Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9670–9674, October 1994
Biochemistry

A nuclear factor containing the leucine-rich repeats expressed in murine cerebellar neurons

(amino acid sequence/molecular cloning/nuclear protein/cerebellar morphogenesis)

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Communicated by Frank W. Putnam, June 13, 1994 (received for review February 15, 1994)

ABSTRACT **A nuclear protein, termed leucine-rich acidic nuclear protein (LANP), has been isolated from among rat cerebellar proteins whose expression was transiently increased during an early stage of postnatal development. The amino acid sequence, deduced from its cDNA, showed that LANP contains 247 amino acids consisting of two distinct structural domains: the N-terminal domain characterized by "leucine-rich repeat," which is found in many eukaryotic proteins and which potentially functions in mediating protein–protein interactions, and the C-terminal domain characterized by a cluster of acidic amino acids with a putative nuclear localization signal. Immunohistochemical study using an antibody against LANP revealed that the protein is localized mainly in nuclei of Purkinje cells. In the rat cerebellum on postnatal day 7, LANP mRNA was expressed moderately in the external granule and Purkinje cells and weakly in the internal granule cells. The expression in these cells, especially in Purkinje cells, increased in the second postnatal week and thereafter decreased to an adult level. The structural characteristics, localization, and the stage- and cell type-specific expression suggest a potential role of LANP in a signal transduction pathway that directs differentiation of cerebellar neurons.**

Morphogenesis of the mammalian central nervous system is a dynamic cellular process that includes neuronal proliferation, differentiation and migration, synaptogenesis, and synaptic rearrangement (1, 2). Several lines of evidence suggest that these morphogenetic changes result from a series of molecular events programed at a transcriptional or posttranscriptional level, such as the control of the expression of proteins during development or posttranslational modifications of expressed proteins frequently by reversible phosphorylation (3–5). Thus, one of the crucial approaches to study the morphogenetic process at the molecular level is to explore molecules that play pivotal roles in the development of the nervous system.

Murine cerebellum is suitable for such an experimental analysis because (*i*) the cerebellum has a highly ordered geometric organization composed of a relatively small number of neuronal populations and much information has been accumulated on the development of its neuronal circuits (1, 2); (*ii*) mutant animals with defects of the cerebellum have become available, which enables investigation of relationships among biochemical, morphological, and physiological changes; and (*iii*) most of the morphogenetic changes in the murine cerebellar cortex are the early postnatal events (1, 2). On the basis of protein maps obtained by two-dimensional (2D) HPLC (6) and 2D electrophoresis (2D PAGE) we screened several hundred proteins with respect to their temporal profiles of expression during the development and identified a group of proteins whose expression transiently reached a maximum level within the first 3 postnatal weeks, the period that is most critical for murine cerebellar morphogenesis. One of those proteins, termed "V-1," was characterized in detail by direct protein analysis, molecular cloning of its cDNA, and *in situ* hybridization histochemistry for the analysis of its expression in normal and aberrant murine cerebella (7, 8). Such studies revealed that V-1 is a member of the cell-cycle motif family that potentially functions in differentiation of cerebellar neurons, particularly of granule cells.

We describe here the identification and characterization of another molecule that comprises leucine-rich sequence repeats and a polyacidic cluster with a putative nuclear targeting signal. This protein was termed leucine-rich acidic nuclear protein (LANP) because of its characteristics in amino acid sequence and localization. We show that LANP is a nuclear protein whose expression suggests a role in the cerebellar morphogenesis.‖

MATERIALS AND METHOD

Analysis of Rat Cerebellar Proteins by 2D PAGE. Wistar rat cerebella (wet weight, 40–150 mg) at various developmental stages [postnatal day (P) 0, 3, 7, 12, 21, 30, and 180] were homogenized with 3-fold volumes of 8 M urea containing 1% Nonidet P-40 and 5% 2-mercaptoethanol. After centrifugation, 5 μl of each supernatant was analyzed by 2D PAGE performed in parallel on a multisample microscale apparatus (9). First-dimension isoelectric focusing was carried out in a capillary gel (1.3 mm i.d. × 35 mm) and second-dimension polyacrylamide gradient (8–17%) gel electrophoresis was carried out in a slab gel (40 × 40 × 1 mm). Protein spots were visualized by Coomassie brilliant blue staining and, where necessary, quantitated by TV camera-based densitometry (10).

Purification of LANP and Determination of Partial Amino Acid Sequence. LANP was purified from bovine brain extracts by the procedure designed to purify a series of brain acidic proteins (11). Briefly, the extracts from 50 brain tissues (20 kg wet weight) were fractionated with ammonium sulfate (30% saturation at pH 7.1 to 85% at pH 4.7) and chromatographed on a DEAE-Sephadex A-50 column (8.5 cm i.d. × 23 cm) eluted stepwise by increasing NaCl concentration in 0.1 M potassium phosphate buffer (pH 7.1). LANP, obtained in a post-calmodulin fraction eluted with 0.5 M NaCl, was

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Abbreviations: 2D, two-dimensional; LANP, leucine-rich acidic nuclear protein; P, postnatal day; LRR, leucine-rich repeat.
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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. D32209).

further purified by HPLC on a TSK-gel DEAE-5PW column (2.0 × 15 cm; Tosoh, Tokyo) eluted isocratically with 50 mM Tris·HCl buffer (pH 7.5) containing 0.26 M NaCl and 5% (vol/vol) CH_3CN.

For the sequence analysis, purified LANP was *S*-pyridylethylated in 8 M urea and digested with lysylendopeptidase (Wako Pure Chemical, Tokyo), arginylendopeptidase (Boehringer Mannheim), *Staphylococcus aureus* V8 proteinase (Cooper Biomedical), or α-chymotrypsin (Sigma) in 50 mM $(NH_4)_2CO_3$ (pH 8.0) at an enzyme/substrate ratio of 1:20–1:100. The resulting peptide fragments were purified by reversed-phase HPLC and sequenced on an automated sequence analyzer (model 477A; Applied Biosystems).

Synthesis of Oligonucleotide Probe. Polymerase chain reaction (PCR) was used to generate a probe specific for LANP. The mixed 28-mer oligonucleotide 5′-TGGGATCCGGNAA(T/C)AA(A/G)AT(T/C/A)AA(A/G)GA(T/C)(T/C)T-3′ (N denoting a mixture of G, A, T, and C) was designed as the 5′ primer for the sequence of bovine brain LANP, GNKIKDL, and the 29-mer oligonucleotide 5′-GCGAATTC(A/G)TCNGGNGC(T/C)TC(T/C)TT(A/G)TC(A/G)TC was designed as the 3′ primer for the sequence DDKEAPD. The reaction mixture for PCR contained cDNA derived from the total RNA of 7-day-old rat brain as a template. PCR amplification was performed with 40 cycles, using a cycle of denaturation for 2 min at 94°C, annealing for 1.5 min at 55°C, and an extension for 1 min at 72°C. The PCR product was digested with *Bam*HI and *Eco*RI, cloned into a pBluescript II SK(+) vector (Stratagene), and sequenced. The cloned 193-mer PCR product was ^{32}P-labeled for cDNA library screening and Northern blot analysis.

Screening of cDNA Library. A 7-day-old rat brain λgt10 library (8) was screened with the cloned PCR product. Independent clones (2.5 × 10^6) were plated in *Escherichia coli* NM514 (Amersham) and replica filters were made using nitrocellulose membrane. The filters were hybridized in 40% formamide, 4× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate), 10% dextran sulfate, 0.8× Denhardt's solution, 250 μg of salmon sperm DNA per ml, and the ^{32}P-labeled 193-mer probe (2× 10^6 cpm/ml) at 42°C for 18 hr. The filters were washed at room temperature in 2× SSC.

DNA Sequencing and Analysis. The cDNA inserts of the clones were subcloned into pBluescript II SK(+) and sequenced by the dideoxy chain-termination method using a modified T7 DNA polymerase (Pharmacia/LKB). The software GENETYX (Software Development, Tokyo) was used to search the GenBank and European Molecular Biology Laboratory databases for nucleotide and protein sequence homology.

Northern Blot Analysis and *in Situ* Hybridization. Total RNA was isolated from rat cerebella at various developmental stages by the acid guanidium thiocyanate/phenol/chloroform procedure (12). The total RNA equivalent to the same wet weight of cerebella (8 mg) at each stage was electrophoresed on 1% agarose gels containing formaldehyde and transferred to nitrocellulose membranes. The probe used was the PCR product spanning nucleotides 289–471 (see Fig. 2*b*). Hybridization and washing were carried out as described above.

In situ hybridization histochemistry was carried out as described (8). The *Eco*RI–*Hae* III fragment of the coding region (nucleotides 134–463; see Fig. 2*b*) was used as a probe for hybridization after labeling with ^{35}S by the random-priming method. After hybridization and dehydration, slides were dipped in Kodak NTB-2 nuclear emulsion and exposed for 4 weeks at 4°C.

Preparation of Antibody and Immunohistochemical Analysis. An anti-LANP serum was raised in New Zealand White rabbits by immunization with purified bovine brain LANP excised from polyacrylamide gel (13) and was affinity-purified as the IgG fraction on a HiPAC protein A column (0.46 × 10 cm; Nihon Gaishi, Tokyo). The purified IgG reacted specifically to a single spot of LANP on Western blotting following 2D PAGE of bovine and rat cerebellar extracts.

Immunohistochemical analysis was performed with rat cerebellum at P14. Animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Dissected rat brain was embedded in a compound (Tissue-Tek, Miles) after postfixation and immersion in 0.1 M PBS containing 20% sucrose. The frozen sections, 8 μm in thickness, were incubated with anti-LANP IgG overnight at 4°C and subsequently immunostained by an avidin-biotinylted enzyme complex method.

RESULTS

Identification, Characterization, and Determination of Partial Amino Acid Sequence of LANP. LANP was first identified by 2D PAGE as a molecule whose expression is transiently increased in an initial stage of postnatal development of rat cerebellum. Fig. 1 shows partial comparison of the protein maps of rat cerebella at different developmental stages. LANP was detected at a map position with an isoelectric point (pI) of 4.0 and a relative molecular mass of 30 kDa. The LANP spot was already detectable at birth, increased gradually by subsequent development to reach a maximal level on P12, and then decreased to an adult level. Quantitative examination of the protein spot showed that LANP is expressed at about 3-fold excess on P12 over the adult level, suggesting that the expression of LANP gene is regulated during the cerebellar development.

To isolate LANP we selected bovine brain as starting material because the bovine tissue is more readily available in large quantity and the tissue apparently contained LANP as examined by 2D PAGE. Thus, about 14 mg of purified LANP was obtained from 20 kg of the brain tissue by the procedure described under *Materials and Method*. The purified protein was thought to be the bovine equivalent of rat LANP because the preparation showed a single spot by 2D PAGE at a map position corresponding to rat LANP and an antibody against the purified protein specifically recognized rat LANP by Western blotting following 2D PAGE of rat cerebellar extracts (results not shown).

Since bovine LANP had a blocked N terminus, the sequence information was obtained from its fragments generated by limited proteinase digestions. By the analysis of >40

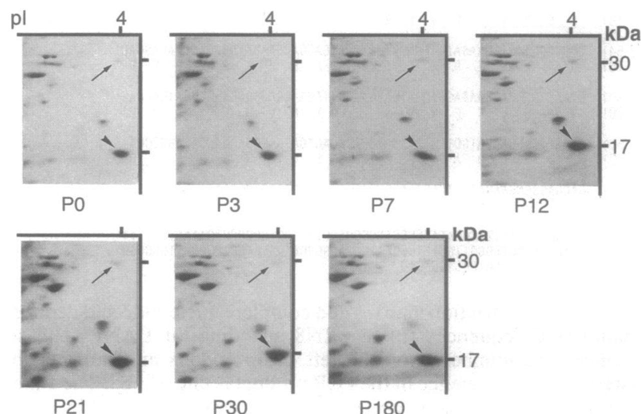

FIG. 1. Partial protein maps of rat cerebella on P0, P3, P7, P12, P21, P30, and P180. 2D PAGE was performed and the profiles of acidic and low molecular mass (kDa) region are shown. LANP is denoted by an arrow. Note that a protein spot identified as calmodulin (indicated by an arrowhead) is present at a constant level during the development.

fragments produced by digestions with a series of proteinases (*Materials and Method*), we determined an internal sequence of 173 residues of bovine LANP (incorporated in Fig. 2).

Nucleotide and Deduced Amino Acid Sequence of LANP. Screening of rat brain cDNA library (2.5×10^6 clones) with a single 193-mer deoxynucleotide probe generated by PCR yielded six positive clones. Because restriction mapping and partial nucleotide sequencing of these clones suggested that all of these clones were derived from a single species of mRNA, the longest clone encoding the entire coding region, pRN-8, was selected for complete nucleotide sequence analysis. Fig. 2 shows the restriction map, the nucleotide sequence of the cDNA, and deduced amino acid sequence. The cDNA sequence contains a total of 1034 bases, including a single open reading frame that extends from nucleotide 1 to nucleotide 741. The sequence of PCR product derived from rat cDNAs is found exactly at positions 289–471. The sequence contains a 93-base 5′ untranslated region including an in-frame stop codon, TGA, at position −48 to −46, and a 183-base 3′ untranslated region.

The continuous open reading frame encodes a polypeptide of 247 amino acids with a calculated molecular mass of 28,565

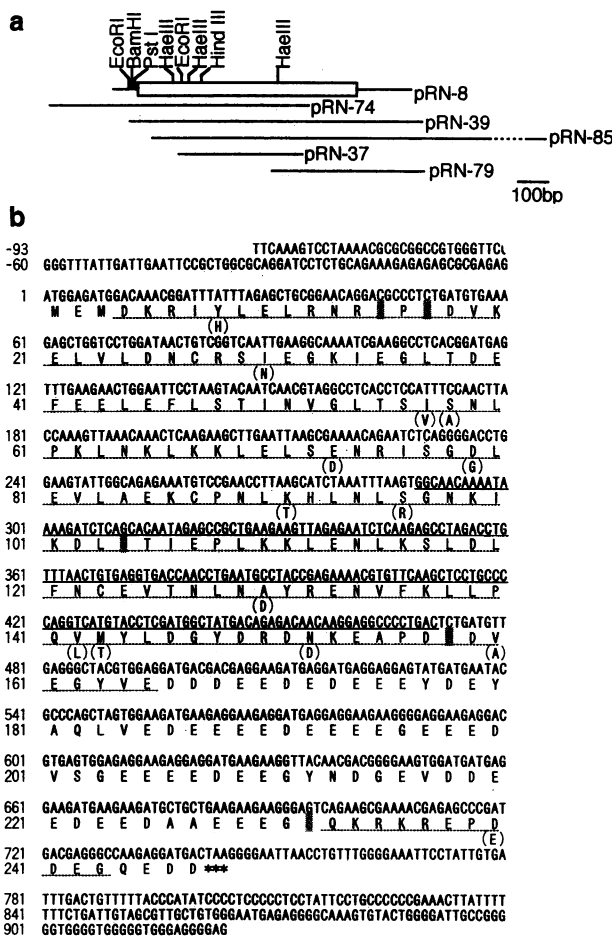

FIG. 2. Restriction map (*a*) and complete nucleotide and deduced amino acid sequences (*b*) of pRN8 encoding rat LANP. The sequences are numbered on the left. Stop codons are indicated by asterisks. The sequence of the PCR product is underlined. The amino acid sequence shown by dotted underline was determined by direct analysis of bovine LANP, where amino acids are different from the rat sequence at 14 positions (amino acids in parentheses indicate the residues found in the bovine LANP). Shaded residues are potential phosphorylation sites: Thr[15] and Ser[17] for Ca^{2+}/calmodulin-dependent protein kinase II; Thr[15], Ser[104], and Ser[158] for casein kinase II; and Ser[232] for Ca^{2+}/diacylglycerol-dependent protein kinase.

daltons and a pI of 3.8, which are in close agreement with the corresponding values of rat LANP determined by 2D PAGE. Because the deduced amino acid sequence also contains the 174-residue sequence of bovine LANP determined by direct protein analysis except for differences at 14 positions (see Fig. 2), we predicted that the cDNA insert in clone pRN-8 encodes rat LANP and that the observed amino acid differences arise from species-specific amino acid substitutions.

Structural Feature of LANP. Computer-assisted retrieval of the deduced sequence revealed that LANP is composed of two structural domains with different characteristics. The N-terminal domain has internally repeated sequence of a 23- to 25-residue segment, rich in leucine, termed previously as "leucine-rich repeat (LRR)" (Fig. 3). The tandem repeats of this motif occur five times along the polypeptide chain, beginning at residue 10 and extending to residue 128, and comprise nearly 50% of the entire chain. This identifies LANP as a member of the LRR-motif superfamily found in a wide range of organisms from yeast to man (*Discussion*). Unlike most of the members of this superfamily, however, LANP appears to be an intracellular protein because the protein contains neither N-terminal signal sequence nor internal hydrophobic segments, which are typical of secreted or transmembrane proteins.

Following a short stretch of relatively hydrophobic segment, a highly repetitive sequence of aspartic and glutamic acids is found in the C-terminal region of LANP (residues 165–247). In fact, 56 amino acids of the total 83 residues in this region are either aspartic or glutamic acid. We also found a putative nuclear targeting sequence (14), KRKR, inside the polyacidic cluster (residues 234–237).

Besides these structural characteristics, LANP contains two asparagines (Asn[13] and Asn[94]) that have potentials for N-linked glycosylation. However, both residues appeared not to be glycosylated at least in the bovine protein as the equivalent residues were identified quantitatively by Edman degradation. We also found five Ser/Thr residues that meet the consensus for Ca^{2+}/calmodulin- or Ca^{2+}/diacylglycerol-dependent phosphorylation or for phosphorylation with casein kinase (Fig. 2, shaded residues).

Developmental Expression and Subcellular Distribution of LANP in Rat Cerebellum. Although LANP was originally identified in rat cerebellum (Fig. 1), later experiments showed that this protein is widely distributed in the rat central nervous system and that the cerebellum is one of the areas where a higher magnitude is detected among examined brain areas (data not shown). Using Northern blot analysis, we examined how the transcription of the LANP gene is regulated at different stages of cerebellar development (Fig. 4). The LANP mRNAs of 2.4 kb and 1.4 kb were found comigrating in all developmental stages examined. Because these RNAs were recognized with probes to the coding and to the 5′ and 3′ noncoding region of the cDNA, we predict that they

FIG. 3. Alignment of the LRRs in the amino acid sequence of LANP. Highly conserved residues are shaded. The consensus sequence is shown at the bottom and compared with LRRs in other members of the superfamily: LRG, leucine-rich α_2-glycoprotein; GPIb, human platelet glycoprotein Ibα; Toll, *Drosophila* Toll; AC, yeast adenylate cyclase; and U2A′, U2 small nuclear RNA-specific A′ protein. α denotes V, I, or L.

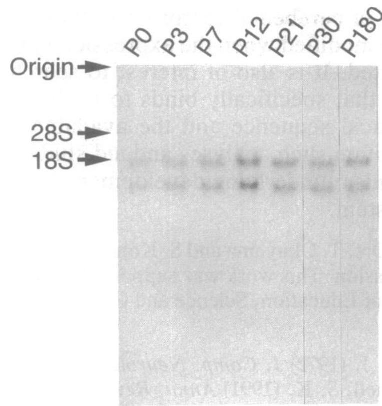

FIG. 4. Northern blot analysis of the expression of LANP mRNA during the postnatal development of rat cerebella. Each lane contained total RNA from 8 mg of wet weight cerebella.

arise from alternative splicing of a single primary RNA transcript, although at present we have no direct evidence that LANP is encoded by a single copy gene in the rat genome. The two RNA species showed parallel developmental changes; they were detectable at birth and then gradually increased to reach a maximum level on P12. The transcripts subsequently decreased on P21 and the suppressed level of expression was maintained in adult rat cerebella. Therefore, changes in the tissue level of LANP mRNA are consistent to that of the mature protein observed by 2D PAGE.

In *in situ* hybridization histochemistry, a moderate level of LANP mRNA expression was detected in the external granular layer and Purkinje cell layer on P7, while the internal granular layer also expressed LANP mRNA weakly (Fig. 5*a*). The expression in these cells, especially in Purkinje cells, increased significantly on P14 (Fig. 5*b*). In the cerebellum on P28 the strong hybridization signal in external granular layer disappeared due to migration of the external granule cells to internal granular layer, while Purkinje and the postmigratory granule cells expressed decreased levels of LANP mRNA (Fig. 5*c*). The hybridization signals were also detected in the molecular layer at this stage, indicating the expression of LANP mRNA in the stellate cells and the basket cells. Thus, *in situ* hybridization histochemistry showed that the temporal profile of LANP mRNA expression is attributed in large part to the increased expression in Purkinje and the granule cells in the second postnatal week and subsequent decrease in these cells and to the disappearance of the external granule cells.

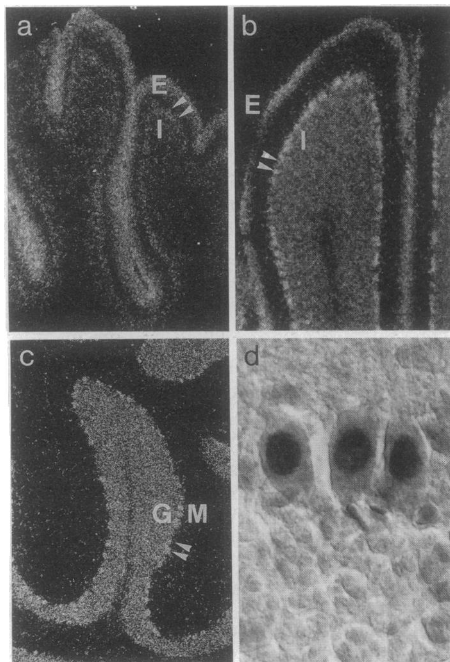

FIG. 5. (*a*–*c*) Cellular distribution of LANP mRNA during the postnatal development of rat cerebella. Shown are dark-field photomicrographs of cerebella on P7 (*a*), P14 (*b*), and P28 (*c*), illustrating hybridization with the ^{35}S-labeled LANP cDNA. E, external granular layer; I, internal granular layer; G, granular layer. Arrowheads indicate Purkinje cell layer. Note the marked increase in hybridization signals in Purkinje cells on P14. (*d*) Immunohistochemical localization of LANP within rat cerebellum on P14. Note the intense positive immunoreactivity in the nuclei of Purkinje cells. (×580.)

Fig. 5*d* shows the subcellular localization of LANP analyzed by the immunohistochemical technique. The positive immunoreactivity was found mainly in the nuclei of Purkinje cells, although a much weaker but detectable level of reaction was observed diffusely in the cytoplasm of Purkinje cells and in the nuclei of granule cells. The predominant nuclear localization of LANP is consistent to the presence of a putative nuclear targeting sequence in the C-terminal region of LANP and identifies LANP as a distinctive nuclear component.

DISCUSSION

We isolated the nuclear protein LANP and deduced its amino acid sequence by cDNA cloning. A significant feature of the sequence is a tandem repeat of the leucine-rich sequence, termed previously as LRR. The repeat motif is clearly distinct from "leucine-zipper," another leucine-rich sequence found in many transcriptional factors (15).

Since the original finding of LRR in human serum leucine-rich α_2-glycoprotein (16), >30 proteins have been identified as members of the LRR superfamily (listed in ref. 17). These proteins occur in a wide range of organisms from yeast to man, contain different numbers of the LRR motif from 1 to 41, and exhibit various cellular and subcellular localizations and functions. For example, *Drosophila* cell surface proteins Toll and connectin contain 15 and 10 LRRs, respectively, in their extracellular domains. Both of these proteins are transiently expressed during the embryonic development of *Drosophila* (18) and function in early morphogenesis, such as the determination of the dorsal–ventral polarity (19). The other cell surface protein in *Drosophila*, chaoptin, contains 41 units of LRR and has a role in photoreceptor cell morphogenesis (20). It has been reported that Toll promotes heterophilic cell adhesion (21), whereas chaoptin and connectin mediate homophilic cell adhesion (18, 22), suggesting that a subgroup of LRR comprises a further class of cell adhesion molecules (22). Another example of this superfamily is yeast adenylate cyclase, in which the LRR motif serves as a site of interaction with RAS protein, a process important for adenylate cyclase activation (23). Likewise, the repeat motif is thought to be the binding site to the von Willebrand factor and thrombin in human platelet glycoprotein Ibα (24, 25) and to the target hormones in the lutropin–choriogonadotropin receptor (26). Thus, despite the wide occurrence of the superfamily, most of the proteins are related to signal transduction pathways and the LRR motif appears to play a role in mediating protein–protein interactions.

Another notable structural feature of LANP is a highly repetitive sequence of acidic amino acids at the C terminus, which should cause the molecule to be very acidic (pI = 4.0) as observed by 2D PAGE. However, such an acidic cluster is not unique in LANP. Nucleoplasmin, the most abundant protein in the *Xenopus* oocyte nucleus, contains an acidic cluster at the C-terminal region (27). Like LANP, this protein has putative nuclear localization signals inside the cluster.

HMG1, a chromosomal non-histone protein found in many eukaryotic cells, also contains a similar polyacidic cluster at the C terminus. In fact, a survey of the group of proteins containing a polyacidic cluster suggests that such a cluster is significantly more common in nuclear than cytoplasmic proteins (28). Although the precise role of the polyacidic cluster is unknown, the structure may serve as a site of electrostatic interaction with other nuclear components, for instance with histones, and thereby determines the microlocalization of proteins in the nuclear compartment. A very recent crystallographic study of porcine ribonuclease inhibitor (29) has shown that 15 LRRs in this protein have the nonglobular, "horseshoe-like" structure of the characteristic β–α units, with an exposed face of the parallel β-sheet for protein–protein interaction. Because the C-terminal polyacidic cluster is likely in an extended conformation, the overall structure of LANP appears to be a tadpole with a compactly folded nonglobular head and an extended tail. On the basis of structural characteristics, we propose that LANP is not an enzyme but is a molecule that mediates protein–protein interaction through binding to its specific target molecules and thereby functions in a signal transduction cascade in the nucleus.

Among proteins in the currently available nucleotide/protein databases, a protein constituent of the U2 small nuclear ribonucleoprotein particle, termed U2 small nuclear RNA (snRNA)-specific A′ protein, is found most homologous to LANP in amino acid sequence. Although the overall homology is relatively low (21% identity), the protein has a molecular mass (28,444 daltons) similar to that of LANP (28,565 daltons) and contains 6 LRRs and a short stretch of polyacidic cluster at the C terminus (30). Besides LANP, the yeast protein sds 22^+ (31) and U2 snRNA-specific A′ protein are the only members of the LRR superfamily that have been identified in the nucleus. The yeast sds 22^+ regulates mitosis through its stimulatory effect on protein phosphatase, and U2 snRNA-specific A′ protein probably functions in the alternative splicing mechanism of RNAs. LANP is most closely related to this latter protein both in structural organization and intracellular localization, but whether a similar function can be attributed to LANP is currently unknown.

Protein (Fig. 1) and mRNA (Fig. 4) analyses revealed a characteristic temporal profile for the expression of LANP in developing rat cerebellum. Namely, the tissue levels of LANP as well as of its mRNA increased transiently around the second postnatal week. *In situ* hybridization histochemistry showed that this temporal pattern of the LANP mRNA expression resulted in large part from the increased expression by Purkinje and granule cells. In the rat cerebellum, the developmental stage of the first to third postnatal week is thought to be critical for its morphogenesis (1) when neuronal differentiation and synaptogenesis actively occur through dynamic cell–cell interactions (32). During this period, the progenitor granule cells migrate from the external to internal granular layer while retaining their axons as the parallel fibers in the molecular layer and form synapses on the dendritic spines of Purkinje cells. Afferent climbing fibers from the inferior olive neurons also form synapses on the Purkinje cells through the transition from multiple to single innervation, and mossy fibers form synapses on the granule cells. Previous studies indicate that these synaptic connections steeply increase around P14 (1, 2). Thus, that the expression of LANP by Purkinje and granule cells increases during this period suggests a potential function of this nuclear factor in these dynamic cellular processes. However, other functional roles of LANP must also be considered because the lower level of the expression persists even after the maturation of the cerebellar neuronal circuit.

In summary, we found a distinctive nuclear factor and predicted a role of this protein in cerebellar morphogenesis. One approach to examine this prediction is to study whether phenotypes of cerebellar neurons and their geometric organization are modified when the expression of the LANP gene is manipulated. It is also of interest to search for a nuclear component that specifically binds to LANP. Knowledge of the amino acid sequence and the availability of its cDNA should facilitate such analyses and aid studies of the molecular mechanism underlying developmental regulation of the nervous system.

We thank Drs. T. Okuyama and S. Konishi for encouragement and helpful discussion. This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan to T. Isobe (05680679).

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