

Identification of Neuronal Cell Lineage-Specific Molecules in the Neuronal Differentiation of P19 EC Cells and Mouse Central Nervous System

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P19 embryonic carcinoma (EC) cells are one of the simplest systems for analyzing the neuronal differentiation. To identify the membrane-associated molecules on the neuronal cells involved in the early neuronal differentiation in mice, we generated two monoclonal antibodies, SKY-1 and SKY-2, by immunizing rats with a membrane fraction of the neuronally committed P19 EC cells as an antigen. SKY-1 and SKY-2 recognized the carbohydrate moiety of a 90 kDa protein (RANDAM-1) and the polypeptide core of a 40 kDa protein (RANDAM-2), respectively. In the P19 EC cells, the expression of RANDAM-1 was colocalized to a part of Nestin-positive cells, whereas that of RANDAM-2 was observed in most Nestin-positive cells as well as β -III-tubulin positive neurons. In the embryonic and adult brain of mice, RANDAM-1 was expressed at embryonic day 8.5 (E8.5), and the localization of antigen was restricted on the neuroepithelium and choroid plexus. The RANDAM-2 expression commenced at E6.0, and the antigen was distributed not only on the neuroepithelium of embryonic brain but on the neurons of adult brain. Collectively, it was concluded that RANDAM-1 is a stage specific antigen to express on the neural stem cells, and RANDAM-2 is constitutively expressed on both the neural stem cells and differentiated neuronal cells in mouse central nervous system (CNS). © 2002 Wiley-Liss, Inc.

Key words: P19 embryonic carcinoma cells; neuronal differentiation; rat monoclonal antibody; membrane-associated molecules; mouse embryo

The murine P19 embryonic carcinoma (EC) cells, one of the pluripotential stem cell lines, can differentiate

into derivatives of all three germ layers in vitro by treatment with chemical inducers (McBurney and Rogers, 1982), and in vivo by transplantation of the cells into embryos (Rossant and McBurney, 1982). Among the chemical inducers, all *trans*-retinoic acid (t-RA) has been well known to induce the neuronal cell differentiation concomitant with the formation of cellular aggregates. The neuronal cells differentiated in this way have been reported to be closely similar to that of mammals in some aspects such as the morphology (Rossant and McBurney, 1982), the functional synaptic formations (McBurney et al., 1988; Morassutti et al., 1994), and the expression of neuron-specific genes for neurotransmitters (Sharma and Notter, 1988; Bain et al., 1993; Turetsky et al., 1993; Ray and Gottlieb, 1993; Staines et al., 1994) and proteins (Jones-Villeneuve et al., 1982; Sharma and Notter, 1988; Tanaka et al., 1992; Hung et al., 1992; Bain et al., 1995; Paterno et al., 1997). Moreover, the aggregated P19 EC cells of t-RA-treated period were found to express some kinds of basic helix-loop-helix (bHLH) genes such as the *Mash-1* (Johnson et al., 1992; Itoh et al., 1997), a mammalian homolog of *achaete-scute*, *Math-1* and *NeuroD* (Itoh et al., 1997), the mammalian homolog of *atonal*, and *Nscl-2* (Itoh et al., 1997). The products of these bHLH genes have been well known to function as positive regulatory

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molecules on the neural fate determination and differentiation. Thus, P19 EC cells have been thought to reflect considerably the early events of neuronal differentiation in vivo, and widely used as an in vitro model system eminently suitable for analyzing the regulation mechanisms of the mammalian neuronal differentiation.

The precise molecular mechanisms of the differentiation process and cellular specification of the t-RA-treated P19 EC cells are largely undefined, although the t-RA-treated period is thought to be essential to get into the differentiation stage committed to the neural fate (Bain et al., 1994), and has thus far found to be regulated by many genes (Ray and Gottlieb, 1996), including the transcriptional genes. It should be pointed, however, out that the analytical aspects of the membrane-associated molecules involved in the neuronal commitment is quite insufficient. The membrane-associated molecules, generally glycoproteins, are understood to involve in many cellular activities, and cell-cell and cell-extracellular matrix (ECM) interactions in embryogenesis and development (Reichardt, 1991; Keynes and Cook, 1995; Gumbiner, 1996; Lauffenburger and Horwitz, 1996). These types of glycoproteins are not only available as surface markers for the presentation of cellular properties, but also thought to play important roles as functional molecules by themselves. Therefore, the identification of these surface glycoproteins specifically expressed during the neuronal differentiation is quite important for finding a clue to elucidate the neural stem cell migrations, intracellular signaling pathways for the neuronal differentiation, and neurogenesis in the mouse developmental processes.

By using P19 EC cells, we analyzed the membrane-associated molecules involved in the early neuronal differentiation in mouse by immunological methods. In this study, we describe the identification and biochemical characterization of two types of membrane-associated glycoproteins, RANDAM-1 and RANDAM-2, specifically expressed during the neuronal differentiation of P19 EC cells detected by specific MAbs, SKY-1 and SKY-2, which were newly generated by immunizing rats with the membrane fraction of the P19 EC cells committed to neuronal differentiation by t-RA treatment. We also show the expression and localization of these molecules during the neuronal differentiation of P19 EC cells and in the central nervous system (CNS) of mouse embryogenesis and adult brain, showing that RANDAM-1 and RANDAM-2 are anticipated to have some functional roles for the early neuronal differentiation in mouse.

MATERIALS AND METHODS

Animals

Female Wistar rats, 3 weeks old, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan). Pregnant and adult BALB/c mice were purchased from the same dealer. All the experiments with animals were performed according to the guidelines of the Animal Welfare Committee of the institute.

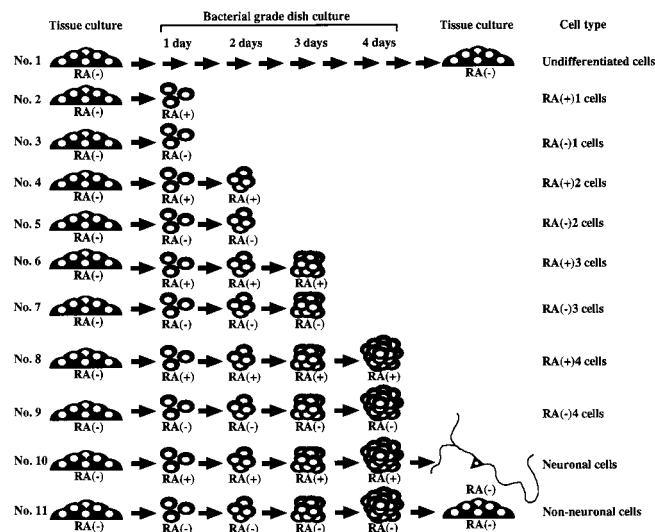


Fig. 1. Schematic diagrams of the culture conditions for P19 EC cells. P19 EC cells were divided into 11 cell types with different culture conditions: No. 1, undifferentiated cells; No. 2–9, aggregated cells treated with (+) or without (−) t-RA for the indicated days on bacterial grade culture dishes (e.g., RA(+)-4 cells for the cells cultured for 4 days in the presence of t-RA); No. 10, neuronally differentiated cells with t-RA treatment for 4 days followed by a culture on tissue grade culture dishes; No. 11, nonneuronally differentiated cells cultured without t-RA for 4 days followed by the same culture as that of No. 10.

Cells and Cell Cultures

Mouse myeloma cells (PAI) were cultured in RPMI-1640 containing 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 µM 2-mercaptoethanol, 1 mM nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS) (Kotani et al., 1993). P19 EC cells were generously provided by Dr. H. Hamada (Osaka University, Osaka, Japan). P19 EC cells were cultured in α-MEM containing 10% FCS. The induction of neuronal differentiation with t-RA was performed according to the protocol of Bain et al. (1993). Briefly, P19 EC cells growing on tissue culture grade dishes were harvested by treatment with a trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and then were cultured onto bacterial grade dishes in the conditioning medium containing 0.5 µM t-RA for 4 days. P19 EC cells aggregated by this treatment were dispersed and replaced as a monolayer culture onto tissue culture grade dishes in the conditioning medium containing 5 µg/ml cytosine arabinoside to remove the glial cells in the absence of t-RA for 1 week, followed by culturing for 5 days to enrich the neuronal cell population in the conditioning medium. The neurite extension was extensively observed within 2–3 days from the commencement of the monolayer culture. The culture conditions for P19 EC cells in this study are summarized in Figure 1.

Preparation of Membrane Fraction

For the preparation of membrane fraction from P19 EC cells, embryos, and various organs, these samples were homog-

enized in 10 vol of 10 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 µM aprotinin, and 1 mM iodoacetoamide. The homogenates were centrifuged at 600 × g for 10 min at 4°C, and the supernatants were centrifuged again at 25,000 × g for 20 min at 4°C. The resulting precipitates were used as a membrane fraction.

Membrane lysates were prepared by lysing the membrane fractions with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40 on ice for 20 min, followed by centrifugation at 25,000 × g for 20 min at 4°C. The supernatant was collected and stored at -80°C until used. Protein concentration was estimated with bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL).

Generation of MAbs

Female Wistar rats, 3 weeks old, were immunized twice in footpad at 5- to 7-intervals with 100 µl immunogen per one footpad prepared by mixing the same volume of cell membrane fraction of RA(+)4 cells (500 µg/ml) and a complete adjuvant Titer Max Gold (CytRx, Norcross, GA). Three days after the final booster immunization, popliteal lymph node cells were fused with PAI myeloma cells using polyethylene glycol 4000 (Merck, Darmstadt, Germany) as described previously (Kotani et al., 1993). Hybridoma cells were screened by Western blotting as described below.

Western Blotting

Cell membrane lysates (10 µg/lane) pretreated under reducing or nonreducing condition without boiling were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) in a 4–20% gradient gel and electroblotted onto a polyvinylidene difluoride (PVDF) membranes (Immobilon; Nippon Millipore, Tokyo) according to Towbin et al. (1979). The blotted membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS), followed by incubation with hybridoma culture supernatant for 1 hr, and incubated with a peroxidase-conjugated goat anti-rat IgG (Fab'_2) (Jackson ImmunoResearch, West Grove, PA; 5,000-fold diluted). The antibody-reacted bands were visualized by a chemiluminescent detection system (ECL; Amersham Pharmashia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol.

For metaperiodate oxidation of the antigens, the PVDF membranes electroblotted with the membrane fraction were treated with or without 25 mM NaIO_4 in 100 mM acetate buffer, pH 4.0, for 30 min at room temperature in the dark. The membranes washed with PBS were subjected to the immunological detection as described above.

Immunocytochemical and Immunohistochemical Analyses

For the surface staining of live cells, the cells were handled in the presence of 0.01% NaN_3 for inhibition of membrane turnover. The preparation of developing embryos was performed according to Beddington (1987). For the staining of fixed cells and frozen sections, RA(+)4 cells, neuronally differentiated P19 EC cells and tissue sections (10 µm) prefixed in cold methanol for 30 min were blocked and incubated with primary antibodies followed by incubation with FITC- or Cy3-

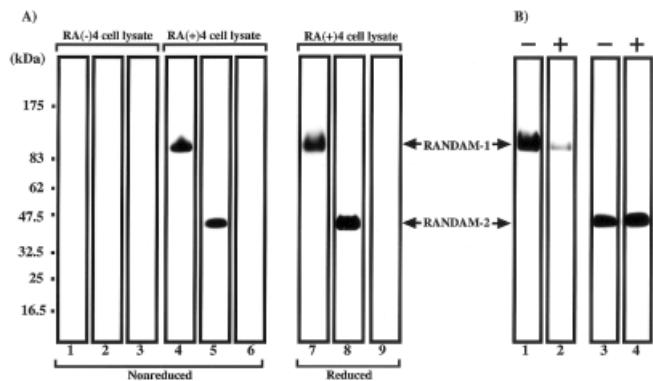


Fig. 2. Specificity of SKY-1 and SKY-2 mAbs. **A:** The membrane lysates of RA(-)4 cells (**lanes 1–3**) and RA(+)4 cells (**lanes 4–9**) were separated by SDS-PAGE under nonreducing (lanes 1–6) or reducing (lanes 7–9) conditions, and electroblotted onto PVDF membranes. The blots were probed with SKY-1 (lanes 1, 4, 7), SKY-2 (lanes 2, 5, 8) or normal rat IgG (lanes 3, 6, 9) followed by incubation with peroxidase-conjugated goat anti-rat IgG for chemiluminescent detection. **B:** The membrane lysate of RA(+)4 cells was separated by SDS-PAGE under nonreducing conditions, and electroblotted onto PVDF membranes. The blots were treated with (**lanes 2 and 4**) or without (**lanes 1 and 3**) NaIO_4 followed by incubation with SKY-1 (lanes 1 and 2) and SKY-2 (lanes 3 and 4). The epitope of SKY-1 MAb is sensitive to NaIO_4 oxidation, but the oxidation does not affect the reactivity of SKY-2. Arrows indicate the SKY-1 and SKY-2 MAb-defined molecules, RANDAM-1 (90 kDa) and RANDAM-2 (40 kDa).

labeled secondary antibodies (Jackson ImmunoResearch; 500-fold diluted) as described previously (Kotani et al., 2000). Other than SKY-1 and SKY-2 MAbs, primary antibodies used were as follows: an affinity-purified goat polyclonal antibody to glial fibrillary acidic protein (GFAP) (Santa Cruz Biotechnology, Santa Cruz, CA; 100-fold diluted); a mouse monoclonal antibody to Nestin (American Research Products, Belmont, MA; 100-fold diluted); a mouse monoclonal antibody to β -III-tubulin isoform (CHEMICON International, Temecula, CA; 100-fold diluted); and a mouse monoclonal antibody to sulfatides (bovine brain) (Kotani et al., 1995). The antibody-stained cells were observed under a microscope (DMRE; Leica, Heidelberg, Germany) equipped with a confocal laser scanning system (TCS-SPII; Leica). Haematoxylin-eosin (H & E) staining was performed routinely.

RESULTS

Identification of the Surface Molecules Specifically Induced With t-RA Treatment in P19 EC Cells

To generate MAbs against the cell membrane-associated molecules upon neuronal differentiation, we prepared the membrane fraction as an immunogen from RA(+)4 cells, which reside in the commitment stage to neural differentiation (Bain et al., 1994). Hybridomas were screened for the antibody reactivity to the membrane lysate from RA(+)4 cells by Western blot analysis, and two hybridoma cell lines were established. The MAbs produced by them were designated SKY-1 and SKY-2 (IgG isotype). As shown in Figure 2A, SKY-1 and SKY-2

MAbs specifically reacted with a single band of a 90 kDa protein, designated RANDAM-1 (retinoic acid-induced neuronal differentiation-associated molecule-1), and a 40 kDa molecule, designated RANDAM-2, respectively, in the membrane lysate prepared from RA(+)4 cells on SDS-PAGE under both reducing and nonreducing conditions. As these MAbs did not react with any band in the membrane lysate from RA(-)4 cells (Fig. 2A), RANDAM-1 and RANDAM-2 were concluded to be specifically induced in P19 EC cells by t-RA treatment.

For addressing the recognition epitope of these MAbs within the antigens, the blots of the membrane lysate from RA(+)4 cells were treated with or without NaIO₄ followed by immunological examination. SKY-1 MAb lost the reactivity to RANDAM-1 (Fig. 2B), whereas the reactivity of SKY-2 MAb was not affected by the same pretreatment to the blot (Fig. 2B). These results suggest that the recognition epitope of SKY-1 and SKY-2 MAbs is the carbohydrate portion of RANDAM-1 and the core polypeptide of RANDAM-2, respectively.

Expression of RANDAM-1 and RANDAM-2 During the Neuronal Differentiation of P19 EC Cells

The expression of RANDAM-1 and RANDAM-2 during neuronal differentiation of P19 EC cells was examined by Western blotting. The RANDAM-1 expression commenced in RA(+)2 cells (Fig. 3B; lane 4). The expression was drastically elevated with the highest level in RA(+)4 cells (Fig. 3B; lane 8), but was decreased to be undetectable in the neuronally differentiated cells (Fig. 3B; lane 10). In the case of RANDAM-2, however, the antigen first appeared in RA(+)3 cells (Fig. 3C; lane 6), and the expression reached the highest level in RA(+)4 cells (Fig. 3C; lane 8), and decreased to a constant level in the neuronally differentiated cells (Fig. 3C; lane 10). These results suggested that RANDAM-1 expression is restricted to the neuronal differentiation process, and that RANDAM-2 is expressed in the neuronal cell lineage population of P19 EC cells.

RANDAM-1 and RANDAM-2 as Membrane-Associated Molecules

To clarify that RANDAM-1 and RANDAM-2 are exposed on the cell surface, we examined an indirect immunofluorescence staining of the neuronally committed P19 EC cells with SKY-1 and SKY-2 MAbs without fixation. SKY-1 only stained RA(+)4 cells (Fig. 4; B), whereas SKY-2 stained not only RA(+)4 cells but neuronally differentiated cells (Fig. 4; E and F). These results, well consistent with the results of Western blotting (Fig. 3B and C), indicate that RANDAM-1 and RANDAM-2 are indeed exposed on the cell surface at least as antibody-recognizable forms.

Identification of Cell Types Showing RANDAM-1 or RANDAM-2 Expression

The types of cells expressing RANDAM-1 or RANDAM-2 were investigated in RA(+)4 cells and neu-

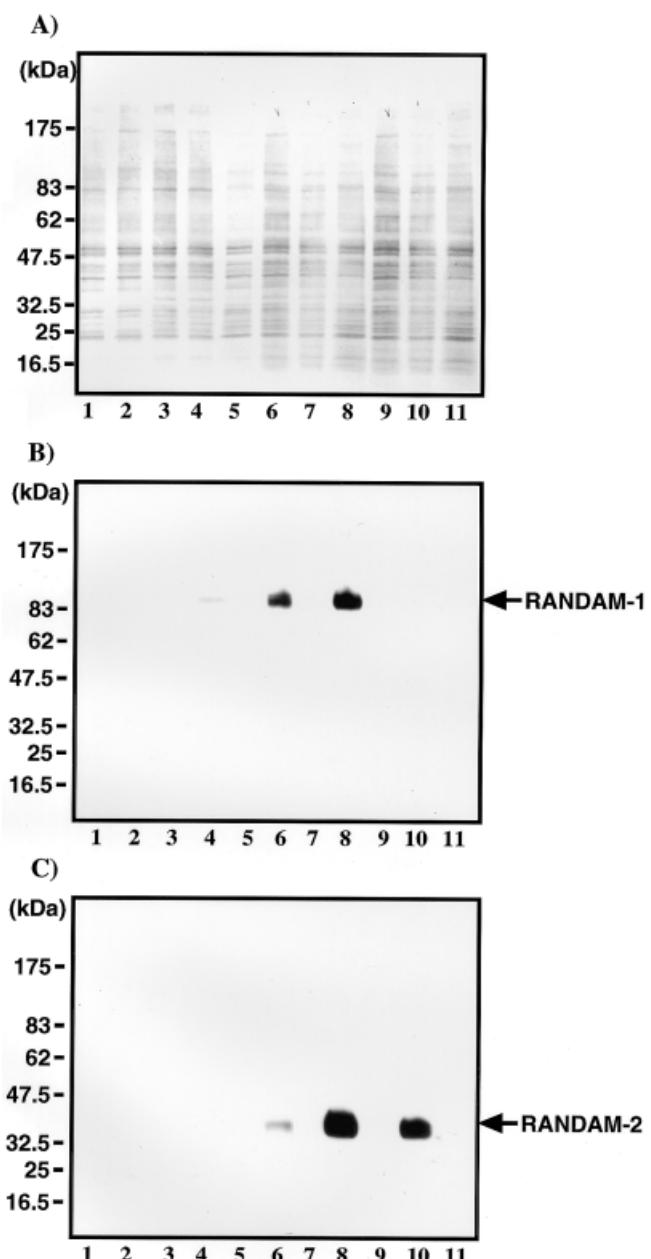


Fig. 3. Expression of RANDAM-1 and RANDAM-2 during neuronal differentiation of P19 EC cells. The membrane lysates were prepared from the undifferentiated P19 EC cells (lane 1), the P19 EC cells treated with or without t-RA for 1 day (lanes 2, 3), 2 days (lanes 4, 5), 3 days (lanes 6, 7), and 4 days (lanes 8, 9), the neuronally differentiated cells (lane 10), and the nonneuronally differentiated cells (lane 11). They were subjected to SDS-PAGE under nonreducing conditions, and electroblotted onto a PVDF membrane. The blots were stained with Coomassie brilliant blue (A), SKY-1 MAb (B) or SKY-2 MAb (C).

ronally differentiated P19 EC cells by using neuronal, astroglial, and oligodendroglial markers. In RA(+)4 cells, SKY-1 MAb costained a part of the cells positive to anti-Nestin antibodies, which have been thought to be

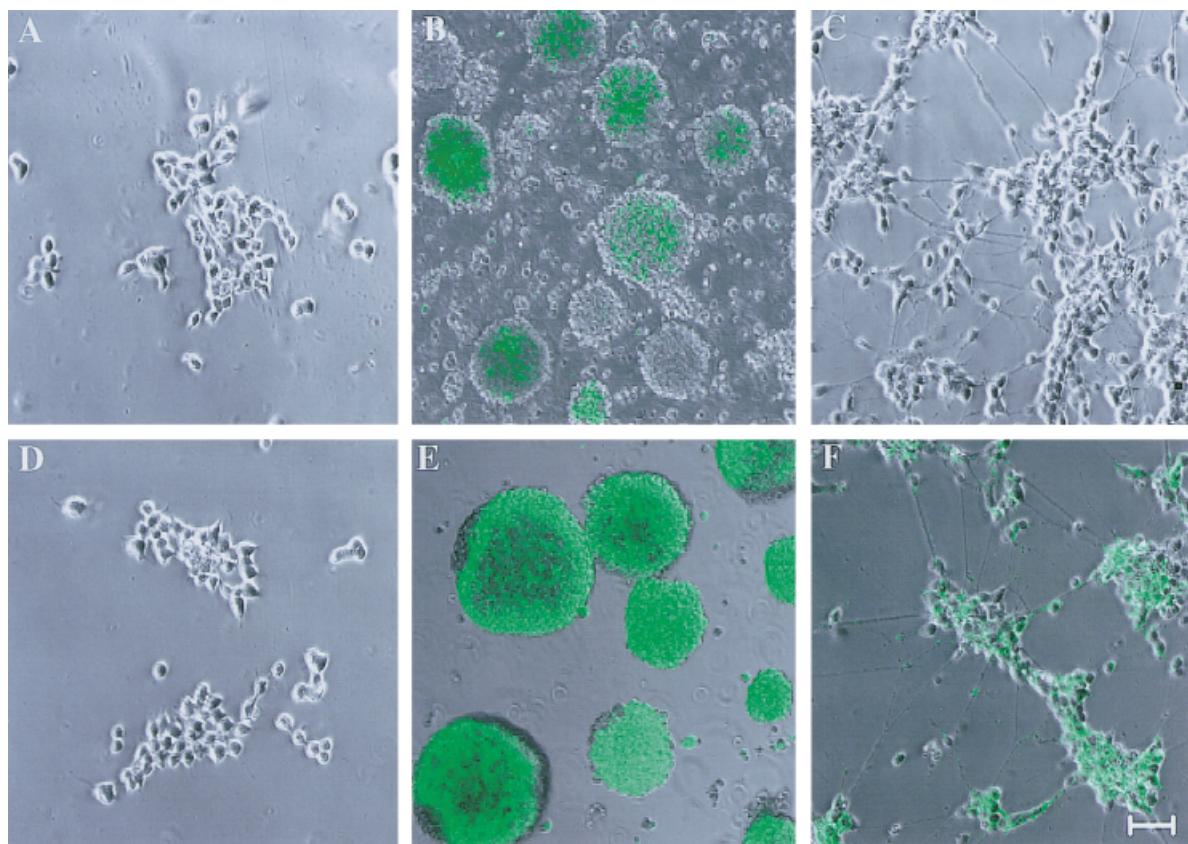


Fig. 4. Surface staining of live P19 EC cells with SKY-1 and SKY-2 mAbs. Live undifferentiated cells (**A**, **D**), RA(+)4 cells (**B**, **E**), and neuronally differentiated cells (**C**, **F**) were stained with SKY-1 (**A–C**) or SKY-2 (**D–F**) followed by microscopic examination. A–F: double-exposed images with phase contrast and laser scanning images. The antigens are exposed on the cell surface. Scale bar = 50 μ m.

undifferentiated neural cells such as neural stem cells and intermediate progenitor cells in mammalian CNS (Hockfield and Mckay, 1985; Cattaneo and McKay, 1990) (Fig. 5A). In contrast, most of the anti-Nestin antibody-positive RA(+)4 cells showed the stainability with SKY-2 MAb (Fig. 5A). Both MAbs did not stain either anti-GFAP antibody-positive cells (astroglial cells) (Halliday et al., 1996) or anti-sulfatide antibody-positive cells (oligodendroglial cells) (Kotani et al., 1995), indicating that the cells reactive with SKY-1 or SKY-2 MAb do not correspond to astroglial or oligodendroglial cells. In neuronally differentiated cells, SKY-1 MAb did not show any immunoreactivity (Fig. 5B). Most of the neuronally differentiated cells showed simultaneous staining with SKY-2 MAb and anti- β -III-tubulin antibody, a specific marker of fully differentiated neurons (Draberova et al., 1998). This consistent with the observations that SKY-2 MAb did not stain the anti-GFAP or anti-sulfatide antibody-positive cells (Fig. 5B). In the double staining of the neuronally differentiated cells with SKY-2 MAb and anti-GFAP or anti-sulfatide antibody, however, some SKY-2 MAb-positive cells also showed positive to anti-GFAP or anti-sulfatide antibody-positive cells (Fig. 5B). This was caused by over-

lapping of the SKY-2 MAb-positive cells with the cells positive to anti-GFAP or anti-sulfatide (Fig. 5B). These results strongly suggested that RANDAM-1 is a stage-specific antigen on neuronal stem cells and RANDAM-2 is an antigen specific to the neuronal cell lineage.

Spatiotemporal Expression of RANDAM-1 and RANDAM-2 During Mouse Embryonic Development

To examine the expression stages of RANDAM-1 and RANDAM-2 during mouse embryogenesis, we carried out Western blot analysis using whole embryos at embryonic day 6.0 (E6.0), E8.5, and E10.5, and only the brain at E12.5, E16.5, and E18. RANDAM-1 was weakly detected as a band of 90 kDa with SKY-1 MAb in the whole embryos at E8.5 and E10.5, and in the brain at E18 (Fig. 6A; lanes 2, 3, and 6). RANDAM-2 was detected as a 40 kDa band at all the stages after E6.0 with SKY-2 MAb (Fig. 6B; lanes 1–6). At E8.5 and E10.5, RANDAM-2 showed the highest expression level (Fig. 6B; lanes 2, 3), followed by a rapid decrease at E16.5 and E18.0 (Fig. 6B; lanes 5, 6).

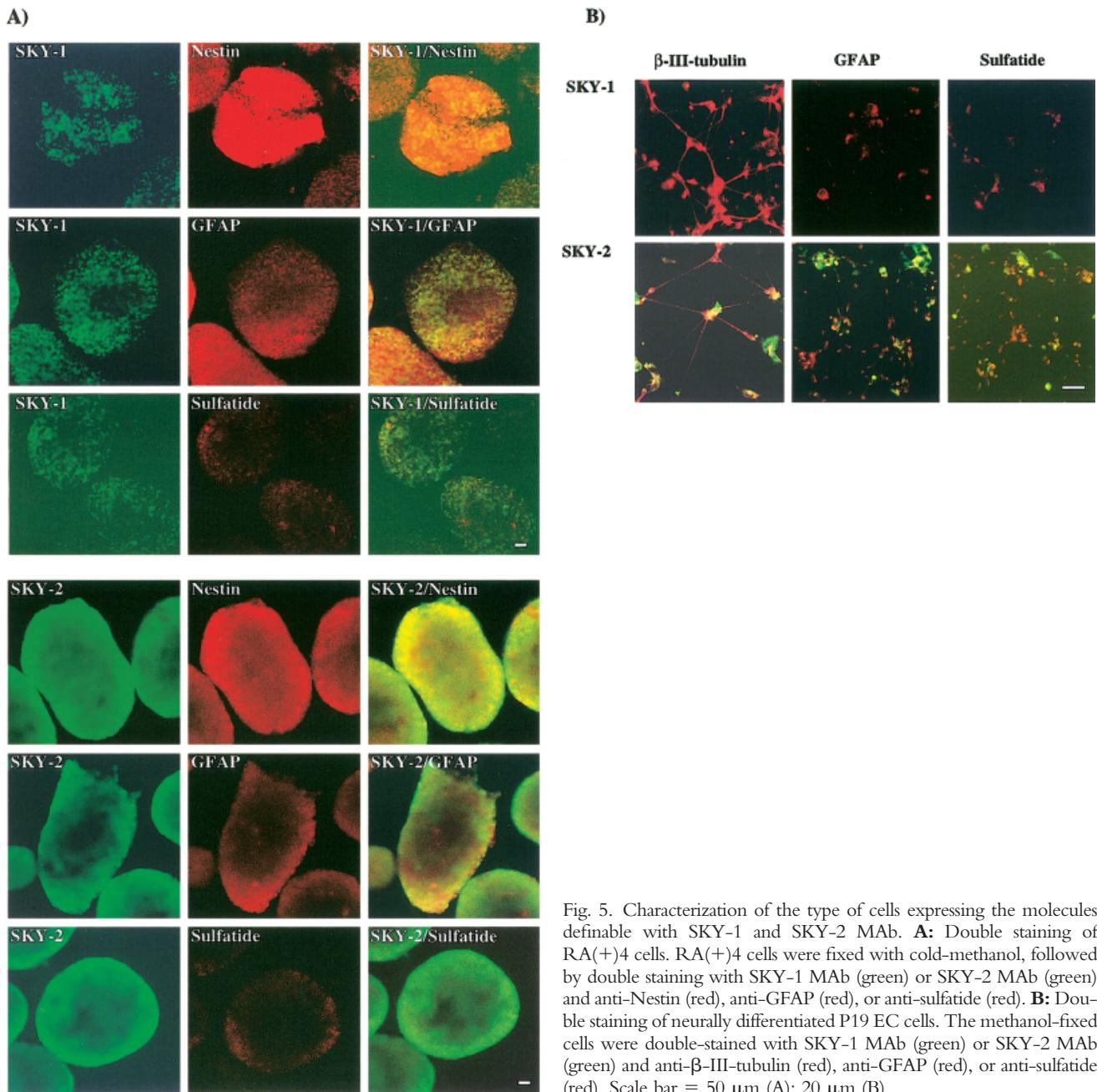


Fig. 5. Characterization of the type of cells expressing the molecules definable with SKY-1 and SKY-2 MAbs. **A:** Double staining of RA(+)4 cells. RA(+)4 cells were fixed with cold-methanol, followed by double staining with SKY-1 MAb (green) or SKY-2 MAb (green) and anti-Nestin (red), anti-GFAP (red), or anti-sulfatide (red). **B:** Double staining of neurally differentiated P19 EC cells. The methanol-fixed cells were double-stained with SKY-1 MAb (green) or SKY-2 MAb (green) and anti- β -III-tubulin (red), anti-GFAP (red), or anti-sulfatide (red). Scale bar = 50 μ m (A); 20 μ m (B).

The expression of RANDAM-1 and RANDAM-2 in the late embryo and post-embryonic organs was analyzed further by Western blot analysis. At E18, RANDAM-1 was detected as a band with the molecular mass of 90–120 kDa in the brain and in some nonneuronal tissues such as lung, liver, spleen, small intestine, and uterus (Fig. 7A, left; lanes 1, 3, 4, 7–9), but not in heart, stomach, or kidney (Fig. 7A, left; lanes 2, 5, 6). At postnatal day 0 (P0), in addition to the organs expressing RANDAM-1 at E18, RANDAM-1 was also detected in the kidney (Fig. 7A, left; lanes 3, 4, 6–9), but the expression in the brain disappeared apparently (Fig. 7A, left; lanes

1, 2, 5). In the adult, RANDAM-1 was detected in all the organs analyzed except for the cerebrum and cerebellum (Fig. 7A, left; lanes 2–9 and lanes a, b). RANDAM-2 was only detected in the brain of E18 (Fig. 7A, right; lane 1). At P0, RANDAM-2 was detected not only in the brain but in the lung (Fig. 7B, right; lanes 1, 3), and the expression pattern of RANDAM-2 was not altered in adult, that is, the restricted expression in the cerebrum, cerebellum, and lung (Fig. 7C, right; lanes a, b, 3).

The localization of RANDAM-1 and RANDAM-2 in the mouse embryos and adult brain were further examined by indirect immunohistochemical analysis. SKY-1

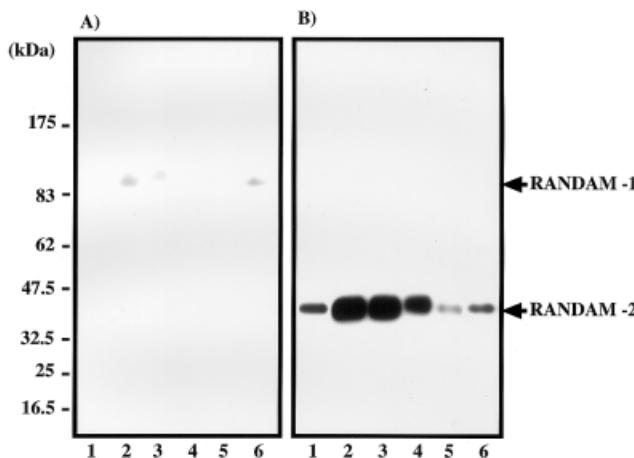


Fig. 6. Expression of RANDAM-1 and RANDAM-2 during mouse embryogenesis. The membrane lysates were from the whole embryo at E6.0 (lane 1), E8.5 (lane 2), and E10.5 (lane 3), and the embryonic brain at E12.5 (lane 4), E16.5 (lane 5), and E18.0 (lane 6). They were subjected to Western blotting with SKY-1 (A) or SKY-2 (B) MAb.

MAb did not show any staining at E12 (Fig. 8A-b). At E14, SKY-1 MAb stained the restricted regions such as diencephalon (anterohypothalamus and neuroepithelium), mesencephalon, myelencephalon, and choroid plexus primodium in CNS (Fig. 8A-e). At E18, SKY-1 only stained the choroid plexus primodium (Fig. 8A-h). In the adult, the SKY-1 MAb-defined RANDAM-1 was only present in the choroid plexus (Fig. 8B-b), but not in the cerebellum, hippocampus formation, or cerebral cortex (Fig. 8B-a, c). SKY-2 clearly stained CNS, including telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon at E12 and E14 (Fig. 8A-c, f). At E18, SKY-2 restrictedly stained the ventricular zones of telencephalon and metencephalon, and the choroid plexus (Fig. 8A-i). In the adult brain, SKY-2 stained the Purkinje cell layer in cerebellum (Fig. 8B-d), the pyramidal and granular layers in hippocampal formation (Fig. 8B-e), the choroid plexus (Fig. 8B-f), and the layers II, III, and V in cerebral cortex (Fig. 8B-g). Furthermore, to elucidate the localization of RANDAM-1 and RANDAM-2, we examined the double-immunostaining of embryonic neocortex with SKY-1 or SKY-2 MAb and an anti-Nestin antibody. As shown in Figure 9, the expression of RANDAM-1 was restricted to the ventricular zone and Nestin was also highly expressed in this region. The two molecules were clearly observed to be colocalized in the ventricular zone. RANDAM-2 was not only expressed in the ventricular zone, but also in other regions (Fig. 9). Within the ventricular zone, however, the localization of RANDAM-2 was well overlapped with that of Nestin. These results are consistent with the results of double-immunostaining (Fig. 5), and indicate that RANDAM-1 is exclusively expressed on the undifferentiated neuronal cells (Figs. 8, 9), and RANDAM-2 is on both the undifferentiated and differentiated neuronal cells in the mouse brain (Figs. 8, 9).

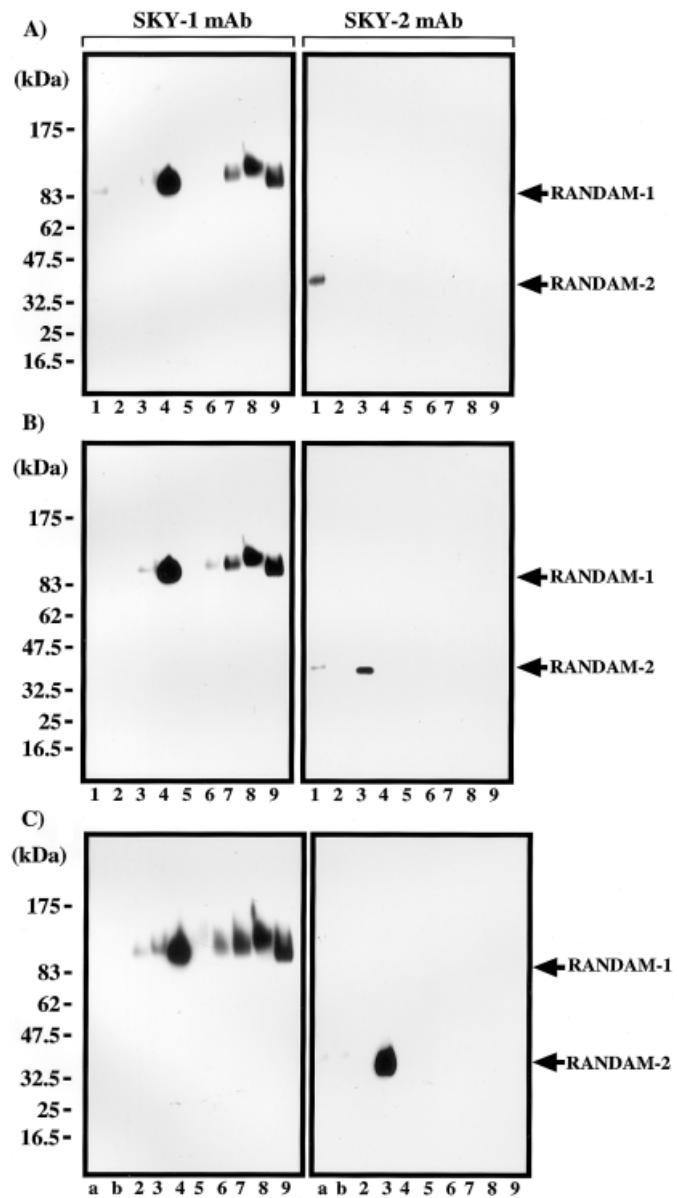


Fig. 7. Expression of RANDAM-1 and RANDAM-2 in various organs from late embryo, neonate, and adult in mouse. The membrane lysates from various organs at E18 (A), P0 (B) and adult (C) were subjected to Western blotting using SKY-1 (left) or SKY-2 (right) MAb. Lanes 1-9 correspond to brain, heart, lung, liver, stomach, kidney, spleen, small intestine, and uterus. The lanes a and b are cerebrum and cerebellum, respectively.

DISCUSSION

The membrane-associated molecules are well known to be involved in many aspects of cell-cell and cell-extracellular matrix interactions. To obtain an immunological clue for analyzing the differentiation signals via direct cell-cell interactions of neuronal cells, we analyzed P19 EC cells at the commitment stage to neuronal cells (RA(+)-4 cells), which form large aggregates and are

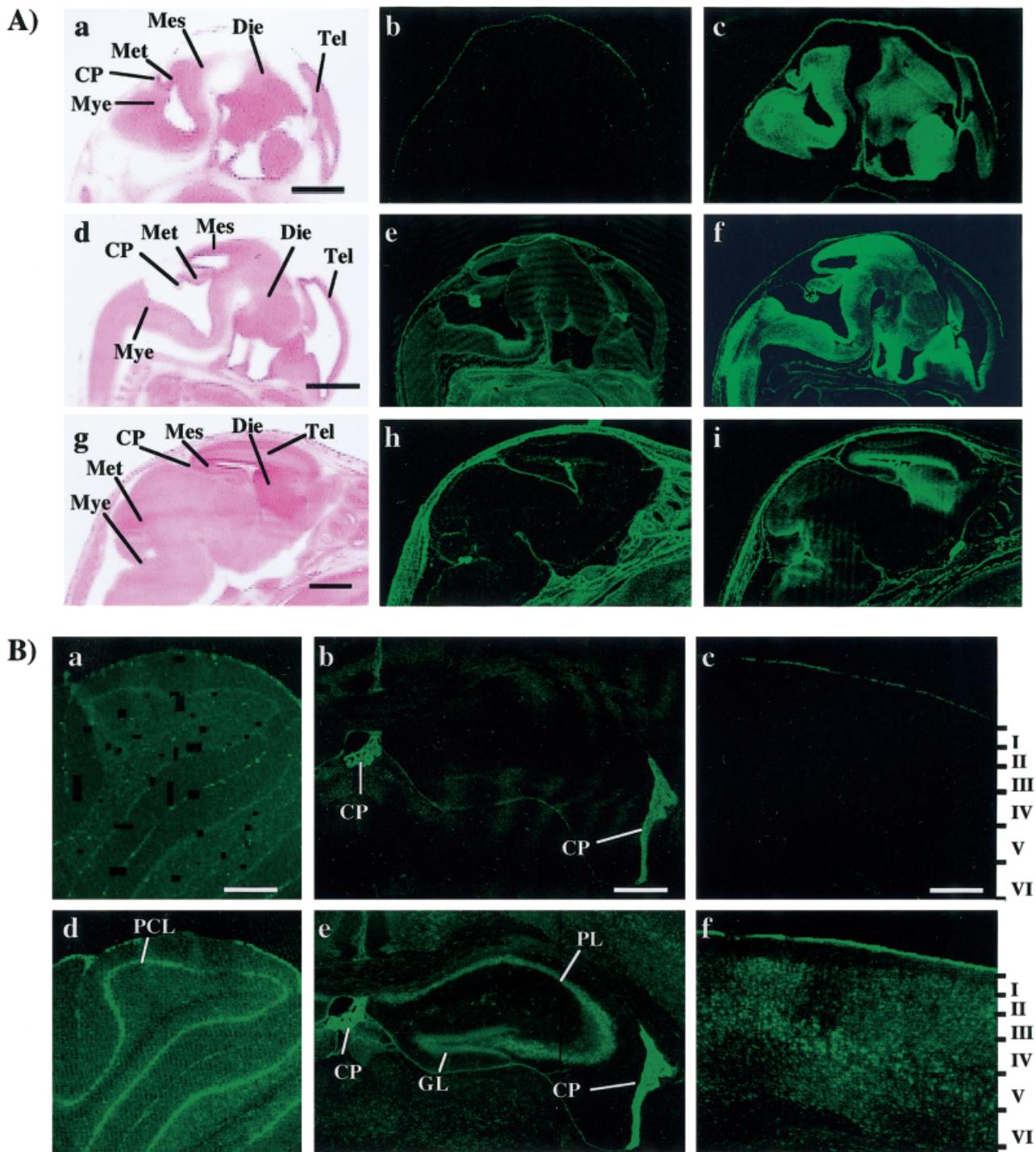


Fig. 8. Localization of RANDAM-1 and RANDAM-2 in mouse CNS. The frozen sections of whole embryos (**A**) at E12 (**a–c**), E14 (**d–f**), and E18 (**g–i**) and adult brain (**B**; **a, d**, cerebellum; **b, e**, hippocampal formation; **c, f**, cerebral cortex) were stained by H & E (**A-a, -d, -g**) and indirect immunofluorescence staining with SKY-1 (**A-b, -e, -h**, and **B-a–c**) and SKY-2 (**A-c, -f, -i**, and **B-d–f**) MAbs. The embryos were selectively focused on the brain region. The detailed

expression of RANDAM-1 and RANDAM-2 in embryonic neocortex (E14) was showed in Figure 9. The edges of the sections sometimes showed a nonspecific staining. CP, choroid plexus; Die, diencephalon; GL, granular layer; Mes, mesencephalon; Met, metencephalon; Mye, myelencephalon; PCL, Purkinje cell layer; PL, pyramidal layer; Tel, telencephalon. Scale bars **A-a, -d, -g** = 1 mm; **A-b, -e, -h** = 80 µm; **B-a–c** = 500 µm.

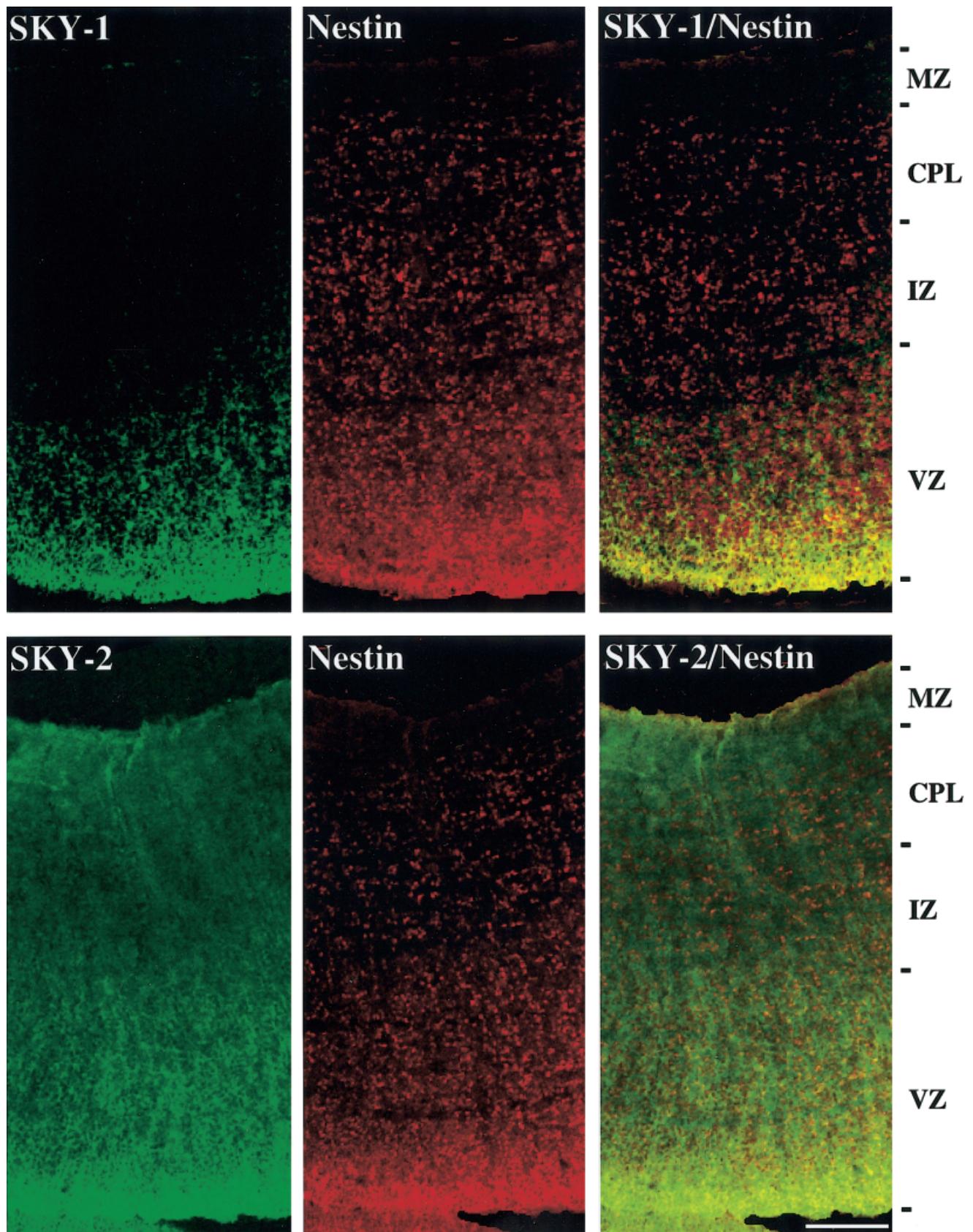


Fig. 9. Localization of RANDAM-1 and RANDAM-2 in embryonic neocortex. The methanol-fixed frozen sections of embryonic brain at E14 were stained with SKY-1 (green) or SKY-2 MAb (green) and an anti-Nestin antibody (red). The embryonic brain was focused on the neocortical region. CPL, cortical plate; IZ, intermediate zone; MZ, marginal zone; VZ, ventricular zone. Scale bar = 200 μ m.

known to interact with each other via cell surface (Bain et al., 1994). By immunizing mice with the membrane fraction from the neuronally committed cells, we obtained two clones producing MAbs, SKY-1 and SKY-2. SKY-1 and SKY-2 specifically reacted with the antigens, RANDAM-1 and RANDAM-2, respectively, which are indeed present on the membrane surface as shown by immunostaining of the live P19 cells in the presence of a respiration inhibitor (Fig. 4). As RANDAM-1 and RANDAM-2 are membrane-associated (Fig. 4), we anticipated that they are involved in cell–cell or cell–ECM interactions. In our preliminary experiments, however, they are not concerned with these interactions as adhesion molecules, as judged by inhibition of the aggregation of P19 EC cells treated with t-RA or the adhesion of RA(+)4 cells and ECM (fibronectin, laminin, vitronectin, collagen type I, and type IV) in the presence of SKY-1 or SKY-2 MAbs (not shown). In addition, based on the molecular weight, expression pattern during the neuronal differentiation of P19 EC cells, and the localization in the mouse brain, both RANDAM-1 and RANDAM-2 did not coincide with other molecules responsible for neuronal differentiation such as retinoic acid receptor (Jong et al., 1992), epidermal growth factor receptor (Jong et al., 1992), bFGF-receptor (Miho et al., 1999), cytokine receptors (van Puijenbroek et al., 1999), and N-CAM (Mauro et al., 1994).

As RANDAM-1 and RANDAM-2 are absent in the membrane fraction from the P19 EC cells cultured without t-RA (RA(-)4 cells) (Fig. 2), they are regarded to be induced as a result of differentiation or commitment into neuronal cells. In the case of RANDAM-1, the antigen is only expressed in the neuronally committed cells but disappears when the cells are differentiated into neuronal cells (Figs. 3–5), indicating the expression of RANDAM-1 as a stage-specific manner in the differentiating P19 EC cells. This was also substantiated by analyzing the expression pattern of RANDAM-1 during the development of mouse brain. Although RANDAM-1 is detectable only in the brain at E18 by Western blotting, immunohistochemical studies revealed that the antigen is widely distributed in the neuroepithelium of CNS at E14. It is restricted in the choroid plexus primodium of CNS at E18, suggesting that RANDAM-1 is expressed on the neuronal stem or neuronal progenitor cells.

In contrast to the expression pattern of RANDAM-1, RANDAM-2 is exclusively expressed in the neuronally-committed P19 EC cells and neuronally-differentiated cells (Figs. 3–5). Consistent with the results, the expression of RANDAM-2 starts as early as at E6.0 in mouse embryogenesis (Fig. 6), and lasts even in the adult brain (Fig. 7). Interestingly, distribution is restricted to the neuroepithelium of the ventricular zone in embryo (Fig. 9) and the pyramidal and granular neurons in adult among CNS (Fig. 8). This is consistent with the distribution patterns of Nestin. Cells collected from the ventricular zone were reported to have the potential differentiating into neurons, astrocytes, and oligodendrocytes (Reynolds

and Weiss, 1992), and the expression of Nestin in this region, a selective marker of undifferentiated neural cells in mammalian CNS, was observed to be significantly high compared to other regions in brain (Kawaguchi et al., 2001). Although the neural induction of vertebrates has been thought to occur close to the mid-gastrula stage (Harland and Gerhart, 1997), Streit et al. (2000) reported recently that it occurs before gastrulation. As the E6.0 embryo used in this study corresponds to the stage just before gastrulation, the expression of RANDAM-2 in brain from E6.0 to the adult supports the notion that RANDAM-2 is constitutively expressed in the early neuronal differentiating cells and neurons.

It is unclear why RANDAM-1 and RANDAM-2 are also expressed in nonneuronal organs. RANDAM-1 is a carbohydrate antigen expressed on protein core(s), whose apparent molecular mass is 90–100 kDa in brain and 100–120 kDa in the nonneuronal organs (Fig. 7). In addition, RANDAM-1 is expressed temporally in brain, but constitutively in the nonneuronal organs. It remains to be elucidated whether the size difference of RANDAM-1 is caused by the different protein core or different modifications of the proteins with carbohydrates or phosphorylation. In RANDAM-2, the RANDAM-2 expressing nonneuronal organ is solely lung (Fig. 7). The reason for the unique expression pattern is unknown. Molecular biological approaches will clarify this point, and therefore the expression cloning of RANDAM-2 with SKY-2 MAb is already in progress in our laboratory.

Until now, there have been some reports concerning the MAbs-specific to the stage-specific cell surface antigens such as Thy-1 (Langley et al., 1982), BSP-2 (Langley et al., 1982), IIC3 (Babiarz and Hathaway, 1986), DSS3 (Denburg and Norbeck, 1989), and EX-1 (Müller-Husmann et al., 1994) in mammalian brain development. We can not, however, obtain any precise data about the biochemical and molecular biological characteristics of these MAb-defined antigens. In addition, no data is available about the expression of the MAb-defined molecules on the neural stem cells or neuronal progenitor cells, unlike the case of SKY-1 and SKY-2. Hence, these MAbs are insufficient as immunological tools to isolate and identify neuronal progenitor cells and to analyze the cell lineage. The MAbs available for the purpose are as follows: anti-Musashi, which reacts with the RNA binding protein specific to the undifferentiated neural cells (Sakakibara et al., 1996); anti-Nestin, which reacts with an intermediate filament specific to the undifferentiated neural cells (Hockfield and McKay, 1985; Cattaneo and McKay, 1990; Lendahl et al., 1990); and anti- β -III-tubulin, which specifically reacts with the neuron-specific tubulin (Drabekova et al., 1998). These MAbs, however, are unsatisfactory for the direct isolation of the neural stem cells because they are directed against the intracellular components. As SKY-1 and SKY-2 MAbs react with the molecules present on the cell surface, they will be useful immunological tools for the investigation of the mouse neuronal differentiation.

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