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A2-Pancortins (Pancortin-3 and -4) Are the Dominant Pancortins During Neocortical Development

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Abstract: We have identified a novel mouse gene named *pancortin* that is expressed dominantly in the mature cerebral cortex. This gene produces four different species of proteins, Pancortin-1–4, sharing a common region in the middle of their structure with two variations at the N-terminal (A1 or A2 part) and C-terminal (C1 or C2 part) sides, respectively. In the present study, we showed that expression of mRNAs for A2-Pancortins (Pancortin species that contain the A2 part, i.e., Pancortin-3 and -4) is more dominant than that of mRNAs for A1-Pancortins (Pancortin species that contain the A1 part, i.e., Pancortin-1 and -2) in the prenatal mouse cerebral neocortex. Using western blot analysis, we found that substantial amounts of both A2-Pancortins were present in the prenatal cerebral neocortex and P19 cells after inducing neuronal differentiation. A2-Pancortins were still present in the cerebral neocortex of the adult, although their mRNAs were hardly detected. In contrast, the amount of A1-Pancortins did not increase after the third postnatal week in spite of their intense gene expression. Furthermore, we showed that recombinant Pancortin-3, one of the A2-Pancortins, was a secreted protein, in contrast to Pancortin-1 (one of the A1-Pancortins). These results suggest that A2-Pancortins are extracellular proteins essential for neuronal differentiation and that their molecular behavior is distinct from that of A1-Pancortins. **Key Words:** Cerebral cortex—Neuroepithelium—Neuronal differentiation—Embryonic carcinoma cell—Olfactomedin.
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What makes the cerebral cortex in mammals distinct from the other regions of the CNS? One biological approach to clarify this issue is to analyze genes that are specifically expressed within the cerebral cortex at various stages of development. In fact, we have already identified several novel genes that are expressed exclusively or dominantly in the developing and/or mature cerebral cortex (Nagano et al., 1994, 1998; Yoneda et al., 1998). Among them is a gene that we identified in mouse and named *pancortin* (Nagano et al., 1994, 1998), whose rat homologue was independently described as a gene encoding "neuron-specific olfactomedin-related glycoproteins" (Danielson et al., 1994).

Our previous study (Nagano et al., 1998) demonstrated four *pancortin* cDNA species, i.e., *pancortin-1* to *-4*. All four *pancortin* cDNAs share a common sequence in the middle of their structure (designated the "B part"), having two alternative sequences at both the 5′ and 3′ ends (designated the "A1 or A2 part" and the "C1 or C2 part," respectively). The composition of each *pancortin* cDNA is indicated in Fig. 1. The deduced products of the *pancortin* B and C1 parts have an amino acid sequence homologous with that of olfactomedin (Yokoe and Anholt, 1993) and the trabecular meshwork-inducible glucocorticoid response (TIGR) gene product protein (Stone et al., 1997; Nguyen et al., 1998). Olfactomedin is an extracellular matrix component originally found in *Xenopus* olfactory neuroepithelium that is thought to be involved in the maintenance, growth, and differentiation of olfactory neuronal processes (Snyder et al., 1991; Yokoe and Anholt, 1993). The TIGR gene is expressed in the trabecular meshwork of the eye, and its mutation causes primary open angle glaucoma (Stone et al., 1997). The TIGR gene product has "olfactomedin domain" near its C-terminus that is especially homologous with the C-terminal half of the Pancortin C1 part (Nguyen et al., 1998). Olfactomedin protein is considered to have two intramolecular disulfide bonds that form a characteristic structure, in which N- and C-termini of the molecule would be "legs," and the rest would be divided as "neck"

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Abbreviations used: E, embryonic day; P, postnatal day; [^{35}S]dATPαS, deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate; TIGR, trabecular meshwork-inducible glucocorticoid response.

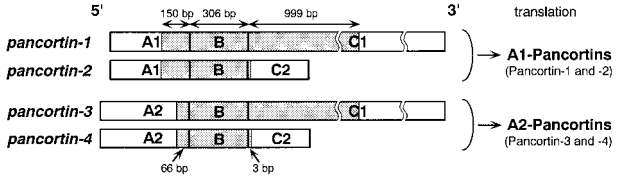

FIG. 1. Schematic drawing of mouse *pancortin* cDNAs. All *pancortin* cDNAs share a common sequence (B part) in the middle of their sequence, having two alternative sequences at both the 5′ (A1 and A2 part) and 3′ (C1 and C2 part) ends, respectively. The putative open reading frame is shaded. Proteins translated from *pancortin-1* and *-2* mRNA are called A1-Pancortins, and proteins translated from *pancortin-3* and *-4* mRNA are called A2-Pancortins.

and "head" (Yokoe and Anholt, 1993; Karavanich and Anholt, 1998). In addition, both legs have a cysteine residue(s) to form disulfide bonds with a neighboring olfactomedin molecule(s). The cysteine residues responsible for the intermolecular disulfide bonds are also present in Pancortins (residues 73 and 75 in Pancortin-1, which are in the B part and common to all Pancortins; residue 409 in Pancortin-1, which is in the C1 part and found only in Pancortin-1 and -3). However, the cysteine residues that form intramolecular disulfide bonds to segregate neck and head are not conserved, suggesting that the structure of Pancortins is substantially different from that of olfactomedin, although Karavanich and Anholt (1998) referred to Pancortin as "murine olfactomedin."

Previously, we showed that mRNAs for A1-Pancortins (Pancortins with the A1 part, i.e., Pancortin-1 and -2) are expressed more dominantly than those for A2-Pancortins (Pancortins with the A2 part, i.e., Pancortin-3 and -4) in the cerebral cortex of the adult (Nagano et al., 1998). Are A1-Pancortins consistently dominant during development? If so, then what is the significance of A2-Pancortins?

To address these questions in the present study, we investigated the expression patterns of mRNAs for A1- and A2-Pancortins in the cerebral cortices of mouse embryos. The results suggested that, in contrast to the adult, mRNAs for A2-Pancortins are dominant in the prenatal cerebral neocortex. However, these results were unable to distinguish mRNAs for the two A2-Pancortins (Pancortin-3 and -4) or those for the A1-Pancortins (Pancortin-1 and -2). Furthermore, the biological significance of a protein is not always reflected in the degree of expression of its mRNA (Alberts et al., 1994). Therefore, we raised an anti-Pancortin antibody that recognizes the four species on western blot analysis and investigated which of the four species is actually present in the mouse cerebral neocortex during development. The assay showed that A2-Pancortins (both Pancortin-3 and -4) were detectable in the prenatal cerebral neocortex. It is surprising that substantial amounts of A2-Pancortins as well as A1-Pancortins were present in the adult cerebral neocortex, although mRNAs for A2-Pancortins were hardly detected. This discrepancy suggests that the turnover rates of A1- and A2-Pancortin proteins are different, which led us to speculate on the possibility that the two groups have distinct dynamics for subcellular distribution. This was further analyzed using alkaline phosphatase-tagged recombinant proteins. Moreover, using an embryonic carcinoma cell line (P19) that can be induced for neuronal differentiation (McBurney and Rogers, 1982), the expression pattern of each Pancortin species was assessed to investigate whether the results obtained from the developing cerebral cortices can be extrapolated to neuronal differentiation as a whole.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized by Japan Bioservice (Saitama, Japan). Deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate ([^{35}S]dATPαS), peroxidase-conjugated anti-rabbit IgG antibody, and the ECL system were from Amersham Pharmacia (Little Chalfont, Buckinghamshire, U.K.). Synthetic peptide was from Kurabo (Osaka, Japan). Dulbecco's modified Eagle's medium, complete and incomplete Freund's adjuvants, and LipofectAMINE PLUS Reagent were from GibcoBRL, Life Technologies (Rockville, MD, U.S.A.). FMP-activated Cellulofine was from Seikagaku Corp. (Tokyo, Japan). Fetal bovine serum was from Cansera International (Rexdale, Ontario, Canada). TransFast Transfection Reagent was from Promega (Madison, WI, U.S.A.). α-Minimal essential medium and all-*trans*-retinoic acid were from Sigma (St. Louis, MO, U.S.A.). Immobilon-P membranes were from Millipore (Bedford, MA, U.S.A.). Skim milk was from Snow Brand (Sapporo, Japan). 5-Bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium chloride were from Roche Diagnostics Boehringer Mannheim GmbH (Mannheim, Germany).

Animals

ICR mice at various embryonic and postnatal ages were used: embryonic day (E) 10.5 (E0.5 is defined as the day of confirmation of the vaginal plug), E12.5, E14.5, E16.5, postnatal day (P) 0 (day of birth), P7, P14, P21, P28, and 6 weeks old. To raise the anti-Pancortin antibody, female New Zealand white rabbits weighing ~2.5 kg were used. These animals were purchased from a local vendor (Japan SLC, Shizuoka, Japan), housed at a constant temperature and humidity, and provided with food and water ad libitum. Diurnal lighting conditions were maintained with lights on from 800 to 2000 h.

In situ hybridization

The following oligonucleotides were prepared as probes to detect mRNAs encoding the A1, A2, C1, and C2 parts, respectively: A1, 5′-ACATCCAGTTGGTGATCATGGCCATGGTGCTTAGCACGACCCCGATCTTCA-3′; A2, 5′-TCTGCATATTTATGGTCCCGGTTCCGGCGCGACTGCCTCTCGGACA-3′; C1, 5′-GGATAACAGACGGACACCTCACGATCTAGCTACAGGGTACGCAAGTTCAA-3′; and C2, 5′-GCATTGACAAGGGTTCAAACTATTCATGGGATGCATCTAGAGTCGCATGC-3′. These oligonucleotides were labeled with [^{35}S]dATPαS by a 3′-end-labeling method and used as probes. Tissue sections of E12.5 and E16.5 mice cerebral cortices were fixed with paraformaldehyde, acetylated with acetic anhydride, dehydrated, and then subjected to hybridization with the labeled probes (1×10^6 cpm of labeled probe/0.5 ml of hybridization buffer per slide). Details of the procedures have been given previously, except for the washing temperature used (65°C) (Sato et al., 1992). The signals were visualized by emulsion autoradiography.

Preparation of anti-Pancortin antibody

A 39-mer peptide (H-CTQRDLQYVEKMENQMKGLET-KFKQVEESHKQHLARQFK-NH_2; corresponding to the C-terminal side of the Pancortin B part) was synthesized and conjugated with diphtheria toxoid. New Zealand white rabbits were then immunized with this peptide–conjugate. Primary immunization with the peptide–conjugate emulsified in complete Freund's adjuvant was followed by booster injections in incomplete Freund's adjuvant on days 24, 29, 36, and 43 after the primary immunization. Forty-seven days after the primary immunization, blood was collected. The resulting antiserum was titrated by ELISA, as described previously (Hornbeck et al., 1992). The antiserum was purified using FMP-activated Cellulofine coupled with the synthetic peptide.

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in 5% CO_2. P19 cells were maintained in α-minimal essential medium containing 10% fetal bovine serum at 37°C in 5% CO_2. For differentiation of P19 cells, 1×10^6 cells were suspended in 8 ml of the above medium supplemented with 500 n*M* all-*trans*-retinoic acid and seeded onto a 100-mm-diameter bacteriological culture dish. Eight milliliters of the medium with retinoic acid was added on day 2. On day 3, aggregated cells were dissociated with 0.025% trypsin and 1 m*M* EDTA in phosphate-buffered saline and plated onto a 100-mm-diameter tissue culture dish in the medium without retinoic acid at a density of $1–3 \times 10^6$ cells per dish.

The cDNA of each Pancortin was subcloned into the pCAGGS mammalian expression vector (Miyazaki et al., 1989). cDNAs of Pancortin-1 and -3 were subcloned into the APtag-2 expression vector (Cheng et al., 1995), which appends alkaline phosphatase without the secretion signal sequence as a tag to the C-terminus of an expressed protein. These vectors were transfected into COS-7 cells and/or P19 cells using TransFast Transfection Reagent and LipofectAMINE PLUS Reagent according to the manufacturers' instructions, respectively.

Western blot analysis

Protein extracts from mouse cerebral cortices (neocortices) were prepared by homogenizing in sample buffer [50 m*M* Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, and 5% 2-mercaptoethanol]. COS-7 cells and P19 cells were similarly homogenized in the sample buffer after either suspension and collection in suspension buffer [50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 5% glycerol, and 1 m*M* phenylmethanesulfonyl fluoride] or fixing with trichloroacetic acid. Aliquots were boiled and resolved by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred onto Immobilon-P membranes by electroblotting. The membranes were first blocked with blocking buffer [10 m*M* Tris-HCl (pH 7.8), 150 m*M* NaCl, 0.1% Tween 20, and 5% skim milk] at 4°C overnight, followed by incubation with the anti-Pancortin antibody diluted in blocking buffer (final immunoglobulin concentration, 0.4 μg/ml) at room temperature for 3 h. The membranes were washed in TBS-T buffer [10 m*M* Tris-HCl (pH 7.8), 150 m*M* NaCl, and 0.1% Tween 20] and then reacted with peroxidase-conjugated anti-rabbit IgG diluted 1:2,000 in blocking buffer at room temperature for 3 h. After washing in TBS-T buffer, immunoreactive bands were visualized with the ECL system.

Detection of alkaline phosphatase activity

Cells that express alkaline phosphatase-tagged protein were stained as described by Cheng and Flanagan (1994). In brief, cells were fixed with 60% acetone and 3% formaldehyde in 20 m*M* HEPES (pH 7.0) for 30 s, followed by incubation at 65°C for 15 min to inactivate endogenous alkaline phosphatase activity. After rinsing with detection buffer [100 m*M* Tris-HCl (pH 9.5), 100 m*M* NaCl, and 50 m*M* $MgCl_2$], they were stained in detection buffer containing 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.33 mg/ml 4-nitro blue tetrazolium for 10 min.

Alkaline phosphatase activity in culture supernatant was determined as described (Flanagan and Leder, 1990): Optical density of the samples at 405 nm was measured after heating them at 65°C for 10 min and incubating in 1 *M* diethanolamine, 0.5 m*M* $MgCl_2$, 10 m*M* L-homoarginine, 0.5 mg/ml bovine serum albumin, and 12 m*M* *p*-nitrophenyl phosphate at 37°C for 30 min. Culture supernatant of COS-7 cells transfected with APtag-2 vector without any cDNA was used as background to measure the optical density. The optical density values were considered as calibrated alkaline phosphatase activities after dividing by the average number of cells that express alkaline phosphatase-tagged Pancortin (detected by alkaline phosphatase staining of the cells) within a circle of 1.1 mm in diameter as a calibration for transfection efficiency. Averages of four experiments were compared.

RESULTS

Dominant expression of mRNAs for A2-Pancortins in prenatal cerebral neocortex

E12.5 and E16.5 mouse sagittal sections were subjected to in situ hybridization histochemistry using synthetic oligonucleotide probes specific to mRNA sequences encoding the *pancortin* A1, A2, C1, and C2 parts, respectively. Hereafter, the signals detected by the probes specific to A1, A2, C1, and C2 parts are referred to as A1, A2, C1, and C2 signals, respectively.

In E12.5 mouse cerebral neocortex, A2 and C1 signals were evident, whereas A1 and C2 signals were faint (Fig. 2). These positive signals were distributed in the neuroepithelium, although they appeared to be weaker in the innermost cellular layer just neighboring the ventricle. These signals were not apparent in the primordial plexiform layer. No regional difference in their distribution along the rostrocaudal axis was evident.

In E16.5 mouse cerebral neocortex, A2 signals were still prominent, and C2 signals as well as C1 signals were also obvious, whereas A1 signals were weaker than the other three signals (Fig. 3). These positive signals were distributed in the neuroepithelium, subplate, and the cortical plate but not evident in the intermediate zone. Also, no regional difference in their distribution along the rostrocaudal axis was noticeable at this age. Positive signals for each part in the E16.5 neuroepithelium were more apparent than those in the E12.5 tissue.

A newly raised antibody can recognize the four Pancortin species

We raised antibodies in rabbits immunized with a synthetic peptide corresponding to the C-terminal side of the B part, which is common to all four Pancortin spe-

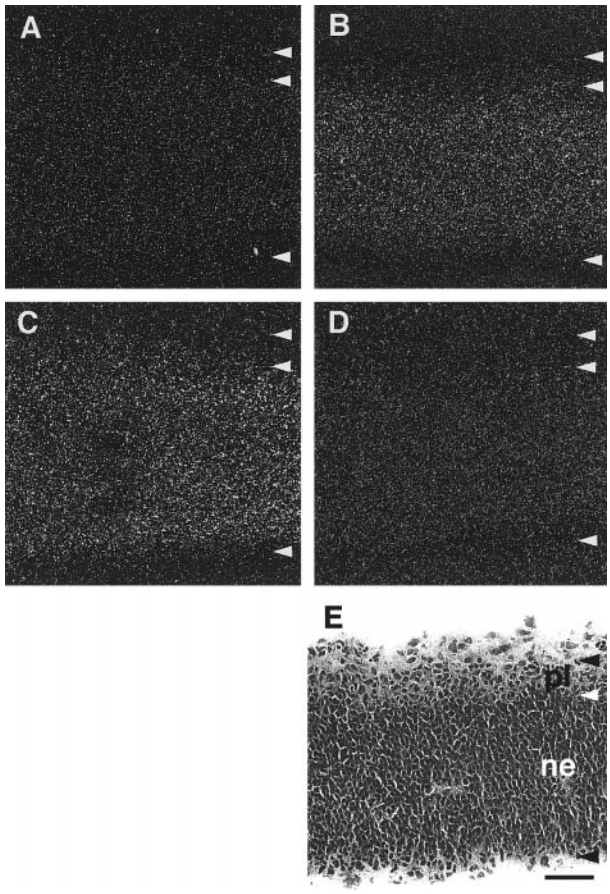

FIG. 2. Part-specific expression profiles of *pancortin* mRNAs in the E12.5 mouse cerebral neocortex. Microautoradiograms are shown of sagittal sections: (**A**) A1 part, (**B**) A2 part, (**C**) C1 part, (**D**) C2 part, and (**E**) thionin staining. pl, primordial plexiform layer; ne, neuroepithelium. Arrowheads indicate the edges of the sections and borders between the layers. Bar = 50 μm.

cies. The immunoreactivity of the antibody was determined using western blot analysis of denatured proteins extracted from the cerebral neocortex of 6-week-old mice and from COS-7 cells transfected with individual *pancortin* cDNA (Fig. 4). The antibody recognized four dominant bands of ~80, 70, 30, and 20 kDa in the cerebral cortical extract from 6-week-old mice (Fig. 4, lane f). On the other hand, COS-7 cells transfected with *pancortin-1* cDNA displayed immunoreactive bands ~80 kDa and smaller (Fig. 4, lane b), whereas mock-transfected COS-7 cells did not show any bands (Fig. 4, lane a). Similarly, dominant immunoreactive bands derived from COS-7 cells transfected with *pancortin-2*, *-3*, and *-4* cDNAs were ~30, 70, and 20 kDa and smaller, respectively (Fig. 4, lanes c–e). These results were consistent regardless of the procedures used to prepare protein samples [homogenizing cells that are either collected in the presence of phenylmethanesulfonyl fluoride or immediately fixed with trichloroacetic acid (data not shown)]. In addition, although undifferentiated P19 cells transfected with each *pancortin* cDNA also yielded more than one band, they express the immunoreactive protein whose size is the same as that of endogenous Pancortin in the cerebral neocortex more dominantly compared with COS-7 cells (Fig. 5).

A2-Pancortins are the dominant Pancortins in prenatal cerebral neocortex, whereas all four Pancortin species are almost equivalent in amount in the adult

We investigated which Pancortin species are expressed at each stage of cerebral cortical development. Homogenates from E10.5, E12.5, E14.5, E16.5, P0, P7, P14, P21, P28, and 6-week-old mouse cerebral cortices (neocortices) were subjected to western blot analysis using the antibody described above (Fig. 6). Pancortin-3 was detected earliest among the four, appearing as early

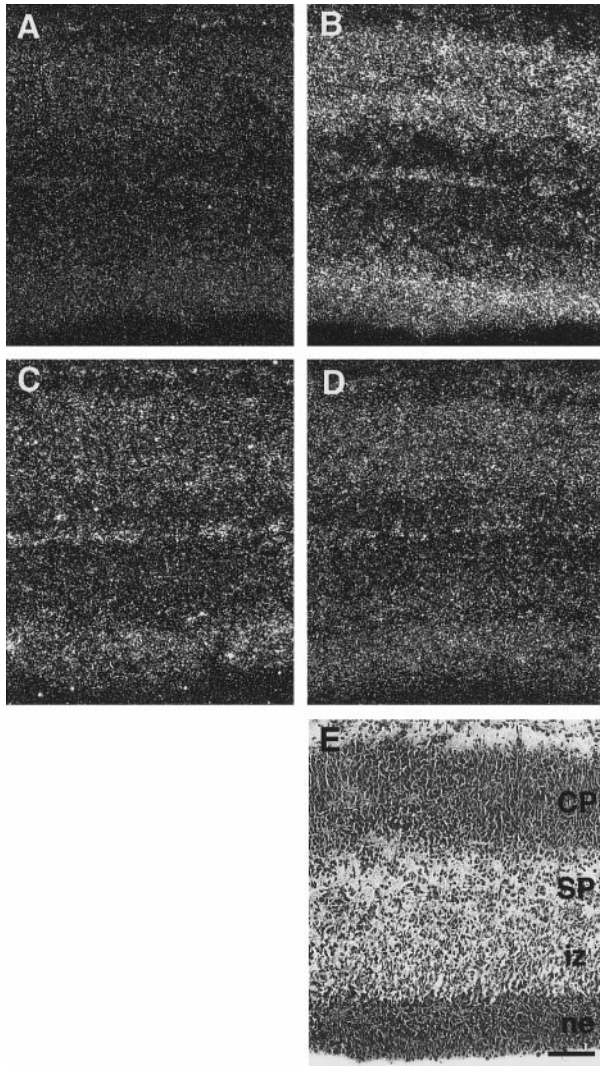

FIG. 3. Part-specific expression profiles of *pancortin* mRNAs in the E16.5 mouse cerebral neocortex. Microautoradiograms are shown of sagittal sections: (**A**) A1 part, (**B**) A2 part, (**C**) C1 part, (**D**) C2 part, and (**E**) thionin staining. CP, cortical plate; SP, subplate; iz, intermediate zone; ne, neuroepithelium. Bar = 100 μm.

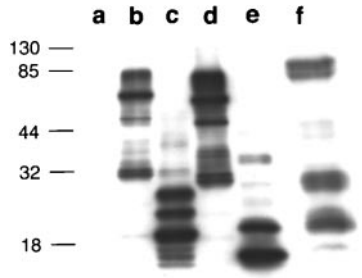

FIG. 4. Western blot analysis to confirm the specificity of the anti-Pancortin antibody. Homogenates prepared from COS-7 cells transfected with vector only (mock transfection; lane a), *pancortin-1* (lane b), *pancortin-2* (lane c), *pancortin-3* (lane d), and *pancortin-4* (lane e) cDNAs, and 6-week-old mouse cerebral cortices (lane f) were used. Migration of size markers is shown to the left in kDa.

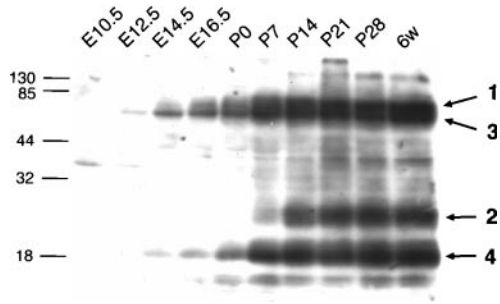

FIG. 6. Western blot analysis with anti-Pancortin antibody of homogenates prepared from mouse cerebral cortices at E10.5, E12.5, E14.5, E16.5, P0, P7, P14, P21, P28, and 6 weeks after birth (6w). Numbers with arrows to the right indicate Pancortin species (1, Pancortin-1; etc.). Migration of size markers is shown to the left in kDa.

as E12.5. Expression of Pancortin-3 increased thereafter as development progressed during the prenatal and postnatal periods before 6 weeks of age. The second species that appeared was Pancortin-4, which was detectable at around E14.5 and also increased in intensity thereafter. In contrast, expression of the other two species (Pancortin-1 and -2, i.e., A1-Pancortins) was not dominant before birth; although Pancortin-1 was detectable at around E16.5, little increase in its expression was observed before P7, and Pancortin-2 did not seem to be present during the prenatal period. Pancortin-1 and -2 became apparent after P14, increasing in intensity thereafter to the level seen at 6 weeks of age.

Alkaline phosphatase-tagged Pancortin-3 is efficiently secreted in contrast to Pancortin-1

COS-7 cells were transfected with APtag-2 vector containing either *pancortin-1* or *-3* cDNA, which allows expression of Pancortin protein tagged with alkaline phosphatase at its C-terminus. Two days later, the culture supernatants and the cells were subjected to quantifying alkaline phosphatase activity and alkaline phosphatase staining. Alkaline phosphatase activity in each culture supernatant was calibrated based on the number of cells expressing alkaline phosphatase-tagged Pancortin (detected by alkaline phosphatase staining) because transfection efficiencies may vary. Table 1 shows the calibrated activity in culture supernatant of COS-7 cells expressing each tagged Pancortin. The concentration of alkaline phosphatase-tagged Pancortin-3 in the culture supernatant was more than that of tagged Pancortin-1, suggesting Pancortin-3 is secreted efficiently from COS-7 cells in contrast to Pancortin-1.

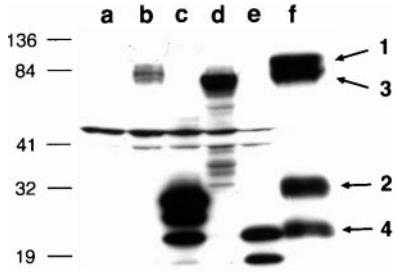

FIG. 5. Western blot analysis with anti-Pancortin antibody to show recombinant Pancortin proteins expressed in undifferentiated P19 cells. Homogenates prepared from undifferentiated P19 cells transfected with vector only (mock transfection; lane a), *pancortin-1* (lane b), *pancortin-2* (lane c), *pancortin-3* (lane d), and *pancortin-4* (lane e) cDNAs, and 6-week-old mouse cerebral cortices (lane f) were used. Numbers with arrows to the right indicate Pancortin species in the cerebral neocortex (1, Pancortin-1; etc.). Migration of size markers is shown to the left in kDa.

Substantial amounts of A2-Pancortins are detected early after induction of neuronal differentiation in P19 cells

Pancortin immunoreactivity was not detected in undifferentiated P19 cells (Fig. 7, lane a). In aggregating P19 cells in suspension culture during retinoic acid treatment, a faint immunoreactive band corresponding to Pancortin-3 was observed (Fig. 7, lane b). After retinoic acid treatment and plating, differentiating P19 cells expressed substantial amounts of A2-Pancortins (Pancortin-3 and -4) on the first, second, and third days after plating (Fig. 7, lanes c–e). Their immunoreactivities increased throughout the first 3 days after plating. In contrast, the immunoreactivities of Pancortin-1 and -2 were quite faint in comparison with those of Pancortin-3 and -4.

TABLE 1. *Comparison of alkaline phosphatase activity in culture supernatant of COS-7 cells transfected with APtag-2 vector containing either* pancortin-1 *or* -3 *cDNA*

Transfection	Calibrated alkaline phosphatase activity
APtag-2-*pancortin-1*	0.016 ± 0.002
APtag-2-*pancortin-3*	0.260 ± 0.074

Culture supernatant of COS-7 cells transfected with APtag-2 vector without cDNA was used as background. Each value was calibrated based on transfection efficiency (see Materials and Methods). Data are average ± SD values of four experiments.

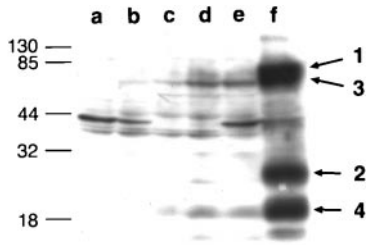

FIG. 7. Western blot analysis with anti-Pancortin antibody of homogenates prepared from undifferentiated P19 cells (lane a), P19 cells in aggregation culture with retinoic acid (lane b), retinoic acid-treated P19 cells 1 day (lane c), 2 days (lane d), or 3 days (lane e) after plating, and 6-week-old mouse cerebral cortices (lane f). Numbers with arrows to the right indicate Pancortin species (1, Pancortin-1; etc.). Migration of size markers is shown to the left in kDa.

DISCUSSION

To assess the specificity of the anti-Pancortin antibody we raised, western blot analysis using COS-7 cell extracts transfected with *pancortin* cDNAs was used. Although each cell extract yielded more than one immunoreactive band, it is apparent that the antibody recognized Pancortin proteins or their fragments because the mock-transfected cell extract showed no immunoreactivity. It is unlikely that these smaller fragments were due to artificial protein degradation during extraction procedure because of the following two reasons: (a) We obtained consistent results using protein homogenates from cells directly fixed with trichloroacetic acid to minimize existing protease activity. (b) Smaller fragments were less dominant in undifferentiated P19 cells than in COS-7 cells transfected with the same *pancortin* cDNAs. This suggests different protein modification processes between COS-7 cells and P19 cells, rather than artificial protein degradation. The dominant immunoreactive band found in each lane was ~80 (for the homogenate of COS-7 cells or undifferentiated P19 cells transfected with *pancortin-1* cDNA), 30 (transfected with *pancortin-2* cDNA), 70 (transfected with *pancortin-3* cDNA), and 20 kDa (transfected with *pancortin-4* cDNA), respectively. On the other hand, the estimated molecular masses of Pancortin-1, -2, -3, and -4 based on their deduced amino acid sequences are ~55, 17, 53, and 15 kDa, respectively. Therefore, the molecular mass of each dominant immunoreactive protein extracted from COS-7 cells or undifferentiated P19 cells transfected with each *pancortin* cDNA was more than expected. This was likely due to posttranslational modification of the proteins because each Pancortin species is expected to have eight (Pancortin-1), two (Pancortin-2), seven (Pancortin-3), or one (Pancortin-4) *N*-glycosylation site(s) (Marshall, 1972). In addition, the cerebral cortical extract showed four dominant immunoreactive bands, each of which was almost the same in size with the maximum-sized dominant immunoreactive recombinant proteins derived from COS-7 cells or undifferentiated P19 cells expressing *pancortin-1, -3, -2,* and *-4,* respectively, in order of molecular mass. Taken together, we conclude that the four dominant immunoreactive bands in the cerebral cortical extract correspond to Pancortin-1, -3, -2, and -4, respectively.

Western blot analysis using this antibody enabled us to investigate the expression profile of individual Pancortin species during cortical development. In prenatal cerebral neocortex, Pancortin-3 was the first of the four Pancortins to appear, being detectable as early as E12.5, and then Pancortin-4 also became detectable at around E14.5. Their intensity of expression increased prenatally, whereas the prenatal expression of Pancortin-1 and -2 was less dominant. These results are consistent with those obtained by in situ hybridization histochemistry of E12.5 and E16.5 cerebral cortices; A2 and C1 signals evident at E12.5 showed that mRNA for Pancortin-3 was dominant. Similarly, the obvious A2, C1, and C2 signals at E16.5 suggest substantial expression of mRNAs for Pancortin-3 and -4 compared with those for Pancortin-1 and -2. These developmental profiles are consistent in neuronally differentiating P19 cells, in which expression of A2-Pancortins is dominant. Therefore, it is likely that A2-Pancortins are essential for neuronal differentiation process and/or the function of differentiated neurons.

The present results also confirmed that all four Pancortin species are expressed in cerebral neocortex after the second postnatal week, suggesting the importance of all four Pancortins in adult cerebral neocortex. We showed previously that few mRNAs for A2-Pancortins were expressed in adult cerebral cortex (Nagano et al., 1998). One possible explanation for this discrepancy is that the degradation of A2-Pancortins is slower than that of A1-Pancortins in postnatal cerebral neocortex. This is likely due to the distinct subcellular and/or extracellular distributions of A1- and A2-Pancortins. Although the C-terminus of the C1 part, SDEL (Ser-Asp-Glu-Leu), is similar to the retention signal for soluble proteins of the endoplasmic reticulum [KDEL (Lys-Asp-Glu-Leu) (Pelham, 1990)], the N-terminal amino acid sequences of the A1 and A2 parts may act independently as sorting signals for Pancortins. As described previously (Danielson et al., 1994), the hydrophobic regions present in the A2 part may act as a signal sequence (of secreted protein). To investigate this issue further, Pancortin-1 or -3 protein tagged with alkaline phosphatase at its C-terminus was expressed in COS-7 cells, and the tagged protein coming out from the cells was quantified by measuring alkaline phosphatase activity in the culture supernatant. We found that tagged Pancortin-3 was secreted more efficiently than tagged Pancortin-1, suggesting that the A2 part is likely to act as a signal sequence for secretion because the only difference between these tagged Pancortins is the A1 or A2 part at their N-termini. Therefore, it is likely that endogenous A2-Pancortins in the cerebral neocortex are also distributed extracellularly, whereas A1-Pancortins stay intracellularly, and this may be a possible explanation for the accumulation of equivalent amounts of A1- and A2-Pancortin proteins without ac-

tive protein translation of A2-Pancortins from their mRNAs in the adult.

Based on the structural analogy of olfactomedin (Karavanich and Anholt, 1998), it is likely that A2-Pancortins could heterogeneously or homogeneously polymerize with neighboring A2-Pancortin(s) through disulfide bonds made by cysteine residues located in the B part (residues 45 and 47 in Pancortin-3 and -4) and/or that in C1 part (residues 381 in Pancortin-3; not present in Pancortin-4). In this polymerization model, Pancortin-3 has two binding sites (residues 45/47 and 381) that enable a homogeneous multimer, whereas Pancortin-4 has only one (residues 45/47) that could bind at the end of Pancortin-3 multimer or form a homodimer with another Pancortin-4. Such multiple polymerization might be the reason why the extracellular A2-Pancortins are stored stably in adult cerebral neocortex.

Then how about A1-Pancortins? The mRNAs for Pancortin-1 and -2 are expressed extensively in postnatal cerebral neocortex (authors' manuscript in preparation; see also Nagano et al., 1998). Therefore, the finding that the protein content of A1-Pancortins in cerebral neocortex stays almost unchanged after P21 suggests enhanced turnover rates of these proteins, in contrast to A2-Pancortins. These two groups differ in their primary structures only in the A1 and A2 parts, and the N-termini of A1-Pancortins are also hydrophobic (Nagano et al., 1998). However, it is unlikely that A1-Pancortins are dominantly secreted as extracellular proteins because alkaline phosphatase-tagged Pancortin-1 expressed in COS-7 cells came out from the cells much less efficiently than tagged Pancortin-3. Although there might be an additional mechanism to regulate secretion of A1-Pancortins, it seems reasonable to speculate that most A1-Pancortins, if not all, stay intracellularly and are degraded rapidly based on their intense gene expression and low secretion efficiency.

The present in situ hybridization study of mice at E12.5 showed that mRNAs for A2-Pancortins are distributed in the neuroepithelium, suggesting that they are expressed in proliferating undifferentiated neuronal stem cells. This was confirmed by the positive signals found in the E16.5 neuroepithelium and also by detection of A2-Pancortins by western blot analysis of P19 cells within the first 3 days after retinoic acid treatment because these P19 cells transiently up-regulate nestin, a marker for undifferentiated neuronal stem cells (Lin et al., 1996). In contrast, stem cells before becoming committed to a neural fate do not appear to express Pancortin because a homogenate of undifferentiated P19 cells, which are not neuronally committed but are multipotential embryonic carcinoma cells, did not yield an immunoreactive band with the anti-Pancortin antibody. On the other hand, the positive A2 signals observed in the subplate and cortical plate of the E16.5 cerebral neocortex suggest that these proteins are also in differentiated neurons. However, it is unlikely that all descendants of neural stem cells express A2-Pancortins. For example, in situ hybridization histochemistry of the E12.5 cerebral neocortex suggested that the Cajal–Retzius cells in the primordial plexiform layer seemed to lack distinct expression of *pancortin* mRNAs. In the E16.5 cerebral neocortex, each signal was weak in the intermediate zone. Therefore, whether migrating neurons and differentiated glial cells express the *pancortin* gene in vivo remains unclear, although Danielson et al. (1994) have reported that cultured rat C6 glioma cells express *pancortin* mRNA. All these findings suggest that A2-Pancortins are likely to be expressed not in stem cells with multipotency but in those committed to a neural fate and their descendants (differentiated neurons and possibly differentiated glial cells).

Then what is the significance of Pancortins? A2-Pancortins, which seem to be extracellular proteins, are present both in the neuroepithelium, where neurogenesis is going on, and in mature cerebral neocortex. Therefore, they may render neuronal cells an appropriate fundamental environment to support their differentiation and/or survival rather than contribute some specific events such as neuronal migration or neurite extension, although there still remains the possibility that the function in the adult is different from that in the developmental stages. On the other hand, A1-Pancortins, which are expressed later than A2-Pancortins in developing cerebral neocortex, seem to have enhanced turnover rates in contrast to A2-Pancortins. A1-Pancortins are quite similar in their primary structure with A2-Pancortins (for example, ~90% of Pancortin-1 is common to Pancortin-3). Therefore, A1-Pancortins, continuously renewed and stored in the cell, may possibly be released extracellularly as supplements or substitutes for A2-Pancortins in the case of cellular damage. Anyway, we have found that the two groups of Pancortins, which are structurally similar, seem to have distinct character and significance. Further studies are necessary to elucidate their functions and relationships to the other.

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