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Biochemical Characterization and Expression Analysis of Neural Thrombospondin-1-like Proteins NELL1 and NELL2[1]

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Two closely related genes coding for NELL proteins (NELL1 and NELL2) have been cloned by the yeast two-hybrid screening of a rat brain cDNA library with the regulatory domain of protein kinase C βI (PKCβI) as bait. The rat NELL proteins show about 55% identity with each other and contain several protein motifs assigned to a secretion signal peptide, an NH_2-terminal thrombospondin-1 (TSP-1)-like module, five von Willebrand factor C domains, and six epidermal growth factor-like domains; the NELL proteins share many protein motifs with TSP-1. The NELL proteins expressed in COS-7 cells are homotrimeric glycoproteins and possess heparin-binding activity. Furthermore, while NELL1 and NELL2 show distinct subcellular localization in cytoplasm, they both are partially secreted into the culture medium of COS-7 cells. Although the *NELL1* mRNA is faintly expressed in adult neural cells, the *NELL2* mRNA is expressed abundantly, particularly in the pyramidal cells of rat hippocampus, showing neuronal high plasticity. During mouse embryogenesis, expression of the *NELL2* mRNA is initiated 7–11 days postcoitum, simultaneously with neural plate formation. These results strongly suggest that the NELL2 protein, similar to but not identical with TSP-1, is involved in the growth and differentiation of neural cells. Additionally, the *NELL1* and *NELL2* mRNAs were found to be expressed abundantly in Burkitt's lymphoma Raji cells and colorectal adenocarcinoma SW480 cells, respectively. Thus, it is likely that the NELL proteins also participate in the growth, differentiation, and oncogenesis of cancer cell lines.

Not less than 100 eukaryotic proteins have so far been found to contain epidermal growth factor (EGF)-like domains, which are composed of 40–50 amino acid residues including 6 conserved Cys residues. These proteins mainly occur in the extracellular fractions (as membrane-associated or secreted proteins) and are related to cell proliferation, growth inhibition, and differentiation (1). It has been revealed that the EGF-like domains participate in the Ca^{2+}-dependent protein-protein interactions; however, target proteins for those domains remain to be identified. In 1995, Matsuhashi *et al.* (2, 3) cloned the gene encoding a novel protein containing 6 EGF-like domains from a chick embryo-derived cDNA library and designated the coded protein as Nel (a protein strongly expressed in neural tissues and containing EGF-like domains). Expression of *Nel* mRNA is initiated after the middle stage (10–17 days old) in all tissues of chick embryos and regulated spatiotemporarily during embryogenesis, but is retained only in neural tissues after hatching. On the other hand, as a part of the Human Genome Project, Watanabe *et al.* (4) cloned a human *Nel* gene (designated

Abbreviations used: EGF, epidermal growth factor; PKC, protein kinase C; TSP, thrombospondin; TGFβ1, transforming growth factor β1; RACE, rapid amplification of cDNA ends; WWW, world wide web; PBS, phosphate buffered saline; NELL-FLAG, COOH-terminally FLAG-tagged NELL; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); TSP-N, NH_2-terminal TSP-like; vWF, von Willebrand factor; knt, kilo nucleotide(s); p.c., postcoitum.

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NELL2) and its homologue (designated *NELL1*).[3] *NELL2* and *NELL1* genes are expressed in human neural tissues and mapped by fluorescence *in situ* hybridization (FISH) to human chromosomal bands 12q13.11–q13.12 and 11p15.1–p15.2, respectively (4). The neural tissue-specific expression of *NELL* mRNAs has thus led to the speculation that the NELL proteins are involved in the growth and differentiation of neural cells. However, it remains unclear how NELL proteins manifest these presumed functions in the neural tissues, because of the lack of their biochemical and molecular analyses.

In this study, we have isolated two *NELL* genes from a rat brain cDNA library by the yeast two-hybrid screening with the regulatory domain of protein kinase C βI (PKCβI) (5, 6) as bait. We here report biochemical properties of NELL proteins, emphasizing the similarity with thrombospondin-1 (TSP-1) (7), and discuss possible roles of NELL proteins in the neural tissues.

MATERIALS AND METHODS

Yeast two-hybrid screening and sequence analysis. As described previously (6), a rat brain cDNA library (Clontech) was screened by the two-hybrid assay using the regulatory domain of rat PKCβI (residues 1–340) as a bait. The cDNA fragments obtained were analyzed with an automated DNA sequencer model 373S (PE Biosystems). Full-length cDNA clones for *NELL1* and *NELL2* genes were obtained by the RACE (rapid amplification of cDNA ends) method.

Immunoprecipitation. For expression of the COOH-terminally FLAG-tagged proteins, a pTB701-FLC plasmid was first constructed by inserting the FLAG epitope sequence under the SV40 early promoter of pTB701 (6). Plasmids pTB701-FLC-NELL1 and pTB701-FLC-NELL2 were constructed by inserting the *NELL1* and *NELL2* cDNA fragments into pTB701-FLC, respectively. COS-7 cells transfected with each plasmid were cultured for 60–72 h. COS-7 cells from a 10-cm plate (about 5×10^7 cells) were suspended in 500 μl of lysis buffer (6). Cleared lysates (500 μl) were incubated on ice for 1 h with 2 μg of an anti-FLAG monoclonal antibody (M2, Eastman Kodak), and then mixed with 20 μl of protein G Sepharose 4 fast flow beads (50% slurry, Pharmacia). After incubation at 4°C for 1 h with shaking, the beads were washed with lysis buffer, and then analyzed by Western blotting.

In vivo protein labeling with [^{35}S]Met. COS-7 cells (about 5×10^7 cells) expressing either NELL1-FLAG or NELL2-FLAG were incubated for 5–6 h in 5 ml of a Met-free DMEM medium (Life Technologies) supplemented with 10% (v/v) dialyzed FBS (Life Technologies) and 0.1 mCi/ml of [^{35}S]Met (>1000 Ci/mmol, Amersham). Secreted NELL-FLAG proteins were immunoprecipitated as described above, subjected to SDS–PAGE, and then fluorographed.

Heparin-Sepharose binding assay. COS-7 cells (about 5×10^7 cells) expressing either NELL1-FLAG or NELL2-FLAG were lysed using 500 μl of the lysis buffer without NaCl. Cleared lysates (500 μl) were incubated on ice for 1 h with 50 μl of heparin-Sepharose resins (50% slurry, Pharmacia). After washing the resins four times with the lysis buffer without NaCl, proteins were eluted successively with the buffer containing 250, 500, 750, and 1000 mM NaCl and subjected to the immunoprecipitation as described above.

Northern blot analysis. Northern blots containing about 2 μg of poly(A)$^+$ RNA per lane were obtained from Clontech. The amount of poly(A)$^+$ RNA in each lane was calibrated using the rat β-actin gene. The sources of poly(A)$^+$ RNA were as follows: eight adult rat tissues, eight human cancer cell lines, and mouse embryos of four different developmental stages (see Fig. 4). The full-length cDNA fragments of *NELL1* and *NELL2* genes labeled with [α-^{32}P]dCTP (>7000 Ci/mmol) were used as probes. Hybridization was carried out under highly stringent conditions. The blots were autoradiographed by using a Bioimage Analyzing System BAS-2000 (Fuji).

In situ hybridization analysis. Brains were removed from the anesthetized rats and rapidly frozen by dry ice. The cryostat sections (20 μm thick) were cut and mounted on poly-Lys coated slides. The sections were rinsed in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) at room temperature for 10 min, then dehydrated by rinsing with graded mixtures of ethanol and chloroform. The air-dried sections were prehybridized at 37°C for 3 h in 200 μl of hybridization buffer [10 mM Tris, pH 7.4, 40% (v/v) formamide, 2 × SSC (0.3 M NaCl, 30 mM sodium citrate), 1× Denhardt's solution (0.02% (w/v) Ficoll-400, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin), 20 μg/ml heat-denatured salmon sperm DNA, 10% (w/v) dextran sulfate, and 250 μg/ml yeast tRNA]. Oligonucleotide probes for *NELL1* mRNA (5′-GTTATTCTCAATACACTCCAGATCCACAGAGCAGCAGACTC-TCCC-3′) and *NELL2* mRNA (5′-CATTGGCACAGCTGTGCCTCCCAGTCCCACATTCA-TCAATATCTT-3′) were complementary to nucleotides 2444–2488 of *NELL1* gene (GenBank U48246) and to nucleotides 1971–2015 of *NELL2* gene (GenBank U48245), respectively. The oligonucleotides were 5′-labeled with [γ-^{32}P]ATP (>7000 Ci/mmol). Each section was hybridized in 200 μl of hybridization buffer containing the ^{32}P-labeled probe (about 1.0×10^6 cpm/ml) at 50°C for 18 h in a humid chamber. The sections were washed four times in 1 × SSC containing 20% (v/v) formamide at 52°C for 15 min. After dipping into 70% (v/v) ethanol, the sections were air-dried and the localization of the bound probe was visualized by autoradiography.

In situ immunocytochemical analysis. The COOH-terminal regions of NELL1 (CSVDLECIENN) and NELL2 (CSVDPQCLQEL) were selected as the specific sequences for raising antisera in female Japanese white rabbits (2.5–3.5 kg). These synthetic oligopeptides were coupled to keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (Wako) and used as antigens. The oligopeptides were also reacted with the activated HiTrap NH resins (Pharmacia) according to the manufacturer's procedure. Each antiserum was applied on the corresponding oligopeptide-immobilized column and washed with phosphate buffered saline (PBS). The specific antibody was eluted from the column with 0.2 M glycine buffer (pH 2.5). Rat brains were fixed by perfusion with 0.5% (v/v) glutaraldehyde, 4% (w/v) paraformaldehyde, and 0.2% (w/v) picric acid in 0.1 M phosphate buffer (PB), pH 7.4. After post-fixation with 4% (w/v) paraformaldehyde and 0.2% (w/v) picric acid in 0.1 M PB, brains were washed in 30% (w/v) sucrose in 0.1 M PB and cut on a cryostat. The cryostat sections (20 μm thick) of the hippocampus were stained with a Vecstatin Avidin Biotin Complex kit (Vector) or by indirect fluorescent immunocytochemistry using the purified antibodies in 1:4000 dilution for anti-NELL1 antibody and 1:1000 dilution for anti-NELL2 antibody.

RESULTS AND DISCUSSION

Cloning of Two Rat Thrombospondin-1-like NELL Genes

By the yeast two-hybrid method using the regulatory domain of PKCβI as bait (6), we have isolated two

[3] *Nel* gene was originally found in chicken by Matsuhashi *et al.* (2), in which the nucleotide sequence contained frame-shift errors. Before correcting the errors (3), Watanabe *et al.* (4) identified two human *Nel* genes and reported them as *Nel*-like genes (*NELL1*, *NELL2*). Based on the sequence similarity, chicken Nel and human NELL2 proteins were concluded to be homologues. In this paper, the nomenclature provided by Watanabe *et al.* (4) is used.

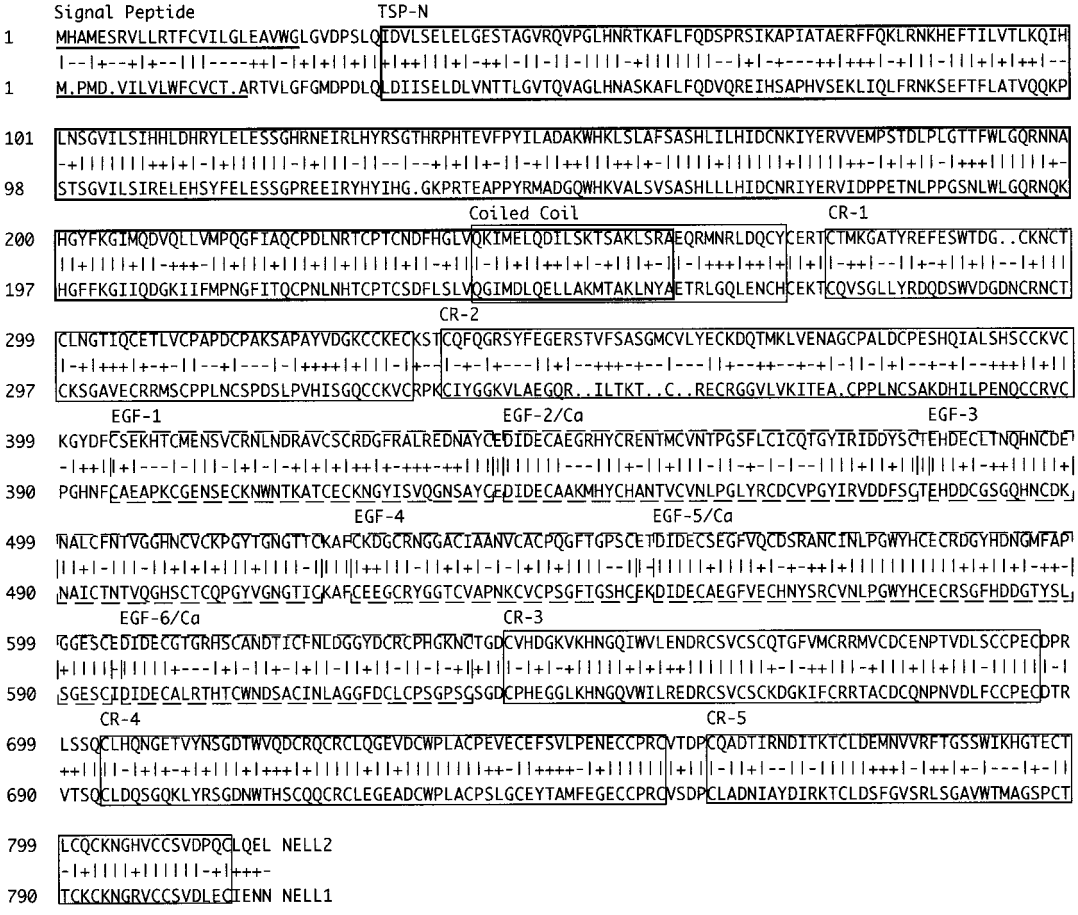

FIG. 1. Sequence alignment of rat NELL2 and NELL1 proteins. Sequence comparison was carried out by the BLAST protocol using a BLOSUM62 amino acid substitution matrix against the non-redundant protein sequence database available at the Genome Net World Wide Web (WWW) site (http://www.genome.ad.jp). Coiled-coil structure was predicted by a software available at the Swiss Institute for Experimental Cancer Research WWW site (http://ulrec3.unil.ch/software/COILS_form.html). Protein motifs were searched in the BLOCKS database accessible at the Fred Hutchinson Cancer Research Center WWW site (http://www.blocks.fhcrc.org/blocks_search.html). Secretion signal was predicted by the PSORT II program at the WWW site of the National Institute for Basic Research (http://psort.nibb.ac.jp/). Gaps (indicated by dots) are introduced to optimize the alignment. Identical and conservatively replaced residues are indicated by vertical bars and plus marks, respectively, and the other residues by minus signs. Potential signal peptide sequences are underlined. The regions predicted to be known protein motifs are *boxed* (*TSP-N*, NH_2-terminal TSP-like module; Coiled Coil, coiled-coil region; *CR*, *v*WF C domain; *EGF*, EGF-like domain; EGF/Ca, Ca^{2+}-binding type EGF-like domain). Amino acid residues are numbered on the left margin.

clones from a rat brain-derived cDNA library. These sequences are different from each other but have the same protein motifs (Fig. 1), i.e., a secretion signal peptide, an NH_2-terminal TSP-like (TSP-N) module (8), a coiled-coil region, five von Willebrand factor (vWF) C domains (9, 10), and three Ca^{2+}-binding and three non-Ca^{2+}-binding EGF-like domains (1, 11, 12). Since one 2618-bp clone (deposited in GenBank, U48245) encoded a polypeptide of 819 amino acid residues with a calculated M_r of 91,402, which was highly homologous with chicken Nel protein (2, 3) (identity, >80%), human NELL2 protein (4) (identity, about 90%), and mouse NELL2 protein (GenBank, U59230) (identity, >90%), the coded protein was identified as a rat homologue of NELL2. Another 2915-bp clone (GenBank, U48246) encoded a polypeptide of 810 residues with a calculated M_r of 89,211, which was highly homologous with human NELL1 protein (4) (identity, about 90%), and hence the coded protein was annotated as a rat homologue of NELL1. Sequence identities between NELL1 and NELL2 proteins are rather low (rat NELL1 vs rat NELL2, about 55%; rat NELL1 vs chicken Nel, about 40%; rat NELL1 vs human/mouse NELL2, about 40%), suggesting that these NELL proteins have diverged in an early stage of molecular evolution.

Heterologous Expression of NELL Proteins in COS-7 Cells

To clarify the biochemical properties of NELL proteins, the NH_2-terminally FLAG-tagged *NELL* pro-

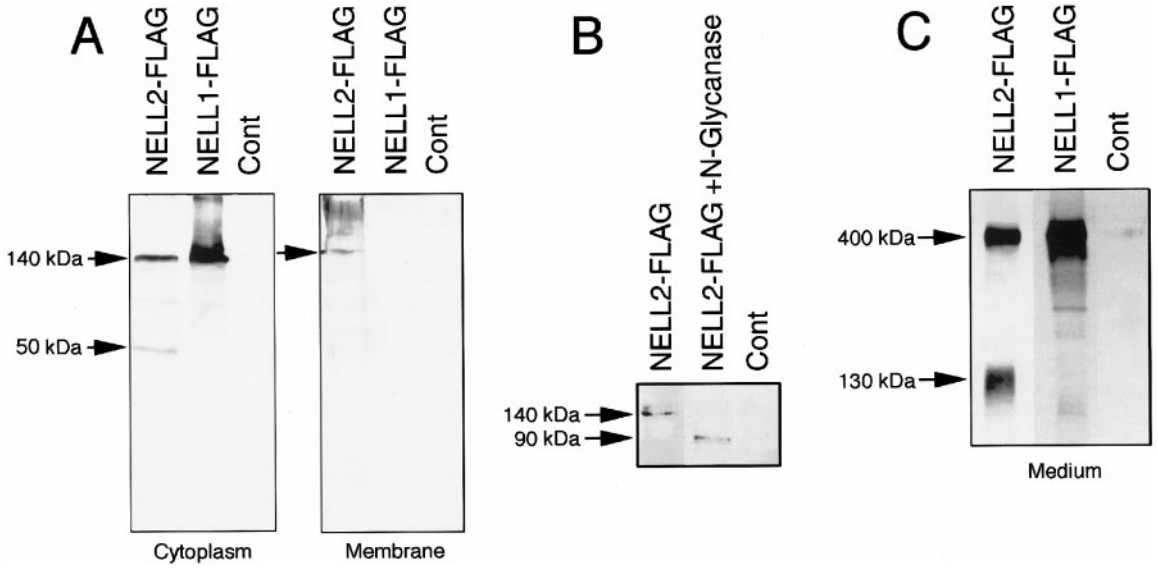

FIG. 2. Expression of NELL proteins in COS-7 cells. (A) Subcellular localization of NELL-FLAG proteins in COS-7 cells. The lysates of COS-7 cells expressing either *NELL2-FLAG* or *NELL1-FLAG* protein were separated into the cytoplasmic and membrane fractions, and analyzed by Western blotting with an anti-FLAG antibody. As a control (Cont), untransfected COS-7 cells were used. Each lane contained the sample derived from about 5.0×10^5 cells. (B) *N*-glycanase treatment of NELL2-FLAG protein. The lysate of COS-7 cells expressing NELL-2-FLAG protein was immunoprecipitated with an anti-FLAG antibody. The precipitates were treated with *N*-glycanase (Genzyme) according to the manufacturer's protocol, and then analyzed by Western blotting with an anti-FLAG antibody. (*C*) *In vivo* labeling of secreted NELL-FLAG proteins. COS-7 cells expressing either *NELL2-FLAG* or *NELL1-FLAG* protein were *in vivo* labeled with [^{35}S]Met for 5–6 h. Secreted NELL-FLAG proteins were immunoprecipitated with anti-FLAG antibody, applied onto SDS–PAGE, and then fluorographed. As a control (Cont), untransfected COS-7 cells were used. The positions of NELL-FLAG proteins are indicated by arrows with their approximate molecular masses (kDa).

teins have been heterologously expressed in COS-7 cells. By Western blotting using anti-FLAG antibody, NELL proteins were detected neither in the cytoplasmic and membrane fractions nor in the culture medium (data not shown). This was thought to be due to the loss of the FLAG epitope moiety presumably by suffering proteolytic cleavage at the NH_2-terminal signal peptide (see Fig. 1, and vide infra). Therefore, we then examined the expression in COS-7 cells of the COOH-terminally FLAG-tagged NELL proteins (NELL-FLAG proteins). In this case, the cells were found to express both NELL proteins (140-kDa) in the cytoplasmic fractions by Western blotting (Fig. 2A). A 50-kDa small protein observed with the NELL2-expressing cells might be produced by either proteolysis or processing. In the membrane fraction, the 140-kDa protein was observed only with the NELL2-expressing cells, suggesting the differences in the intracellular localization of NELL proteins. Smeared bands of more than 200 kDa were likely generated by oligomerization of NELL proteins (see below). When the expressed NELL proteins were treated with *N*-glycanase, the molecular mass of NELL2 changed from 140 to 90 kDa (Fig. 2B), in good accordance with its calculated molecular size without the signal peptide. Because there are many potential sites for *N*-linked glycosylation (Asn-Xaa-Thr/Ser; Xaa is any residue other than Pro) in NELL1 (10 sites) and NELL2 (7 sites) proteins, the 140-kDa NELL2 protein is considered to possess about a 50-kDa *N*-linked carbohydrate moiety. NELL1 protein also was found to contain an about 50-kDa *N*-linked carbohydrate moiety (data not shown).

Occurrence of the NH_2-terminal signal peptide in NELL proteins prompted us to investigate their secretion into the culture medium of COS-7 cells. After *in vivo* labeling with [^{35}S]Met of the NELL-expressing COS-7 cells, the medium was subjected to immunoprecipitation with an anti-FLAG antibody. As shown in Fig. 2C, two protein bands with approximate sizes of 130 and 400 kDa were detected in the medium of the NELL2-expressing cells, and a 400-kDa protein in the medium of the NELL1-expressing cells. These 400-kDa proteins were converted to 130-kDa proteins by prolonged denaturation before applying to SDS–PAGE (data not shown). The 130-kDa protein is about 10 kDa smaller than those of the cytoplasmic NELL proteins (140 kDa), suggesting further processing (including deglycosylation) or proteolysis during or after secretion. The 130-kDa monomers are assumed to associate intermolecularly into homotrimers through either the coiled-coil region or vWF C domains (7). Taken altogether, the newly synthesized NELL proteins are sorted into the endoplasmic reticulum lumen by the presence of signal peptide, glycosylated in the Golgi complex, and finally some portions are secreted into

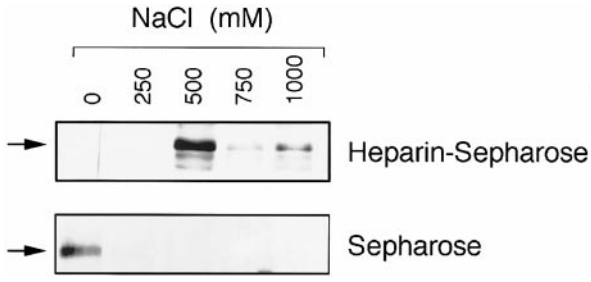

FIG. 3. Binding of NELL2-FLAG protein to heparin-Sepharose. The lysate of COS-7 cells expressing NELL2-FLAG protein was incubated with heparin-Sepharose (upper panel) and Sepharose (lower panel) beads followed by stepwise elution with NaCl at the indicated concentrations. Lane 1, unbound fraction; lanes 2–5, fractions eluted with 250, 500, 750, and 1000 mM NaCl, respectively. The position of NELL2-FLAG protein is indicated with arrows.

the medium with the rest being retained intracellularly.

Heparin-Binding Activity of NELL Proteins

The TSP-N module in TSP-1 has been implicated in the interaction with the cell surface receptors including heparan sulfate proteoglycans (13, 14). Since NELL proteins contain the TSP-N module (see Fig. 1), the NELL2-FLAG protein has been examined for interaction with the heparin-Sepharose beads. As shown in Fig. 3, NELL2-FLAG protein (140 kDa) is retained in the beads upon elution with the buffer containing NaCl at less than 250 mM, and the majority of bound NELL2-FLAG is eluted with the buffer containing 500 mM NaCl. In contrast, the control Sepharose beads could not retain NELL2-FLAG protein even with the buffer containing no NaCl. These results show that NELL2 protein is capable of binding with sulfated glycoconjugates at a physiological salt concentration. The NELL1-FLAG protein also showed similar heparin-binding activities (data not shown). These heparin-binding activities of NELL proteins strongly suggest that the secreted NELL proteins interact with the heparan sulfate proteoglycan on the cell surface.

Northern Blot Analyses of NELL mRNAs

Although the neural tissue-specific expression of *NELL2* mRNA was demonstrated in adult chick (2) and adult human (4), there has been no report describing the expression of *NELL1* mRNA in adult tissues. Northern blot analysis of adult rat tissues has shown that a *NELL2* mRNA of about 4.0 knt is expressed strongly in brain and faintly in testis, while *NELL1* mRNA is undetectable under the same conditions (Fig. 4A). When the exposure time for autoradiography was extended about 10 times, a 4.0-knt *NELL1* mRNA was detected again only in brain (Fig. 4B). These results indicate that both *NELL* mRNAs are preferentially expressed in adult neural tissues, although the amount of *NELL1* mRNA is less than that of *NELL2* mRNA.

During the chick embryogenesis, the *NELL2* mRNA is expressed in all tissues after the middle stage (10 days old). After hatching, the expression is retained only in neural tissues and disappears from other tissues, indicating that the expression of *NELL2* mRNA is regulated spatiotemporarily (2). The expression of *NELL1* mRNA during the embryogenesis was investigated using mouse embryos. As shown in Fig. 4D, a 4.0-knt *NELL1* mRNA is faintly observed in the embryo after 11-day p.c. In contrast, a 4.0-knt *NELL2* mRNA is efficiently expressed after the middle stage of embryogenesis [11-day postcoitum (p.c.)], and a 3.0-knt *NELL2* mRNA is weakly expressed throughout the embryogenesis (Fig. 4C). Development of the nervous system in mouse embryo is known to begin during 7-11 day p.c., concomitantly with the neural plate formation. Therefore, the present results suggest that NELL proteins (especially NELL2 protein) bear important roles in the development of embryo, particularly in the neural tissues.

Recently, the *NELL* mRNAs were shown to be expressed in hemopoietic cells and developmentally regulated in the B lineage (15). The expression of *NELL* mRNAs has been investigated also in human cancer cell lines. Colorectal adenocarcinoma SW480 cells express a 4.0-knt and lung carcinoma A549 cells weakly express 4.5-knt *NELL2* mRNA (Fig. 4E). On the other hand, Burkitt's lymphoma Raji cells express a 9.0-knt *NELL1* mRNA strongly and 4.0- and 5.0-knt *NELL1* mRNAs weakly (Fig. 4F). Differences in the mRNA size may be attributed to alternative splicing; the size of *NELL2* mRNA was reported to be 3.5–3.6 knt in adult human tissues (4). The amounts of *NELL2* mRNA in SW480 cells and *NELL1* mRNA in Raji cells were approximately the same, giving similar intensities under the same conditions of autoradiography (Figs. 4E and 4F). These results show that the expression of *NELL* mRNAs is not restricted to neural cells, and suggest that the NELL proteins may play a more general role(s) in cell actions, e.g., cell growth and oncogenesis.

Localization of NELL mRNAs and Proteins in Rat Brain

Cellular localization of *NELL* mRNAs in adult rat brain was analyzed by *in situ* hybridization. Significant signals for *NELL2* mRNA were detected in the coronal sections of the brain (Fig. 5A). *NELL2* mRNA was most abundant in the hippocampus, and moderate signals were seen in the cerebral cortex, amygdaloid complex, thalamus, and ventromedial hypothalamic nucleus. These areas in the brain are considered to exhibit high neuronal plasticity. As for *NELL1* mRNA, faint signals were visible in several areas (Fig. 5B).

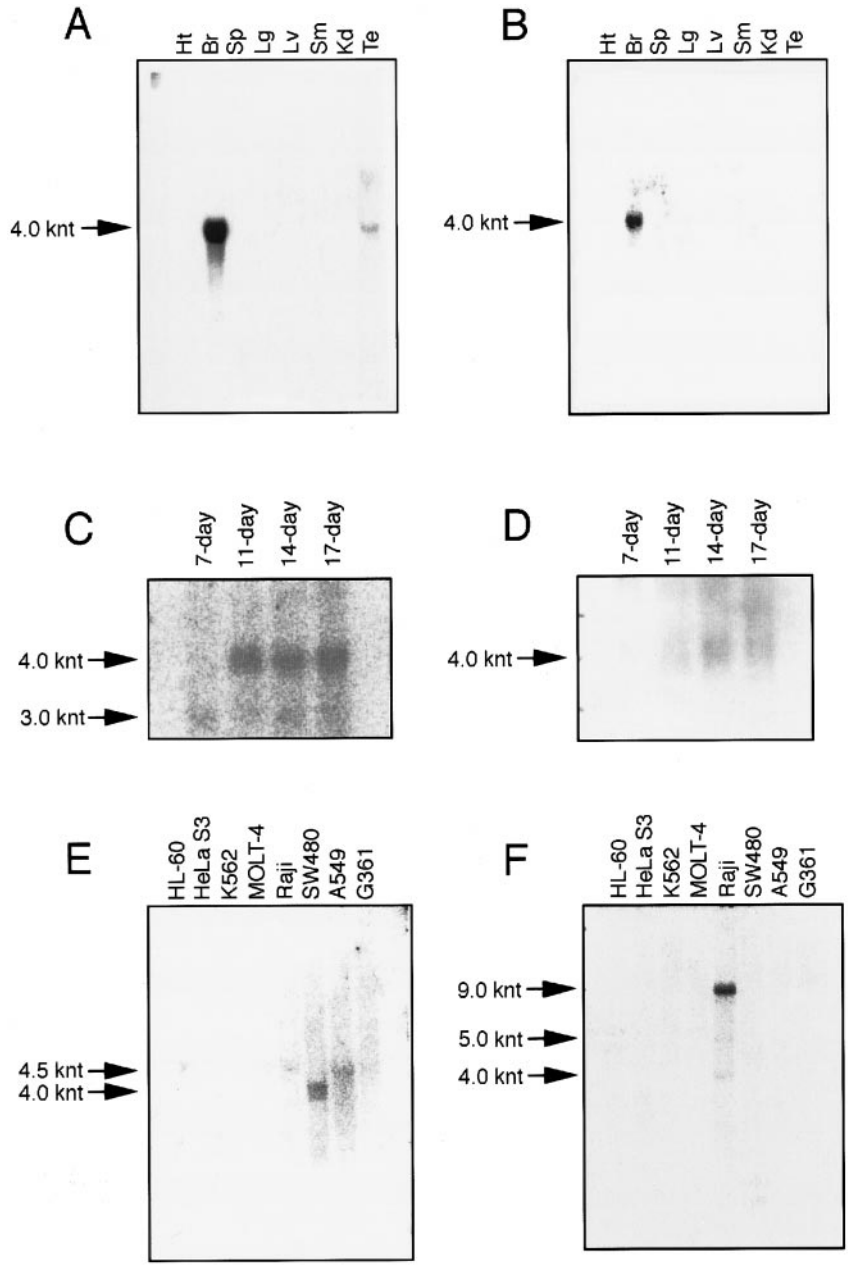

FIG. 4. Northern blot analysis of *NELL* mRNAs. (A and B) Detection of *NELL2* and *NELL1* mRNAs, respectively, in adult rat tissues. Ht, heart; Br, brain; Sp, spleen; Lg, lung; Lv, liver; Sm, skeletal muscle; Kd, kidney; Te, testis. (C and D) Detection of *NELL2* and *NELL1* mRNAs, respectively, in developing mouse embryos. 7-, 11-, 14-, and 17-day indicate days p.c. (E and F) Detection of *NELL2* and *NELL1* mRNAs, respectively, in human cancer cell lines. HL-60, promyelocytic leukemia HL-60 cells; HeLa S3, cervix adenocarcinoma HeLa cells S3; K562, chronic myelogenous leukemia K562 cells; MOLT-4, lymphoblastic leukemia MOLT-4 cells; Raji, Burkitt's lymphoma Raji cells; SW480, colorectal adenocarcinoma SW480 cells; A549, lung carcinoma A549 cells; G361, melanoma G361 cells. The positions of *NELL* mRNAs are indicated by arrows with their approximate sizes (knt).

Then, to identify the cells expressing NELL proteins in brain, we carried out an *in situ* immunocytochemical observation. Two types of antibodies were prepared by immunizing the rabbits with the synthetic peptides corresponding to the COOH-termini of NELL proteins. By Western blotting analysis of the COS-7 cells expressing NELL proteins, these antibodies were confirmed to recognize specifically each NELL protein without cross reactivity (M. Oyasu, S. Kuroda, M. Nakashita, M. Fujimiya, U. Kikkawa, and N. and Saito, submitted for publication). Although no signal was observed by the NELL1-specific antibody, most pyramidal cells in the hippocampus were densely stained by the NELL2-specific antibody (Fig. 5C). The dendrites

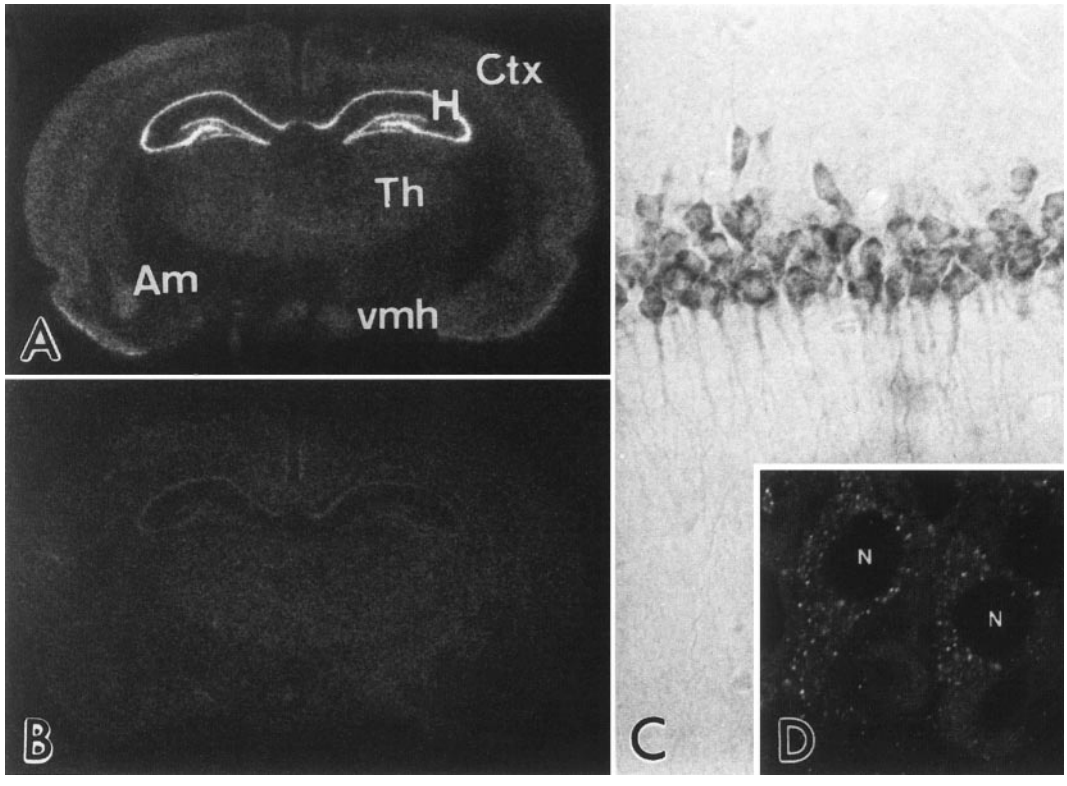

FIG. 5. *In situ* hybridization of *NELL* mRNAs in rat brain (A, B) and immunocytochemical staining of NELL2 protein in the rat hippocampus (C, D). (A and B) *In situ* hybridization of *NELL2* and *NELL1* mRNAs, respectively, with coronal sections of the brain (H, hippocampus; Ctx, cerebral cortex; Am, amygdaloid complex; Th, thalamus; vmh, ventromedial hypothalamic nucleus). (C) immunocytochemical staining of NELL2 protein. (D) An observation under a confocal laser scanning microscopy.

were less stained and glial cells were not. Under the confocal laser scanning microscopy, the NELL2-derived immunoreactivity in the pyramidal cells was found as dots within the perikarya but not in the nuclei (Fig. 5D), indicating that NELL2 protein is localized in the specific intracellular organelle. Such intracellular occurrence of an EGF-like domain-containing protein is not unprecedented; although most proteins containing those domains are extracellular proteins (1), some proteins are known as intracellular proteins, e.g., amphiregulin present in nucleus (16) and prostaglandin endoperoxide H synthases existing in endoplasmic reticulum and nuclear envelopes (17).

Possible Molecular Functions of NELL Proteins

As shown in Fig. 6, the NELL proteins have been revealed to share many protein motifs with a homotrimeric glycoprotein TSP-1, which is expressed in various tissues and partly secreted from the cells. Additionally, NELL proteins interact with heparan sulfate proteoglycans. TSP-1 also interacts with various receptors, cytokines, proteases, and extracellular molecules (e.g., heparan sulfate proteoglycans, sulfatides, low-density-lipoprotein receptor-related protein (LRP), CD36, integrins, the 52-kDa integrin-associated protein, latent TGFβ1, and cathepsin G), and exhibits versatile cell-specific effects on adhesion, migration, and proliferation (for review, see Ref. 7). Although NELL proteins lack some of the TSP-1-specific sequence motifs, such as type I TSP (properdin) repeats, type III TSP Ca^{2+}-binding repeats including an Arg-Gly-Asp (RGD) cell attachment site, and the COOH-terminal domain (7), the biochemical properties of NELL proteins described above strongly suggest that the molecular functions of NELL proteins are similar to but not identical with those of TSP-1.

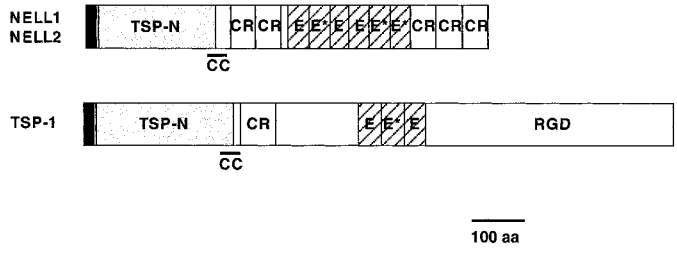

FIG. 6. Schematic structures of rat NELL proteins and mouse TSP-1. Signal peptides (closed), TSP-N modules (TSP-N, shaded), vWF C domains (CR, open), and EGF-like domains (E, hatched) are boxed. Coiled-coil regions (CC, bars), Ca^{2+}-binding type EGF-like domains (asterisks), and the cell attachment sequence in TSP-1 (Arg-Gly-Asp, RGD) are also indicated.

Among the neuronal tissues, TSP-1 is preferentially expressed in the pyramidal cells in hippocampus, like NELL2 protein (see Fig. 5C). During the early degeneration of Alzheimer's disease, it is known that the expression of TSP-1 in the pyramidal cells is considerably decreased (18). TSP-1 has also been revealed to exert neural tissue-specific functions during embryogenesis, including neurulation, neurite outgrowth, and axon guidance (7, 19–22). Thus, it is speculated that NELL2 protein is involved in both the neurogenesis and the maintenance of neuronal plasticity.

Recently, Ting *et al.* (23) reported that the expression of human *NELL1* mRNA is up-regulated in the development and remodeling of cranial intramembranous bone and neural tissues (both having a neural crest cell origin). They also communicated a preliminary finding that the ectopic expression of NELL1 protein in osteoblastic MC3T3-E1 cells induces mineralization (K. Ting, unpublished results). It is interesting to note that TSP-1 is also up-regulated during the osteogenic differentiation of MC3T3-E1 cells (24). TSP-1 has been shown to be a major activator of TGFβ1 (transforming growth factor β1), which bind to and activate the latent form of TGFβ1 extracellularly (25). Since TGFβ1 is known as a negative regulator of osteoclastogenesis (26) and neural crest cell diversification (27), the NELL1 protein may also activate the TGFβ superfamily extracellularly as observed for TSP-1 (25). To prove this hypothesis, a TGFβ superfamily protein(s) which interacts with NELL proteins should be identified.

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