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Isolation, sequence, and developmental profile of a brain-specific polypeptide, PEP-19

(cerebellum/development/HPLC/S100 homologue/neural marker)

R. ZIAI*, Y.-C. E. PAN†, J. D. HULMES†, L. SANGAMESWARAN*, AND J. I. MORGAN*‡

*Department of Neurosciences, Roche Institute of Molecular Biology, and †Department of Protein Biochemistry, Roche Research Center, Nutley, NJ 07110

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ABSTRACT **By comparing the HPLC profiles of cerebellar extracts from adult and neonatal rats, a developmentally regulated polypeptide, termed PEP-19, was identified. The concentration of PEP-19 rose from 0.1 nmol/g of cerebellum at birth to 2 nmol/g at 20 days postpartum. The polypeptide could also be detected at lower levels in olfactory bulbs of adult rats but was absent in cerebral cortex, brain stem, and all nonneural tissues examined. HPLC-purified PEP-19 contained 61 amino acids and had a molecular size of 7.6 kDa. The native polypeptide is blocked at its amino terminus but was sequenced following proteolytic and chemical fragmentation. The primary amino acid sequence was determined to be: X (S-E) R Q S A G A T N G K D K T S G D N D G Q K K V Q E E F D I D M D A P E T E R A A V A I Q S Q F R K F Q K K K A G S Q S. PEP-19 has a unique sequence, but shares some homology with several calcium binding proteins including the β chain of S100 and intestinal calcium binding protein. This polypeptide is the primary translation product of cerebellar poly(A)$^+$ mRNA.**

The maturation of the nervous system is accompanied by alterations in the quantitative and qualitative expression of specific gene products. These gene products in their turn contribute to the differentiated phenotype of specific cell types within the brain. While a number of developmentally expressed brain proteins have been reported (1–9), there has been no systematic attempt made to date to identify such molecules. Indeed, the fact that a number of neural proteins were shown to have differential expression during development was only realized some time after their initial discovery. Because we have shown (10, 11) that HPLC mapping techniques combined with rapid isolation methods can be used to identify brain region-specific peptides, we felt that the same approach would be amenable to the identification of developmentally regulated polypeptides. Thus a database was constructed of the retention times of polypeptides present in neonatal and adult rat cerebellum using the HPLC systems described (10, 11).

A number of developmentally regulated polypeptides were readily identified using this approach that either appeared or disappeared during cerebellar maturation. We describe here the characterization and sequence of a major developmentally regulated cerebellar polypeptide termed PEP-19 in the database. This molecule was selected since it was not only developmentally regulated, appearing after birth, but also was restricted to nervous tissues and in particular the cerebellum; a valuable feature in our studies of cerebellar development. Based on sequence homologies, PEP-19 seems to be related to the S100 proteins and may be a form of brain calcium binding protein.

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MATERIALS AND METHODS

Preparation of Tissue Samples for HPLC Analysis. CD Sprague–Dawley-derived rats (Charles River Breeding Laboratories) were decapitated, and their brains and several other tissues were dissected, flash frozen in liquid nitrogen, and homogenized in 6 M guanidine hydrochloride as described by Slemmon *et al.* (10). Each tissue homogenate was diluted with an equal volume of 0.2% orthophosphoric acid and centrifuged at 30,000 × *g* at 4°C for 45 min. The supernatants were decanted, and 10 ml was desalted by passing over a 1-ml preparative reverse-phase cartridge (Sep-Pak C_{18}, Waters Associates) that had been preactivated and equilibrated with 0.1% trifluoroacetic acid (F_3CCOOH) according to the manufacturer's instructions. The loaded cartridge was washed with 20 ml of 0.1% F_3CCOOH, and peptides were eluted with 5 ml of 0.1% F_3CCOOH containing 80% (vol/vol) acetonitrile. The eluate was dried overnight in a vacuum centrifuge (Speed Vac, Savant) and subsequently analyzed by HPLC.

HPLC Analysis. Tissue homogenates were resuspended in 1 ml of 0.1% F_3CCOOH and centrifuged at 10,000 × *g* for 5 min at room temperature. The clear supernatant was loaded on a Vydac C_{18} reverse-phase HPLC column (Rainin Instruments, Ridgefield, NJ) preequilibrated with 0.1% F_3CCOOH. The column was washed with 0.1% F_3CCOOH, and peptides were eluted with a linear gradient of acetonitrile in 0.1% F_3CCOOH with a continuous monitoring of the effluent at 214 nm. Preparative HPLC was performed on the same column using various gradients of acetonitrile as described in the appropriate figure legends.

Amino Acid Analysis and Sequence Determination. The amino acid composition of peptides was determined by hydrolysis of approximately 300 pmol of peptide in 6 M HCl at 110°C for 24 hr (12). Peptides or their fragments were sequenced using an Applied Biosystems model 470A sequencer. The sequence of the carboxyl terminus was carried out by a timed carboxypeptidase Y digestion of 200 pmol of peptide as described by Jones *et al.* (13).

***In Vitro* Translation of Rat Cerebellum mRNA.** Total rat cerebellum RNA was prepared as described by Auffray and Rougeon (14). The poly(A)$^+$ mRNA was then isolated over an oligo(dT)-cellulose column (15) and translated in a rabbit reticulocyte lysate system (Amersham) supplemented with [^{35}S]methionine. Labeled lysates were incubated with rabbit anti-PEP-19 antiserum and subsequently precipitated with immobilized protein A. Precise details of the production and characterization of the anti-PEP-19 antisera will be described elsewhere. Briefly, a peptide was synthesized corresponding to residues 43–55 of PEP-19 and coupled to bovine thyroglobulin as described (11). This region of PEP-19 has no

‡To whom reprint requests should be addressed.

sequence homology with any protein in the data base.§ Antibodies were detected by their ability to immunoprecipitate synthetic peptide labeled with ^{125}I at a tyrosine added at position 42 of PEP-19.

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE was carried out according to the method of Laemmli (16). Gels, loaded with radioactive samples, were fixed, immersed in EN^{3}HANCE (New England Nuclear), dried, and autoradiographed as described by Laskey and Mills (17).

RESULTS

In an effort to detect developmentally regulated neuropeptides of rat cerebellum, homogenates obtained from neonatal (3-day-old) and young adult (30-day-old) rat cerebella were analyzed by reverse-phase HPLC. By comparing the chromatograms of the two samples, a peptide, referred to as PEP-19 (indicated with an arrow in Fig. 1 *A* and *B*) was detected. This peptide appeared to be at least 10 times more abundant in the adult rat cerebellum than in a comparable weight of cerebellum obtained from newborn rats (as judged by the peak height of the respective HPLC profiles). PEP-19 was subsequently purified to homogeneity by pooling the fractions under the peptide peak (Fig. 1*B*) followed by a further chromatographic separation on the same reverse-phase column using an extended gradient of acetonitrile (Fig. 1*C*). The isolated peptide preparation was homogeneous as determined by amino acid composition analysis (Table 1) and by ion-pairing HPLC analysis (data not presented). Amino acid composition analysis of PEP-19 revealed the presence of 61 amino acid residues per mol of peptide (Table 1). This was in close agreement with a molecular size of 7.6 kDa obtained by NaDodSO$_4$/PAGE (data not presented).

Initial attempts at sequencing the intact peptide were unsuccessful indicating the presence of a blocked amino terminus. Thus specific chemical and proteolytic cleavages were employed to obtain fragments that could be sequenced. Fragmentation of PEP-19 with cyanogen bromide produced two peptides, BR1 and BR2 (Fig. 2), one of which (BR2) was sequenced. Amino acid composition of peptides BR1 and BR2 is shown in Table 1. Digestion of intact PEP-19 with clostripain cleaved the peptide at all three arginine residues and generated four peptides, three of which (C2–4) were sequenced. The blocked amino terminus tripeptide (C1) was subjected to amino acid composition analysis, timed carboxypeptidase Y digestion, and treatment with pyroglutamate aminopeptidase. The most likely sequence for the fragment C1 was determined to be Ser-Glu-Arg, serine being the blocked residue (Fig. 2). It was also shown that the pyroglutamate aminopeptidase did not deblock intact PEP-19. The sequence of the carboxyl terminus of the intact peptide was determined by carboxypeptidase Y digestion and was shown to be Ser-Gln-Ser. This sequence was identical to the one obtained by gas-phase Edman degradation of the peptide C4 (Fig. 2). The amino acid composition of peptides C1–4 is shown in Table 1. Combining these various sequences yielded the complete primary structure of PEP-19 shown in Fig. 2. A computer search of the Protein Identification Resource Protein Sequence Database§ showed PEP-19 to have a unique amino acid sequence. However, a limited homology to another neural polypeptide, the β chain of S100, was indicated. Specifically, the amino terminus of the rat β chain of S100 has homology with residues 36–61 of PEP-19.

To investigate the possibility of the existence of a precursor protein for PEP-19, poly(A)$^+$ mRNA was isolated from the adult rat cerebellum and translated in a rabbit reticulocyte lysate system supplemented with [^{35}S]methionine. The labeled translation products were immunoprecipitated with specific anti-PEP-19 antiserum and analyzed by NaDodSO$_4$/PAGE. As shown in Fig. 3, a polypeptide with molecular size of 7.8 kDa was specifically precipitated with only a minor cross-reactivity being observed with larger proteins (Fig. 3, lane 1). This indicated that the isolated PEP-19 may indeed represent the primary gene product.

To obtain a developmental profile of PEP-19 in the rat

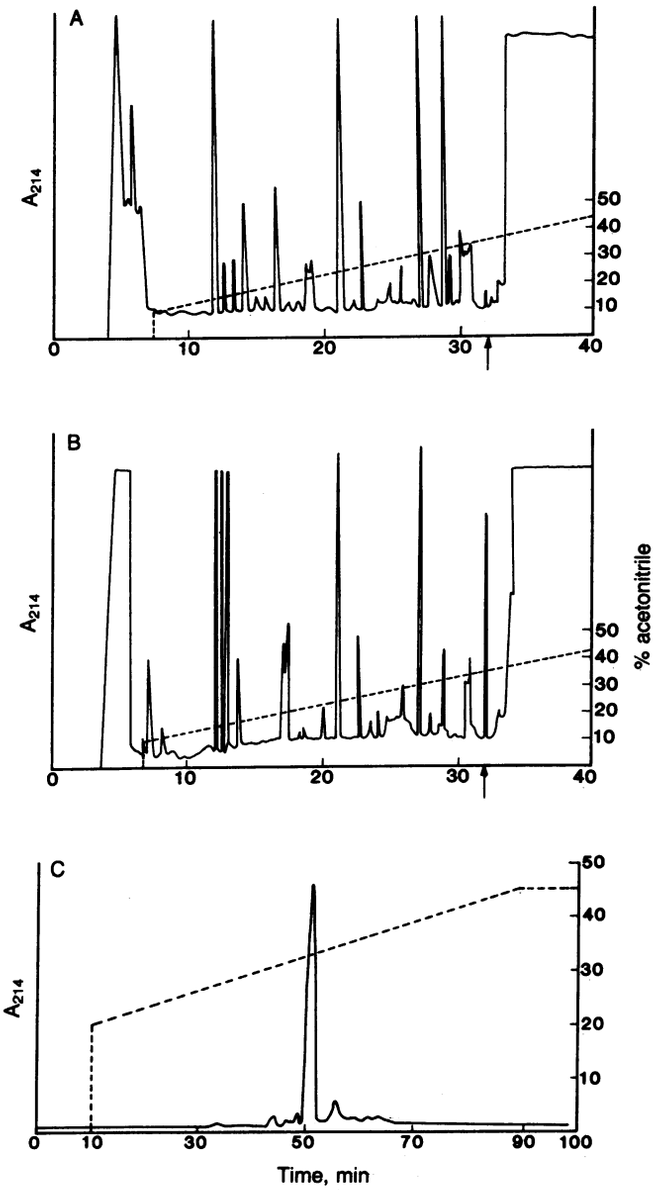

FIG. 1. The reverse-phase HPLC analysis of the rat cerebellum extract. Equivalent weights of the neonatal (*A*) and young adult (*B*) rat cerebellum were homogenized in 6 M guanidine·HCl, and the desalted homogenates were loaded on a reverse-phase HPLC column. The peptides were eluted with a linear gradient of acetonitrile, and the chromatograms were compared. The peptide PEP-19 (indicated with an arrow) was detected in the adult cerebellum extract (*B*) at approximately 10 times more abundance than in the neonatal rat cerebellum (*A*). The column fractions under the peak representing the peptide PEP-19 were pooled, dried under vacuum, resuspended in 0.1% F_3CCOOH (1.0 ml), and loaded on the same reverse-phase HPLC column as *A* above that had been preequilibrated with 0.1% F_3CCOOH. The peptides were eluted with a linear gradient of acetonitrile (20–45%) (*C*), and the fractions containing the peptide PEP-19 were pooled and dried under vacuum.

§National Biomedical Research Foundation (1986) Protein Identification Resource Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC 20007), Release No. 7.0.

Table 1. Amino acid composition analysis of the neuropeptide PEP-19 and its fragments

Amino acid	Peptide						
	PEP-19	BR1	BR2	C1	C2	C3	C4
Asx	8	7	1	0	8	0	0
Thr	3	2	1	0	3	0	0
Ser	6	3	3	1	2	1	2
Glx	12	6	6	1	7	2	2
Gly	5	4	1	0	4	0	1
Ala	7	2	5	0	3	3	1
Val	2	1	1	0	1	1	0
Met	1	1*	0	0	1	0	0
Ile	2	1	1	0	1	1	0
Leu	0	0	0	0	0	0	0
Tyr	0	0	0	0	0	0	0
Phe	3	1	2	0	1	1	1
His	0	0	0	0	0	0	0
Lys	8	4	4	0	4	0	4
Arg	3	1	2	1	1	1	0
Pro	1	0	1	0	1	0	0
Cys	0	0	0	0	0	0	0

Amino acid composition analysis of the intact neuropeptide PEP-19 or its fragments generated by CNBr treatment (BR1 and BR2) or by clostripain digestion (C1–4) was carried out on approximately 300 pmol of each peptide following a complete hydrolysis in 6 M HCl. The ratio of the amino acids to peptide for the intact PEP-19 has been calculated on the basis of a molecular size of 7.6 kDa.

*The CNBr cleavage of the methionine residue generated the fragment BR1 that contained one mol of homoserine per mol of methionine present in the intact PEP-19.

cerebellum, comparable amounts of cerebellum homogenate prepared from rats of different ages were analyzed by HPLC. For the purpose of quantitation of the peptide in various HPLC profiles, a sample of pure PEP-19 was subjected to amino acid analysis, and its concentration was accurately determined. The HPLC chromatograms were subsequently calibrated using such a preparation, and the peak heights were measured on that basis. As shown in Fig. 4, the concentration of the neuropeptide PEP-19 progressively increased after birth from 0.1 nmol/g of tissue to approximately 1.8 nmol/g at 20 days postpartum. The concentration of the peptide remained steady thereafter and did not exceed 2 nmol/g even in 30-week-old rats (data not presented).

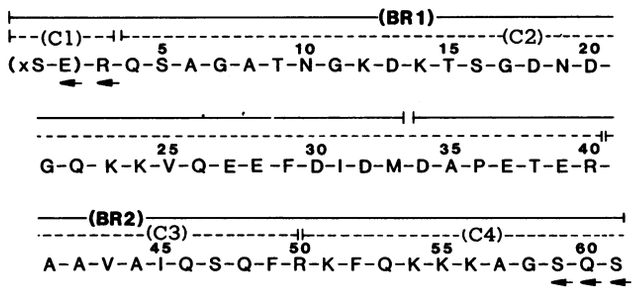

FIG. 2. Sequence determination of the peptide PEP-19. The purified peptide PEP-19 (2 nmol) was cleaved with 200 μg of CNBr in 100 μl of 70% (vol/vol) F_3CCOOH at 20°C for 24 hr, and the peptide fragments (BR1 and BR2) were purified by HPLC and subjected to sequence determination. Digestion of the peptide PEP-19 (2 nmol) with clostripain (1 μg) was carried out in 100 μl of 0.1 M NH_4HCO_3/10 mM dithiothreitol, pH 7.8, at 20°C for 24 hr, and the fragments (C1–4) were purified by HPLC and sequenced. The carboxyl terminus of the intact peptide, and also the sequence of the peptide C1 were determined by a timed digestion with carboxypeptidase Y (arrows). The blocked amino terminus is indicated by x.

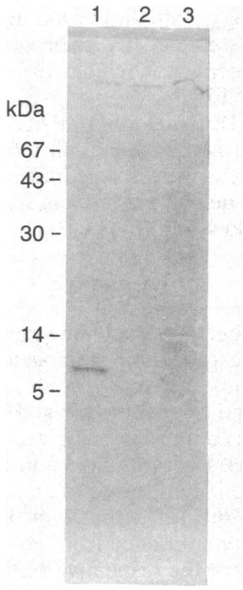

FIG. 3. $NaDodSO_4$/PAGE analysis of the *in vitro* translation products of PEP-19. The [^{35}S]methionine-labeled products of the *in vitro* translation of adult rat cerebellum poly(A)$^+$ mRNA were immunoprecipitated with rabbit anti-PEP-19 antiserum (lane 1), rabbit anti-PEP-19 antiserum preabsorbed with 5 nmol of pure PEP-19 (lane 2), or a normal rabbit serum (lane 3). The immunoprecipitates were electrophoresed on a 15% polyacrylamide gel and autoradiographed. Positions of molecular size marker proteins are shown on the figure.

PEP-19 was undetectable in brain extracts obtained from rats at 17–21 days of gestation (data not presented).

The tissue distribution of PEP-19 was studied by comparing the chromatograms obtained following the analysis of homogenates from various brain regions and several nonneural tissues. PEP-19 was undetectable in the HPLC profile of cerebral cortex or brain stem, but it was present in the olfactory bulbs at approximately 20% of the level found in the cerebellum. The peptide was undetectable in the liver, kidney, heart, spleen, lung, adrenal, and testis, indicating a

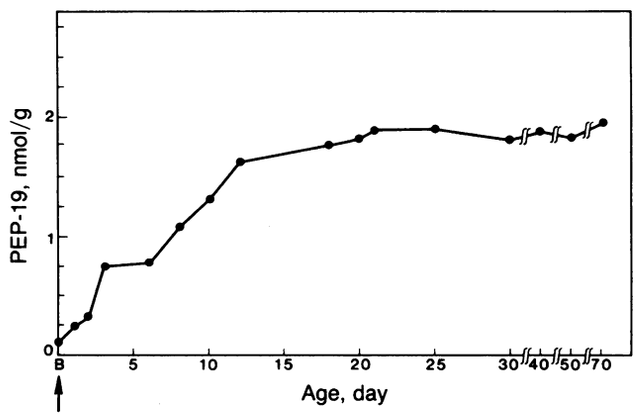

FIG. 4. Developmental profile of the neuropeptide PEP-19 in the rat cerebellum. Cerebellum tissue (500 mg) was obtained from rats at different stages of development and homogenized. Each sample was loaded on a reverse-phase HPLC column, and peptides were eluted with a gradient of acetonitrile as in Fig. 1. The height of the peak representing the peptide PEP-19 was measured in each chromatogram, and the concentration of PEP-19 in the tissue was determined by calibrating the chromatogram with a known concentration of PEP-19. The level of the peptide PEP-19 is shown at birth (B) and in the subsequent developmental stages.

predominant enrichment of the peptide in the cerebellum. Adult mouse cerebellum also contained PEP-19 at the identical retention time on the HPLC chromatograms and at a comparable level to that of the adult rat cerebellum (data not presented).

DISCUSSION

The cellular maturation of the cerebellum has been extensively documented in mammals (18–20). This permits one to correlate the developmental profile of PEP-19 with specific cellular events occurring in the cerebellum as well as with the expression of other known markers. PEP-19 appears around the time of birth and rises to adult levels by days 15–20 (Fig. 4). At the cellular level this pattern of increase correlates with both the formation of the internal granular layer (21) and the maturation of Purkinje cells (22). Indeed, preliminary immunocytochemical evidence shows PEP-19 to be present only in Purkinje cells and stellate neurons of rat cerebellum (data not presented). Further, antibodies to S100 protein (which has homology to PEP-19) are not localized with PEP-19 antibodies, but rather are found with glial cells (data not presented). This is in agreement with the developmental expression of S100 in rat cerebellum that plateaus about 1 week later than PEP-19 (ref. 2 and Fig. 4). Thus, despite the homology between S100 and PEP-19 their cellular locations are quite distinct, the former being glial and the latter neuronal.

Another Purkinje cell marker peptide, cerebellin, shows a similar time course of appearance but decreases in concentration after day 25 postpartum until a stable adult level is attained (11). PEP-19 does not exhibit this characteristic decline in concentration (Fig. 4), suggesting that the level of expression is regulated by mechanisms different from those controlling cerebellin biosynthesis.

As noted above, PEP-19 shares a limited sequence homology with S100 protein (23). This homology also extends to a number of other proteins that might be broadly classified as calcium binding proteins and includes intestinal calcium binding protein (24, 25) and tropomyosin (26). While the overall homology between PEP-19 and these proteins is not substantial, there is a region of sequence identity shared among all species of these polypeptides that is thought to be involved in calcium binding (27). In fact, PEP-19 appears to possess a so-called "EF-hand" structure (28) indicative of a calcium binding domain between residues 22 and 49 (Fig. 2). It is, therefore, conceivable that PEP-19 represents a neuron-specific calcium binding polypeptide. Cerebellar Purkinje cells also contain relatively high concentrations of a 28-kDa vitamin D-dependent calcium binding protein (29). In contrast to PEP-19, this latter protein is also abundant in kidney (ref. 29 and data not presented). Furthermore, the deduced amino acid sequence for the 28-kDa protein from chicken has no sequence homology with PEP-19 (30). Thus, it is concluded that PEP-19 is not a fragment of this larger protein because the *in vitro* translation assay had no detectably larger forms of PEP-19 (Fig. 3). Why an identified single cell should contain two distinct molecular forms of putative calcium binding proteins is unclear. However, it will be of interest to establish whether their expression is coordinately controlled.

A number of S100-like polypeptides have been described, one of which has neuritogenic properties (31), while another S100 homologue has been found complexed with a protein that is a substrate for a tyrosine-specific protein kinase (32, 33). Interestingly the computer homology analysis showed PEP-19 to be most related to residues 180–241 of the c-*sis* gene transforming protein (34). What the significance of this result is, if any, remains to be clarified. Clearly it will be important to establish whether PEP-19 has similar properties to the above molecules. Many of the questions regarding the similarity of structure and function of these S100-like proteins may be resolved by the molecular cloning of PEP-19 and the subsequent comparison of its gene organization with that of the above molecules.

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