

## EXPRESSION AND CHARACTERIZATION OF DISULFIDE BOND USE OF OLIGOMERIZED A2-PANCORTINS: EXTRACELLULAR MATRIX CONSTITUENTS IN THE DEVELOPING BRAIN

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**Abstract**—The region-specific characteristics of the extracellular matrix are crucial for diverse functions in the brain. Pancortins/neuron-specific olfactomedin-related glycoproteins are components of the extracellular matrix. They comprise four alternatively spliced variants, Pancortin-1 to -4, which share a common portion, the B part, in the middle of their structure, have two pairs of alternatively spliced 5' regions, A1 and A2, and 3' regions, C1 and C2. Here we demonstrate that in mice, Pancortin-3 (A2-B-C1) and Pancortin-4 (A2-B-C2), which we have grouped together the A2-Pancortins, were transcribed early during the development of the brain in a region specific manner and were expressed very stably *in vivo*. They are N-glycosylated and secreted. Furthermore, we examined their ontogenetical expression profiles in the developing thalamus using antiserum against the common B region, since transient expressions of their mRNAs were notable there. In the developing thalami, they lasted long in oligomerized form even after the transcription of their mRNAs decreased to an undetectable level. Further analyses revealed that cysteine residues that are located in the common B part are important for homo- and hetero-oligomer formation of A2-Pancortins. When we substituted cysteine residues 45 and 47 with serine residues in that common B part, oligomerization of the A2-Pancortins was highly disturbed. © 2005 Published by Elsevier Ltd on behalf of IBRO.

**Key words:** development, dimer, myocilin, noelin, olfactomedin, thalamus.

The constituents of the extracellular matrix in the brain are unique compared with other tissues. As a consequence of the existence of the glial limiting membrane of astrocytes, which surrounds the capillaries and the brain surface, there is

no considerable number of fibroblasts in the extracellular matrix there. In addition, because of the blood–brain barrier, neurons and glias substantially produce all of the components of the extracellular matrix that surrounds them, creating a unique environment. Furthermore, the extracellular matrix in the brain is known to exhibit region-specific characteristics. The heterogeneous composition of the major constituents suggests that the extracellular matrix is specifically adapted to the functional domain of the brain.

Pancortins/neuron specific olfactomedin-related glycoproteins that are produced by neurons are members of the extracellular matrix constituents, which belong to a large family (Snyder et al., 1991; Kubota et al., 1997; Karavanich and Anholt, 1998; Kulkarni et al., 2000; Green and Klein, 2002; Torrado et al., 2002; Tsuda et al., 2002; Hillier and Vacquier, 2003; Loria et al., 2004). Some of them have been identified as molecules that are expressed predominantly in the mature cerebral cortex of the mouse and rat (Nagano et al., 1998, 2000; Danielson et al., 1994). The sole Pancortin gene produces four alternatively spliced species of translated products, Pancortin-1 to -4, which share a common region (B part) in the middle of their structure, with two splice variations at the N-terminal (A1 or A2 part) and C-terminal sides (C1 or C2 part; Fig. 1). Therefore, in the mouse, Pancortin-1 comprises A1-B-C1; Pancortin-2, A1-B-C2; Pancortin-3, A2-B-C1, and Pancortin-4, A2-B-C2 (Fig. 1). In the rat, each part of Pancortin is differently called from the mouse; mouse A1 part is called B in the rat, A2 part is A, B part is M, C1 part is Z and C2 part is Y, respectively (Fig. 1).

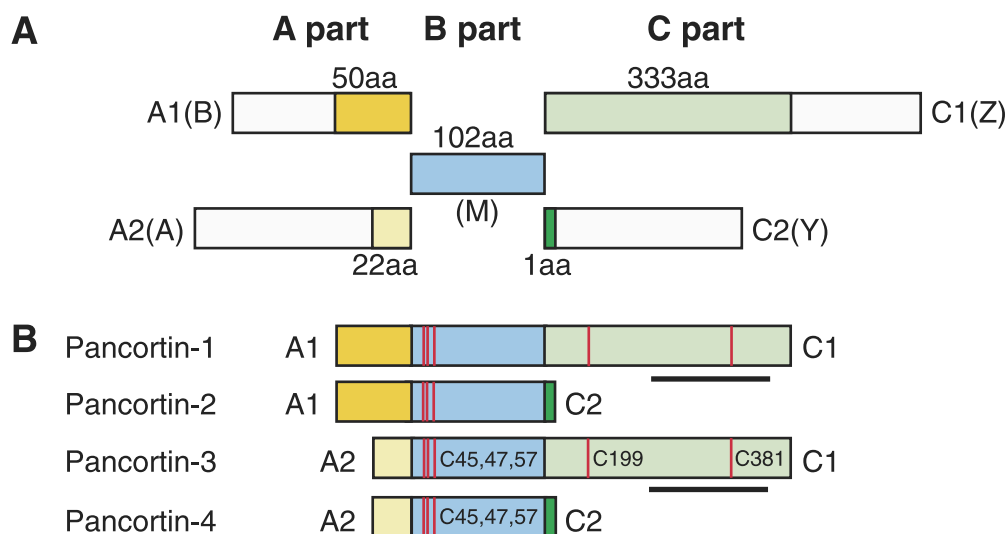
The deduced amino acid product of the Pancortin C1 part has homology to olfactomedin domain (Yokoe and Anholt, 1993). Olfactomedin is the major extracellular matrix component in the olfactory epithelium, and is thought to be involved in the maintenance, growth, and differentiation of olfactory neuronal processes (Yokoe and Anholt, 1993). It has been demonstrated that chick orthologs of the Pancortins play an essential role in neural crest cell migration during the early developmental stages (Barembaum et al., 2000) and *Xenopus* ortholog promotes neurogenesis (Moreno and Bronner-Fraser, 2001).

We have already demonstrated that the expression of A2-Pancortins (the species of Pancortin for which the N-terminus is composed of the A2 part; Pancortin-3 and Pancortin-4) mRNAs is more dominant than that of A1-Pancortins (the species of Pancortin for which the N-terminus is composed of the A1 part; Pancortin-1 and Pancortin-2) mRNAs in the prenatal mouse cerebral cortex, despite the fact that levels of the former are very low in

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**Abbreviations:** AP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; HEK, human embryonic kidney; P, postnatal day; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20.



**Fig. 1.** Schematic representation of cDNA structure of the Pancortin species. (A) PCT share a common structure (B part) in the middle of their sequence, having two alternative sequences at both the 5' (A1 and A2) and 3' (C1 and C2) ends. The putative open reading frames are shown tinted. Different nomenclature is used for each part of the rat PCT orthologue, which is shown in parentheses (Danielson et al., 1994). (B) Structure of PCT-1 to PCT-4. The B part shown in blue is common to all PCT species. The olfactomedin domain is underlined, and the red lines indicate the location of cysteine residues, which are capable of forming disulfide bonds and were mutated in this study.

the adult. We have also demonstrated that Pancortin-3 (one of the A2-Pancortins) is secreted more efficiently than Pancortin-1 (one of the A1-Pancortins) from COS-7 cells, suggesting that the secretion of these two varieties of Pancortin is regulated differently (Nagano et al., 2000).

In the study presented here, we demonstrated a transient but robust expression of *A1*- and *A2*-Pancortin mRNAs and their translated molecules in the developing brain, especially in the thalamus, although almost no *Pancortin* mRNAs were detected in the adult thalamus. Furthermore, the molecular aspects of these apparently important constituents in the extracellular matrix were investigated. Then, we found that the Pancortins form oligomers through their common B part.

## EXPERIMENTAL PROCEDURES

### Animals

ICR mice were purchased from local vendors (Japan SLC, Hamamatsu, Japan; Clea Japan, Tokyo, Japan). Animals at various

embryonic and postnatal ages were used; embryonic days (E) E10, E12, E14, and E16, and postnatal days (P) P0, P7, P14, P21, P28, and P42. The day that the presence of vaginal plug was confirmed was defined as E0. For pups, the day of birth was designated P0. Before experiments, all animals were deeply anesthetized by hypothermia (for P0–P5) or by i.p. injection of sodium pentobarbital (40 mg/kg). For embryos, their dams were deeply anesthetized with sodium pentobarbital (40 mg/kg). All experiments were conducted in accordance with the guidelines for animal experiments of Fukui Medical University (Faculty of Medical Sciences, University of Fukui, Japan) which meet the requirements of the international guidelines on the ethical use of animals. We minimized the number of animals used and their suffering as much as possible.

### In situ hybridization

The following synthetic oligonucleotides were labeled with  $^{35}\text{S}$ -dATP by the 3'-end labeling method and used as probes to detect mRNAs encoding the A1, A2, C1 and C2 parts, respectively, as described previously (Nagano et al., 1998). A1: 5'-ACATCCAGT-TGGTGATCATGGCCATGGTGCTTAGCACGACCCCGATC-TTCA-3', A2: 5'-TCTGCATATTTATGGTCCCGGTTCCGGC-GCGACTGCCTCTCGGACA-3', C1: 5'-GGATAACAGACGGA-

### Abbreviations used in the figures

|     |                                 |            |                                                                  |
|-----|---------------------------------|------------|------------------------------------------------------------------|
| Cb  | cerebellum                      | Pros       | prosencephalon                                                   |
| Cor | cortex                          | Rhom       | rhombencephalon                                                  |
| DRG | dorsal root ganglion            | SC         | superior colliculus                                              |
| Hip | hippocampus                     | SP         | spinal cord                                                      |
| IC  | inferior colliculus             | sup GFP-IP | supernatants that were immunoprecipitated with anti-GFP antibody |
| IP  | immunoprecipitation             | Tg         | tegmentum                                                        |
| LGN | lateral geniculate nucleus      | Th         | thalamus                                                         |
| LV  | lateral ventricle               | Thy        | thymus                                                           |
| Md  | medulla oblongata               | 5Gn        | trigeminal ganglion                                              |
| Mes | mesencephalon                   | VMH        | ventromedial hypothalamic nucleus                                |
| MH  | medial habenular nucleus        | VPN        | ventral posterior nucleus of the thalamus                        |
| MN  | midline nucleus of the thalamus | WB         | Western blot analysis                                            |
| OB  | olfactory bulb                  | ZI         | zona incerta                                                     |
| PCT | Pancortin                       |            |                                                                  |
| Pir | piriform cortex                 |            |                                                                  |

CACCTCACGATCTAGCTACAGGGTACGCAAGTTCAA-3', C2: 5'-GCATTGACAAGGGTCAAACCTATTCATGGGATGCATCTAGAGTCGCATGC-3'. Details of the procedures used to prepare the sections and for *in situ* hybridization have been described previously (Sato et al., 1992). Briefly, sections were fixed with paraformaldehyde, acetylated with acetic anhydride, dehydrated and then subjected to hybridization with the labeled probes ( $3.7 \times 10^6$  Bq probe/slide). The signals were visualized by emulsion autoradiography.

### Plasmid construction and mutagenesis

The full-length cDNA of Pancortin-3 was subcloned into the APTag-2 expression vector (Gen Hunter, Nashville, TN, USA) or into the pEGFP-N1 vector (BD Bioscience, San Jose, CA, USA) in order to generate fusion proteins of alkaline phosphatase (AP) or enhanced green fluorescent protein (EGFP) tagged to the C-terminus of Pancortin-3. For mutagenesis, the cysteine residue at position 199 or 381 of Pancortin-3 was replaced by serine with a mutagenesis primer: 5'-TGCATGTATGCAAACTAGCCTG/CCGGGA-3' (for cysteine 199 to serine) or 5'-CCGGTGAAGCCTTCATCATCTG/CCGGCACC-3' (for cysteine 381 to serine). Pancortin-4 was also subcloned into pEGFP-N1 and cysteine was replaced by serine at positions 45 with a mutagenesis primer 5'-GAGGGCAGGTG/CTATCTGCACAGTGGTC-3' or 47 with 5'-GAGGGCAGGTGATCTG/CCACAGTGGTC-3' or 57 with 5'-GTGCGCCCCCAGCAGACCATGTG/CTTCGCGGATGCCCGCA-3' within the B part by the Transformer site-directed mutagenesis kit (BD Bioscience). The success of these mutations was confirmed by sequencing in both directions.

### Database inquiries

The structural analyses of the Pancortins were subjected to CYPRED prediction software on web site (<http://cubic.bioc.columbia.edu/predictprotein/>). Homology research was carried out using PROSITE software on web site (<http://www.expasy.ch/prosite/>).

### Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 10% fetal bovine serum (Cell Culture Technologies, Gravesano, Switzerland) at 37 °C and with 5% CO<sub>2</sub>. Cells at ~80% confluence were placed in 100 mm dishes and transfected with 12 µl of FuGENE6 (Roche Applied Science, Indianapolis, IN, USA) containing 4 µg of expression vectors, according to the manufacturer's instructions. Transfected HEK 293 cells were maintained for 24 h in the same medium and then the medium was switched to serum-free DMEM. After 72 h, the culture supernatant and the cells were harvested separately.

### Western blot analysis

Homogenized protein extracts were prepared from mouse brains or cultured cells with the sample buffer (50 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol). Aliquots were boiled in the presence of 2-mercaptoethanol for the Western blot analysis under reduced conditions, and in the absence of 2-mercaptoethanol for the analysis under the non-reduced condition. For analyzing the glycosylation of Pancortins, samples (culture supernatant of HEK 293 cells expressing Pancortin-3-EGFP) were treated with PNGase F (*N*-glycosidase F, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. They were then resolved by electrophoresis on 10 or 12.5% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore Japan, Tokyo, Japan) by electroblotting. The membranes were blocked with 5% skimmed milk (Snow Brand,

Tokyo, Japan) in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% Tween 20) at 4 °C overnight, followed by incubation with the primary, anti-Pancortin antibody (0.4 mg/ml), as described previously (Nagano et al., 2000). The membranes were washed in TBST buffer and then reacted with peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) diluted to 1:2000 in the TBST buffer with 5% skimmed milk at room temperature for 3 h. After washing in the TBST buffer, immunoreactive bands were visualized with the ECL system (Pharmacia Biosciences).

### Immunoprecipitation

The cultured cells transfected with various constructed vectors were homogenized in LBB buffer (30 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM CaCl<sub>2</sub>) with 26G needle, and incubated for 3 h with rotation, followed by centrifugation at  $10,000 \times g$  for 20 min. The cell lysate and culture supernatant were subjected to immunoprecipitation by incubating them with anti-EGFP- or anti-AP-antibody-coated Dynabeads M-280 that had been pre-conjugated with anti-rabbit or anti-mouse IgG (Invitrogen (Dyna), Carlsbad, CA, USA) for 12 h at 4 °C. The following antibodies were used in this procedure: an anti-AP antibody (mouse monoclonal MIA1802, Seradyn, Indianapolis, IN, USA), and an anti-green fluorescent protein (GFP) antibody (Invitrogen (Molecular Probe), Carlsbad, CA, USA). The beads were washed in LBB buffer three times and then three times in 0.1 M phosphate-buffered saline (PBS, pH 7.4), with the aid of a magnet stand (Promega, Madison, WI, USA). After washing, the beads were suspended in the same sample buffer as was used for the Western blot analysis, and proteins were released from the beads by boiling. Collected proteins were separated on 10% SDS-polyacrylamide gels by electrophoresis, and transferred onto Immobilon-P membranes. Membranes were then subjected to Western blot analyses with the anti-Pancortin antibody, as described above. For an *in vitro* binding assay, plasmids containing Pancortin-3-EGFP (*PCT-3-EGFP*) or Pancortin-3-AP (*PCT-3-AP*) cDNA were transfected independently into HEK293 cells, and each supernatant was collected separately. One milliliter of each supernatant was mixed and incubated for 1 h at 37 °C and then subjected to the immunoprecipitation procedure described above.

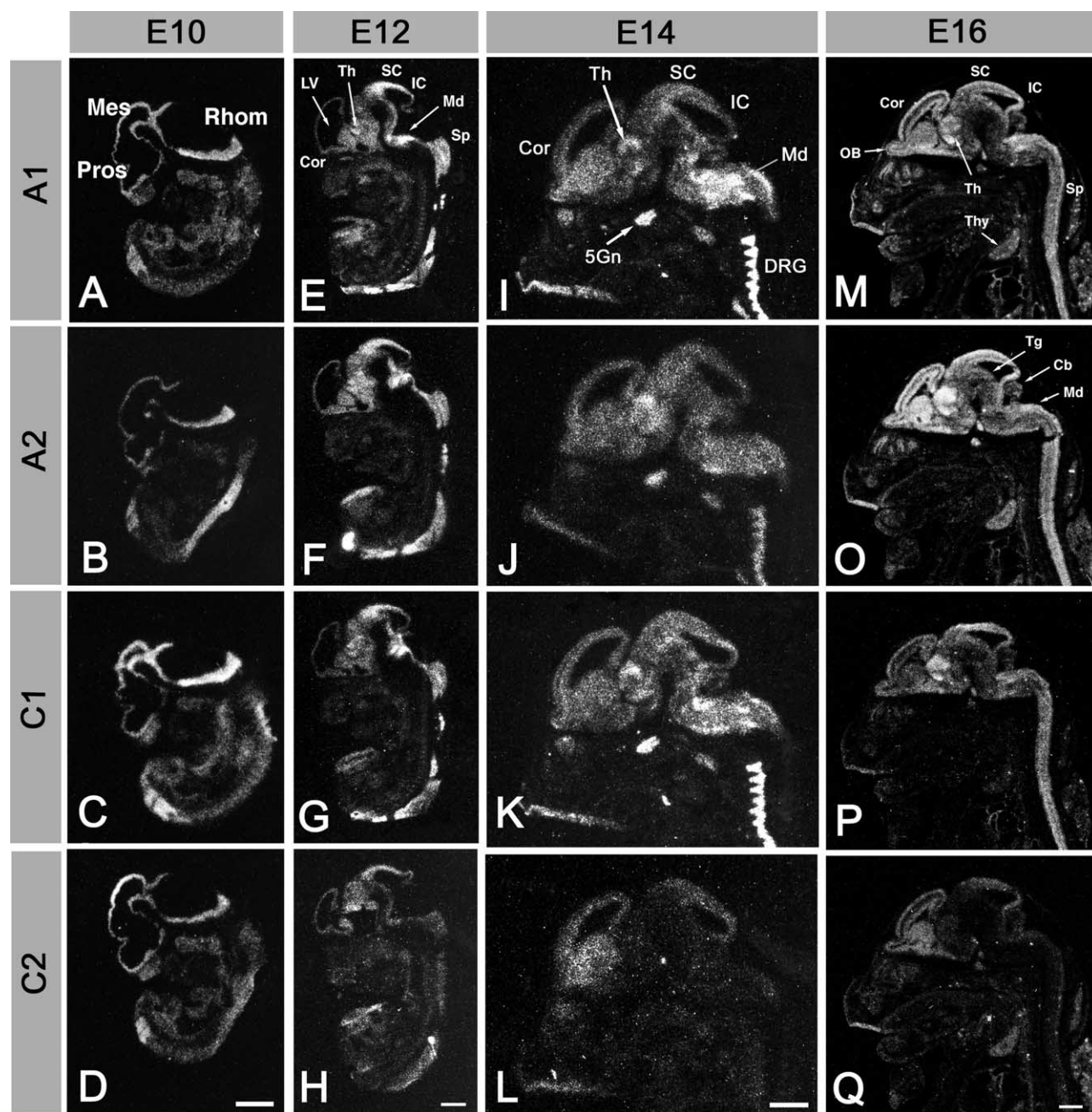
## RESULTS

### Pancortin-3 mRNA is expressed notably but transiently in the developing brain, especially in the thalamus

Mouse brain sections were subjected to *in situ* hybridization using synthetic oligonucleotide probes specific to mRNA sequences encoding the Pancortin A1, A2, C1, and C2 parts. Hereafter, mRNA expressions detected by the probes specific to the A1, A2, C1, and C2 parts will be referred to as A1, A2, C1, and C2 signals, respectively. It is noteworthy that A2 and C1 signals were expressed transiently in the brain during embryogenesis, notably in the thalamus in the prosencephalon, in addition to the neocortex (Nagano et al., 1998) (Figs. 2, 3). In addition, localization of their signals was observed clearly in the rhombencephalon. Their temporal expression profiles in the brain are as follows.

All types of signals (A1, A2, C1, and C2) were detected in the prosencephalon, mesencephalon, and rhombencephalon on E10 (Fig. 2A–D). In general, signals in the ventral prosencephalon were weak, while those in the dorsal prosencephalon and the rhombencephalon were strong. In addition, C1 signals in the mesencephalon were clearly visible (Fig. 2C).

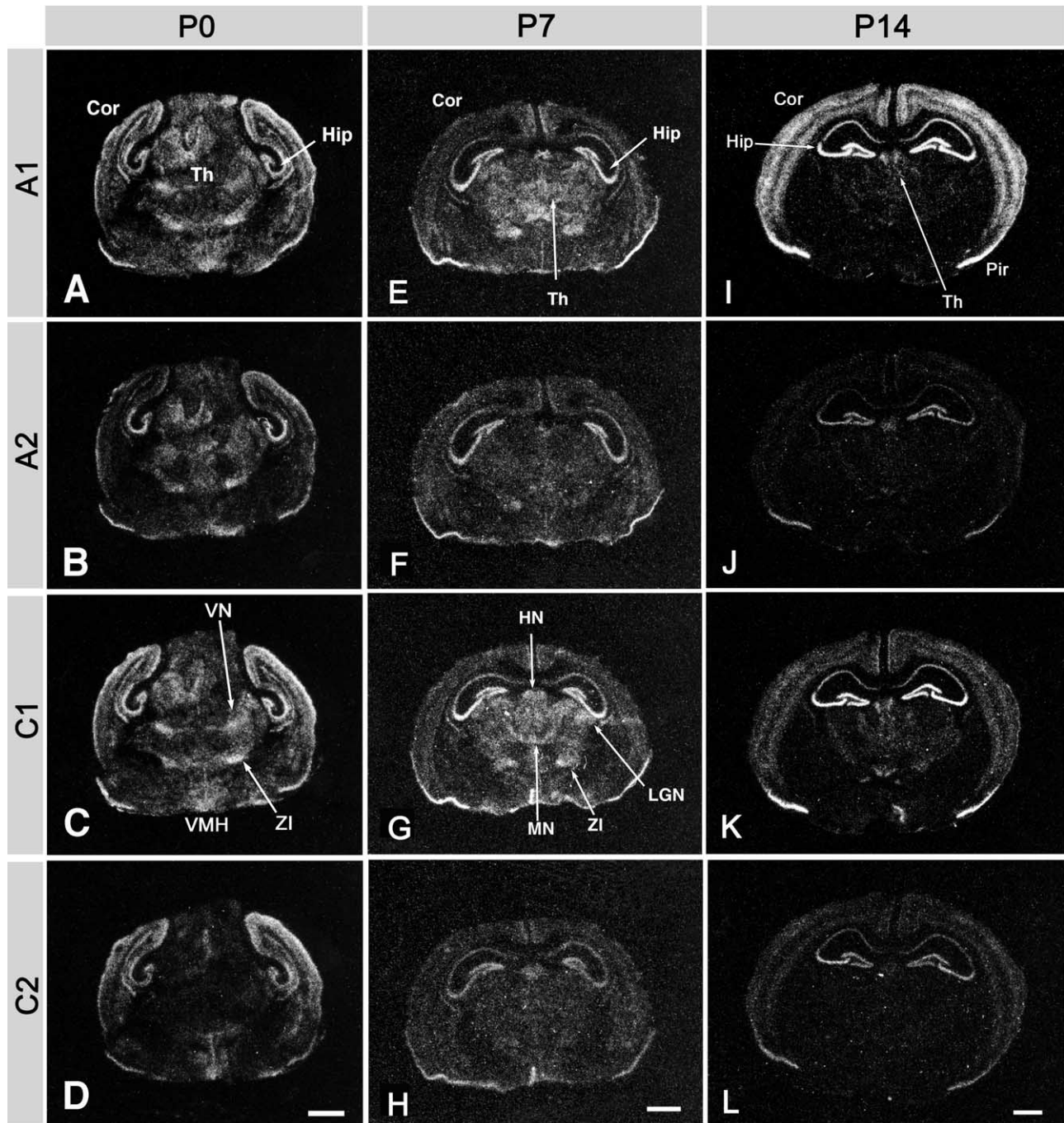




**Fig. 2.** Localization of A1, A2, C1, and C2 part mRNAs in the developing embryos at E10, 12, 14, and 16. ICR mice of E10 (A–D) and E12 (E12, E–H) were used. mRNA signals are shown in white. A1 signals; A, E, I, M: A2 signals; B, F, J, O: C1 signals; C, G, K, P: C2 signals; D, H, L, Q. Note that all mRNA signals were observed on E10. Strong expression of A2 signals was observed on E16. Scale bar=1 mm for each column.

On E12, A1–C2 signals were equally well observed as on E10. Although C2 signals were detected in the thalamus and the superior colliculus, they were weak in intensity. On the other hand, distinct A1, A2, and C1 signals were noticed in the thalamus, the superior colliculus, and widely in the medulla oblongata (Fig. 2E–G). On E14, A2 signals were observed in the thalamus (Fig. 2J), while A1 and C1 signals were strong in the medulla oblongata (Fig. 2I, K). On E16 and later, in addition to the areas where A1 and C1 signals were observed on E14, strong A1 and C1 signals were seen in the

cerebral cortex, the septum, and the thalamus. Robust expression of A2 signals was continuously observed in the thalamus (Fig. 2M–O). C2 signals were detected in the cerebral cortex, the septum, and in the superior and inferior colliculi (Fig. 2P). At birth, all four part-specific signals, A1, A2, C1, and C2 were observed in the cerebral cortex and the hippocampus (Fig. 3A–D). In addition, A1, A2, and C1 signals were noticed in the ventral nucleus, including the ventral posterior nucleus, of the thalamus, the ventral thalamus, especially in the zona incerta, and the ventromedial hypothal-



**Fig. 3.** Localization of A1, A2, C1, and C2 part mRNAs in the forebrain at P0, 7, and 14. A1 signals, A, E, I; A2 signals, B, F, J; C1 signals, C, G, K; C2 signals, D, H, L. The expression of A2 signals decreased compared with that on E16. The intensity of A1 signals increased during the postnatal development while A2 signals decreased. Note that the expression of A1 and C1 signals in the Th decreased at P14. Scale bar=1 mm for each column.

lamic nucleus. By contrast, C2 signals were barely detectable in the thalamus. On P7, A1 and C1 signals in the thalamus were strong, while A2 and C2 signals were very weak (Fig. 3E–H). It was of particular interest that on P14, the expression patterns of A1, A2, C1, and C2 signals (Fig. 3I–L) resembled those in the adult. In the hippocampus, strong A1, C1, and C2 signals were observed, while A2 signals were faint. All four signals were weak in the thalamus. On the other

hand, strong expression of A1, C1, and C2 signals was noted in the cerebral cortex.

#### Each *Pancortin* species is differentially regulated during development

Since transient expression of *Pancortin* mRNAs in the developing thalamus was remarkable, we next explored the ex-



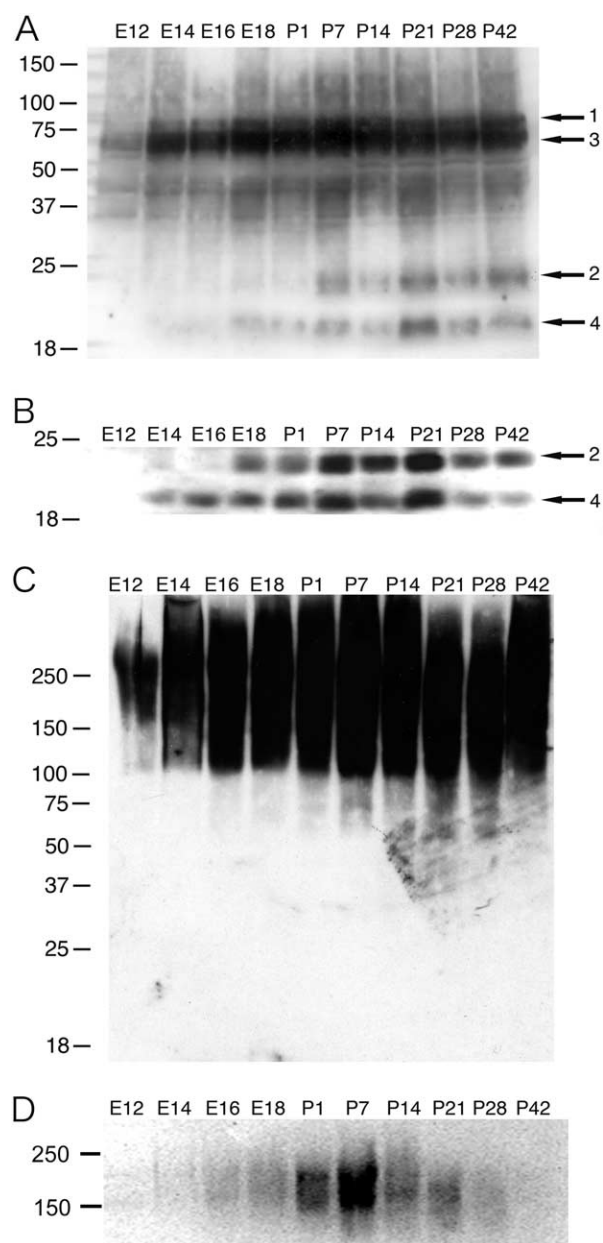
pression profiles of each Pancortin species there. Protein extracts prepared from the thalami at various embryonic stages were subjected to Western blot analyses using an anti-Pancortin antibody that recognizes the four Pancortin species simultaneously (Fig. 4A, B). During development, Pancortin-3 (A2-B-C1) was detected earliest among the four species; it could already be detected on E12. The expression of Pancortin-3 increased toward P7 as development advanced, and then decreased gradually thereafter. Pancortin-1 (A1-B-C1) and Pancortin-4 (A2-B-C2) were detected from E14, after which levels increased progressively, the highest expression on P7. Pancortin-4 was barely detectable on P42. By contrast, expression of Pancortin-2 (A1-B-C2), which was first detected on E18, was apparent on P7, and then decreased after P21. We also re-estimated the molecular sizes of Pancortins precisely using fine protein size markers. In our previous paper, we roughly estimated the sizes of Pancortin-1 to -4 are approximately 80, 30, 70, 20 kDa (Nagano et al., 2000). We re-estimated sizes of Pancortin-1 to -4 using the same antisera and they were approximately 75, 22, 58, 18 kDa, respectively. Detected signals of Pancortins were broader in the adult mice compared with those in embryonic ones (Fig. 4).

### Pancortins are glycosylated

The members of olfactomedin family have been revealed to be glycosylated (Bal and Anholt, 1993; Moreno and Bronner-Fraser, 2001). Since molecular sizes of Pancortins were bigger than the calculated molecular weights, it is highly likely that Pancortins are glycosylated. Pancortins have one possible glycosylated site in the B domain and 6 sites in the C1 domain (Nagano et al., 2000). Our Western blot analyses under non-reducing condition suggested that Pancortins are glycosylated, because the bands in Fig. 4B migrated diffusely compared with the result under reducing condition. To confirm this possibility, Pancortin-3 tagged with EGFP was expressed in COS-7 cells and the cellular extracts were treated with PNGase F (N-glycosidase F) in order to remove sugars. The resultant size of Pancortin-3 tagged with EGFP is shorter than that without glycosidase treatment (Fig. 5A). Therefore, Pancortins are glycosylated.

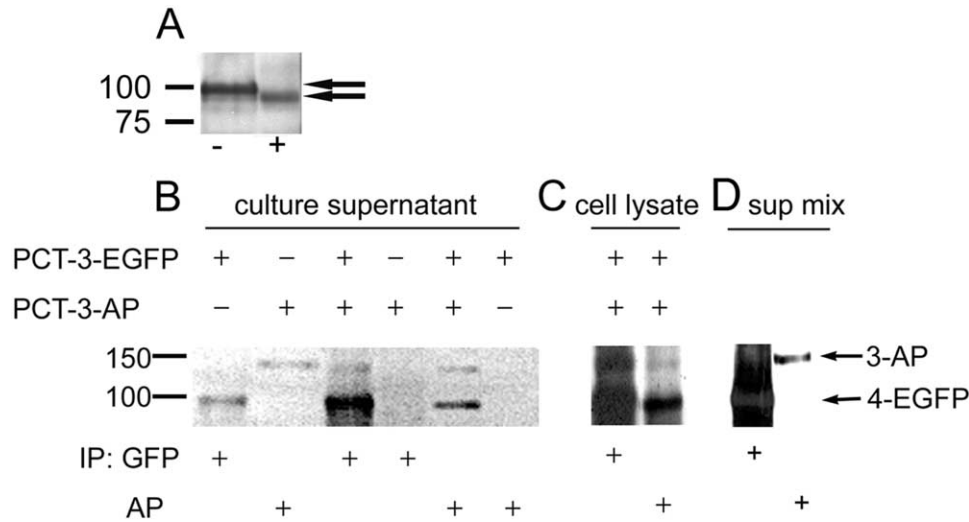
### Pancortins form a large complex *in vivo*

The expression of Pancortin-3 protein was highest on P7 in the thalamus, while the expression of A2 and C1 mRNA peaked on E16. There is a delay of approximately 10 days between peaks of mRNA and protein expression of Pancortin-3. This suggests that Pancortin-3 exists in a stable form *in vivo*. To explore this further, we examined the molecular form of Pancortins *in vivo*. Our Western blot analyses carried out under non-reducing conditions with the thalamic extracts revealed that Pancortins form a complex of large molecular mass (Fig. 4C, D). It is of interest that we could not detect any single form of the Pancortins *in vivo* (Fig. 4C, D); although the biggest Pancortin (Pancortin-1) is approximate 75 kDa (Fig. 4A), only structures with a molecular weight larger than 100 kDa were detected. This implies that Pancortins exist in the oligomerized forms (dimer or multimer). In fact, some Pancortins of



**Fig. 4.** Ontogenetic expression profiles of PCT in the Th. WB were carried out using thalamic extracts prepared from mice at various developmental stages (E12–P42). The numbers on the left side show molecular weights in kDa, while numbers 1–4 on the right side indicate each PCT species (PCT-1 to PCT-4, respectively). Blotted membranes were reacted with anti-PCT antibody and signals were detected by chemiluminescence. (A) WB carried out under reducing conditions. (B) Ontogenetic changes of PCT-2 and PCT-4 were examined to reveal their onset with a longer exposure time. Independent experiment from A. Samples were run under reducing conditions. (C) WB carried out under non-reducing conditions. (D) An image of the same membrane as in panel C, but with a shorter exposure time.

molecular weight  $\approx 240$  kDa were observed in the E12 lane (Fig. 4A, B), when only Pancortin-3 expression was confirmed (Fig. 4A). On P7, bands of approximately 240 kDa and 160 kDa were detected (Fig. 4C, D). We pursued this issue further in the following experiments.



**Fig. 5.** PCT-3-EGFP are glycosylated and are capable of forming homo-oligomer with PCT-3-AP prior to be secreted. (A) *PCT-3-EGFP* was transfected into HEK293 cells, and its culture supernatant was treated with (+) or without (–) PNGase F (glycosidase F). Apparently, molecular weight was reduced by PNGase F treatment indicating that PCT-3-EGFP was *N*-glycosylated. Arrows indicate the recognized band in each lane. (B–D) *PCT-3-EGFP* and *PCT-3-AP* were cotransfected or transfected separately into HEK293 cells, and then the resultant culture supernatants (B) or the cell lysates (C) were subjected to IP with anti-GFP antibody (GFP) or anti-AP antibody (AP). Note that PCT-3-EGFP (approximate 100 kDa) and PCT-3-AP (approximate 140 kDa) were coimmunoprecipitated. D, *PCT-3-EGFP* and *PCT-3-AP* were transfected discretely and the each culture supernatant was mixed *in vitro* (sup mix), followed by IP. PCT-3-EGFP and PCT-3-AP were not coimmunoprecipitated. Molecular weights (kDa) are shown on the left side.

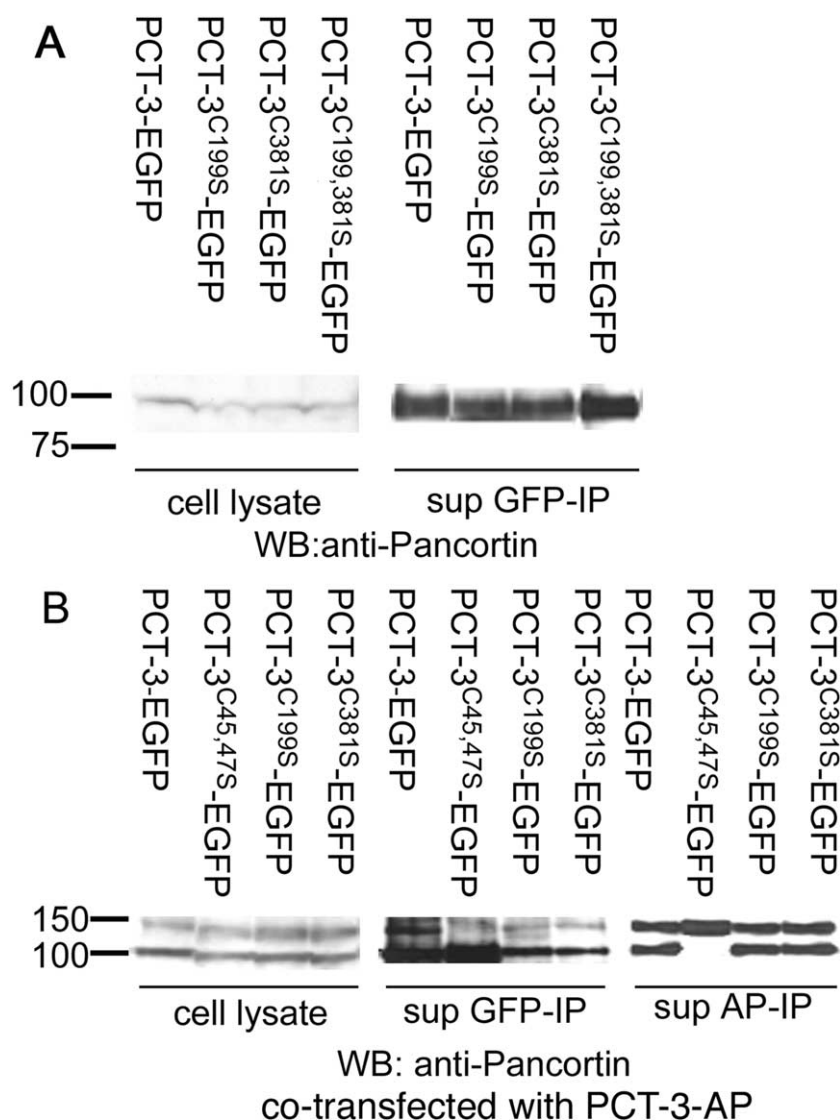
### Pancortin-3 is capable of forming oligomers

In order to verify whether or not Pancortins form oligomers, we constructed Pancortin-3 expression vectors tagged with different marker proteins: EGFP (*PCT-3-EGFP*) and AP (*PCT-3-AP*). HEK 293 cells, which do not express Pancortins endogenously, were co-transfected *PCT-3-EGFP* and *PCT-3-AP* expressing vectors. Three days later, the culture supernatant and the cell lysate were subjected to immunoprecipitation with an anti-EGFP or an anti-AP antibody, and then Western blot analyses with the anti-Pancortin antibody were carried out. PCT-3-EGFP, with the molecular weight of approximately 100 kDa, was co-immunoprecipitated with PCT-3-AP, with the molecular weight of approximately 140 kDa, and vice versa. Therefore, differentially tagged Pancortin-3 structures were bound to each other (Fig. 5B, C). However, PCT-3-EGFP and PCT-3-AP did not form dimer when we had transfected *PCT-3-EGFP* or *PCT-3-AP* alone and then mixed separately harvested these supernatants, indicating that they are fully oligomerized before secretion and not able to be bound to each other after secretion (Fig. 5D). Therefore, it is possible that the Pancortins do not form dimer or oligomer non-covalently after being secreted, rather do covalently while being processed within a cell, probably through disulfide bonds.

### The cysteine residues in the C1 part, where the olfactomedin domain exists, are not essential for oligomerization or secretion

In the case of Myocilin/TIGR (trabecular meshwork inducible glucocorticoid response), one olfactomedin-

related molecule, the disulfide bonds in the olfactomedin domain are important for precise folding and secretion (Jacobson et al., 2001; Fautsch et al., 2004). In the Pancortins, the olfactomedin domain is located in the C1 part (amino acid residues 271–434 of Pancortin-3, Fig. 1). Since the secreted Pancortins form a large complex, we substituted cysteine 381 in the C1 part of Pancortin-3 for serine, PCT-3<sup>C381S</sup>, to examine whether cysteine residue in the olfactomedin domain is critical for the secretion and/or oligomerization (dimerization or multimerization) of Pancortins *in vitro*. In addition, we generated mutated Pancortin-3 harboring serine 199 instead of cysteine 199 (PCT-3<sup>C199S</sup>), for which the bonding probability was the highest among cysteines in the C1 part; bonding probabilities of cysteines in the C1 part (residues 193, 199, and 381 in Pancortin-3) were 0.272, 0.391 and 0.107, respectively *in silico*. Mutated Pancortin-3 tagged with EGFP (*PCT-3<sup>C199S</sup>-EGFP* or *PCT-3<sup>C381S</sup>-EGFP*) was transfected into HEK 293 cells and then the culture supernatant was subjected to immunoprecipitation. We found that PCT-3<sup>C199S</sup>-EGFP and PCT-3<sup>C381S</sup>-EGFP were secreted from cells in addition to PCT-3-EGFP, suggesting that cysteine residues in the olfactomedin domain of Pancortin are not essential for secretion (Fig. 6A). Furthermore, we generated doubly mutated Pancortin-3, *PCT-3<sup>C199, 381S</sup>*, in which both cysteines in the olfactomedin domain were substituted for serines, and examined whether or not it is secreted. This double-mutated Pancortin is predicted not to be capable of being secreted, if these cysteines are required for precise folding necessary for secretion. However, this molecule was secreted as well as the



**Fig. 6.** No substantial role in the secretion or oligomerization of PCT-3 was revealed for cysteine 199 or 381 in the C1 part. (A) Plasmids containing *PCT-3-EGFP* were modified to express serine(s) instead of cysteine(s) (*PCT-3<sup>C199S</sup>-EGFP*, *PCT-3<sup>C381S</sup>-EGFP*, or *PCT-3<sup>C199,381S</sup>-EGFP*) were transfected into HEK293 cells, and then the resultant cell lysates or culture sup GFP-IP were subjected to WB with anti-PCT antibody under reducing conditions. Corresponding bands were observed in all cases. (B) *PCT-3-AP* and wild or mutated *PCT-3-EGFP* (*PCT-3-EGFP*, *PCT-3<sup>C199S</sup>-EGFP*, *PCT-3<sup>C381S</sup>-EGFP*, or *PCT-3<sup>C45,47S</sup>-EGFP*) were cotransfected into HEK293 cells, and then culture sup GFP-IP or with anti-AP antibody were subjected to WB with anti-PCT antibody under reducing conditions. *PCT-3<sup>C199S</sup>-EGFP* and *PCT-3<sup>C381S</sup>-EGFP* were coimmunoprecipitated with *PCT-3-AP*, while *PCT-3<sup>C45,47S</sup>-EGFP* was not.

native *PCT-3-EGFP* (Fig. 6A). Subsequently, we examined whether or not these cysteines are required for forming oligomer. We co-transfected *PCT-3<sup>C199S</sup>-EGFP* together with *PCT-3-AP* or *PCT-3<sup>C381S</sup>-EGFP* together with *PCT-3-AP* into HEK293 cells. The culture supernatant was then subjected to immunoprecipitation with an anti-GFP or an anti-AP antibody. Either *PCT-3<sup>C199S</sup>-EGFP* or *PCT-3<sup>C381S</sup>-EGFP* was coimmunoprecipitated with *PCT-3-AP*, and vice versa, indicating that neither cysteine 199 nor cysteine 381 is essential for forming oligomer (Fig. 6B). This is in harmony with our in silico analyses, in which cysteine residues in the C1 part (residues 193, 199, and 381 in Pancortin-3) showed low bonding probabilities.

#### The common B part is responsible for oligomerization and is important for secretion

Since cysteine residues in the C1 part were not critical for oligomerization, it is possible that cysteine residues in the common B part are responsible for oligomer formation. Furthermore, our in silico analyses revealed that three cysteine residues (residues 45, 47, and 57 in Pancortin-3 and Pancortin-4) in the common B part may be disulfide bonded, since their bonding probabilities were 0.716, 0.691 and 0.659, respectively. To investigate whether cysteine residues in the B part are important for oligomerization, we made mutants of Pancortin-3 tagged with EGFP by replacing cysteine residues at 45 and 47 (*PCT-3<sup>C45,47S</sup>-EGFP*).



EGFP) for serine residues. Then we co-transfected *PCT-3<sup>C45, 47S</sup>-EGFP* together with *PCT-3-AP* into HEK293 cells. The culture supernatant was then subjected to immunoprecipitation with an anti-GFP or an anti-AP antibody. *PCT-3<sup>C45, 47S</sup>-EGFP* was found in the culture supernatant but it was not co-immunoprecipitated with *PCT-3-AP* (Fig. 6B). Therefore, cysteine residues at 45 and 47 in the B part are crucial for oligomerization and the B part is highly likely to be essential for oligomer formation.

To confirm this assumption, we constructed the expression vector of Pancortin-4 tagged with EGFP (*PCT-4-EGFP*); because there is only one amino acid residue in the C2 part, Pancortin-4 was sufficient to study the potency of the common B part. Since the B part is common to all four Pancortin species, it is also likely that different Pancortin species form hetero-dimers through the common B part. We first tested this possibility with Pancortin-3 and Pancortin-4, and found that not only individual Pancortin species form homo-dimers, but they also tied up with each other, forming hetero-dimers. We transfected *PCT-4-EGFP* and *PCT-3-AP* into HEK293 cells, then the collected supernatants were subjected to immunoprecipitation with anti-EGFP or anti-AP antibodies, and probed by the anti-Pancortin antibody. *PCT-3-AP* was detected when we immunoprecipitated the supernatant with the anti-GFP antibody and *PCT-4-EGFP* was detected with the anti-AP antibody (Fig. 7A). This result indicates that Pancortins are capable of forming hetero-dimers through the B part. We then examined the role of cysteine residues 45 and 47 using mutated *PCT-4-EGFP*, of which cysteine residues 45 and 47 were substituted for serine residues (*PCT-4<sup>C45, 47S</sup>-EGFP*). *PCT-4<sup>C45, 47S</sup>-EGFP* was recognized in the culture supernatant but its amount was very small (Fig. 7A). This indicated that *PCT-4<sup>C45, 47S</sup>-EGFP* was secreted inefficiently compared with *PCT-4-EGFP*. In the culture supernatant of cells transfected with *PCT-3-AP* and *PCT-4<sup>C45, 47S</sup>-EGFP*, *PCT-3-AP* and *PCT-4<sup>C45, 47S</sup>-EGFP* were not detected simultaneously after immunoprecipitation with the anti-GFP antibody or with the anti-AP antibody, showing that cysteine residues 45 and 47 are essential for hetero-dimerization (Fig. 7A). Furthermore, when *PCT-4-EGFP* was transfected into HEK293 cells, oligomers were surely formed, however, neither dimer nor higher multimer of *PCT-4<sup>C45, 47S</sup>-EGFP* was observed in that cell lysate by our Western blot analysis under non-reducing condition (Fig. 7B). This clearly demonstrated that *PCT-4<sup>C45, 47S</sup>-EGFP* were not capable of forming homo-dimers, either.

To investigate which cysteine residue in the B part is important for oligomerization, we made mutants of Pancortin-4 by replacing cysteine residue at 45 (*PCT-4<sup>C45S</sup>-EGFP*), 47 (*PCT-4<sup>C47S</sup>-EGFP*) or 57 (*PCT-4<sup>C57S</sup>-EGFP*) for serine residue. These mutated *Pancortin-4* tagged with EGFP were cotransfected with *PCT-3AP* into HEK 293 cells. Cell lysate was subjected to Western blot analyses and the culture supernatant was subjected to immunoprecipitation with the anti-GFP antibody. *PCT-3-AP* was co-immunoprecipitated with *PCT-4-EGFP* or *PCT-4<sup>C57S</sup>-EGFP*, but not with *PCT-4<sup>C45S</sup>-EGFP* or *PCT-4<sup>C47S</sup>-EGFP* (Fig. 7C). Therefore, it is likely that disulfide bonds through

cysteine residue 45 and 47 are essential for the oligomer formation of Pancortin. In addition, apparently cysteine residue 57 plays a role for oligomerization, since the co-immunoprecipitated amount of *PCT-3-AP* with *PCT-4<sup>C57S</sup>-EGFP* was small compared with that with *PCT-4-EGFP* (Fig. 7C).

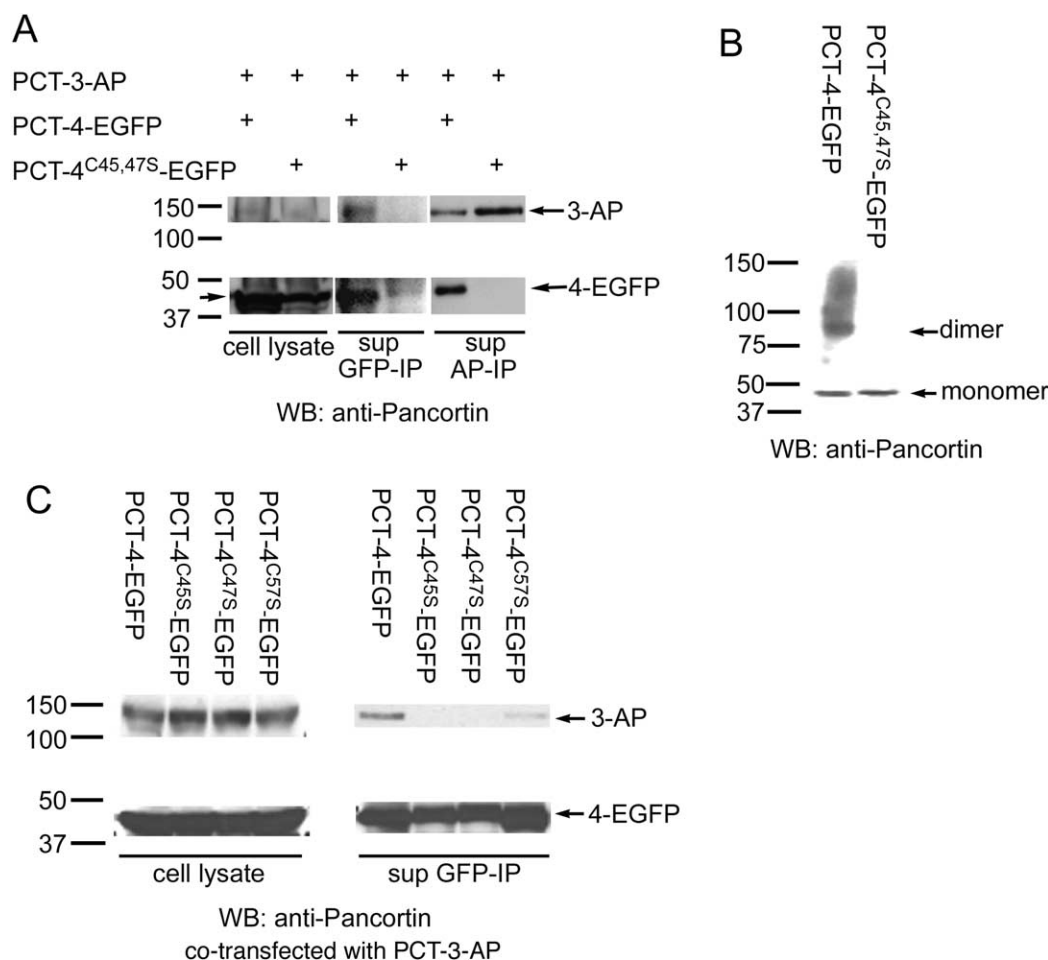
## DISCUSSION

There are no specific sequences for individual Pancortin species; the B part is common to all four Pancortins, and the other parts are shared by two of them. We therefore employed synthetic oligonucleotide probes specific to the A1, A2, C1, and C2 part for *in situ* hybridization. In general, since oligonucleotide probes were tagged with <sup>35</sup>S-dATP by the 3'-end labeling method, their specificities and sensitivities may vary. This was actually the case for the probe for the C2 part. There are only three nucleotides for encoding one amino acid in the C2 part, we therefore looked for an appropriate sequence in their 3' non-coding region. Different C2 probes gave different intensities (data not shown). However, our B part-specific anti-Pancortin antibody enabled us to observe and compare the expressions of each Pancortin species at the protein level, simultaneously.

Moreno and Bronner-Fraser (2002) have examined the expression of Noelin-1/2 (Pancortin orthologues in the *Xenopus* that corresponds to Pancortin-1 and Pancortin-3, respectively) mRNAs at E8–E10 using a probe against the C1 part of Pancortins in mice, and have reported Noelin-1/2 mRNA expression in the neural plate. Our study, in a sense, extended their study using oligonucleotide probes specific to the A1, A2, C1, and C2 and examined their expression from E10 to postnatal ages, revealing the mRNA expression patterns of all Pancortin species there. We observed C1 mRNA expression already at E10, and this observation was consistent with their report.

Pancortins are suggested to be important for neurogenesis in *Xenopus* (Moreno and Bronner-Fraser, 2001). Therefore, it is highly likely that Pancortins play an important role for brain development in mice and that they are highly expressed during the development. Consistently, in the middle of embryogenesis, *Pancortin* mRNAs were distributed widely in the brain. This expression became confined to specific nuclei as the development progressed, suggesting that the extracellular matrix is specifically adapted to the physiological specialties of the specific cell populations. In our previous *in situ* hybridization study (Nagano et al., 1998), we found that no substantial levels of mRNAs of any Pancortin species were recognized in the thalamus of the adult. However, to our surprise, the present study revealed high levels of Pancortin expression in the thalamus during development. We demonstrated previously that the A2-Pancortins are secreted from a cell efficiently (Nagano et al., 2000). Therefore, the long lifetime of translated A2 Pancortins, suggests that they work as extracellular matrix components in the thalamus throughout the development.

On E12, only Pancortin-3 (approximately 58 kDa) was expressed in the thalamus. Subsequent Western blot analyses under non-reducing conditions revealed the sole expres-



**Fig. 7.** Hetero- and homo-oligomer formation of PCT through cysteine 45 and 47 in the B part. (A) *PCT-3-AP* and *PCT-4-EGFP* or *PCT-4<sup>C45, 47S</sup>-EGFP* were cotransfected to the HEK293 cells, and then each supernatant was immunoprecipitated with GFP antibody (GFP-IP) or AP antibody (AP-IP) and subjected to WB. *PCT-3-AP* was not coimmunoprecipitated with *PCT-4<sup>C45, 47S</sup>-EGFP*, while it was coimmunoprecipitated with *PCT-4-EGFP*. All materials were simultaneously electrophoresed in the same gel, but an image of 'GFP-IP' was obtained by shorter exposure time to a film than others. Position of 4-EGFP is also indicated from the left side by an arrow between the size markers of 37 and 50 kDa. (B) Cell lysates of HEK293 cells transfected with *PCT-4-EGFP* or *PCT-4<sup>C45, 47S</sup>-EGFP*, were subjected to WB under non-reducing condition. *PCT-4<sup>C45, 47S</sup>-EGFP* did not form oligomer. The molecular weight of *PCT-4-EGFP* is approximately 45 kDa. (C) *PCT-3-AP* and *PCT-4-EGFP*, *PCT-4<sup>C45S</sup>-EGFP*, *PCT-4<sup>C47S</sup>-EGFP* or *PCT-4<sup>C57S</sup>-EGFP* were cotransfected into the HEK293 cells, and each supernatant was immunoprecipitated with GFP antibody (GFP-IP) then was subjected to WB. *PCT-3-AP* was not coimmunoprecipitated with *PCT-4<sup>C45S</sup>-EGFP* or *PCT-4<sup>C47S</sup>-EGFP*. Only small amount of *PCT-3-AP* was immunoprecipitated with *PCT-4<sup>C57S</sup>-EGFP*. Molecular weight (kDa) are shown on the left side (A–C).

sion of an approximately 240 kDa molecule. Assuming that no other species of Pancortins except for Pancortin-3 are involved in this complex formation, it is probable that Pancortin-3 forms molecular complex by itself. Pancortin-4 is also likely to form molecular complex, with itself and/or other molecules, *in vivo*, since Pancortin-4 is approximate 18 kDa and no bands smaller than 100 kDa were detected there under non-reducing condition (Fig. 4C). In addition, Pancortin-4 tagged with EGFP (*PCT-4-EGFP*) forms the large complex (Fig. 7B).

Many molecules that contain an olfactomedin domain have been cloned and most of them have been identified as extracellular matrix protein or cell surface receptors (Green and Klein, 2002; Wentz-Hunter et al., 2002; Nagy et al., 2003). It is of interest that mutated Myocilin, which lacks the olfactomedin domain, accumulates inside the cell and reduces secretion of endogenous Myocilin (Caballero et al.,

2000). Furthermore, mutation in of its olfactomedin domain leads to misfolding of Myocilin, probably because of disturbance of forming intra-domain disulfide bonds (Nagy et al., 2003), and to disturbance of secretion (Fautsch et al., 2004), resulting in its accumulation within cells (Stone et al., 1997; Jacobson et al., 2001). In the study presented here, we demonstrated that the olfactomedin domain is not crucial for the secretion of Pancortins; Pancortin-3 was secreted even when its olfactomedin domain was mutated. In addition, Pancortin-4 that does not have the olfactomedin domain was also secreted. Nagy et al. (2003) have characterized the olfactomedin domain of Myocilin and found that its olfactomedin domain contains an intra-molecular disulfide bond connecting cysteine 245 and cysteine 433 of Myocilin. Since equivalent cysteine residues are conserved in the Pancortin-3 as cysteine residues 199 and 381, it is possible that these cysteines are involved in intra-molecular disulfide bond, rather

than inter-molecular bond. Anyhow, it should be noted that in some case, the olfactomedin domain or the C1 part, which contains the olfactomedin domain, plays a role for the secretion, because the secretion of PCT-4<sup>C45, 47S</sup>-EGFP, which lacks the C1 part, was highly disturbed but that of PCT-3<sup>C45, 47S</sup>-EGFP, which has the C1 part, was not highly bothered.

A common structural feature shared by Pancortins and their related proteins is that they have some protein-interacting domain, in addition to the olfactomedin domain (Green and Klein, 2002; Wentz-Hunter et al., 2002; Nagy et al., 2003). Wentz-Hunter et al. (2002) have demonstrated that Myocilin can form homo-multimers between the leucine-zipper domains in the N-terminal region, independent of the olfactomedin domain. It is possible that such protein-interacting domain, which is also found in the B part of Pancortin, facilitates the formation of specific disulfide bonds in this region.

Our mutagenesis studies revealed that cysteine residues located in the B part (residues 45 and 47 in Pancortin-3 and Pancortin-4) are essential for the oligomerization of Pancortins with other Pancortin(s), while, as mentioned above, cysteine residues 199 and 381 are unlikely. However, the definitive conclusion is not finally drawn with the methodologies that we employed in this study. Particularly, the involvement of cysteine residue 57 in inter- and/or intra-molecular disulfide bond still remains open. All of the mutagenesis studies were carried out with A2-Pancortins; but our results are applicable to the A1-Pancortins (Pancortin-1 and Pancortin-2), because they also have the common B part, suggesting that A1-Pancortins also form large molecular complex through this part.

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