American Journal of Pathology, Vol. 155, No. 5, November 1999

High Expression of Doublecortin and KIAA0369 Protein in Fetal Brain Suggests Their Specific Role in Neuronal Migration

Masashi Mizuguchi,* Jiong Qin,† Mitsunori Yamada,‡ Kazuhiko Ikeda,§ and Sachio Takashima*†

From the Department of Pediatrics, Jichi Medical School, Tochigi; the Department of Mental Retardation and Birth Defect Research,† National Institute of Neuroscience, NCNP, Kodaira; the Department of Pathology,‡ Brain Research Institute, Niigata University, Niigata; and the Department of Ultrastructure and Histochemistry,§ Tokyo Insitute of Psychiatry, Tokyo, Japan*

The X-linked subcortical laminar heterotopia and lissencephaly syndrome is a disorder of neuronal migration caused by a mutation in *XLIS*, a recently cloned gene on chromosome Xq22.3-q23. The predicted protein product for *XLIS*, doublecortin (DC), shows high homology to a putative calcium calmodulin-dependent kinase, KIAA0369 protein (KI). Here we identified DC and KI in the brains of human and rat fetuses by immunochemical and immunohistochemical means. In this study, Western blotting demonstrated that both DC and KI are specific to the nervous system and are abundant during the fetal period, around 20 gestational weeks in humans and embryonic days 17 to 20 in rats. Immunostaining of the developing neocortex disclosed localization of DC and KI immunoreactivities in neuronal cell bodies and processes in the zones of ongoing neuronal migration. Although KI showed a somewhat wider distribution than DC, the temporal and spatial patterns of their expression were similar. These results suggest that DC and KI participate in a common signaling pathway regulating neuronal migration. *(Am J Pathol 1999, 155:1713–1721)*

In the developing mammalian cerebral cortex, neurons arise in the ventricular zone and move outward along the radial glia to reach their final destination, the cortical plate. This process is referred to as neuronal migration and occurs mainly from the second to sixth months of gestation in the neocortex of human fetuses[1] and from embryonic day 14 (E14) to around birth in that of rat fetuses.[2] An arrest in neuronal migration may cause various brain anomalies, which are collectively referred to as neuronal migration disorders. Classical lissencephaly is a prototype of these malformations, characterized pathologically by a markedly thickened cerebral cortex consisting of four coarse layers.[3,4] This condition is subdivided clinically into Miller-Dieker syndrome (MDS) and isolated lissencephaly (ILS), according to the presence or absence of associated facial and somatic anomalies.

Recent advances in molecular genetics have revealed the gene defects causing many cases of lissencephaly. MDS is a contiguous gene syndrome that results from a microdeletion of chromosome 17p13.3 encompassing the *LIS1* gene.[5,6] This gene encodes the β subunit of brain type platelet-activating factor acetylhydrolase (PAFAH1B1),[7] which is present in abundance in normal brains but is deficient in those of patients with MDS.[8,9] On the other hand, ILS consists of heterogeneous conditions. Approximately 40% of sporadic ILS patients have a deletion of 17p13.3 involving the *LIS1* gene. Among those without such a deletion, 40% have a mutation or an intragenic deletion in the *LIS1* gene, whereas the other 20% have a mutation in the *XLIS* (or *DCX*) gene located on chromosome Xq22.3-q23.[10,11] Most of the latter patients with X-linked lissencephaly are male and hemizygous for the mutated *XLIS* gene. Morphological features of the brains of patients with an *XLIS* mutation resemble those with a *LIS1* mutation except for several minor differences.[11,12] X-linked female patients heterozygous for the same mutation usually show a milder phenotype, termed subcortical laminar heterotopia (SCLH), band heterotopia, or double cortex syndrome.[13] The spectrum of these X-linked neuronal migration disorders is therefore called X-linked SCLH and lissencephaly syndrome.

The *XLIS* gene has recently been cloned.[14,15] The predicted gene product, doublecortin (DC), is a novel protein with a molecular weight of about 40 kd. DC includes a potential Abl phosphorylation site at tyrosine residue 70 and other sites of potential phosphorylation. The absence of a signal peptide and a transmembrane domain suggests that DC is a hydrophilic, intracellular protein. DC shows high homology to KIAA0369 (KI),

Supported by a Grant-in-Aid for Scientific Research (10670753) from the Ministry of Education, Science and Culture, Japan, and by Grants for Research on Brain Science (H10-Brain-28), on Nervous and Mental Disorders (8A-10–37) and on Neurocutaneous Diseases (3310) from the Ministry of Health and Welfare, Japan.

Accepted for publication July 15, 1999.

Address reprint requests to Masashi Mizuguchi, M.D., Department of Pediatrics, Jichi Medical School, 3,311–1 Yakushiji, Minamikawachi, Kawachi-gun, Tochigi, 329-0498 Japan.

which has previously been isolated from a human fetal brain cDNA library.[16] KI is an 80-kd protein encoded by a gene on chromosome 13q13-q14.1, consisting of an amino-terminal DC-like domain and a carboxy-terminal calcium calmodulin-dependent kinase-like domain.[17] The DC and KI mRNAs are expressed at high levels in the fetal brains, which is compatible with their roles in brain morphogenesis.[14,15,17] It has been speculated that the DC and KI proteins interact in a competitive fashion in a signaling pathway regulating neuronal migration.[14,15,18] With regard to the protein expression, however, there has been no information available about the identification and characterization of these proteins.

In this study, we produced antibodies that specifically recognize DC and KI and used them to delineate the temporal and spatial patterns of expression of these proteins in normally developing brains of humans and rats.

Materals and Methods

Antibody Preparation

Antisera were raised in rabbits against peptides synthesized according to the sequence deduced from human DC and KI cDNAs.[14,15,16] The peptide fragments of DC consisted of the amino acid residues 1–10 plus a carboxy-terminal cysteine (peptide DC1; MELDFGHFDEC) 347–360 plus an amino-terminal cysteine (peptide DC2; CPLSLDDSDSLGDSM) and 271–284 (peptide DC3; CRVMKGNPSATAGP), and those of KI consisted of the residues 1–12 plus a carboxy-terminal cysteine (peptide KI1; MSFGRDMELEHFC) and 276–287 (peptide KI3; CRVVKSTSYTKI). The DC3 and KI3 peptides were mutually homologous: 5 of the 14 amino acids in the former and 6 of the 12 in the latter were shared by DC and KI.[14–16] Peptide synthesis and immunization, as well as the collection and titration of the antisera, were performed as described previously.[8] Some aliquots of sera were absorbed with the corresponding peptides and used for negative control experiments.[8]

Western Blotting

Human tissues for Western blotting were obtained from 10 patients ranging in age from 12 gestational weeks (GW) to 85 years. These patients died of non-neurological diseases, and necropsy was done within 12 hours postmortem. The tissues were kept frozen at −80°C until use.

Cerebral tissues were also removed from Wistar rats ranging from E14 to postnatal day 60 (P60) of age under anesthesia by inhalation of ether.

To study protein expression in tissue cultures, rat cerebral neurons and astrocytes were isolated as described previously[19] and cultured for 3 days *in vitro*. We also used human neural cell lines, such as the neuronally differentiated NT2 teratocarcinoma cells,[20] NB1 and TGW-III neuroblastoma cells, and I-23 and Bu17 glioma cells, as well as non-neural cells, such as the HeLa cervical carcinoma cells and U937 monocytic leukemia cells.

Protein extraction, sodium dodecyl sulfate polyacrylamide electrophoresis, and Western blotting were performed as described previously.[8,21] Briefly, proteins were extracted with Tris/saline buffer containing 1% Triton X-100, and were assayed by Bradford's method. They were then separated on a 10% polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane. To detect DC and KI immunologically, the membrane was probed with one of the anti-DC/KI antibodies overnight at 4°C. The dilution used was 1:1200 for anti-DC1 and anti-DC2, 1:200 for anti-DC3 and anti-KI3, and 1:500 for anti-KI1. Successsive incubations were then done with biotinylated anti-rabbit IgG (Vector, Burlingame, CA; diluted 1:1,000) and a horseradish peroxidase-conjugated avidin-biotin complex (Vector), both at room temperature, for 1 hour. Diaminobenzidine HCl (DAB; Dojin, Osaka, Japan) was then used as the chromogen. To confirm that the samples loaded onto each lane contained an equal amount of protein (40 μg), unused portions of the transfer membranes were stained with Coomasie Brilliant Blue R-250.

Subcellular Fractionation

Crude subcellular fractionation was performed by differential centrifugation, as described elsewhere.[8] Each fraction was cytochemically identified by Feulgen, methyl-green-pyronin, and Janus green stainings.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were prepared from tissues of 12 control patients without a neurological disease, from 6 GW to 75 years of age. For experiments in rats, brain tissues of Wistar rats from E11 to P60 of age were fixed overnight in Bouin's solution and embedded in paraffin.

Immunoperoxidase staining was performed by the biotin-streptoavidin method.[22] Briefly, the slides were incubated with the first antibodies against DC and KI (diluted 1:200), and then processed using an S-HRP immunostaining kit (Seikagaku, Tokyo, Japan). The immunoproducts were visualized with DAB. No counterstaining was done.

Results

Specificity of Antibodies

The specificity of the anti-DC/KI antibodies was first determined by Western blotting of cerebral tissue proteins. On the blots of human fetal cerebral homogenates, the anti-DC and anti-KI antibodies yielded a doublet of bands at about 45 and 80 kd, respectively (Figure 1A). These proteins were identified as DC and KI, because they were consistently recognized by the appropriate antibodies and the molecular weights coincided with those predicted previously.[14–16] The anti-KI antibodies occasion-

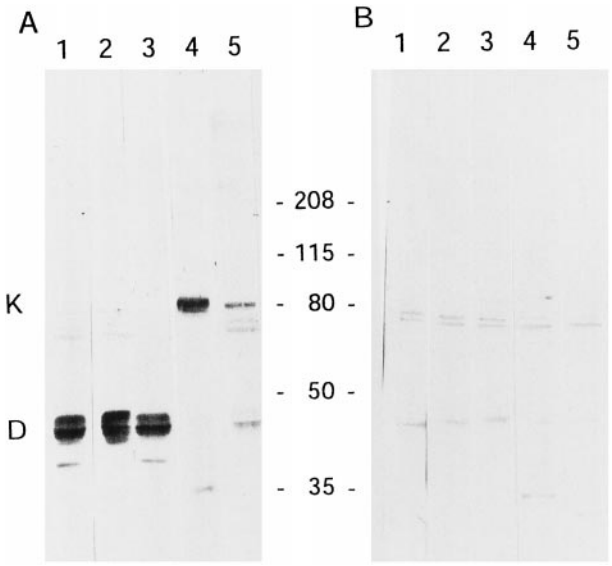

Figure 1. Western blots of a homogenate of the cerebrum of a human fetus at 18 gestational weeks (GW) probed with the anti-DC1 (**Lane 1**), anti-DC2 (**Lane 2**), anti-DC3 (**Lane 3**), anti-KI1 (**Lane 4**), and anti-KI3 (**Lane 5**) antibodies before (**A**) and after (**B**) preabsorption with their corresponding peptides. **A:** The antibodies against doublecortin (DC) yielded a doublet of bands at 45 kd (**Lanes 1–3**) and those against KIAA0369 (KI) at 80 kd (**Lanes 4** and **5**). The anti-KI3 also showed weak cross-reactivity with a 45-kd protein (**Lane 5**). **B:** All these bands disappeared after preabsorption of the antisera.

ally yielded an additional, weak 45-kd band, which may have resulted from a cross-reaction with DC. No band appeared on the blots probed with the preimmune or preabsorbed antisera (Figure 1B).

Immunochemistry

Tissue distribution of DC and KI was studied by Western blotting of various organs of human fetuses (Figure 2). Both antigens were present in the central nervous system (CNS) and were most abundant in the cerebrum. Other organs did not contain a detectable amount of these proteins, although the adrenal glands showed a weak KI immunoreactivity.

We next examined the expression of DC and KI by cultured cells of humans and rats (Figure 3). The anti-DC and anti-KI antibodies cross-reacted with the rat anti-

Figure 2. Western blotting of tissue homogenates of a human fetus at 23 GW, with 40 μg of protein loaded onto each lane. Blots of protein extracted from the cerebrum (**Lane 1**), cerebellum (**Lane 2**), pons (**Lane 3**), spinal cord (**Lane 4**), muscle (**Lane 5**), heart (**Lane 6**), lung (**Lane 7**), thymus (**Lane 8**), spleen (**Lane 9**), liver (**Lane 10**), kidney (**Lane 11**), adrenal gland (**Lane 12**), and jejunum (**Lane 13**) probed with the anti-DC1 (D) and anti-KI1 (K) antibodies. Both DC and KI were contained in the central nervous system (CNS) (**Lanes 1–4**) and were most abundant in the cerebrum (**Lane 1**). KI was also expresssed at a low level in the adrenal gland (**Lane 12**), whereas DC was restricted to the CNS (**Lanes 1–4**).

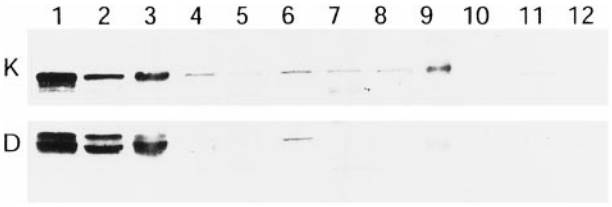

Figure 3. Western blotting of tissue homogenates and cell extracts of humans and rats. Blots of proteins of the human (**Lane 1**, 23 GW) and rat (**Lane 2**, E18) fetal cerebrum, cultured rat cerebral neurons (**Lane 3**) and astrocytes (**Lane 4**), NT2 teratocarcinoma cells before (**Lane 5**) and after (**Lane 6**) neuronal differentiation, NB1 (**Lane 7**) and TGW-III (**Lane 8**) neuroblastoma cells, I-23 (**Lane 9**) and Bu17 (**Lane 10**) glioma cells, HeLa cervical carcinoma cells (**Lane 11**), and U937 monocytic leukemia cells (**Lane 12**), probed with the anti-DC1 (D) and anti-KI1 (K) antibodies. Both DC and KI are produced by the CNS neurons in culture (**Lanes 3** and **6**). Some neuroblastoma and glioma cell lines (**Lanes 7–9**) also expressed KI, but not DC, at low levels.

gens. In tissue culture, the rat cerebral neurons and neuronally differentiated human NT2 cells contained both DC and KI. DC was undetectable in the cultures of non-neural cells, whereas a small amount of KI was present in the astrocytic and neuroblastoma cells.

The subcellular distribution of DC and KI was studied in the human fetal cerebrum (Figure 4). Both proteins were recovered in all of the fractions, but were most abundant in the mitochondrion-rich and microsome-rich fractions.

Developmental changes of DC and KI were studied in the human and rat cerebrum (Figure 5). In humans, both the proteins were present in fetuses, and were most abundant at about 20 GW. DC was undetetectable in the postnatal brains, whereas a low level of KI expression persisted until adulthood (Figure 5A). Similar changes were observed in rats. Both DC and KI were abundant in the fetal brains and decreased in amount during the postnatal period. There was a detectable amount of KI, but not of DC, in the adult brain (Figure 5B).

Immunohistochemistry

The anti-DC1, anti-DC2, anti-KI1, and anti-KI3 antibodies positively labeled the CNS of rat (Figures 6-8) and human (Figure 9) fetuses. Histologically, immunoreactivity for DC and KI was localized mainly in the perikarya of cortical neurons and in the processes in the cortical plate and intermediate zone. The staining patterns were essentially

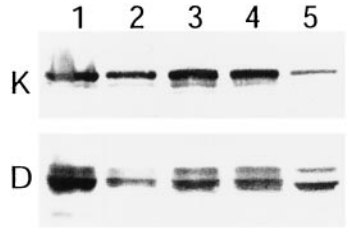

Figure 4. Results of subcellular fractionation. Blots of the cerebral homogenate of a 23-GW-old human fetus (**Lane 1**), the nucleus-rich (**Lane 2**), mitochondrion-rich (**Lane 3**), microsome-rich (**Lane 4**), and cytosolic (**Lane 5**) fraction, incubated with the anti-DC1 (D) and anti-KI1 (K) antibodies. Both DC and KI were contained in all of the fractions, but were most abundant in the mitochondrion-rich and microsome-rich fractions (**Lanes 3** and **4**).

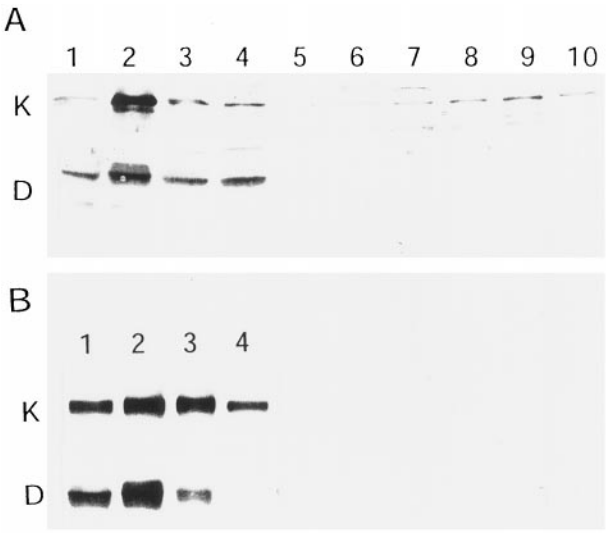

Figure 5. Developmental study in humans (**A**) and rats (**B**), using the anti-DC1 (D) and anti-KI1 (K) antibodies. **A:** Blots of human cerebral homogenates from fetuses at 12 GW (**Lane 1**), 21 GW (**Lane 2**) and 30 GW (**Lane 3**), a neonate born at 40 GW (**Lane 4**), children at 6 months (**Lane 5**), 1 year (**Lane 6**) and 9 years (**Lane 7**), and adults at 16 (**Lane 8**), 31 (**Lane 9**), and 85 years (**Lane 10**). **B:** Blots of rat cerebral homogenates from E17 (**Lane 1**) and E20 (**Lane 2**) fetuses, a P5 pup (**Lane 3**), and a P60 adult (**Lane 4**). In both human (**A**) and rat (**B**) cerebrum, DC and KI were abundant during the fetal period (**Lanes A1-A3** and **B1-B2**). The amount of both proteins decreased after birth. In the adult cerebrum, the expression of DC was undetectable, but that of KI persisted at low levels (**Lanes A4-A10** and **B3-B4**).

the same with the anti-DC1 and anti-DC2 antisera. This was also the case with the anti-KI1 and anti-KI3, although the intensity was stronger with the former antibody (Figure 7). The immunohistochemical distributions of DC and KI were similar (Figures 7–10). In negative control experiments with preimmune or preabsorbed sera, there was no positive labeling (data not shown).

Developmental changes in DC and KI expression were first studied in the rat cerebrum, because preliminary experiments revealed that the staining quality was superior in the rat compared to human specimens. The rat CNS at E11 was negative for DC and KI (data not shown), but at E14 the entire thickness of the developing neocortex was positively stained (Figure 8, A and E). The most intense immunoreactivity was localized in the cell body of the Cajal-Retzius cells, neurons that appear first in the cortical plate (Figure 9, A and E). At E17, the ventricular zone lost the immunoreactivity for DC, whereas the cortical plate and intermediate zone remained strongly positive (Figure 8B). Intensely labeled neuronal cell bodies were lined up in the innermost layer of the cortex (Figure 9B). A small number of DC-positive cell bodies were also present in the intermediate zone. Strongly immunoreactive processes ran vertically in the cortex and horizontally in the intermediate zone (Figures 8B and 9B). Immunoreactivity for KI showed a similar distribution, although a low level of labeling persisted in the ventricular zone (Figures 8F and 9F). At E20-P0, neuronal perikarya became negative for DC and KI. The immunoreactive processes broke up, resulting in a fine granular labeling that was most prominent in the subpial layer (Figures 8C, 8G, and 9C). After P5, the only DC-positive structure was a subset of astrocytes which were most numerous in the subpial layer (Figures 8D and 9D). There was no immunoreactivity for KI (Figure 8H).

We next performed a developmental study in the human brain. In the cerebrum of a 6-GW-old fetus, there was no immunoreactivity for DC and KI (data not shown). At 12 to 17 GW, the entire thickness of the cerebrum was stained strongly for DC and moderately for KI. The labeling was most intense in the cortex. These distribution patterns were reminiscent of those in rat aged E14–17, although the labeling in humans was mostly confined to the perikarya and proximal processes (Figure 10, A-D). DC and KI immunoreactivities became restricted to the cortex at 22 to 26 GW (Figure 10, C and G) and was mostly lost from the brain at 33 GW and thereafter (Figure 10, D and H), except for scattered astrocytes that were moderately positive for DC (data not shown).

Discussion

Several previous studies have examined the spatial and temporal patterns of DC and KI expression at the mRNA

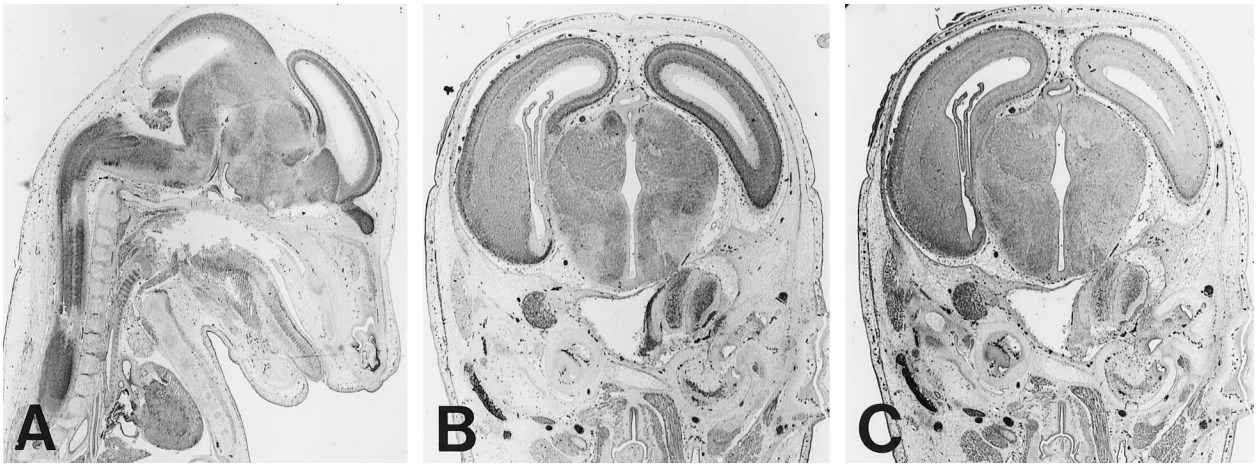

Figure 6. Localization of DC and KI immunoreactivities in the rat central nervous system. Sagittal (**A**) and coronal sections (**B, C**) of an E17 fetus immunostained with the anti-DC2 (**A, B**) and anti-KI1 (**C**) antibodies. Original magnifications, ×6 (**A**) and ×10 (**B, C**).

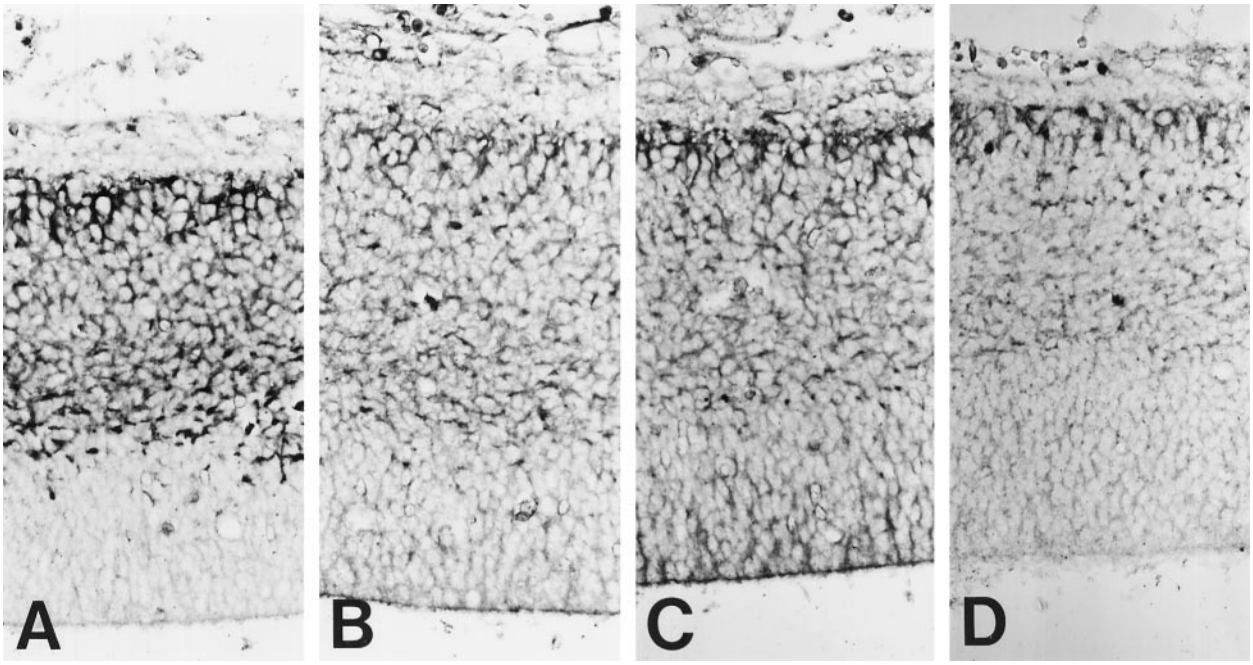

Figure 7. Results of immunostaining using different antibodies. The subicular cortex of an E17 rat probed with the anti-DC1 (**A**), anti-DC2 (**B**), anti-KI1 (**C**), and anti-KI3 (**D**) antisera. The cerebral surface is shown at the top, and the ventricular epithelium at the bottom. The perikarya of cortical neurons and the processes in the cortical and intermediate zones showed immunoreactivities for DC (**A** and **B**) and KI (**C** and **D**). The staining patterns were similar for two different anti-DC (**A** and **B**) as well as two different anti-KI (**C** and **D**) antisera, but the intensity was stronger with anti-KI1 (**C**) than anti-KI3 (**D**). In the cortical and intermediate zones, the distribution of DC and KI was similar, whereas the ventricular zone was positive for KI (**C** and **D**) but not for DC (**A** and **B**). Original magnification, ×250.

level, demonstrating that both transcripts are highly expressed in the fetal brain. Northern blot analyses of human fetal tissues showed that DC mRNA is expressed exclusively in the brain.[14,15] An *in situ* hybridization study of the cerebrum of a 21-GW-old human fetus detected strong labeling by a DC antisense probe in the cortical plate and ventricular zone, and moderate labeling in the intermediate zone.[14] The production of DC mRNA by cultured mouse fetal cerebral neurons, but not by glial cells, was detected by a reverse transcription-polymerase chain reaction (RT-PCR) analysis, suggesting a neuronal localization of DC.[14] DC mRNA is abundant in the cerebrum of a 20-GW-old human fetus, but is not detectable in an adult brain.[14,15,17] A similar developmental change has been observed in mouse, where the expression of DC mRNA is maximal at E11, is down-regulated thereafter, and reaches an undetectable level by adulthood.[14,15,17]

KI mRNA is also abundant in the fetal brain. Unlike DC, the KI transcript is expressed at a low level in the adult brain.[14,15,17] In a Northern blot analysis of adult human tissues, the expression of the KI mRNA was confined to the nervous system.[17] The cell type-specific localization of KI has not been studied.

The present study further extended our knowledge about the developmental expression pattern of DC and KI by investigating their distibution at the protein level. We identified the DC and KI proteins for the first time by means of Western blotting using multiple antibodies that recognize different epitopes. The molecular weights of DC and KI were estimated to be 45 and 80 kd, respectively, values close to those which were expected based on the results of cDNA cloning.[14–16] DC and KI were recognized on Western blots as doublet proteins, which may have resulted from alternative splicing[14,15,17] or posttranslational modification. The reported splicing of the DC gene, however, does not affect the predicted open reading frame,[14] and a Northern blot analysis detected only one message size.[15] Studies are underway in our laboratory to reveal whether DC and KI undergo phosphorylation and other posttranslational modifications by producing additional antibodies, including those raised against phosphorylated and non-phosphorylated peptides corresponding to their potential phosphorylation sites.

Regarding the temporal pattern of expression, our Western blotting data on the DC and KI proteins showed patterns essentially identical to those reported previously for their mRNAs.[14,15,17] These proteins resembled each other in that they were most abundant during the period of neuronal migration. The immunohistochemical distributions of DC and KI were also similar. In the early phase of neuronal dispersion (E14 in rats and 12–17 GW in humans), a robust expression of these proteins was noted in all of the zones: ventricular, intermediate, and cortical. During the subsequent stages (E17–20 in rats and 22–26 GW in humans), their immunoreactivities gradually became confined to the superficial layers. The strong DC and KI immunoreactivities of certain subsets of rat cortical neurons, such as the Cajal-Retzius cells at E14 and neurons in the innermost layer at E17, suggest a pivotal role of these cells in regulating cortical lamination. After the main surge of migrating neurons had arrested (after P5 in rats and after 33 GW in humans), the two proteins

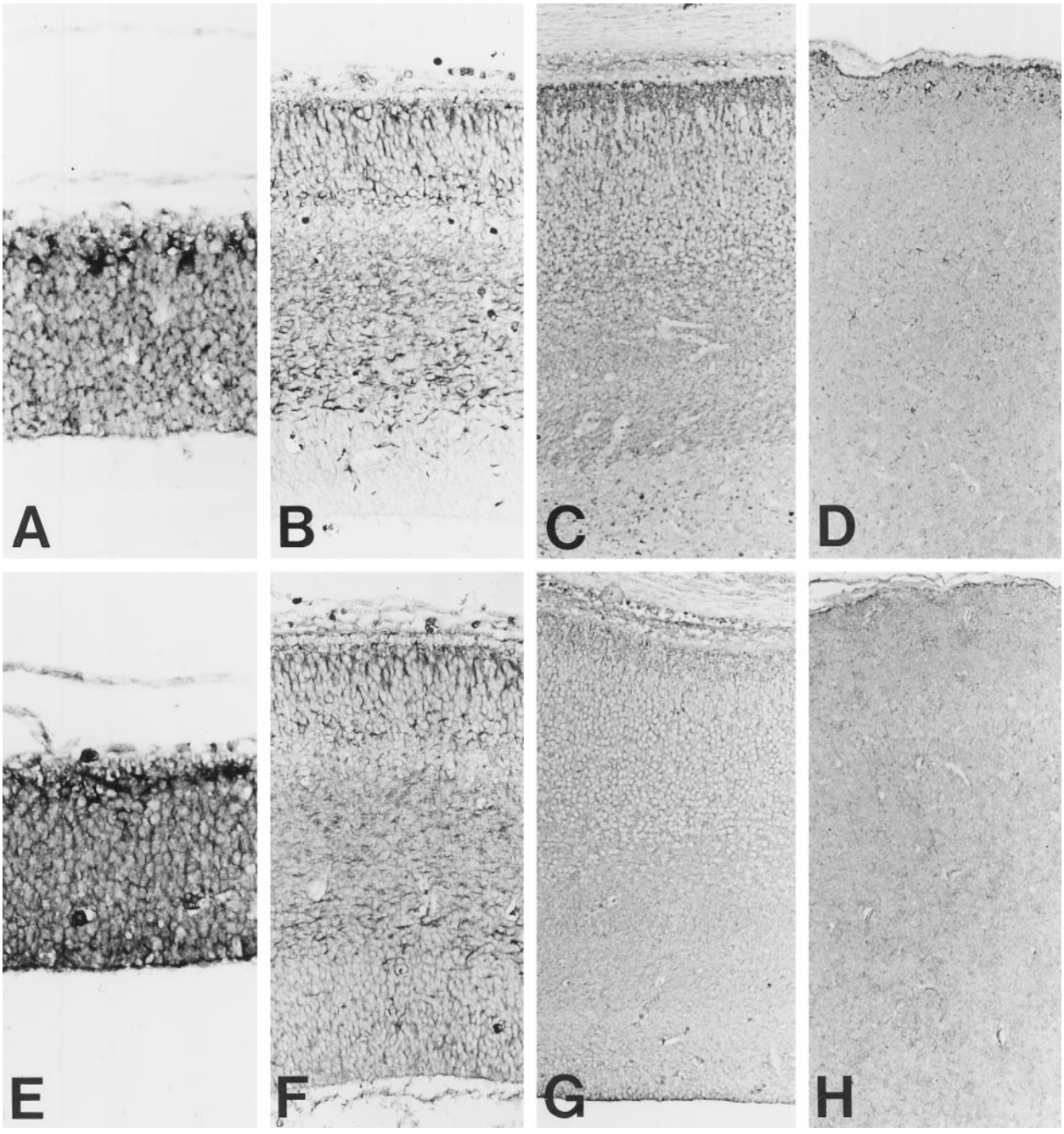

Figure 8. Development of DC and KI immunoreactivity in the rat cerebrum, low magnification views. Sections of the neocortex at E14 (**A** and **E**), E17 (**B** and **F**), E20 (**C** and **G**), and P10 (**D** and **H**) immunostained with the anti-DC2 (**A-D**) and anti-KI1 (**E-H**) antibodies. At E14, intense immunoreactivities for DC (**A**) and KI (**E**) were present in both the cortical plate and ventricular zone. The most intense labeling was localized in the cortical neurons. At E17, strong immunoreactivity for DC (**B**) was localized in the cortical plate and intermediate zone. Positively labeled cellular processes ran vertically in the cortex and horizontally in the intermediate zone. The ventricular zone was negative for DC. Immunoreactivity for KI (**F**) showed a similar distribution, but weak labeling was present in the ventricular zone. At E20, labeling was mostly restricted to the subpial layer, and was moderate for DC (**C**) and weak for KI (**G**). In the rest of the cortex, staining was minimal. At P10, no immunoreactivity was noted for DC (**D**) or KI (**H**), except for scattered DC-positive astrocytes. Original magnifications, ×250 (**A** and **E**), ×165 (**B** and **F**), ×100 (**C, D, G, H**).

became hardly detectable on immunohistochemical sections. Thus, DC and KI tended to colocalize in the zones of ongoing neuronal migration, suggesting that they participate in the same signaling pathway regulating the entirety of this developmental process.

The immunohistochemical findings were somewhat different in the rat and human cerebrum. In rats, many processes distant from the cell bodies were positive for DC and KI (Figure 9, B and F), whereas in humans, labeling tended to be restricted to the perisomal area (Figure 10, A and E). This difference may have resulted from agonal and postmortem changes to which human specimens are liable. We also noted several minor discrepancies between the Western blotting and immunostaining results. First, immunohistochemistry failed to detect the KI protein in the mature cerebrum (Figure 8H),

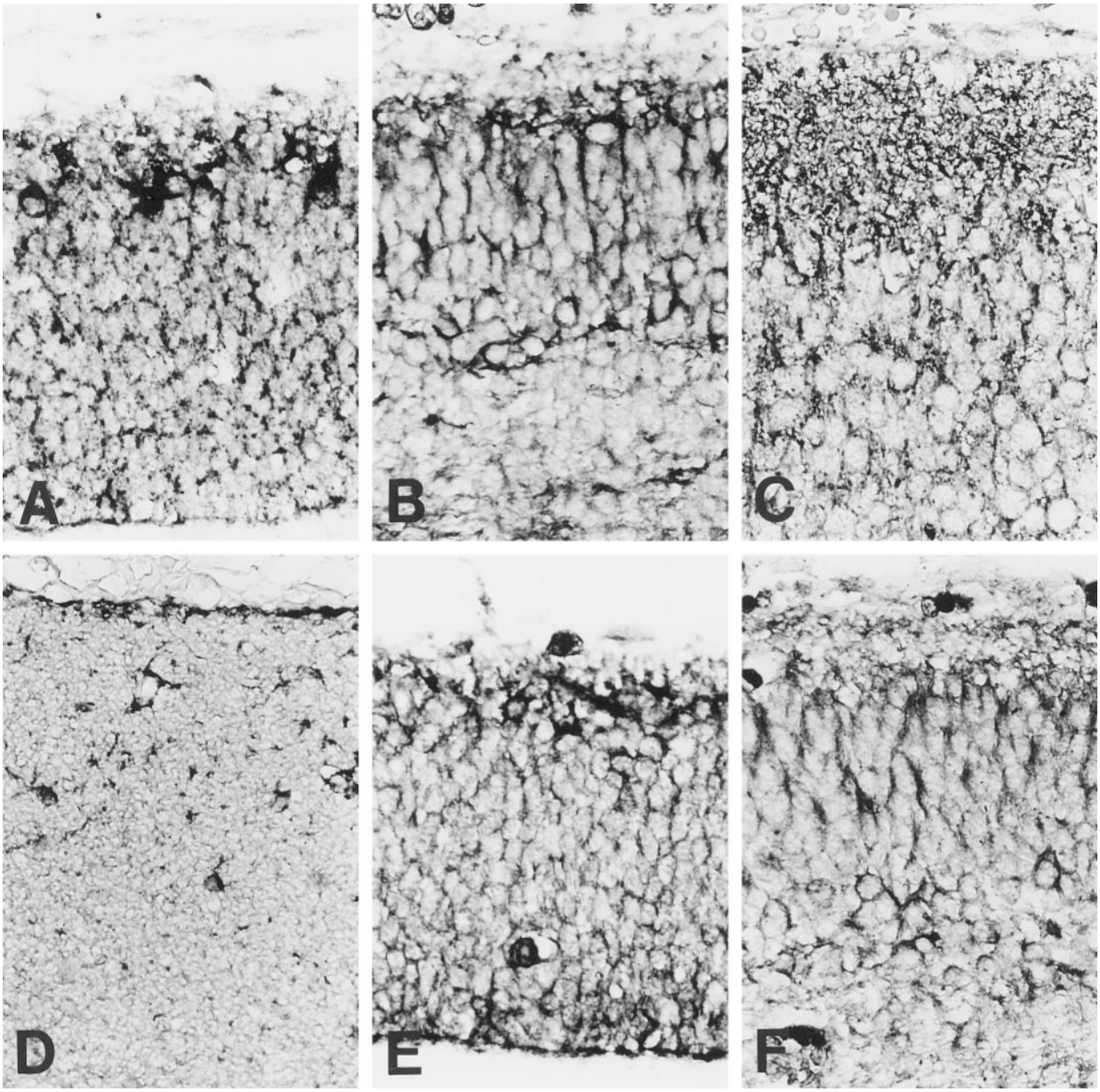

Figure 9. Development of DC and KI immunoreactivity in the rat cerebral cortex, high magnification views. Sections of the neocortex at E14 (**A** and **E**), E17 (**B** and **F**), E20 (**C**) and P10 (**D**) immunostained with the anti-DC2 (**A-D**) and anti-KI1 (**E** and **F**) antibodies. At E14, cell bodies and processes of the Cajal-Retzius cells were intensely immunostained for DC (**A**) and KI (**E**). At E17, neurons in the innermost zone of the cortex showed strong immunoreactivities for DC (**B**) and KI (**F**) in their perikarya and apical dendrites. Vertical processes were also labeled intensely. At E20, the subpial layer was positively labeled for DC in a fine granular fashion (**C**). There were no intensely labeled cell bodies. At P10, DC-positive astrocytes were most numerous in the subpial layer (**D**). Original magnification, ×500 (**A-F**).

where it was shown by Western blotting to remain in a small amount (Figure 5). Second, Western blotting detected DC and KI in the nucleus-rich fraction (Figure 4), whereas the nuclei were unlabeled on the immunohistochemical sections (Figures 8–10). These discrepancies are probably due to the different sensitivities of the two methods, although the second may have resulted from impurity of the subcellular fraction. Third, a subset of astrocytes disclosed a DC-like immunoreactivity on the immunohistological sections of the cerebrum at the postmigratory stages (Figure 9D), which was not detected by the Western blotting (Figure 5). Although the astrocytic immunoreactivity could have resulted from a cross-reactivity with a different antigen, it appears worth examining the possibility that some astrocytes can indeed express DC.

It is interesting that KI showed a somewhat wider distribution on Western blots compared to DC, both temporally and spatially. KI remained present in the brain until adulthood, whereas DC became undetectable during early postnatal development (Figure 5). During the fetal period, DC was expressed exclusively in the CNS, whereas KI was also present in the adrenal gland (Figure 2). In tissue culture, the expression of DC was confined to CNS neurons, whereas KI was produced also by astrocytic and neuroblastoma cells (Figure 3). The presence

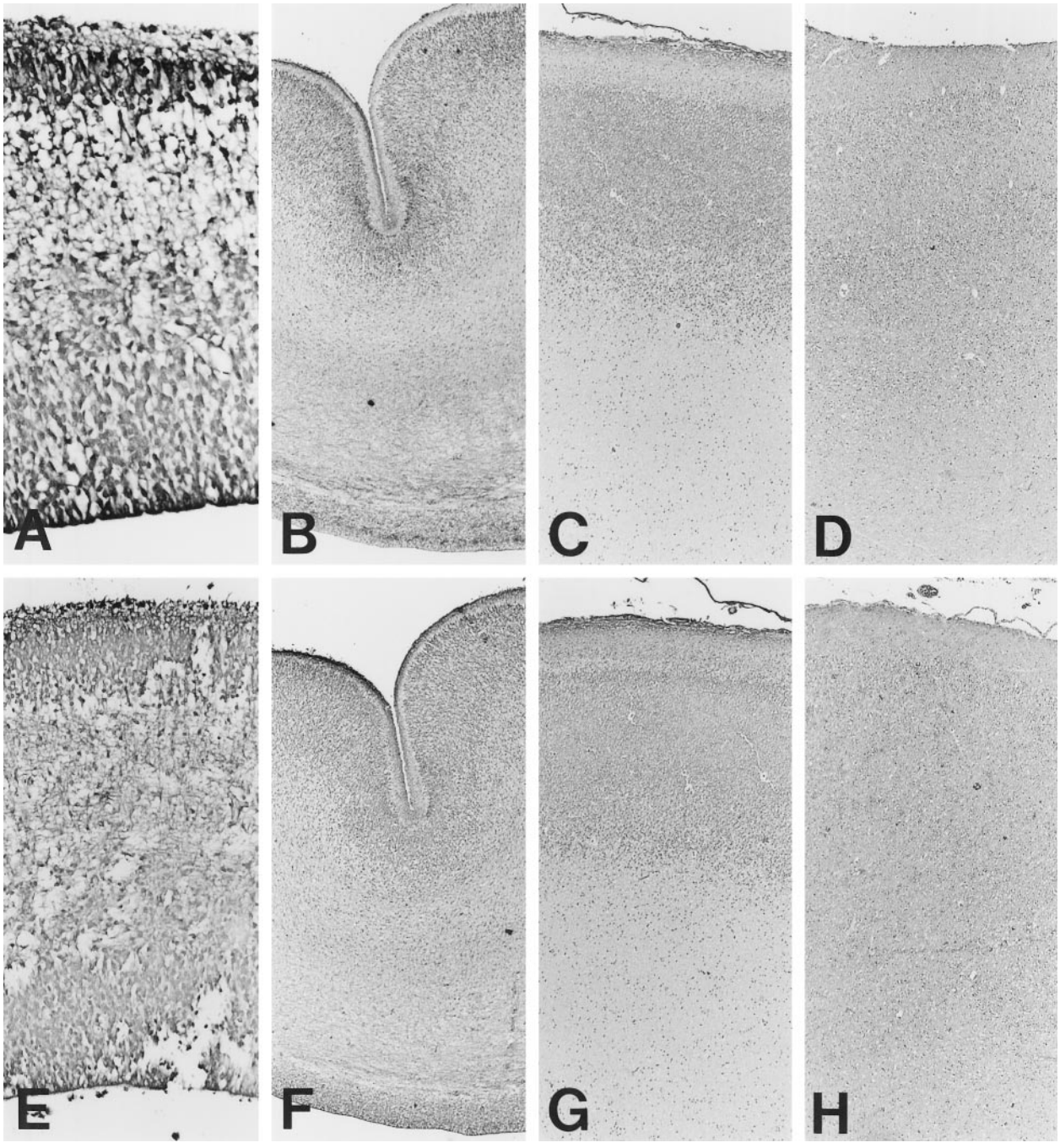

Figure 10. Development of DC and KI immunoreactivity in the human cerebrum. Sections of the frontal cortex at 12 GW (**A** and **E**), 17 GW (**B** and **F**), 26 GW (**C** and **G**) and 33 GW (**D** and **H**) immunostained with the anti-DC2 (**A-D**) and anti-KI1 (**E-H**) antibodies. At 12–17 GW, there were diffuse immunoreactivities that were strong for DC (**A** and **B**) and moderate for KI (**E** and **F**). At 26 GW, immunoreactivities for DC and KI were confined to the cortex (**C** and **G**), and at 33 GW, no immunoreactivities were noted (**D** and **H**). Original magnifications, ×200 (**A** and **E**), ×33 (**B-D** and **F-H**).

of small amounts of KI in these cells suggests that KI is involved in their modest motility or in their nonmigratory activities during the postnatal period. By contrast, the function of DC appears to be specific to the migration of fetal neurons.

The mechanism by which DC and KI regulate neuronal migration will be the main subject for future studies. An attractive theory is that DC regulates the function of KI via a competition for binding to an effector protein, or via a modulation of the kinase activity of KI.[14,15] The identification of this effector would be difficult, but PAFAH1B1 and other molecules involved in neuronal migration disorders could be candidates. A clue to this issue may be obtained by studying the subcellular localization of DC and KI and correlating the findings with those of the candidate proteins. It also appears important that DC is a potential substrate for Abl and a possible substrate for members of the mitogen-activated protein kinase family.[14,15] To elucidate the precise roles of DC and KI in CNS development, it may be worth performing two lines of

experiments: analysis of their Abl-dependent phophorylation and immunoelectron microscopic observations.

Acknowledgments

We thank Kazu Iwasawa for technical assistance.

References

1. Sidman RL, Rakic P: Neuronal migration, with special reference to developing human brain: a review. Brain Res 1973, 62:1–35
2. Bayer SA, Altman J, Russo RJ, Zhang X: Embryology: Pediatric Neuropathology. Edited by Dukett S. Baltimore, Williams and Wilkins, 1995, pp 54–107
3. Jellinger K, Rett A: Agyria-pachygyria (lissencephaly syndrome). Neuropadiatrie 1976, 7:66–91
4. Houdou S, Kuruta H, Konomi H, Takashima S: Structure in lissencephaly determined by immunohistochemical staining. Pediatr Neurol 1990, 6:402–406
5. Reiner O, Carrozzo R, Shen Y, Wehnert M, Faustinella F, Dobyns WB, Caskey CT, Ledbetter DH: Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. Nature 1993, 364: 717–721
6. Chong SS, Pack SD, Roschke AV, Tanigami A, Carrozzo R, Smith ACM, Dobyns WB, Ledbetter DH: A revision of the lissencephaly and Miller-Dieker syndrome critical regions in chromosome 17p13.3. Hum Mol Genet 1997, 6:147–155
7. Hattori M, Adachi H, Tsujimoto M, Arari H, Inoue K: Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor acetylhydrolase. Nature 1994, 370:216–218
8. Mizuguchi M, Takashima S, Kakita A, Yamada M, Ikeda K: Lissencephaly gene product: localization in the central nervous system and loss of immunoreactivity in Miller-Dieker syndrome. Am J Pathol 1995, 147:1142–1151
9. Clark GC, Mizuguchi M, Antalffy B, Barnes J, Armstrong D: Predominant localization of the LIS family of gene products to Cajal-Retzius cells and ventricular neoepithelium in the developing human cortex. J Neuropathol Exp Neurol 1997, 56:1044–1052
10. Ross ME, Allen KM, Srivastava AK, Featherstone T, Gleeson JG, Hirsch B, Harding BN, Andermann E, Abdullah R, Berg M, Czapansky-Bielman D, Flanders DJ, Guerrini R, Motte J, Mira AP, Scheffer I, Berkovic S, Scaravilli F, King RA, Ledbetter DH, Schlessinger D, Dobyns WB, Walsh CA: Linkage and physical mapping of X-linked lissencephaly/SBH (XLIS): a gene causing neuronal migration defects in human brain. Hum Mol Genet 1997, 6:555–562
11. Pilz DT, Matsumoto N, Minnerath S, Mills P, Gleeson JG, Allen KM, Walsh CA, Barkovich AJ, Dobyns WB, Ledbetter DH, Ross ME: LIS1, and XLIS (DCX) mutations cause most classical lissencephaly, but different patterns of malformation. Hum Mol Genet 1998, 7:2029–2037
12. Berg MJ, Schifitto G, Powers JM, Martinez-Capolino C, Fong C-T, Myers GJ, Epstein LG, Walsh CA: X-linked female band heterotopia-male lissencephaly syndrome. Neurology 1998, 50:1143–1146
13. Dobyns W, Andermann E, Andermann F, Czpansky-Beilman D, Dubeau F, Dulac O, Gerrini R, Hirsch B, Ledbetter D, Lee N, Motte J, Pinard J-M, Radtke RA, Ross ME, Tampieri D, Walsh CA, Truwit CL: X-linked malformations of neuronal migration. Neurology 1996, 47: 331–339
14. des Portes V, Pinard JM, Billuart P, Vinet MC, Koulakoff A, Carrie A, Gelot A, Dupuis E, Motte J, Bernard-Netter Y, Catala M, Kahn A, Beldjord C, Chelly J: A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. Cell 1998, 92:51–61
15. Gleeson JG, Allen KM, Fox JM, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA: Doublecorin, a brain-specific gene mutated in human X-linked lissencephaly, and double cortex syndrome, encodes a putative signal protein. Cell 1998, 92:63–72
16. Nagase T, Ishikawa K, Nakajima D, Ohira M, Seki N, Miyajima N, Tanaka A, Kotani H, Nomura N, Ohara O: Predilection of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res 1997, 4:141–150
17. Omori Y, Suzuki M, Ozaki K, Harada Y, Nakamura Y, Takahashi E-I, Fujiwara T: Expression and chromosomal localization of KIAA0369, a putative kinase structurally related to Doublecortin. J Hum Genet 1998, 43:169–177
18. Sossey-Aaoui K, Hartung AJ, Guerrini R, Manchester DK, Posar A, Puche-Mira A, Andermann E, Dobyns WB, Srivastava AK: Human doublecortin (DCX), and the homologous gene in mouse encode a putative Ca2+-dependent signaling protein which is mutated in human X-linked neuronal migration defects. Hum Mol Genet 1998, 7:1327–1332
19. Mizuguchi M, Yamada M, Kim SU, Rhee SG: Phospholipase C isozymes in neurons, and glial cells in culture: an immunocytochemical and immunochemical study. Brain Res 1991, 548:35–40
20. Pleasure SJ, Page C, Lee VMY: Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. J Neurosci 1992, 12:1802–1815
21. Mizuguchi M, Ikeda K, Asada M, Mizutani S, Kamoshita S: Expression of Bcl-2 protein in murine neural cells in culture. Brain Res 1994, 649:197–202
22. Mizuguchi M, Kato M, Yamanouchi H, Ikeda K, Takashima S: Tuberin immunohistochemistry in brain, kidneys and heart with or without tuberous sclerosis. Acta Neuropathol 1997, 94:525–531