The developmental changes of mRNA levels for a cerebellar protein (spot 35 protein) in rat brains

T. Yamakuni, K. Araki and Y. Takahashi*

Department of Neuropharmacology, Brain Research Institute, Niigata University, Niigata 951, Japan

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Our previous papers described a protein called spot 35 found in the cerebellar cytosol of adult rats by two-dimensional gel electrophoresis and localized in the Purkinje cells by immunohistochemical methods. Here we describe the biosynthesis of this spot 35 protein using a reticulocyte lysate cell-free system containing rat cerebellar mRNA. The developmental changes of mRNA-dependent protein biosynthesis were also examined. During postnatal 10–30 days, a rapid increase of mRNA levels for spot 35 protein was observed. The application of the new ^{45}Ca-binding assay procedure revealed that this protein is a Ca-binding protein.

Cerebellar protein *mRNA* *Translation* *Brain development*

1. INTRODUCTION

In a previous paper, Yoshida and Takahashi [1] described the developmental changes of soluble proteins in the rat cerebral mantle, cerebellum and brain stem using two-dimensional gel electrophoresis. They found a protein called spot 35 protein with p*I* around 5.3 and M_r around 27 000, that appears in the adult cerebellum but does not appear in fetal and newborn rats. Yamakuni et al. [2] isolated this protein and made an antibody against it. Furthermore, the immunohistochemical examination of spot 35 protein revealed its localization in Purkinje cells. Since this localization was very interesting, we attempted to characterize the properties of the protein and observe the developmental changes of mRNA levels in the rat cerebellum.

2. MATERIALS AND METHODS

2.1. *Preparation of spot 35 protein and its antiserum*

The isolation of spot 35 protein and the preparation of its antisera were previously described. Briefly, bovine cerebellum (200 g) was homogenized in 5 vols of 20 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and the homogenate was centrifuged at $10^5 \times g$ for 90 min. After solid ammonium sulfate was added to the supernatant to achieve 85% saturation, the suspension was stirred for 24 h and recentrifuged at $16\,000 \times g$ for 40 min. To the resulting supernatant, solid ammonium sulfate was added to achieve 100% saturation. The resulting precipitated protein contained the spot 35 protein. Further purification of this protein was achieved by 2 cycles of DEAE-Sephadex A50 column chromatography. However, the protein fraction still contained about 10% contaminant proteins, according to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, 1.0–1.5 mg of this protein fraction was applied to a preparative slab SDS-PAGE (12.5%) and the protein band corresponding to spot 35 was cut from the gel and the protein extracted electrophoretically. Antiserum was prepared by subcutaneous injection of the purified protein (250, 250, 400 and 400 μg) mixed with Freund complete adjuvant to a rabbit once a fortnight. The Ouchterlony double diffusion technique was used for the antiserum analysis.

* To whom correspondence should be addressed

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2.2. *^{45}Ca-binding assay*

This assay was carried out according to Maruyama et al. [3] with a slight modification. Briefly, after the proteins were separated using SDS-PAGE (8–18% gradient gel), the proteins were transferred onto a nitrocellulose membrane by electroblotting. The membrane was soaked in a solution containing 5 mM $MgCl_2$, 60 mM KCl and 10 mM imidazole-HCL (pH 6.8). Then the membrane was incubated in 25 ml of the same buffer solution containing $^{45}CaCl_2$ (1 mCi/l) for 10 min and then rinsed in 50% ethanol solution for 5 min. The membrane was dried at room temperature for 3 h and then subjected to autoradiography.

2.3. *Isolation of rat brain mRNA*

Postnuclear RNA was extracted from cerebra or cerebella of fetal, newborn, 10-day-old, 30-day-old and adult rats by the phenol-chloroform extraction procedure as described [4]. Guanidinium chloride extraction of rat total cerebellar RNA was also carried out [5]. Poly(A) RNA was isolated from postnuclear RNA or total cerebellar RNA by oligo(dT)-cellulose chromatography [6].

2.4. *Translation of poly(A) RNA in a rabbit reticulocyte lysate cell-free system and analysis of the translation products*

In vitro translation was carried out in a nuclease-treated reticulocyte lysate cell-free system with cerebellar poly(A) RNA as described [7,8]. mRNA-dependent incorporation of [^{35}S]methionine (1000 Ci/mmol, Amersham, England) into trichloroacetic acid-insoluble proteins was determined using an aliquot of the translation products. An aliquot was applied to SDS-PAGE (12.5%) to examine the [^{35}S]methionine incorporation pattern into total proteins. Furthermore, to an aliquot of the translation products, anti-spot 35 protein antiserum and spot 35 protein were added and then immunoprecipitates were applied on SDS-PAGE or two-dimensional PAGE followed by fluorography. These procedures have been detailed in [8].

3. RESULTS

3.1. *Characteristics of spot 35 protein and its antiserum*

Purified spot 35 protein shows a single band with M_r around 27 000 in comparison with M_r marker proteins. Furthermore, two-dimensional gel electrophoretic analysis of purified spot 35 protein reveals that this protein shows a single spot with M_r 27 000 and pI 5.3 (fig.1). Other properties have been described in [2]. During the course of the studies on S-100 protein and calmodulin we used ^{45}Ca-binding assay for identification of these proteins. Then we applied this assay procedure for the studies of our purified proteins. Fig.2 shows

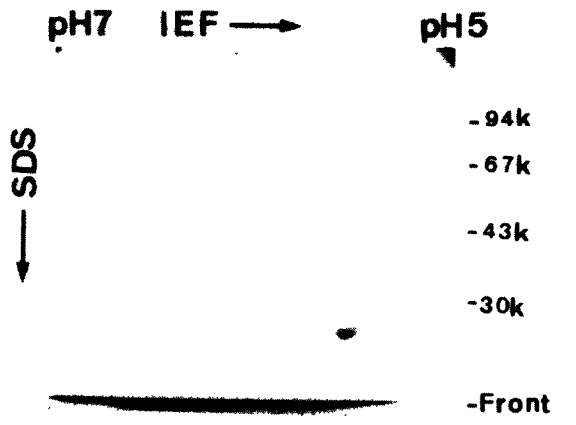

Fig.1. Two-dimensional gel electrophoresis of spot 35 protein. The second dimensional gel was 10%.

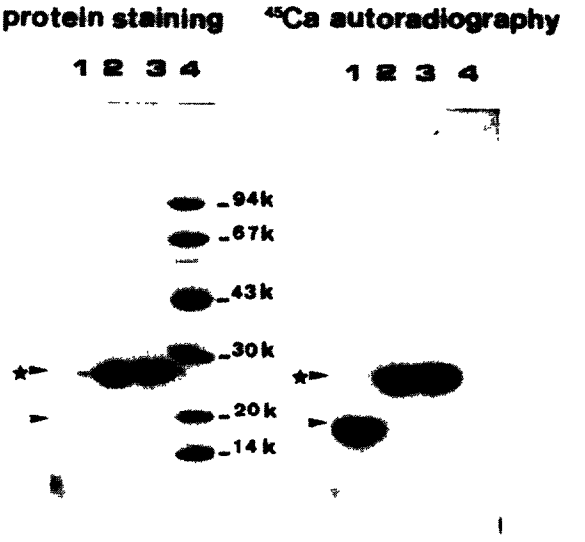

Fig.2. ^{45}Ca autoradiography of spot 35 protein. Experimental procedures are described in section 2. (1) Bovine brain calmodulin, (2) rat spot 35 protein, (3) bovine spot 35 protein, (4) molecular mass markers.

that spot 35 proteins purified from rat and bovine cerebella are Ca-binding proteins. Ouchterlony double diffusion analysis shows that antisera against this protein gave the precipitin line to the antigen and cerebellar soluble protein fraction, but did not react with the soluble protein fractions of cerebrum, brain stem and liver (not shown).

3.2. *Biosynthesis of spot 35 protein in a reticulocyte lysate cell-free system*

Translation of poly(A) RNA from developing and adult rat brains showed incorporation of [^{35}S]methionine into polypeptides with heterogeneous molecular mass values which spanned about 100 kDa. Endogenous incorporation of [^{35}S]methionine into the proteins was very low. Specific immunoprecipitation of spot 35 protein synthesized in vitro was done as described in section 2 and the precipitates were examined by SDS-PAGE and fluorography or by two-dimensional gel electrophoresis, followed by fluorography. Fig.3a demonstrates the one-dimensional SDS gel electrophoretic pattern of total translation products (lane 1) and the labeled band of immunoprecipitated protein with similar M_r to spot 35 (lane 2). Fig. 3b also shows that the labeled spot of immunoprecipitated protein possesses similar p*I* and M_r values to the authentic spot 35 by two-dimensional gel electrophoresis. Further, we could purify mRNA for spot 35 protein from rat cerebellar free polysomes using an immunoadsorption procedure [9] (unpublished). Therefore, it is clear that spot 35 protein was synthesized in our system.

3.3. *Developmental changes in the mRNA level for spot 35 protein*

Poly(A) RNA was extracted from the cerebella of newborn, 10-, 30- and 90-day-old rats and translated in a reticulocyte lysate system. Labeled spot 35 protein was immunoprecipitated from

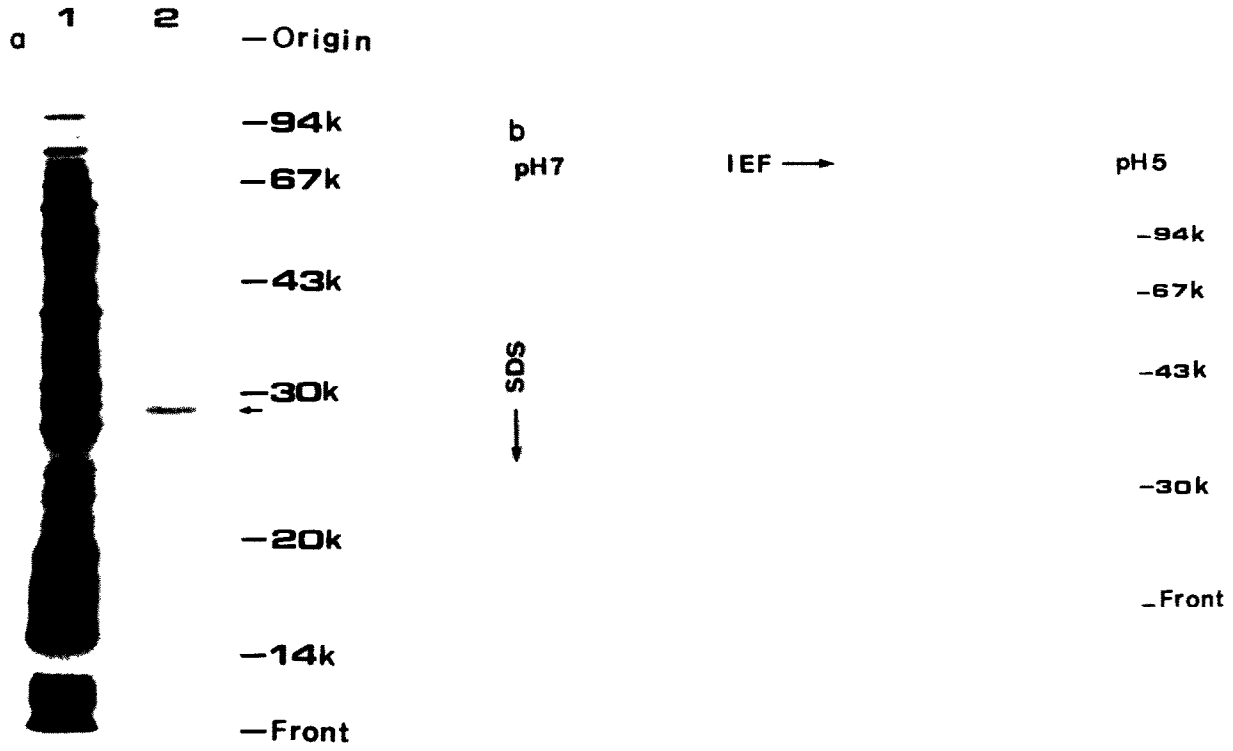

Fig.3. Fluorogram of translation products in a reticulocyte lysate directed with adult rat cerebellar mRNA. Experimental conditions are described in section 2. (a) One-dimensional gel electrophoresis of total translation products (lane 1) and the immunoprecipitates from the total translation products (lane 2), and (b) two-dimensional gel electrophoresis of the immunoprecipitates from the translation products.

equal counts of the translational products as described in section 2 and was examined on SDS-PAGE, followed by fluorography. Fig.4 shows that one labeled band corresponding to spot 35 protein was clearly found in 10-90-day-old rats. The density of this band increased rapidly with the development of rats after 10 days postnatal. Poly(A) RNA extracted from cerebral tissue was not translated into spot 35 protein during every developmental age as shown in fig.4.

4. DISCUSSION

In the previous study [10], we observed the biosynthesis of total soluble cerebellar protein in cerebellar slices and in a reticulocyte lysate cell-free system using two-dimensional gel electrophoretic analysis. In that study the protein having similar p*I* and M_r to spot 35 protein seemed to be synthesized, although identification with antiserum against it was not carried out. Morrison et al. [11] and Soreq et al. [12] also described the biosynthesis of total cerebellar protein in a cell-free system. The results of this paper clearly demonstrate the mRNA-dependent cell-free biosynthesis of spot 35 protein by using specific immunoprecipitation and SDS-PAGE or two-dimensional gel electrophoresis. It was further found that the levels of mRNA for spot 35 protein showed a great increase during cerebellar development. In a separate paper it was described that the antiserum against spot 35 protein specifically stained Purkinje cells [2]. Furthermore, our unpublished study revealed the appearance of positive PAP staining of rat cerebellar Purkinje cells on around the third day after birth. These results suggest that spot 35 protein may be a marker protein of maturation of Purkinje cells. The recent introduction of the new ^{45}Ca-binding assay procedure showed that this protein is a Ca-binding protein. Further, our neurophysiological study revealed the involvement of this protein in modulation of Purkinje cell activity of the rat cerebellum [13].

Fig.4. Developmental changes in the fluorogram of translation products. (1) Total translation products (500 000 cpm) with cerebellar poly(A) RNA; (2–5) fluorogram of immunoprecipitates from the total translation products with cerebral poly(A) RNA; (2) newborn, (3) 10-day-old, (4) 30-day-old, (5) 90-day-old; (6–9) Fluorograms of immunoprecipitates from the total translation products with cerebellar poly(A) RNA: (6) newborn, (7) 10-day-old, (8) 30-day-old, (9) 90-day-old.

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