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ACTIN MOLECULES PROMOTE NEURITE OUTGROWTH OF CHICK TELENCEPHALIC NEURONS *IN VITRO*

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SUMMARY: Chick brain extract contains protein factors for neurite outgrowth from embryonic chick telencephalic neurons in dissociated cell cultures. In the course of purification of the factors using a bioassay system, a 41 kDa protein was detected to be one of the factors. Immunoblot analysis showed that this protein is identical to actin. In addition, G-actin purified from rabbit skeletal muscle exhibits neurite outgrowth activity in the same dose-dependent manner as the 41 kDa protein. The same bioassay carried with other purified proteins did not show such a remarkable activity.

Elucidating the mechanisms underlying morphogenesis of highly complicated neuronal networks containing specific connections is one of the most important challenges in neurobiological sciences today. Although the mechanisms of brain construction are yet almost unknown, the trophic factor hypothesis (1-4) is widely accepted, providing a plausible basis for a theory of brain development. Our studies on trophic factors have shown that certain factors derived from chick muscle extracts exclusively promote survival of and neurite outgrowth from telencephalic neurons in primary dissociated cell cultures of chick embryonic brain (5). Due to spatial separation, however, it is not probable that these factors direct telencephalic neuronal morphogenesis.

As a more plausible hypothesis, we assumed that proteins similar to the muscle-derived factors exist in or near the developing brain and function in neuronal morphogenesis. When brain-derived extracts were prepared from telencephalic areas of embryonic chick brain, these extracts were found to contain almost the same neurite promoting activity (5). We could detect several fractions showing the activity after partial purification of the factors. In this report, it is shown that one of these fractions contains actin as a major component and actin molecules promote neurite outgrowth from chick telencephalic neurons *in vitro*.

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MATERIALS AND METHODS

Neuronal cell culture:Telencephalic regions from embryos of chick (White leghorn) were dissected out in Ca^{2+},Mg^{2+}-free phosphate buffer saline supplemented 10 mM glucose. These embryos develop to the stage 25 defined by Hamburger and Hamilton (6). The tissue from 2 embryos were treated with 0.125% trypsin (Gibco) in the same medium for 30 min at 37°C. These tissues were then rinsed in Ham's F12 medium supplemented glucose (10 mM), glutamin (2 mM), insulin (2.5 μg/ml) and antibiotics, and dissociated in this medium by gentle trituration using a disposable plastic pipette (Falcon). Dissociated telencephalic neurons ($5x10^4$ cells per well) were plated in 16-mm wells containing 0.4 ml of the same F12 medium. Neurons were cultured at 37°C in 5% CO_2 and saturating humidity.

Preparation of brain-derived extracts:Chick brain hemispheres dissected from embryos after 12-13 days incubation were mixed with 5 volumes of Hepes-Na buffer containing 10 % glycerol, 50 mM Hepes-Na (pH 7.0), 2 mM EGTA, 2 mM EDTA, and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain). These hemispheres were gently homogenized in ice using teflon homogenizer and the homogenate was centrifuged at 1.0×10^5 *g* for 1 h at 4°C. The clear supernatant was stored at -80°C before use.

Neurite outgrowth assay:Serial dilutions of brain extract were tested in duplicate for their effect on neurite outgrowth. Extract or a protein diluted with culture medium was added into the well of a culture dish, and incubated for 2 h at 37°C. The well was then washed several times with culture medium. After 24 hours of culture, the total number of surviving cells and the number of cells bearing one or more neurites more than twice the cell diameter in length were counted for quantification of neurite outgrowth.

Sodium dodecylsulfate polyacrylamide gel electrophoresis(SDS-PAGE):Electrophoresis was carried out on 12.5% polyacrylamide gels using the buffer system of Laemmli (7).The gels were stained with Coomassie Brilliant Blue R-250.

Immunoblot analysis:Proteins in a gel after SDS-PAGE were electrically transferred onto a nitrocellulose sheet using semi-dry transfer system. The sheet was incubated with 5% (w/v) skim milk and 5% (v/v) calf serum in 150 mM NaCl and 50 mM Tris, pH 7.6 for 1 h to block non-specific binding sites, and then washed with the NaCl-Tris buffer. The sheet was incubated with the antibodies in the same buffer for 3 h at room temperature.The antibodies bound to antigens on nitrocellulose sheet were reacted with horse radish peroxidase-conjugated anti-rabbit IgG and the proteins were visualized by incubation with dimethylaminoazobenzene and H_2O_2 after washing the sheet.

Protein concentration assay:Protein concentrations were determined by the method of Bradford (8) using the reagent of Bio-Rad.

RESULTS

Brain extract containing proteins at a concentration of 2.0 mg/ml was applied on a column of Sephadex G-25 (PD-10, Pharmacia) to change the buffer solution to 10 mM sodium phosphate buffer (pH 6.8) and then, 25% saturation (final) of ammonium sulfate was added. After incubation for 15 min at 4°C, this extract was centrifuged at 14,000 rpm for 15 min at 4°C using the microcentrifuge. The supernatant was carefully collected and filtered with the durapore membrane (Millipore). This clear supernatant was applied on a column for hydrophobic interaction

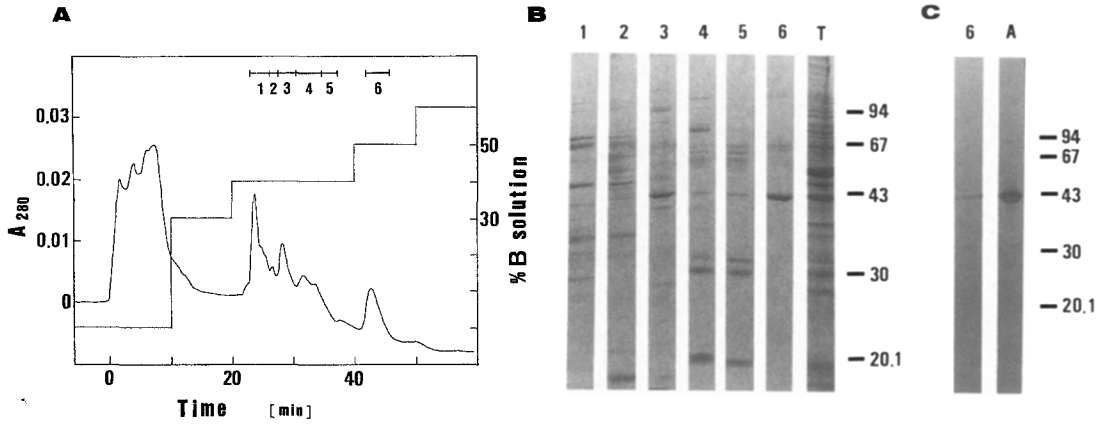

Fig.1 (A) Elution profile of hydrophobic interaction chromatography. Supernatant after precipitation with 25% ammonium sulfate containing 5 mg protein was applied to a column(7.5x75 mm), TSK Phenyl-5PW (Tosoh), equilibrated with A solution composed of 25% saturation of ammonium sulfate and 10 mM sodium phosphate buffer (pH 6.8). The proteins were eluted with a discontinuous gradient of B solution composed of 50%(v/v) ethylene glycol and 10 mM sodium phosphate buffer (pH 6.8), at the flow rate of 0.5 ml/min. Absorbance at 280 nm (curved line) and % B solution (step-like line) are shown in the figure. (B) SDS-PAGE (12.5% polyacrylamide gels) analysis of the eluates. The numbers on the top of the lanes correspond to these in Fig.1(A) and indicate the fraction numbers of the chromatography. The lane marked with T shows the total proteins applied on the column. The proteins on the gel were stained by Coomassie Brilliant Blue R-250. (C) Immunoblot analysis of fraction No.6 and purified G-actin. After SDS-PAGE of these samples, the proteins were electrophoretically blotted on a nitrocellulose filter. The proteins treated with a rabbit anti-actin antibody (BioMakor) and a second antibody conjugated with horse radish peroxidase were visualized by incubation with dimethylaminoazobenzene and H_2O_2. The numerals at the right side of Fig.1(B) and (C) indicate the molecular sizes (kDa) of standard proteins.

chromatography, TSK Phenyl-5PW (Tosoh), which was integrated in HPLC system (LKB) and equilibrated with A solution composed of 10 mM sodium phosphate buffer (pH 6.8) and 25 % saturation of ammonium sulfate. As shown in Fig.1A, proteins were eluted with a discontinuous gradient of B solution composed of 10 mM sodium phosphate buffer (pH 6.8) and 50 %(v/v) ethylene glycol. Of the eluates, four fractions, No.3, 4, 5 and 6, were found to contain neurite outgrowth activity. Fig.2B and Fig.3B show the neurite promoting activity in fraction No.6. The activity in the other active fractions was almost the same as that in fraction No.6. We decided to continue purifying the substance responsible for the activity in the fraction No.6 because of the simplicity of protein components.

SDS-PAGE elucidated that the major component in the fraction No.6 is a 41 kDa protein, as shown in Fig.1B. Since this protein is one of the major components in brain extract (Fig.1B), it is supposed to be a popular protein. Immunoblotting (Western blotting) using anti-actin polyclonal antibody (BioMakor) clearly revealed that the 41 kDa protein is identical to an actin molecule. A

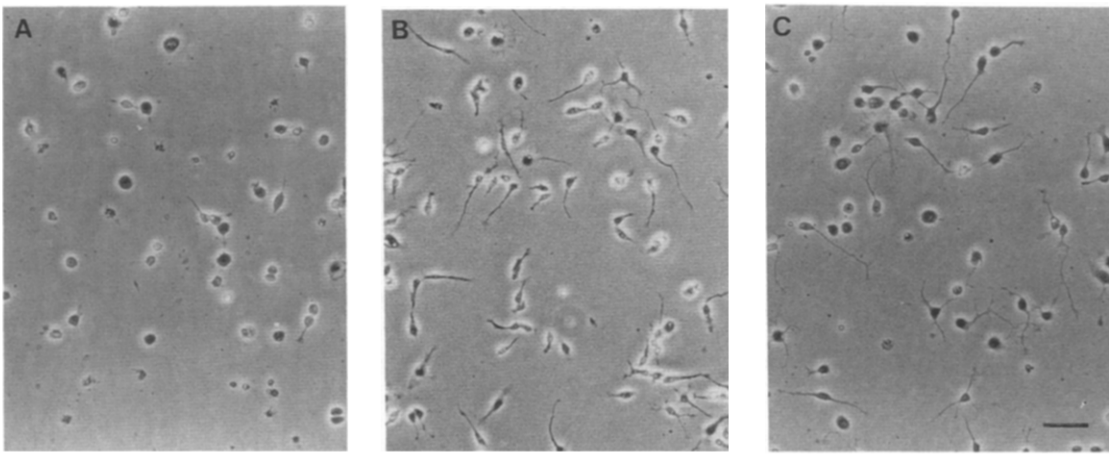

Fig.2. Neurite outgrowth of chick telencephalic neurons. Dissociated cells from telencephalons of E5 chick embryos were cultures for 24 hours on the 16 mm well of a cluster dish which absorbed no protein (A), 0.6 μg protein of the fraction No.6 shown in the previous figure (B) or 1.0 μg purified G-actin (C). The cell density in the well was 25,000/cm^2. The bar indicates 50 μm.

test using the *in vitro* assay system was made to confirm the result, whether purified G-actin could promote neurite outgrowth. Fig.2C and Fig.3 show that G-actin purified from rabbit skeletal muscle promotes neurite outgrowth from chick telencephalic neurons *in vitro* in the same dose-dependent manner as the fraction No.6.

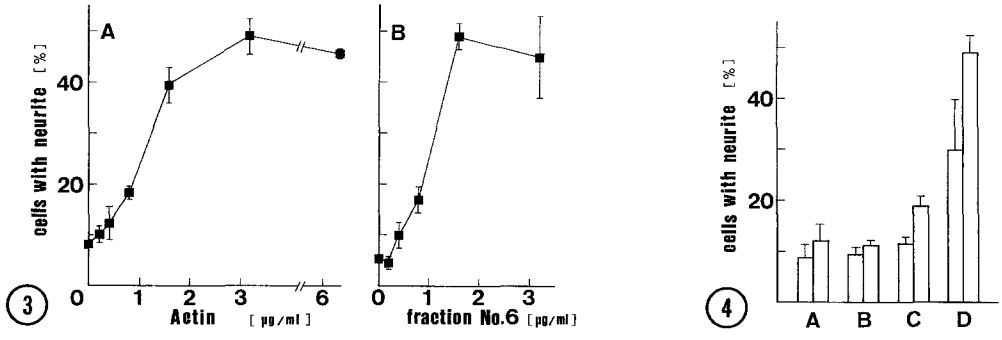

Fig.3. Dose-response curves for neurite outgrowth in response to the fraction No.6 and purified G-actin. Dissociated cells from chick embryonic telencephalons were cultured on the 16 mm well of a cluster dish which absorbed various amounts of the fraction No.6 (A) or purified G-actin (B). After 24 hours of culture, the total number of surviving cells and the number of cells with neurite in 3 microscopic fields per well were used to calculate % cells with neurite (mean ± S.D., n=4 wells). The total number of cells are normally about 400 per well.

Fig.4. Effect of purified proteins on neurite outgrowth. Dissociated neurons from chick embryonic telencephalons were cultured on the 16 mm well of a cluster dish which absorbed bovine serum albumin (A), ovotransferin (B), insulin (C) or G-actin (D). The ratios of cells with neurite after 24 hours of culture were calculated and described in Fig.3 (mean ± S.D., n=4 wells). The left and right columns for each protein indicate the ratios in the wells which absorbed 0.4 μg protein and 2.0 μg protein, respectively.

These results were, furthermore, supported by the affinity chromatography of the fraction No.6 using deoxyribonuclease I (DNase I)-conjugated Sepharose 4B. The content of the 41 kDa protein in the fraction No.6 which passed through the affinity column decreased extremely and the neurite outgrowth activity in this fraction diminished to almost the level of control experiments (data not shown). In addition, both G-actin and fraction No.6 had no activity after freezing and thawing (data not shown).

To eliminate the possibility that the activity is a general property of proteins, neurite outgrowth assays were performed with other proteins such as bovine serum albumin, ovotransferin, and insulin. These proteins did not show the activity as actin molecules in the range of protein concentrations to at least 5 μg/ml (Fig.4).

DISCUSSION

Laminin is a protein which is well-known for having the potent neurite promoting activity. In the previous study (5), it was shown that this protein is also active in the same *in vitro* assay system using chick embryonic telencephalic neurons. A small region of 5 amino acids, IKVAV, in A chain of laminin molecule was found to be a minimum unit for neurite outgrowth activity (9). Although no identical amino-acid sequence exists in the actin molecule, three similar sequences which contain one positively charged amino acid and four hydrophobic amino acids are found in actin at the positions of 27-31 (PRAVF), 94-98 (LRVAP) and 326-330 (IKIIA). These oligopeptides are located in the surface areas of actin by X-ray analysis (10). These regions can, therefore, be considered to be responsible for the novel function of G-actin *in vitro*.

We think that actin shows the neurite outgrowth activity as monomer form (G-actin) for two reasons: firstly, the concentration of actin during the incubation on the culture dish surface was at most 5 μg/ml which is lower than critical actin concentration for polymerization (11); secondly, absorbed actin in distilled water with extremely low ionic strength showed comparative activity for the neurite outgrowth (data not shown).

From these results, it is evident that actin molecules promote neurite outgrowth from chick telencephalic neurons *in vitro*. However, it is not probable that actin plays the same physiological role *in vivo* since there is no evidence that a trace amount of actin is localized in extracellular regions, nor that an actin gene and/or an actin-like pseudogene in chromosomes is linked with a signal sequence.

Chick brain extract passed through a column of DNase I-coupled Sepharose 4B still retained the activity, whereas the content of 41 kDa protein substantially decreased (data not shown). This

result indicates that (a) certain protein(s) other than actin, promote(s) neurite outgrowth in the extract and further purification of the factor is now in progress.

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