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Molecular cloning of cDNA encoding the rat neural cell adhesion molecule L1

Two L1 isoforms in the cytoplasmic region are produced by differential splicing

Masayuki Miura, Masaaki Kobayashi, Hiroaki Asou and Keiichi Uyemura

Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjukuku, Tokyo 160, Japan

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We have isolated and sequenced a full-length cDNA encoding the rat neural cell adhesion molecule L1. The deduced amino acid sequence as a whole shows high homology to mouse L1 sequence. In addition to this complete form of L1, we found an isoform, L1cs, which lacks four animo acid residues (RSLE) in the cytoplasmic domain and probably is derived from the same single L1 gene by tissue-specific alternative splicing. While L1 mRNA was predominantly expressed in the brain, L1cs mRNA was found exclusively in peripheral nervous tissue. Differential splicing in the highly conserved cytoplasmic domain may play an important role in modulating the function of L1 in different cells.

Neuron, Adhesion molecule, L1, Immunoglobulin superfamily, cDNA cloning

1. INTRODUCTION

A correct neural network formation is fundamentally important for the higher functional activity of the nervous system. Neural network formation is the result of many phenomena during brain development, namely neuronal migration, differentiation, synaptogenesis and myelination. Ample evidence has accumulated in the recent years emphasizing the functional importance of cell surface glycoproteins, such as cell adhesion molecules in these morphogenetic processes in the brain [1]. Among these molecules, unique glycoproteins expressed in specific neuronal subtype are of particular interest. We attempted to study the molecular basis for the function of the neural cell-adhesion molecule L1 during brain development. L1 is a 200 kDa glycoprotein which is involved in neuronal migration [2], adhesion [3], neurite outgrowth [4], fasciculation [5] and myelination [6].

Complementary DNA (cDNA) cloning of mouse L1 has revealed that this molecule is a member of the immunoglobulin gene superfamily and contains multiple type III domains of fibronectin [7]. Rat NILE (nerve growth factor (NGF) inducible large external glycoprotein) is suggested to be the corresponding molecule to mouse L1 as judged by immunological analysis and partial amino acid sequence from NILE cDNA [8]. In this report, we describe the isolation of cDNA clones encoding rat L1 protein and the analysis of its complete cDNA sequence: we found an isoform generated by alternative splicing of mRNA from a single L1 gene. Different distribution of isoforms of L1 among various neural cells and tissues suggests that alternative use of an exon may modulate the multiple functions of L1 in different cells during neural development.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X59149.

Correspondence address: M. Miura, Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan. Fax: (81) (3) 3357 5445.

2. MATERIALS AND METHODS

2.1. *Isolation of cDNA and determination of the cDNA sequences*

Complementary DNA libraries were constructed in the λgt10 or λgt11 vector with mRNA from the adult rat brain (a kind gift from Dr T. Yamauchi and Dr H. Tomura) and in the λgt11 vector with mRNA from the adult rat hippocampus and neonatal rat brain (CLONTECH Lab. USA). These libraries were screened with mouse L1 cDNA probes described below. To obtain the cDNA probes, a polymerase chain reaction (PCR) was performed using 5 μg total RNA from the mouse brain and synthetic primers against 1–21 (PL4), 325–366 (PL3), 3564–3598 (PL2), and 3763–3783 (PL1) nucleotide position described by Moos et al. [7]. The conditions for amplification of the mRNA sequence from cDNA by PCR were according to Kawasaki [9]. The PCR was run for 40 cycles of denaturation (94°C, 2 min), annealing (55°C, 2.2 min), and extension (72°C, 3.4 min). The amplified fragments (1–366 or 3564–3783) were cloned into the *Sma*I site of the pBluescript SK vector. Inserted fragments were isolated and labeled by a random primer method using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I [10]. The hybridization solution was 5 × SSPE (1 × SSPE: 180 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50% formamide, 0.1% SDS, 0.1 mg/ml heat-denatured herring sperm DNA. The filters were hybridized with labeled L1 cDNA probes overnight at 42°C, and washed twice in 2 × SSC (1 × SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 0.1%

SDS at room temperature for 10 min and subsequently washed twice in 0.1 × SSC, 0.1% SDS at 50°C for 20 min. The filters were exposed to Kodak XAR5 film with an intensifying screen at −70°C. Positive clones were selected and *Eco*RI inserts were cloned into pUC119 vector. DNA sequence was determined by the dideoxy chain termination procedure using the T7 polymerase system (Pharmacia).

2.2. *Southern and Northern blot analysis*

Chromosomal DNA was extracted from rat liver according to Maniatis et al. [11]. The genomic DNA (10 μg) was digested with each of *Kpn*I, *Pst*I, *Pvu*II, and *Sac*I, respectively. The digests were then separated by electrophoresis on 1.0% agarose gels, blotted to a nylon membrane (Biodyne B, Pall, USA) and hybridized at 42°C overnight with random-primed ^{32}P-labeled rat L1 cDNA. The conditions of hybridization, washing and detection were similar to those described above.

Total cellular RNA was isolated from various cells and tissues by the guanidium-CsCl method [11]. RNA (15 μg) was electrophoresed on 1.2% agarose-formamide gel, blotted and hybridized as described above, with random-primed ^{32}P-labeled rat L1 cDNA in 50% formamide, 5 × Denhardt's solution, 5 × SSC, 50 mM sodium phosphate pH 6.5, 0.1% SDS, 0.1 mg/ml heat-denatured herring sperm DNA and 10% dextran sulfate. The membrane was washed twice in 2 × SSC and 0.1% SDS at room temperature for 10 min, twice in 0.1 × SSC and 0.1% SDS at 60°C for 20 min. The blot was exposed at −70°C to X-ray film with an intensifying screen.

2.3. *PCR detection of isoforms of L1*

Amplification of the mRNA sequence from cDNA was performed as described above using 5 μg of total cellular RNA with a synthetic primer at position 3541–3558 (PL11) and 3671–3687 (PL12).

3. RESULTS

3.1. *Cloning and sequence analysis of rat L1*

Four rat brain cDNA libraries were screened and 29 independent clones were isolated. From the λgt11 rat brain cDNA library, 6 clones were positive out of 1.5 × 10^6 screened, the longest insert being pRL1 (2.5 kb); from the other libraries the positive clones were as follows: λgt10 rat brain cDNA library, 4/2.4 × 10^6 screened, pRL3 (2.8 kb insert); λgt11 newborn rat brain cDNA library, 5/1.5 × 10^6 screened, pRL13 (2.8 kb insert); λgt11 rat hippocampus cDNA library, 14/1.5 × 10^6 screened, pRL10 (3.7 kb insert). The boundaries of these inserts were determined by DNA sequencing. Plasmids pRL1, pRL3 and pRL13 contained 3′-portion of cDNA including a poly(A) stretch. A cDNA clone including the first ATG codon was isolated only by the screening of random-primed library (rat hippocampus library). The plasmid pRL10 contained the first ATG and most of the coding region. This clone and the other three clones had overlapping sequences, therefore we could determine the entire nucleotide sequence of rat L1 cDNA (Fig. 1). The whole sequence includes the entire coding region corresponding to 1259 amino acids with a 3′-untranslated region of 1225 nucleotides. Rat L1 consists of six immunoglobulin C2-like domains followed by five fibronectin type III domains, a single membrane-spanning region and a cytoplasmic domain. Two RGD (Arg-Gly-Asp) sequences which are involved in the adhesion of cells to the extracellular matrix were conserved in the fourth C2-like domain in rat as in mouse. Twenty potential sites for asparagine-linked glycosylation (Asn-X-Ser/Thr) were found. The coding sequence exhibits a 95.7% homology with mouse L1 sequence, while the amino acid sequences of rat and mouse are 96.7% identical. The cytoplasmic domain of rat L1 is identical to that of mouse L1. Among the cDNA clones sequenced, we found a 12 bp deletion corresponding to the RSLE sequence in Fig. 1 in the cytoplasmic domain in pRL13, but no other addition or deletion to the sequence was found in all other clones sequenced. We have designated this RSLE deleted form as L1cs (L1 cytoplasmic domain short). These results indicate that rat neural tissue expresses two types of L1 which differ in the cytoplasmic region.

3.2. *Southern blot analysis*

Southern blot analysis was performed to investigate the gene organization of L1 (Fig. 2). Rat genomic DNA was digested with *Kpn*I, *Pst*I, *Pvu*II and *Sac*I, respectively. Only one hybridization band was detected in the *Pst*I, two bands in the *Kpn*I and *Pvu*II, and three bands in the *Sac*I digest. These simple band patterns indicate that rat L1 is encoded by a single gene as previously reported in mouse and human [12].

3.3. *Northern blot analysis*

We analyzed L1 expression by Northern blotting (Fig. 3). In both rat and mouse brain, a single band of about 6 kb was detected. To investigate the cell specificity of L1 expression, total RNA from various cell lines was analyzed. While we could not detect any signal in C6 rat glioma, NIH3T3 mouse fibroblast and F9 mouse embryonal carcinoma cells, a high level expression of L1 was observed in both differentiated and non-differentiated rat phaeochromocytoma PC12 cells, followed by expression in N18TG and NG108-15 cells. No obvious changes in expression of L1 in PC12 cells were observed after treatment of 50 ng/ml for 6 days with NGF (Takara). Therefore, we conclude that the expression of L1 is exclusively restricted to cells of neuronal origin.

3.4. *Differential splicing of L1*

We examined the cell and tissue distribution of isoforms of L1 by PCR analysis of cellular RNA (Fig. 4). In both rat and mouse brain, the major PCR product observed corresponded to L1 but not L1cs. The pattern of L1 expression did not change during postnatal development of the rat brain. In contrast, L1cs was the major PCR product in the rat sciatic nerve. Among the cell lines, NG108-15 cells expressed exclusively L1, Neuro2a cells expressed only L1cs and N18TG cells expressed both types of L1. PC12 cells predominantly expressed L1 even after the treatment of NGF.

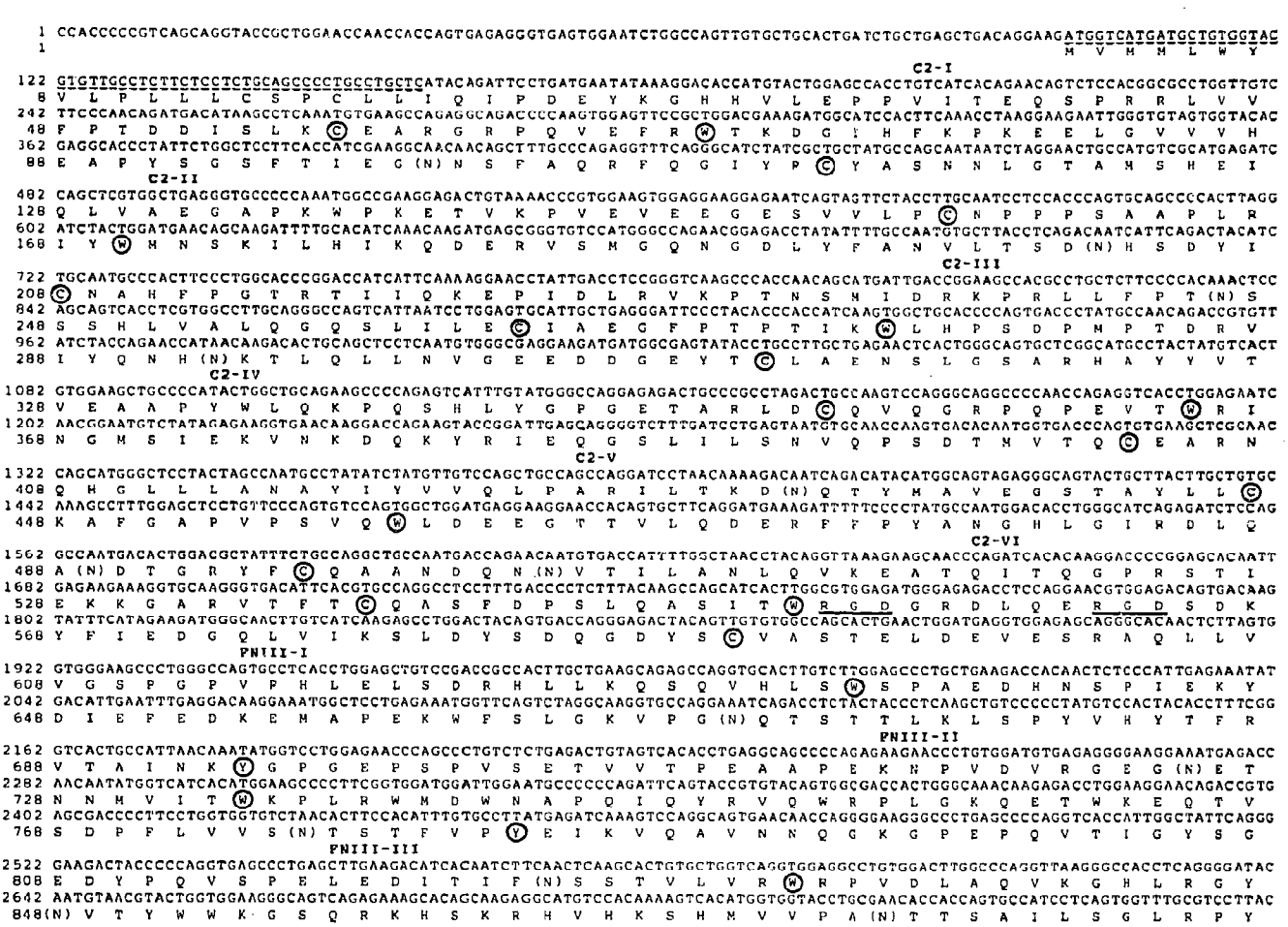

Fig. 1. Nucleotide sequence and deduced amino acid sequence of rat L1. The two hydrophobic regions representing the signal peptide (1–19) and the transmembrane region (1123–1145) and poly(A) addition signal (nucleotide number 5068–5073) are marked by broken lines. The RGD sequence are underlined. The potential sites of asparagine-linked glycosylation are marked with (N). The immunoglobulin C2-like domains are numbered from I to VI, and the conserved tryptophan with the characteristic cysteins are indicated by circles. The fibronectin-type III repeating units are numbered I to V with the characteristic tryptophan and tyrosine (or phenylalanine) residues marked with circles.

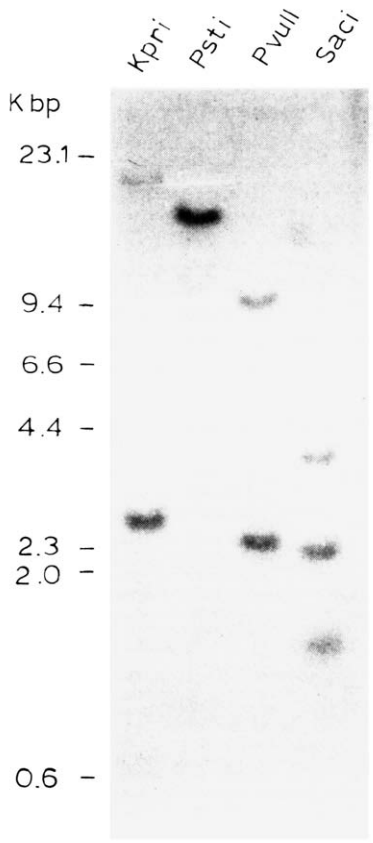

Fig. 2. Southern blot analysis of rat genomic DNA by using a rat L1 cDNA probe. The cDNA region used for probe is the 3017–3861 nucleotide sequence as described in Fig. 1.

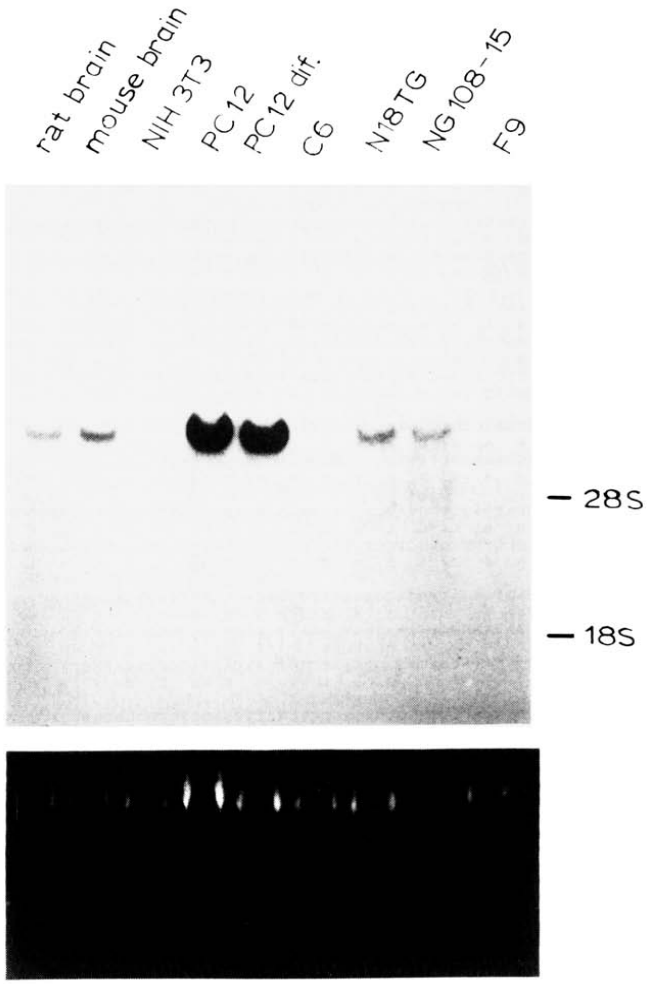

Fig. 3. Northern blot analysis of L1 expression. Intensities of rRNA bands were revealed by ethidium bromide staining of the gel before transfer. The same probe was used for Southern blotting. Total RNA isolated from various cell lines and adult rat and mouse brain were used for experiment.

4. DISCUSSION

We have cloned and determined the entire sequence of rat L1 cDNA. Compared with mouse L1, the overall structure of rat L1 was highly conserved, particularly in the cytoplasmic domain. The cytoplasmic domain contains several potential phosphorylation sites. For instance, one recognition motif (Ser/Thr-X-Arg/Lys) for phosphorylation of protein kinase C [13] is located at residues 1154 and 1232, another recognition motif (Ser-X-X-Asp/Glu/Ser) for casein kinase II [14,15] is located at 1154, 1180, 1183, 1206 and 1209, respectively. There are several reports on phosphorylation of L1. L1 from cultures of mouse cerebellar neurons or of explants of fetal rat forebrain is metabolically phosphorylated [16,17]. Kinase activities are co-purified with L1 immunoprecipitates [18]. Serine residues are the predominant phosphorylation sites and this phosphorylation is associated with the 30 kDa and 80 kDa fragments both of which contain the cytoplasmic domain [18]. We can speculate that L1 is phosphorylated on the cytoplasmic domain by a L1-associated kinase. The L1cs isoform, generated by alternative splicing, is lacking one of the putative phosphorylation sites by casein kinase II. Thus, alternative splicing may affect the L1 phosphorylation.

The cytoplasmic domain of E-cadherin can affect the binding affinity of its extracellular domain [19]. It has been found that E-cadherin associates with several cytoplasmic or cytoskeletal proteins [20]. The 180 kDa form of N-CAM has a unique 261 amino acid insert in the cytoplasmic domain [21] which interacts specifically with spectrin [22]. In the case of L1, this molecule is concentrated in the filopodia of neuronal growth cones and is co-localized with actin and integrins [23]. Association of L1 cytoplasmic domain with cytoskeletal components may be critical for its function such as cell migration and/or neurite outgrowth. The cytoplasmic domain of L1cs, which exhibits a deletion, possibly has different functional activities in such a process.

Two types of the so-called neuroglian, a *Drosophila* homolog of L1, are also generated by alternative splicing in the cytoplasmic domain [24]. The short term of neuroglian is expressed in a wide range of tissues but the long form is restricted to neurons in both central and peripheral nervous system, and to some support cells in peripheral nervous system. L1 and L1cs are differen-

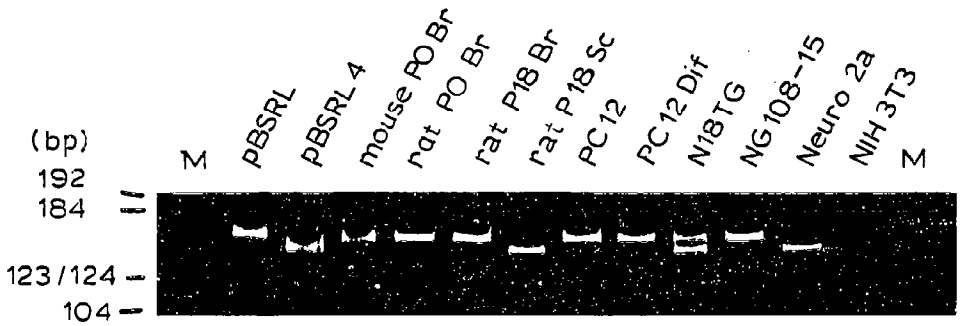

Fig. 4. Differential expression of L1 isoforms in various cells and tissues. Total cellular RNA was processed for cDNA synthesis followed by PCR. PCR was also done by using plasmid pBSRL3 and pBSRL4. Plasmid pBSRL3 contains full length rat L1 cDNA cloned into *Sma*I site of the pBluescript SK vector including the 153 bp 5'- and 117 bp 3'-non-coding region. Plasmid pBSRL4 is the same as pBSRL3 except for the deletion of 12 bp nucleotide sequence coding for RSLE motif. The bands corresponding to L1 and L1cs are 147 bp and 135 bp, respectively. The symbols, P0, P18, Br and Sc, show postnatal day 0, day 18, brain and sciatic nerve, respectively.

tially expressed in the nervous tissues. L1 is expressed predominantly in brain, while L1cs is detected in sciatic nerve tissue (Fig. 4). As Schwann cells are considered to be a major source of L1cs, it is probable that L1cs is a non-neuronal type of L1. The partial sequence of human L1 by cDNA cloning from cDNA library of the M21 human melanoma cell line has been reported, and it was found that the RSLE sequence is absent in the cytoplasmic domain [8], which supports the hypothesis presented above. Recently we ascertained that human brain L1 contains the RSLE sequence (Kobayashi, M. et al., submitted). Therefore the lack of the RSLE sequence reported for human melanoma L1 is possibly due to the different cell origin (neuronal or non-neuronal) rather than to interspecific differences.

The evidence that the cytoplasmic domain of molecules of the L1 family from various species such as *Drosophila* neuroglian [25] and chicken Ng-CAM [26] is highly conserved, suggests that this domain is functionally important. In this domain, the RSLE sequence may be one of the pivotal motifs and differential splicing of L1 mRNA may be involved in the modulation of L1 function in different cells.

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