Two Acidic Proteins Associated with Brain Development in Chick Embryo

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Abstract: The developmental changes in protein composition of the chick optic tectum were analyzed by two-dimensional gel electrophoresis. Staining with Coomassie Brilliant Blue R revealed 54 major proteins, eight of which remarkably changed their abundance during development: Four of these proteins (S8, S14, S30, and S54) increased and two of them (S7 and S37) decreased in the course of the brain development. The other two proteins (S5 and S6) appeared at specific embryonic stages and were not detected in the adult. The abundance of S5 protein was highest at day 7, and that of S6 protein at days 9–18. The two proteins were present in other regions of the embryonic brain but were not detected in the embryonic liver. The proteins were purified from the soluble fraction of embryonic chick brains by pH 5.5 precipitation, DEAE-Sepharose column chromatography, ammonium sulfate precipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weights of S5 and S6 proteins were 95,000 and 100,000, respectively, and their isoelectric points were about 4.5. They were compared by peptide mapping using V8 protease and found to share 11 common peptides out of 17 distinct ones. This indicates a strong degree of structural homology between these two proteins. Key Words: Chick embryo brain—Acidic proteins S5 and S6—Structural homology. Shirao T. and Obata K. Two acidic proteins associated with brain development in chick embryo. J. Neurochem. 44, 1210–1216 (1985).

In the course of the development of the nervous system, neurons are produced by proliferation of matrix cells in the ventricular zone, migrate from there to their permanent location, extend axonal and dendritic processes in the proper direction, and form synapses selectively with their appropriate partners. These processes have been investigated mainly with morphological methods, including electron microscopy and autoradiography. Birthdate of each neuron, layer formation, and subsequent maturation have been disclosed in mammalian cerebral (Angevine and Sidman, 1961; Rakic, 1972) and cerebellar cortices (Fujita et al., 1966; Altman, 1972) and in chick optic tectum (LaVail and Cowan, 1971a,b), but the molecular mechanisms of these processes are yet to be clarified.

One approach to understanding developmental processes at the molecular level is to select a key function in each developmental process, and to look for the protein that will mediate that function. Neuron adhesion is important in brain development. Edelman’s group has developed a specific immunological assay for the molecules involved in cell adhesion (Brakenbury et al., 1977) and purified molecules that mediate neural cell adhesion (Hoffman et al., 1982). The survival and axon growth of neurons in tissue culture requires some neurotrophic factors. In addition to nerve growth factor (Levi-Montalcini and Angeletti, 1968; Ihouen and Barde, 1980), several trophic factors have been partially purified for the neurons in the CNS (Kligman, 1982; Berg, 1984).

Another approach is to isolate the proteins that appear specifically in the developmental processes, to purify them, and then to identify their functions. Skene and Willard (1981) hypothesized that axon extension requires unique substances that are not found in mature axons, and identified several proteins, which appear characteristically in growing and regenerating axons, by using two-dimensional gel electrophoresis (2DGE). Sperry hypothesized the presence of two orthogonal gradients of molec-

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Abbreviations used: Bisacrylamide, N,N'-Methylene-bis-
acrylamide; CBB, Coomassie Brilliant Blue R; 2DGE, Two-dimensional gel electrophoresis; IEF, Isoelectric focusing; PMSF, Phenylmethylsulfonyl fluoride; SA buffer, 70 mM Sodium acetate–acetic acid buffer, pH 5.5 containing 1 mM EDTA; SDS, Sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
cules on retinal ganglion neurons and corresponding gradients of complementary molecules in the optic tectum. These molecules might determine the specific connections between retinal and tectal neurons (Sperry, 1963). Trisler et al. (1981) identified an antigen that is distributed in a dorsal-ventral topographic gradient in chick embryo retina by using a monoclonal antibody. Further investigation should be carried out to elucidate the functions of these novel proteins.

Since existence of other unique proteins that appear specifically in the developmental processes is expected, this approach is still promising. Therefore, we have analyzed protein composition of the chick optic tectum from the fourth day of incubation to adulthood by 2DGE and found that two acidic proteins appear characteristically in the developing brain tissue: one (S5 protein) increased from day 4 to day 7 and then decreased, and the other (S6 protein) increased from day 7 to day 9 and decreased after day 18 in the chick embryo optic tectum. Further, we purified and characterized these two acidic proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following materials were purchased from the sources indicated: Ampholines from LKB (Bromma, Sweden); sodium dodecyl sulfate (SDS) from BDH (Poole, England); acrylamide, N,N'-methylene-bis-acrylamide (bisacrylamide) from Eastman Kodak (Rochester, NY); urea from Schwarz/Mann (Spring Valley, NY); phenylmethylsulfonyl fluoride (PMSF) and Coomassie Brilliant Blue R (CBB) from Sigma (St. Louis, MO); Nonident P-40 and 2-mercaptoethanol from Nakarai Chemicals (Kyoto, Japan); Staphylococcus aureus V8 protease from Miles Laboratories (Elkhart, IN); molecular-weight standards and DEAE-Sepharose CL 6B from Pharmacia Fine Chemicals (Uppsala, Sweden).

**Preparation of the sample for electrophoresis**

Fertilized eggs of chickens and about 1.5-year-old chickens (White Leghorn) were obtained from a local poultry farm. These eggs were incubated in a draft incubator at 38°C. Embryos were staged according to the number of days of incubation and were within the normal developmental stages of Hamburger and Hamilton (1951). Parts of brain or other tissues were taken from embryos at various stages, from newly hatched (0-day-old) chicks, and from 1.5-year-old chickens.

Tissues were isolated in ice-cold saline, weighed, and directly homogenized with a micro tissue grinder in 10 volumes of isoelectric focusing (IEF) lysis buffer containing 9 M urea, 2% Nonident P-40 (w/vol), 2% Ampholine (1.6% pH range 5–7 and 0.4% pH range 3.5–10), and 5% 2-mercaptoethanol (O’Farrell, 1975). More than 90% of the total protein was solubilized in this procedure. To ascertain whether proteolytic degradation did not occur during the preparation of samples for electrophoresis, tissues were homogenized in IEF lysis buffer with 1 M PMSF, 1 mM disopropyl fluorophosphatase, 1 mM p-tosyl-L-lysine chloromethyl ketone hydrochloride, and 5 μg/ml leupeptin, and then electrophoresed. The electrophoretic patterns of the samples were identical with those of samples prepared in the absence of the above protease inhibitors.

**Two-dimensional gel electrophoresis**

The procedure of O’Farrell (1975) was carried out with the modifications using the minigel system as described by Mikawa et al. (1981). Three microliters of the sample were loaded. For the run the first-dimension (IEF) electrophoresis was carried out for 30 min at 100 V, 1 h at 200 V, 1 h at 300 V, and then 15 min at 400 V. After the run, the gel was equilibrated with 62.5 mM Tris-HCl (pH 6.7) containing 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 1 mM EDTA. For the run in the second dimension a discontinuous SDS gel system was used as described by Laemmli (1970). The resolving gel contained 12.5% acrylamide/bisacrylamide (from a 30% stock solution composed of 29.2% acrylamide and 0.8% bisacrylamide). The gel was subjected to electrophoresis at 10 mA/plate, fixed, and stained with CBB (Chrambach et al., 1967) or silver (Oakley et al., 1980). For comparison of the amount of a specific protein between two samples, both cylindrical IEF gels of the two samples were placed on a single slab gel plate. Therefore the staining and destaining conditions of the 2DGE gels were the same, and the staining intensity of each spot was taken as a measure of the amount of each protein. Reproducible electrophoretic patterns were obtained either from multiple two-dimensional separations of the same tissue preparation or from different preparations of the same tissue. At no time were randomly variable proteins detected.

The molecular weights were estimated on SDS gels using phosphorylase b (MW 94K), bovine serum albumin (67K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and a-lactalbumin (14.4K) as standards.

The isoelectric points were estimated on an IEF gel as described by O’Farrell (1975).

**Purification of the two acidic proteins**

All operations were carried out at 4°C. Whole brains of 11-day chick embryos were homogenized in 3 volumes of 0.32 M sucrose, 0.1 mM PMSF, and 1 mM EDTA. The homogenate was centrifuged at 1,000 × g for 10 min. The supernatant was removed and centrifuged at 100,000 × g for 2 h. The supernatant was removed and brought to pH 5.5 by dialysis against 20 mM sodium acetate–acetic acid buffer (pH 5.5) containing 1 mM EDTA (SA buffer). The suspension was centrifuged at 100,000 × g for 1 h. The supernatant was applied to a DEAE-Sepharose CL 6B column previously equilibrated in SA buffer. Following washing with SA buffer, bound material was eluted stepwise with 0.25 M, 0.5 M, and 1.2 M NaCl in SA buffer. The two acidic proteins were recovered in 0.5 M NaCl fraction. This fraction was brought to 20% saturation with ammonium sulfate. The suspension was centrifuged at 7,000 × g for 15 min and the supernatant was decanted and brought to 30% saturation with ammonium sulfate. The 30% suspension was centrifuged at 100,000 × g for 60 min and the precipitate was dissolved in a minimum volume of SDS-sample buffer composed of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1 mM EDTA, 40 mM Tris, and 240 mM glycine at pH 8.5. This solution was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and CBB staining. The two stained bands (MW 95K and 100K) were excised from the wet gel sep-
Developmental changes in protein composition

The developmental changes in protein composition of the chick optic tectum from the fourth day of incubation to hatching were analyzed by 2DGE. Because protein from the same amount of tissue was applied in each 2DGE, the staining intensity of each spot in the electrophoretogram represented the abundance of each specific protein in the tissue (Fishbein, 1972). There are significant differences in the rate of development of the ventrolateral and dorsomedial portions of the optic tectum on the one hand and its rostral and caudal regions on the other (LaVail and Cowan, 1971a). Therefore, we separate the optic tectum into three dorsoventral levels corresponding to its dorsal, intermediate, and ventral thirds, and only the dorsal portions of optic tecta were used for this analysis. Many protein spots were detected in the electrophoretogram. Fifty-four principal spots were numbered as illustrated in Fig. 1 and analyzed for changes in their staining intensities during development. Among them several spots were identified by their migration coordinates or comigration analysis as follows: spots 8 (S8) and 14 (S14) as neurofilament proteins (Czosnek and Soifer, 1980; Tapscott et al., 1981); S21 as β-tubulin; S22 and S23 as α-tubulins (Strocchi et al., 1981); and S33 as actin (Mikawa et al., 1981).

There were eight spots (S5, 6, 7, 8, 14, 30, 37, and S4) that changed their staining intensities remarkably during embryonic development. These are shown as black spots in Fig. 1. Figure 2 shows the electrophoretograms of chick optic tecta from a 7-day chick embryo (A) and a newly hatched chick (B). Several consistent differences were observed: S5 and S37 stained intensely on day 7, but were not or were hardly detected after hatching. On the other hand S14 and S30 stained faintly on day 7, but stained intensely after hatching. These eight spots were divided into three groups: Group I was S8 (MW 150K), S14 (70K), S30 (45K), and S34 (110K).

FIG. 1. Composite diagram of major proteins of developing chick optic tectum. The proteins were separated by 2DGE as described in Experimental Procedures. Forty-four major spots were numbered. Filled spots are those spots that remarkably changed their staining intensities during development.

Peptide mapping

Peptide mapping of proteins in spots from two-dimensional gel, using S. aureus V8 protease, was carried out by the procedure of Cleveland et al. (1977).
S5 AND S6 PROTEINS IN CHICK EMBRYO BRAIN

FIG. 3. Comparison of the optic tectum (A) and the liver (B) in an 11-day chick embryo. Panels are selected parts of 2DGE. Arrowheads indicate landmark spots that were common to both tissues. 5, S5 protein; 6, S6 protein. Silver stain.

S8, S14, and S30 proteins appeared by day 7 and increased in staining intensity with a similar time course during development. S4 protein appeared by day 15 and then increased. The proteins of Group I stained intensely in the electrophoretogram from an adult chicken. They could not be detected in the liver at any embryonic stages. Group II was S7 (120K) and S37 (38K) which stained intensely in the electrophoretogram from 4-day embryos and then decreased in staining intensity gradually during development. Group III was S5 (95K) and S6 (100K). They stained intensely only at specific embryonic stages (see below).

Comparison of Groups I, II, and III proteins in the optic tectum and in the liver

Each protein of Groups I, II, and III was present in the spinal cord, the cerebellum, the cerebrum, the optic tract, and the retina, as well as in the optic tectum. However, in an 11-day chick embryo from which the CNS (the brain, the spinal cord, and the eyes) was removed, the proteins of Groups I and III were not or were hardly detected.

We therefore compared the proteins of Groups I, II, and III systematically in 2DGE of the liver as a nonnervous tissue and of the optic tectum at various developmental stages. Each protein was identified by comigration analysis. While the proteins of Group II (S7 and S37) were present in the liver and decreased gradually with embryonic age as in the optic tectum, the proteins of Group I (S8, S14, S30, and S54) and Group III (S5 and S6) were not detected in the liver at any embryonic stages.

FIG. 4. Developmental changes of S5 protein and S6 protein in the chick optic tectum. Panels are selected parts of 2DGE. A: Day 4, B: Day 7, C: Day 15, D: Newly hatched. CBB stain.

Figure 3 shows the portions of silver-stained two-dimensional electrophoretograms of the optic tectum (A) and the liver (D) from an 11-day embryo. S5 and S6 proteins were detected neither by CBB staining nor by silver staining in the liver, though two other common proteins (arrowheads) could be detected by silver staining in both the optic tectum and the liver.

Developmental changes of S5 and S6 proteins

In the dorsal portion of the optic tectum S5 protein appeared by day 4, reached a maximum level on day 7, and then decreased. S6 protein was not detected on day 4 by CBB staining, and appeared by day 7. It reached a maximum level on day 9, retained this level until day 18, and then decreased (Fig. 4A, B, and C). At hatching S6 protein was decreased to a low level and S5 protein was not detected (Fig. 4D). In adult chickens (1.5 years old) neither S5 nor S6 protein was detected by CBB staining.

The developmental changes of S5 protein and S6 protein occurred earlier in the rostral portion than in the caudal portion of the optic tectum from day 6 to day 12. Figure 5 shows the difference between the rostral portion and the caudal portion of the optic tectum in an 8-day chick embryo. S14 protein (neurofilament protein) and S6 protein were more abundant in the rostral (Fig. 5A) than in the caudal portion (Fig. 5B) as compared with the same intensity of actin and tubulin between the portions. The difference of S5 protein was not remarkable on day 8.

The amounts of S5 and S6 proteins and their developmental changes varied between regions in the brain; in the cerebellum S5 protein increased less strikingly but could be detected at a later stage of development than in the optic tectum. Figure 6 shows the portions of the electrophoretograms from the analyses of the cerebellum and the optic tectum in a 13-day embryo. S5 protein was present in the cerebellum (Fig. 6A), in contrast to the optic tectum where S5 protein was hardly detected (Fig. 6B).

Purification of S5 and S6 proteins

The homogenate of 61 brains of 11-day chick embryos was centrifuged. The supernatant contained 154 mg of protein and was fractionated by pH 5.5 precipitation. Although about 70% of the total pro...
tein was precipitated, S5 and S6 proteins were recovered in the supernatant. This supernatant (46 mg protein) was chromatographed on a DEAE-Sepharose column. Although most proteins were eluted with 0.25 M NaCl in SA buffer, S5 and S6 proteins remained adsorbed. They were eluted with 0.5 M NaCl in SA buffer. This eluate (2.3 mg protein) was fractionated by ammonium sulfate precipitation. S5 and S6 proteins were precipitated at 30% saturation. This precipitate was subjected to SDS-PAGE. S5 and S6 proteins separated on the gel were identified by 2DGE (Fig. 7). Eleven percent of the total protein in the 30% ammonium sulfate fraction was determined to be S5 protein and 14% was S6 protein by densitometric estimation of SDS-PAGE gel stained with CBB. S5 and S6 proteins were eluted from the gel of SDS-PAGE. Figure 8 shows SDS-PAGE of purified S5 protein and S6 protein.

The molecular weights of S5 and S6 proteins were 95K and 100K, respectively, and their isoelectric points were determined to be about 4.5 although S5 protein was slightly more acidic than S6 protein.

Structural homology of S5 and S6 proteins

S5 and S6 proteins were compared by gel electrophoresis peptide mapping using S. aureus V8 protease. They had at least 11 common peptides and 6 distinct ones (Fig. 9). This indicates a strong degree of structural homology between S5 and S6 proteins.

**DISCUSSION**

The present study has disclosed that eight proteins in the chick brain remarkably changed their abundance during development. They can be classified into three groups. The proteins of Group I (S8, S14, S30, and S54 proteins) appeared in the course of the development, then increased, and were present in the adult brain. S8 and S14 proteins belonging to this group were identified as neurofilament proteins. Neurofilament proteins in the rat brain were also reported to increase during development (Shaw and Weber, 1982). S30 protein increased with a similar time course to S8 and S14 proteins during development. It was not detected in the embryonic liver. Therefore S30 protein may also be a neuro-specific protein like neurofilament proteins (S8 and S14 proteins). The proteins of Group II (S7 and S37 proteins) were found not only in the brain but also in the liver of the embryo, and they decreased during development. We suggest that these proteins are involved in some developmental function but are not specific for the nervous system.

Group III contains S5 and S6 proteins, which are characterized by their abundance at specific stages of the development. In the optic tectum of the chick embryo S5 protein increased from day 4 to day 7; thereafter it decreased. S6 protein, on the other hand, increased from day 7 to day 9, and decreased after day 18. These proteins increased at specific stages in all brain regions so far examined, but they were not detected in liver tissue at any embryonic stages. Therefore they are likely to be highly concentrated in the nervous system. Although they were present only in trace amounts in a chick embryo without the CNS tissue, this slight content of S5 and S6 proteins may be explained by the existence of the proteins in the peripheral nervous system.

In the optic tectum of the chick embryo the cell proliferation reaches maximum level on day 6 and then decreases (Cowan et al., 1968). From day 6 to
day 12 the differentiated cells migrate heavily from the ventricular zone, and the neurons grow their axons extensively from day 6 to day 18 (LaVall and Cowan, 1971a). Thus S5 protein is present when cells are proliferating and migrating, and S6 protein is present when neurons are extending their axons. The changes in the amount of S5 and S6 proteins occurred earlier in the rostral parts of the optic tectum than in the more caudal region, which is consistent with the rostrocaudal gradients of development in the optic tectum (LaVall and Cowan, 1971a). Further, in the cerebellum, where the proliferation and migration of cells continues later than in the optic tectum (Hanaway, 1967), S5 protein could be detected later than in the tectum. All of these findings suggest that S5 and S6 proteins have some relation to neuronal differentiation and network formation.

The peptide mapping evidence indicates that S5 and S6 proteins form a family of structurally related proteins. It is not yet clear whether the two proteins are each coded by unique, but homologous, mRNA species or the two forms arise as a result of post-translational modification of a single protein precursor. It will be of interest in future investigation to elucidate how the developmental changes of the two acidic proteins are regulated.

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REFERENCES


Cowen W. M., Martin A. H., and Winger E. (1968) Mitotic patterns in the optic tectum of the chick during normal devel-

FIG. 9. Peptide mapping of S5 protein (lane a) and S6 protein (lane b). Each protein spot was cut out from the two-dimensional gel of 30% ammonium sulfate precipitate and treated with S. aureus V8 protease (3.0 μg per slot). The peptides were separated on 15% acrylamide/bisacrylamide. (Open arrows), Intact S5 and S6 proteins; (closed arrows), distinct peptides; (lines), common peptides; (A), origin; (Δ), front of V8 protease band was stained diffusely in the 27,000-dalton region, where two other common peptides were also stained. Silver stain.