000; Molecular Probes), anti-PKC (1:20; Oncogene Research Product) and anti-Ki67 (1:200; BD Biosciences). The primary antibodies were visualized using anti-rabbit IgG–Alexa Fluor® 488 or anti-mouse IgG–Alexa Fluor® 546 (Molecular Probes) as secondary antibodies. All the samples were sealed using VectaShield mounting medium (Vector Laboratories) containing DAPI (4′,6-diamidino-2-phenylindole) for nuclear staining.

BrdU (bromodeoxyuridine) labelling and detection

Retinal explants were incubated with 5 μM BrdU for 24 h at 3 or 4 days after retrovirus infection, harvested and fixed with 4 % PFA. The samples were embedded in OCT compound and frozen-sectioned. Then, the sections were treated with 1 unit/μl of DNase (Takara) in PBS for 1 h at 37 °C, and the incorporated BrdU was visualized immunohistochemically using anti-BrdU monoclonal antibody (Roche) and appropriate secondary antibodies.

NIH 3T3 cells

NIH 3T3 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with 10 % (v/v) FCS (foetal calf serum). The cells were plated on to 8-well plates (1×10^4 cells/well) and then transfected the next day with either pMXc-IRES-EGFP or pMXc-full-length MLP-IRES-EGFP (0.5 μg/well) using FuGENE™ 6 transfection reagent (Roche). After 24 h of culture, the medium was replaced with DMEM supplemented with 1 or 2.5 % FCS. Cells were harvested after another 24 h of culture, fixed with 4 % PFA and then immunostained with anti-Ki67 and anti-GFP antibodies.

RESULTS

Preparation of membrane proteins

To identify comprehensive expression patterns of cell membrane proteins during the development of the mammalian retina, we first optimized the experimental conditions using adult neural retina of ICR mice. As described in the Materials and methods section, we separated the cell lysate into eight fractions by sucrose-density-gradient centrifugation. To identify which of the fractions contained membrane proteins, we immunostained our Western blots for the marker protein gp130, which is one of the cytokine receptors localized in the plasma membrane. The distribution of gp130 indicated that fractions 3 and 4 were enriched with membrane proteins (Figure 1A); these fractions were then combined and processed to prepare a tryptic peptide mixture for MS identification. The fully automated nanoflow LC separation of the tryptic peptides followed by data-dependent MS/MS analysis generated ~2500 MS/MS spectra in a single analysis, from which 500 non-redundant peptides were identified by a database search against Refseq with criteria as described previously [18]. The peptides were ultimately assigned to 326 proteins in the adult retina (see Supplementary Table 1). We then subjected the membrane fractions from E17 retinas to proteomic analysis, from which 310 proteins were identified (Supplementary Table 1). We classified the proteins from adult and embryonic retinas into seven groups and one unclassified group, according to information found in the GO database and the previous literature. The proteins in each of the seven groups were then subcategorized into either membrane or non-membrane proteins. Only 5 % of proteins from embryonic retinas and 14 % of proteins from adult retinas appeared to be plasma membrane proteins. However, when membrane proteins from all seven groups were taken into account, it was revealed that approx. 30 % of the identified proteins from embryonic retinas and 50 % of identified proteins from adult retinas were membrane proteins (Table 1). Our results from adult retina are comparable with those from a previous study using a similar protocol to prepare membrane fractions from breast cancer cells [11]. Adam et al. [11] reported that 51 % of total proteins were membrane-related in some way, including transmembrane, membrane-associated and integral membrane proteins. However, a fraction of only 30 % membrane-related proteins was detected in the embryonic retina, suggesting that the structural and/or physical characteristics of cellular membranes in the retina could differ depending on the developmental stage.

Proteins expressed specifically in the embryonic retina

The purpose of the present study was to identify surface markers on retinal progenitor cells and thus identify a tool for the purification of retinal progenitors and the analysis of early retinal development. Therefore we focused our efforts on the proteins identified specifically in the embryonic retina. Among

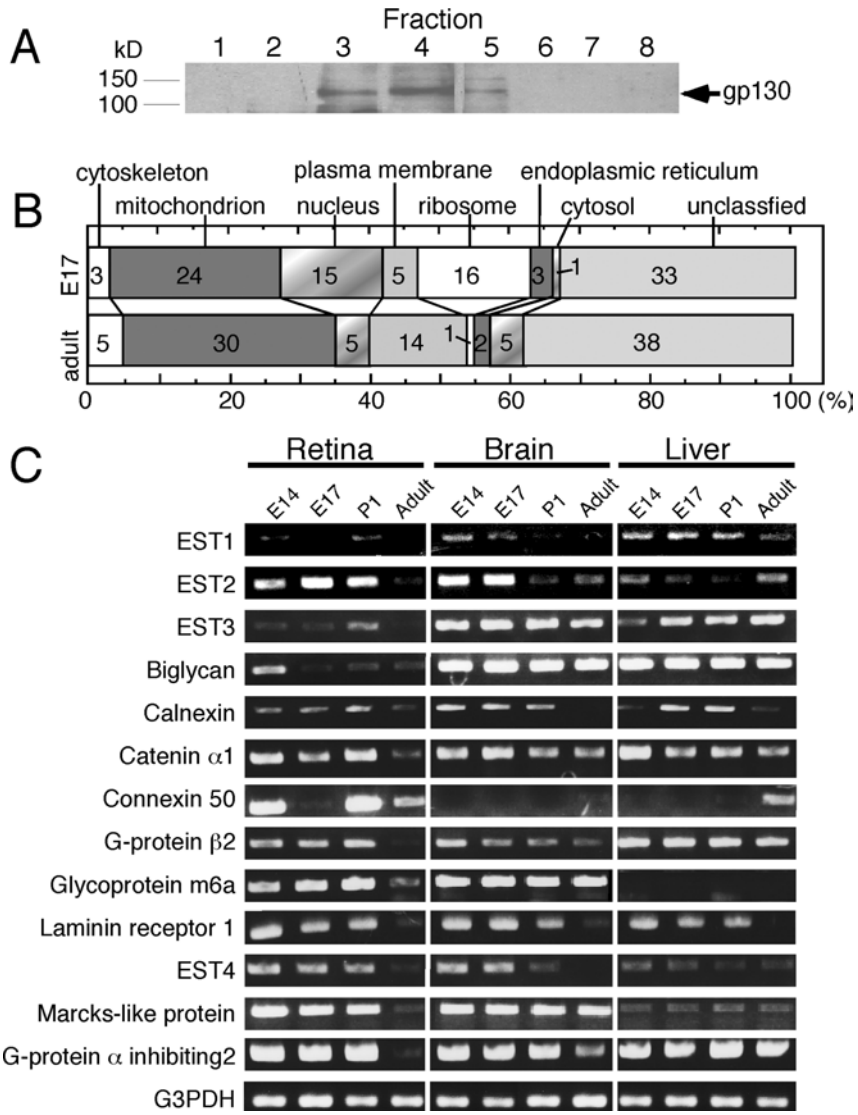

Figure 1 Comprehensive proteomic analysis of membrane proteins in the developing retina

(**A**) Western-blot analysis of fractionated proteins using an anti-gp130 antibody. Aliquots of each fraction were subjected to Western blotting. Protein size was estimated against a protein molecular mass marker. Arrow indicates gp130 (130 kDa). (**B**) Classification of proteins identified from retinal membrane preparations. Proteins were classified into the eight groups illustrated using the GO database. (**C**) Semi-quantitative RT–PCR results from representative genes. Total RNA was purified from neural retina, brain or liver at the indicated developmental stages, and semi-quantitative RT–PCR was conducted. Bands were visualized by ethidium bromide. EST, expressed sequence tag; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

the 310 proteins found in the E17 retina, 203 proteins were predominantly found in the embryonic retina. To confirm the MS data and identify candidates for further biological study, 13 of the 203 proteins were selected from the protein list based on the frequency of peptide hits in embryonic retina. The proteins were selected from the top of the list but on condition that proteins are classified as membrane or membrane-related proteins. In the large-scale proteomic analysis, since the peptide-hit number is assumed to correspond to the expression level, it was surmised that we selected candidate proteins that are highly and specifically expressed in the embryonic retina. Expression levels in neural retina, brain and liver at different developmental stages were examined by semi-quantitative RT–PCR (Figure 1C). As expected, expression levels of these proteins in the retina decreased throughout retinal development, confirming the reliability of our proteomic analysis. Notably, connexin 50 showed a biphasic expression pattern, i.e. the strong expression at E14 decreased at E17 and further expression was observed at P1. In addition, the results suggest that expression of these proteins is regulated at the level of transcription. Similar changes in expression levels were also observed in the brain for some of these proteins. However, the expression levels of biglycan, MLP and glycoprotein m6a mRNA did not decrease in the brain, as they did in the retina, suggesting that transcription of these genes is regulated specifically in the retina and that the resultant protein products are involved in retinal development.

MLP is specifically expressed in the developing retina

Among the three proteins implicated in retinal development, we focused on MLP for further analysis. We first examined the expression of MLP mRNA over time, using semi-quantitative RT–PCR (Figure 2A). MLP was expressed in E14 mouse retina; after birth, expression started to decrease until only a faint band

Table 1 Classification of proteins identified from embryonic and adult neural retina cell membrane preparations

Proteins were classified into seven groups and one unclassified group as shown in Figure 1(B). Each group was further subcategorized into membrane and non-membrane proteins. The numbers indicate the number of proteins in each subcategory.

	Embryo		Adult	
	Membrane	Non-membrane	Membrane	Non-membrane
Mitochondrion	29	51	26	54
Endoplasmic reticulum	4	4	0	5
Cytoplasmic		77		50
Nuclear	2	55	2	10
Cytoskeleton	5	32	4	9
Plasma membrane	18		40	
Unspecified membrane	18		44	
Subtotal	76	219	116	128
Unclassified	47		93	
Total	342*		337*	

* Since some proteins are double classified into more than one category, the total number is less than the sum of all categories.

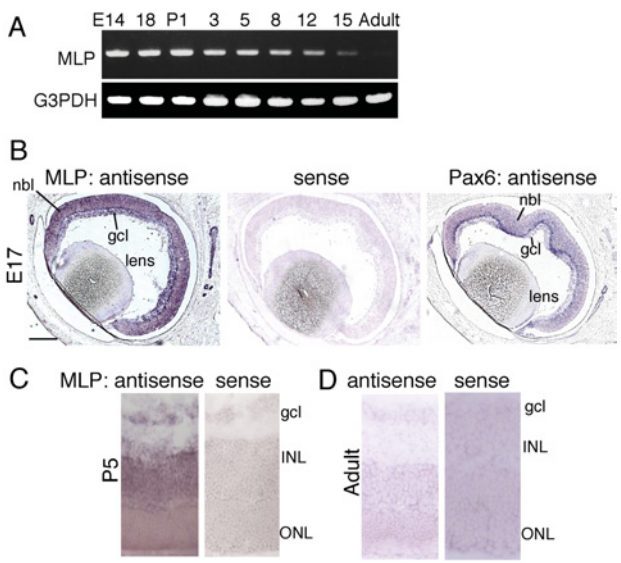

Figure 2 mRNA expression of MLP in the retina at various developmental stages

(**A**) Semi-quantitative RT–PCR using RNA purified from neural retina at various developmental stages. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control. Bands were visualized by ethidium bromide. (**B**–**D**) *In situ* hybridization of the mouse retina at E17 (**B**), P5 (**C**) and adult (**D**) stages. Probes used are antisense- and sense-MLP and antisense-Pax6 cDNAs. Scale bar, 200 μm. nbl, neuroblastic layer; gcl, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

was visible at P15. Since a previous study reported that the neural tube expresses MLP [7], we speculate that MLP transcripts are expressed continuously during the neural tube stage. Since previous investigations of retinal MLP mRNA expression in neonatal mice [7] did not examine expression patterns in detail, we performed *in situ* hybridization of MLP in sections of mouse retina at various developmental stages. In mouse E17 retina, MLP mRNA was expressed broadly in the neuroblastic layer and ganglion cell layer (Figure 2B), in contrast with the expression of Pax6, which appeared in the neuroblastic layer but not in the ganglion cell layer (Figure 2B, right-hand panel). At the P5 stage, MLP expression was apparent in all layers of the retina (Figure 2C); in contrast, hybridization signals could not be detected in the adult retina (Figure 2D).

MLP overexpression does not affect cell fate or subretinal localization of retinal precursors

We then examined the role of MLP in retinal development using embryonic retinal explant cultures, which provide an excellent model to monitor differentiation *in vivo* [16]. Retinas were isolated from mouse embryos on day 17 and placed on a filter with the ganglion cell layer facing up. By this developmental stage, ganglion cells and a few other cells types had begun to differentiate; after 2 weeks in culture, all of the retinal subpopulations had differentiated properly. We overexpressed MLP in the E17 mouse retina by infecting the cells with a retrovirus vector containing either MLP–EGFP or EGFP only (control), and then cultured the retina for 2 weeks. Since the retrovirus infects only mitotic cells, retinal precursor cells were assumed to be the major targets for gene transfer. The explanted retinal cells differentiated during culture, and the virus-infected cells could be identified by analysing for EGFP-positive cells in frozen sections. We first examined frozen sections of the explants to determine the subretinal localization of virus-infected cells. We found that the cells expressing EGFP and MLP–EGFP were distributed similarly among the three layers of the retina (Figure 3A). We then examined the differentiation of the virus-infected cells by immunostaining frozen sections with antibodies against various marker proteins for retinal cell subpopulations (Figure 3C). The antibodies used were anti-rhodopsin for rod photoreceptors, anti-HuC/D for retinal ganglion cells and amacrine cells, anti-PKC for bipolar cells, and anti-(glutamine synthetase) for Muller glia. The population of EGFP–MLP-overexpressing cells that also stained positive for rhodopsin was slightly smaller than the population of rhodopsin-positive control cells, but the difference was not significant (Figure 3B). None of the other retinal cell populations was affected by MLP overexpression.

MLP promotes the proliferation of retinal precursor cells

Next, we examined the proliferation of retinal cells expressing EGFP or MLP by measuring BrdU incorporation. Retinal explants were infected with retrovirus encoding MLP–EGFP or EGFP (control) and cultured for 4 or 5 days. BrdU (5 μM) was added in the last 24 h of culture, and the incorporation of BrdU was examined by immunostaining with an anti-BrdU antibody. Among EGFP-expressing cells, nearly 11 % of the population was BrdU-positive by day 3–4, after which the BrdU-positive population decreased to 8 % by day 4–5 (Figure 4A). Among MLP-overexpressing cells, the population of BrdU-positive cells had increased by day 3–4 and day 4–5, with values that were nearly twice as high as the control samples (Figure 4A). To confirm these results, we stained the retinal explants for Ki67, a nuclear antigen expressed in proliferating cells [19]. The proportion of Ki67-positive cells was also significantly higher in MLP-overexpressing cells than in control cells after 4 or 6 days of culture (results not shown and Figure 5B). Then, we examined whether the MLP-induced enhancement of retinal cell proliferation leads to an increase in cell number using isolated EGFP-positive cells in re-aggregation culture, which is an excellent model to investigate proliferation and differentiation of retinal progenitors *in vitro* [4]. Dissociated E17 retinas, infected with either MLP–EGFP or EGFP retrovirus, were mixed with an excess amount of dissociated retinal cells from the same stage. The proliferative

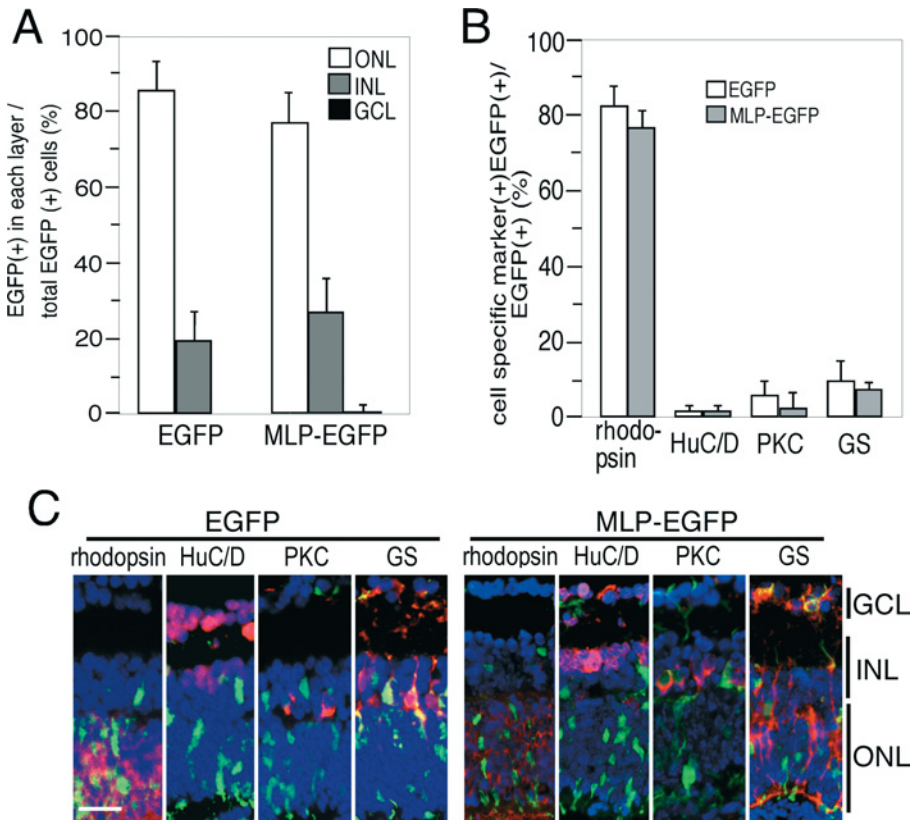

Figure 3 Migration and differentiation of retinal progenitor cells expressing MLP

(**A**) Retinal explants were infected with retroviruses encoding either EGFP control- or MLP–EGFP. After 2 weeks of culture, cell distribution in the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) was examined in frozen sections. (**B**, **C**) Cell differentiation was examined by immunostaining with antibodies against specific marker proteins to identify subpopulations of retinal cells. The markers used are: rhodopsin for rod photoreceptors, HuC/D for amacrine and ganglion cells, glutamine synthetase (GS) for Muller glia cells and PKC for bipolar cells. Populations of the differentiated immunoreactive cells expressing either EGFP (control) or MLP–EGFP are shown in (**B**). Immunostained fluorescent images of EGFP-expressing retinal sections (control; left-hand panel) and MLP–EGFP-expressing retinal sections (right-hand panel) are shown in (**C**). Nuclei are stained with DAPI. The values shown are the means for three samples with S.D. Scale bar, 50 μm.

ability of the transplanted cells was assayed by counting the number of cells in each colony derived from a single founder cell after 5 days of culture. More than 80 % of the colonies derived from control cells remained as single cells; in contrast, only 60 % of the colonies remained as single cells when the donor cells overexpressed MLP (Figure 4B). Only 7.6 % of colonies founded by control cells had more than three cells/colony; in contrast, 21.9 % of colonies founded by MLP-overexpressing donors had more than three cells. The average colony size of control compared with MLP-expressing retinal cells was also significantly different (Figure 4C). The above findings indicate that MLP promotes not only the proliferation of retinal progenitor cells, but also an increase in cell numbers.

MLP overexpression does not affect apoptosis in retinal explants

To elucidate the possible mechanism by which MLP promotes proliferation of retinal precursors, we investigated whether MLP overexpression had any effect on apoptosis. *In situ* detection of apoptotic cells was performed by immunohistochemical staining with an anti-active caspase 3 antibody. The number of positive cells was counted under a microscope. The percentage of apoptotic cells among MLP-overexpressing retinal cells was not significantly different from that among control cells (Figure 4D). This suggests that the ability of MLP to promote the proliferation of retinal progenitor cells is not via regulation of apoptosis.

MLP does not alter proliferation in NIH 3T3 cells

Next we examined whether MLP affects proliferation in non-retinal cell types. We transfected NIH 3T3 fibroblasts with plasmids encoding EGFP (control) or MLP–EGFP, and examined the effect on their proliferation as indicated by the expression of the Ki67 antigen. After 48 h of the culture, cells were harvested and the expression of Ki67 in EGFP-positive cells was analysed by immunostaining with anti-Ki67 and anti-EGFP antibodies. As shown in Figure 4(E) (left-hand panel), nearly 80 % of cells were Ki67-positive in the control samples. Likewise, approximately the same proportion of MLP-overexpressing cells was Ki67-positive, suggesting that MLP does not affect proliferation in NIH 3T3 cells. Next, we repeated this experimental design with the following modification: after 24 h of transfection, the concentration of FCS, which may itself promote substantial background proliferation, was reduced from 2.5 to 1 % (Figure 4E, right-hand panel). The population of Ki67-positive cells in the control decreased as expected; however, MLP-overexpressing cells responded in a similar manner, supporting the idea that MLP does not affect proliferation activities in NIH 3T3 cells.

Myristoylation is necessary for the function of MLP, and phosphorylation neutralizes its function

As a member of the MARCKS family of PKC substrates, MLP shares three conserved domains with its related proteins: a

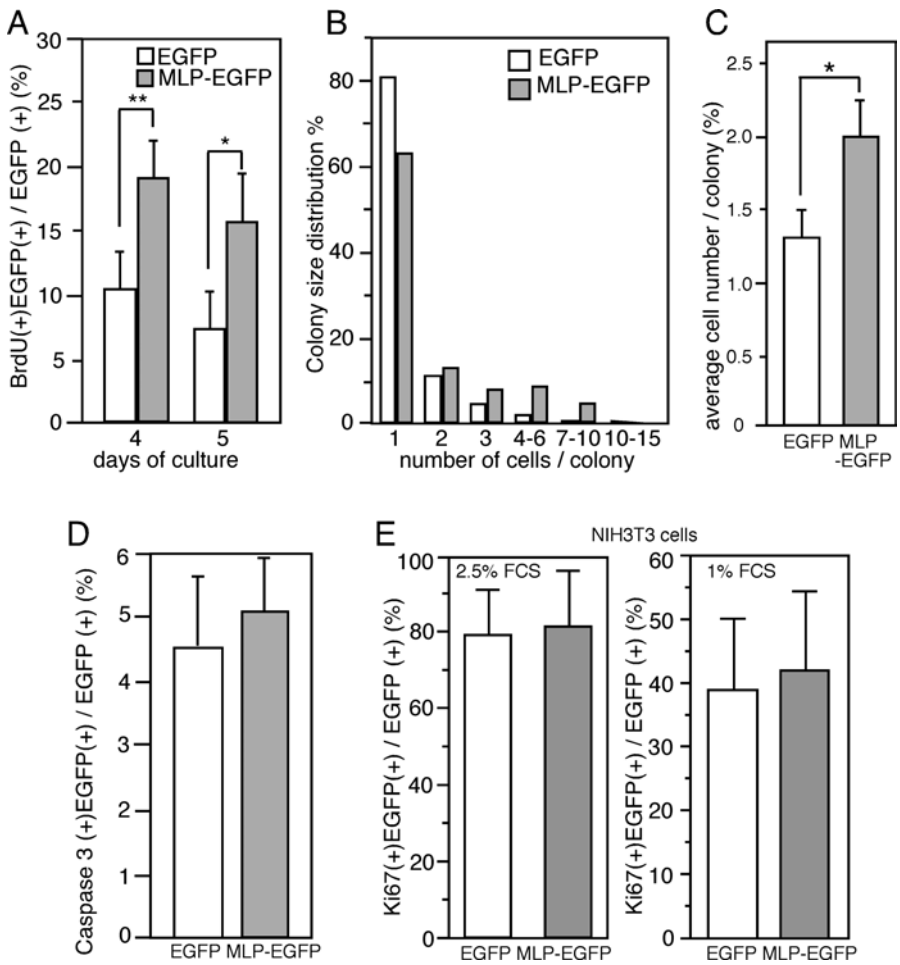

Figure 4 Effect of retrovirus-mediated expression of MLP on retinal proliferation

(**A**) BrdU incorporation into infected retinal cells. Retinal explants were infected with either the control or MLP–EGFP virus and cultured for 4 or 5 days. BrdU at 5 μM was added during the last 24 h. The percentage of BrdU-positive cells was measured by immunostaining with an anti-BrdU antibody. The values shown are the means for three samples with S.D. (**B**) Colony-forming analysis of control- or MLP-IRES–EGFP-virus-infected retinal cells. Virus-infected retinal explants were subjected to re-aggregation culture. After 5 days of culture, colony size (number of cells in each colony) was examined under a fluorescence microscope. More than 100 colonies were examined for each sample. Distribution of colony size is shown in (**B**), and average colony size with S.D. is shown in (**C**). Experiments were performed at least three times, and we obtained essentially the same results. (**D**) Apoptotic cells were examined by immunostaining with anti-active caspase 3 antibody. Population of fragmented caspase-3-positive cells in control-EGFP- or MLP–EGFP-expressing cells is shown. The values shown are the means for three samples with S.D. (**E**) Proliferation of NIH 3T3 cells expressing either control-EGFP or MLP–EGFP. NIH 3T3 cells were transfected with plasmids encoding either EGFP or MLP–EGFP. After 2 days of culture, cell proliferation in the presence of either 1 or 2.5 % FCS was examined by immunostaining of an anti-Ki67, proliferation antigen, antibody. The values shown are the means for three samples with S.D. $*P < 0.05$; $**P < 0.01$.

membrane-binding myristoylation domain, an MH2 (Mad homology 2) domain of unknown function and an ED (effector domain) that binds calmodulin and actin. To further dissect the mechanism of MLP in the enhancement of retinal proliferation, we constructed various mutants of MLP as indicated in Figure 5(A). wt-MLP is the full-length wild-type protein, whereas S3A-MLP contains amino acid substitutions in the ED domain. In this mutant, three serine residues that are known to be PKC targets in MARCKS were replaced by alanine residues to create a molecule that could not be phosphorylated. In the mutant S3D-MLP, the same three serine residues were replaced by aspartate residues to mimic constitutive phosphorylation by PKC. The mutant GA-MLP had an N-terminal glycine residue replaced for an alanine residue to create a form of MLP that could not be myristoylated. E17 retinal cells were infected with IRES–EGFP retroviruses encoding one of these mutants or wt-MLP. The proliferating cells were then stained with an anti-Ki67 antibody after 4 days of *in vitro* culture (Figure 5B). Overexpression of wt-MLP increased the population of Ki67-positive cells as expected, and S3A-MLP expression induced similar proliferation although to a lesser extent than wt-MLP. However, when cells were infected with the GA-MLP or S3D-MLP expression, the population of cells was not significantly different from the control cells. These results indicate that MLP positively affects retinal precursor cell proliferation in a manner related to phosphorylation within the ED. In addition, the lack of proliferative ability displayed by the GA-MLP mutant, in which myristoylation was abolished but the ED was intact, suggests that myristoylation is necessary for MLP to promote proliferation.

DISCUSSION

Construction of a database of membrane protein expression profiles in embryonic and adult neural retinas

The membrane properties of cells play important roles in integrating extrinsic information as it is relayed through ligand/receptor systems, the extracellular matrix and adherent molecules.

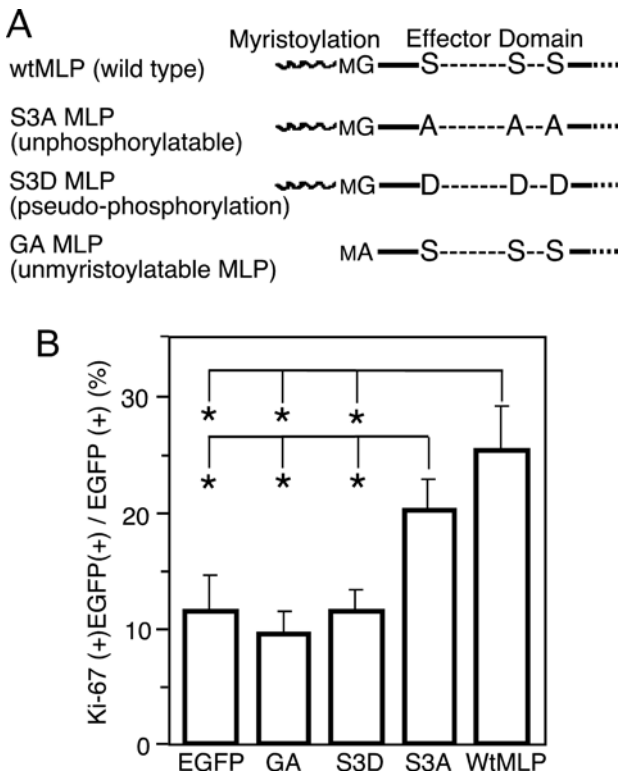

Figure 5 Expression of MLP mutants in retinal explant

(**A**) Schematic representation of MLP mutants. (**B**) Proliferation was examined by immunostaining with an anti-Ki67 antibody, which recognizes the proliferation antigen. Retinal explants were infected with retrovirus encoding wild-type or mutant forms of MLP and then harvested after 4 days of culture. The expression of Ki67 was examined by immunostaining frozen sections. The values shown are the means for three samples with S.D. $^{*}P < 0.05$.

This information is used to modulate cell-intrinsic properties, such as proliferation, survival and differentiation. In the present study, we identified more than 300 proteins from the membrane fractions of adult and embryonic mouse retinas. Results from mRNA analysis of 13 representative proteins showed clear agreement with protein expression patterns in embryonic and adult retinas obtained through proteomics, demonstrating that the strategy described here is an efficient means of characterizing the cell surface subproteome in the developing neural retina. Nearly half of the total proteins found in the adult retina were identified as membrane proteins according to Gene Ontology annotation (GO database). However, our strategy did not yield membrane proteins free of contamination. Resultant protein fractions contained abundant housekeeping molecules, such as ribosomal constituents, cytosolic and nuclear molecules, especially in the fractions derived from the embryonic retina. Although the yield of membrane-associated proteins from total protein in the adult retina was comparable with that previously reported from breast cancer [11], the results suggest that this purification protocol should be modified to adapt for the physical characteristics of plasma membrane in the case of the embryonic retina.

MLP promotes the proliferation of retinal progenitor cells

We found MLP protein expression in mouse retina derived from embryonic stages, but not from adult stages. Since our RT–PCR results also showed immature stage-specific expression of MLP mRNA, the expression of MLP may be mainly regulated at the level of transcription. The strong expression of MLP mRNA in the neuroblastic layer, which consists mostly of retinal progenitor cells, coincides with our findings that MLP promotes cell proliferation during retinal development. To date, no report has been published about the role of MLP in proliferation; however, the reduced size of the retina observed in MLP-knockout mice supports our findings [9]. MARCKS affects proliferation negatively in different cell types such as Swiss 3T3 and some tumours [20–22]. On the other hand, MARCKS deficiency in mice causes abnormal brain development, resulting in decreased head and brain size and increased ventricular volume [7]. These results imply that MARCKS plays a positive role in proliferation and that this activity acts in a neuro-specific manner. Similar conclusions concerning MLP can be surmised from reports that MLP-knockout mice show reduced brain size, enlarged ventricles, and a thinner, compressed retina [9]. Our results show that MLP expression does not affect proliferation in NIH 3T3 fibroblasts, which supports the idea that MLP-induced proliferation is cell-type-specific. Given that the conserved domains of MARCKS and MLP are very similar, promotion of proliferation in certain cells may be a common feature of these proteins.

Myristoylation of MLP functions in combination with ED

Using mutation analysis, we found that myristoylation is critical for MLP to promote proliferation in retinal progenitor cells. Myristoylation affects the function of many signalling molecules, such as p60src and p56lck in cell transformation [23]. MARCKS binds to membranes through electrostatic and hydrophobic interactions in both *in vitro* and *in vivo* studies [24,25]. However, *in vitro* studies of MLP binding to membranous structures have shown a weak co-operativity between the ED and myristoylation [26]. In contrast, phosphorylation may be involved in the translocation of MLP [27]. We observed that unphosphorylatable MLP promotes retinal cell proliferation, but that unmyristoylated or pseudo-phosphorylated MLP do not. This suggests that demyristoylation or phosphorylation causes MLP to translocate into the cytosol, thus inhibiting its function. Introduction of various mutants of MARCKS into MARCKS-knockout mice has been reported [28,29]. A non-myristoylatable, pseudo-phosphorylated form of MARCKS was able to complement almost all cerebral anatomical abnormalities. However, these mice also exhibited a novel phenotype: profound retinal ectopia. Although we did not observe any additional phenotypic changes in our infected retinal explants, our results and the work of others indicate that MLP-regulated signal transduction may act in a cell-type-specific manner.

Possible role of the PIP2 (phosphatidylinositol 4,5-bisphosphate) signalling pathway

The EDs of MARCKS and MLP have a high affinity for PIP2 [30]. MARCKS and MLP may regulate PIP2 availability through non-specific local electrostatic interactions in response to upstream signalling that activates PKC and/or Ca^{2+}/calmodulin [31]. Modulation of local PIP2 concentration leads to activation of PLD (phospholipase D) [32]. PLD functions in several ways to affect proliferation [33] such as overcoming cell cycle arrest, preventing apoptosis, or enhancing cell cycle progression in various cells. Based on our results, we can speculate that MLP promotes proliferation through elevation of the local levels of PIP2 and subsequent activation of PLD signalling. We speculate that unmyristoylatable MLP or pseudo-phosphorylated MLP cannot dock to the membrane in retinal progenitors and thus is unable to capture as much PIP2 as a wild-type protein can. In contrast, unphosphorylatable MLP binds to the membrane for a longer time and, as a result, the mutant is able to promote proliferation

of retinal precursors in a fashion similar to wild-type MLP. To our knowledge, the present study is the first showing that MLP plays a role in the proliferation of neural precursor cells. Further research into the mechanism of MLP function during retinal development is in progress.

We are grateful to R. Whittier and Y. Ouchi for discussions and C. Itagaki for technical support on LC–MS/MS analyses and data processing. This work was supported a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1 Marquardt, T. and Gruss, P. (2002) Generating neuronal diversity in the retina: one for nearly all. Trends Neurosci. **25**, 32–38
2 Jean, D., Ewan, K. and Gruss, P. (1998) Molecular regulators involved in vertebrate eye development. Mech. Dev. **76**, 3–18
3 Koso, H., Satoh, S. and Watanabe, S. (2007) c-kit marks late retinal progenitor cells and regulates their differentiation in developing mouse retina. Dev. Biol. **301**, 141–154
4 Koso, H., Ouchi, Y., Tabata, Y., Aoki, Y., Satoh, S., Arai, K. and Watanabe, S. (2006) SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells. Dev. Biol. **292**, 265–276
5 Aderem, A. (1992) The MARCKS brothers: a family of protein kinase C substrates. Cell **71**, 713–716
6 Umekage, T. and Kato, K. (1991) A mouse brain cDNA encodes a novel protein with the protein kinase C phosphorylation site domain common to MARCKS. FEBS Lett. **286**, 147–151
7 Stumpo, D. J., Eddy, Jr, R. L., Haley, L. L., Sait, S., Shows, T. B., Lai, W. S., Young, III, W. S., Speer, M. C., Dehejia, A., Polymeropoulos, M. and Blackshear, P. J. (1998) Promoter sequence, expression, and fine chromosomal mapping of the human gene (MLP) encoding the MARCKS-like protein: identification of neighboring and linked polymorphic loci for MLP and MACS and use in the evaluation of human neural tube defects. Genomics **49**, 253–264
8 Stumpo, D. J., Bock, C. B., Tuttle, J. S. and Blackshear, P. J. (1995) MARCKS deficiency in mice leads to abnormal brain development and perinatal death. Proc. Natl. Acad. Sci. U.S.A. **92**, 944–948
9 Wu, M., Chen, D. F., Sasaoka, T. and Tonegawa, S. (1996) Neural tube defects and abnormal brain development in F52-deficient mice. Proc. Natl. Acad. Sci. U.S.A. **93**, 2110–2115
10 Watarai, H., Inagaki, Y., Kubota, N., Fuji, K., Nagafune, J., Yamaguchi, Y. and Kadoya, T. (2000) Proteomic approach to the identification of cell membrane proteins. Electrophoresis **21**, 460–464
11 Adam, P. J., Boyd, R., Tyson, K. L., Fletcher, G. C., Stamps, A., Hudson, L., Poyser, H. R., Redpath, N., Griffiths, M., Steers, G. et al. (2003) Comprehensive proteomic analysis of breast cancer cell membranes reveals unique proteins with potential roles in clinical cancer. J. Biol. Chem. **278**, 2482–2489
12 Watanabe, S., Aoki, Y., Nishijima, I., Xu, M.-J. and Arai, K. (2000) Analysis of signals and functions of the chimeric human granulocyte-macrophage colony-stimulating factor receptor in Ba/F3 cells and transgenic mice. J. Immunol. **164**, 3635–3644
13 Nunomura, K., Nagano, K., Itagaki, C., Taoka, M., Okamura, N., Yamauchi, Y., Sugano, S., Takahashi, N., Izumi, T. and Isobe, T. (2005) Cell surface labelling and mass spectrometry reveal diversity of cell surface markers and signalling molecules expressed in undifferentiated mouse embryonic stem cells. Mol. Cell. Proteomics **4**, 1968–1976
14 Shinkawa, T., Taoka, M., Yamauchi, Y., Ichimura, T., Kaji, H., Takahashi, N. and Isobe, T. (2005) STEM: a software tool for large-scale proteomic data analyses. J. Proteome Res. **4**, 1826–1831
15 Morita, S., Kojima, T. and Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. **7**, 1063–1066
16 Tabata, Y., Ouchi, Y., Kamiya, H., Manabe, T., Arai, K. and Watanabe, S. (2004) Retinal fate specification of mouse embryonic stem cells by ectopic expression of Rx/rax, a homeobox gene. Mol. Cell. Biol. **24**, 4513–4521
17 Watanabe, T. and Raff, M. C. (1990) Rod photoreceptor development *in vitro*: intrinsic properties of proliferating neuroepithelial cells change as development proceeds in the rat retina. Neuron **4**, 461–467
18 Mawuenyega, K. G., Kaji, H., Yamuchi, Y., Shinkawa, T., Saito, H., Taoka, M., Takahashi, N. and Isobe, T. (2003) Large-scale identification of *Caenorhabditis elegans* proteins by multidimensional liquid chromatography-tandem mass spectrometry. J. Proteome Res. **2**, 23–35
19 Gerdes, J., Schwab, U., Iemke, H. and Stein, H. (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer **31**, 13–20
20 Herget, T., Brooks, S. F., Broad, S. and Rozengurt, E. (1993) Expression of the major protein kinase C substrate, the acidic 80-kilodalton myristoylated alanine-rich C kinase substrates, increases sharply when Swiss 3T3 cells move out of cycle and enter G0. Proc. Natl. Acad. Sci. U.S.A. **90**, 2945–2949
21 Brooks, G. (1994) The role of 80K/MARCKS, a specific substrate of protein kinase C, in cell growth and tumour progression. Pigment Cell Res. **7**, 451–457
22 Manenti, S., Malecaze, F., Chap, H. and Darbon, J. M. (1998) Overexpression of the myristoylated alanine-rich C kinase substrate in human choroidal melanoma cells affects cell proliferation. Cancer Res. **58**, 1429–1434
23 Magee, A. I., Gutierrez, L., Marshall, C. J. and Hancock, J. F. (1989) Targeting of oncoproteins to membranes by fatty acylation. J. Cell Sci. **11** (Suppl.), 149–160
24 Abraham, N. and Veillette, A. (1990) Activation of p56lck through mutation of a regulatory carboxy-terminal tyrosine residue requires intact sites of autophosphorylation and myristylation. Mol. Cell. Biol. **10**, 5197–5206
25 Myat, M. M., Chang, S., Rodriguez-Boulan, E. and Aderem, A. (1998) Identification of the basolateral targeting determinant of a peripheral membrane protein, MacMARCKS, in polarized cells. Curr. Biol. **8**, 677–683
26 Schmitz, A. A., Schleiff, E., Rohring, C., Loidl-Stahlhofen, A. and Vergeres, G. (1999) Interactions of myristoylated alanine-rich C kinase substrate (MARCKS)-related protein with a novel solid-supported lipid membrane system (TRANSIL). Anal. Biochem. **268**, 243–253
27 Jin, T., Yue, L. and Li, J. (2001) *In vivo* interaction between dynamitin and MacMARCKS detected by the fluorescent resonance energy transfer method. J. Biol. Chem. **276**, 12879–12884
28 Swierczynski, S. L., Siddhanti, S. R., Tuttle, J. S. and Blackshear, P. J. (1996) Nonmyristoylated MARCKS complements some but not all of the developmental defects associated with MARCKS deficiency in mice. Dev. Biol. **179**, 135–147
29 Kim, J., Shishido, T., Jiang, X., Aderem, A. and McLughlin, S. (1994) Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. J. Biol. Chem. **269**, 28214–28219
30 Sundaram, M., Cook, H. W. and Byers, D. M. (2004) The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. Biochem. Cell Biol. **82**, 191–200
31 Glaser, M., Wanaski, S., Buser, C. A., Boguslavsky, V., Rashidzada, W., Morris, A., Rebecchi, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D. et al. (1996) Myristylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. J. Biol. Chem. **271**, 26187–26193
32 Powner, D. J. and Wakelam, M. J. (2002) The regulation of phospholipase D by inositol phospholipids and small GTPases. FEBS Lett. **531**, 62–64
33 Foster, D. A. and Xu, L. (2003) Phospholipase D in cell proliferation and cancer. Mol. Cancer Res. **1**, 789–800

Received 21 June 2007/7 August 2007; accepted 10 August 2007
Published as BJ Immediate Publication 10 August 2007, doi:10.1042/BJ20070826