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IN VITRO SYNTHESIS OF NERVE GROWTH FACTOR RELATED GLIOMA C_6 CELL POLYPEPTIDES

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SUMMARY: Rat glioma C_6 cell polyribosomal preparations were tested in a heterologous in vitro system for their ability to direct the synthesis of nerve growth factor related polypeptides. Two major polypeptides of MW ~ 21,000 and ~ 43,000 respectively were found, both of which were immunoprecipitable with specific anti-mouse 2.5S nerve growth factor serum. After incubation of in vitro synthesized proteins with submaxillary gland extract the bulk of these protein species was converted into immunoprecipitable material of MW ~ 13,000, which comigrated in sodium dodecyl sulfate/polyacrylamide gel electrophoresis with mouse 2.5S nerve growth factor.

Nerve growth factor (NGF)[1], a hormone like protein found in vertebrates, is thought to effect the morphological and metabolic differentiation of sympathetic neurons and the development of some sensory neurons in a pleiotypic manner (1,2). NGF can be isolated from its principal source, the adult male mouse submaxillary gland, as part of a high MW complex, 7S NGF (3), but all the NGF-stimulating activity resides in a smaller subunit of MW ~ 26,000. This subunit, called 2.5S NGF, is a dimer of two identical noncovalently linked polypeptide chains, each one having a MW of 13,000 (4). Like other secretory proteins (5), submaxillary gland NGF is initially synthesized as precurser. Berger and Shooter (6) identified a 22,000 MW protein as direct precurser of the 13,000 MW NGF polypeptide, and their results were indicative of a second, even larger precurser.

[1] Abbreviations: NGF, nerve growth factor;
SDS, sodium dodecyl sulfate.

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A variety of in vitro cultured cell lines including transformed and untransformed fibroblasts (7,8), neuroblastoma (9) and glioma cells (10) has been shown to synthesize and secrete biologically active NGFs. Although the high MW NGF complexes secreted by these cell cultures generally differ in molecular properties from those of submaxillary gland 7S NGF, at least one of them, L-cell NGF, has been shown to contain as part of its structure a molecule that is closely similar in size and electrophoretic properties to 2.5S NGF (11).

An in vitro protein synthesizing system programmed by components of established cultured cell lines would be of advantage for studying the synthesis and processing of NGF molecules, particularly in view of possible cell type specific regulatory mechanisms. In the present report we show that polyribosomes isolated from rat glioma C_6 cells direct the synthesis of two polypeptides crossreacting with antibody to mouse 2.5S NGF which both are bigger in size than the 2.5S NGF polypeptide chains.

MATERIALS AND METHODS

A mixture of 15 uniformly labeled ^{14}C-amino acids (^{14}C-mix), ^{14}C-leucine (312 mCi/mmol), ^{14}C-aspartic acid (208 mCi/mmol), ^{14}C-glutamic acid (250 mCi/mmol), and ^{14}C-alanine (10 mCi/mmol) were obtained from New England Nuclear Corp. The preparation of rabbit anti-mouse submaxillary gland 2.5S NGF serum is described in (12), that of ^{125}J-2.5S NGF in (13). Goat anti-rabbit IgG serum was purchased from Miles Laboratories, Inc., and ribonuclease from Sigma Chemical Co.

Preparation of polyribosomes: Rat glioma C_6 cells (14), purchased from American Type Culture Collection, were grown in roller bottles supplemented with Dulbecco's modified Eagle medium, 10 units/ml of penicillin, 10 µg/ml of streptomycin sulfate, and 10% fetal calf serum. Six hours prior to harvest the original growth medium was routinely replaced with fresh one. For lysis, cells were chilled in ice, washed free of growth medium, and treated with 15 ml/bottle of 50 mM tris/HCl, pH 7.4, 100 mM KCl, 5mM $MgCl_2$ and 0.5% NP40 (Shell Chemical Company) while still attached to the roller bottles (15). Lysates were centrifuged at 12,000 x g and 4° for 10 min, supernatants layered on discontinuous sucrose gradients (0.5 and 0.2 M sucrose in lysis buffer without NP40), and the ribosomal material was pelleted at 100,000 x g and 4° for 4 h.

Preparation of submaxillary gland cell extract: Freshly excised mouse submaxillary glands were homogenized in 5 vol 50 mM tris/HCl, pH 7.4, 5 mM $MgCl_2$ and 100 mM KCl at 4°, and the homogenate centrifuged at 15,000 x g and 4° for 20 min. The resulting supernatant was incubated with ribonuclease (2.5 mg/ml) at 37° for 15 min and stored at -20° until use.

In vitro protein synthesis: The protein synthesis reaction mixture contained per ml: 50 µmole tris/HCl, pH 7.4, 5 µmole $MgCl_2$, 100 µmole KCl, 0.25 µmole DTT, 1 µmole ATP, 1 µmole GTP, 20 µmole creatine phosphate, 100 µg creatine kinase, 500 µg rat liver transfer RNA (General Biochemical), 0.4 A_{280} units rat brain microsomal wash fraction (16), 4.5 A_{280} units of As_{70} rat brain fraction (16), ^{24} A_{260} units C_6 polyribosomes, 6 µCi ^{14}C-amino acid mix, 2 µCi ^{14}C-leucine, 2 µCi ^{14}C-aspartic acid, 2 µCi ^{14}C-glutamic acid, 0.2 µCi ^{14}C-alanine and 100 nmole of each: asparagine, methionine, glutamine, tryptophan and cysteine. Incubations were performed at 37° for 90 min, mixtures subsequently centrifuged at 100,000 x g for 120 min and supernatants tested for material crossreacting with NGF antiserum.

Immunoprecipitation and SDS polyacrylamide gel electrophoresis: 300 µl-Aliquots of antigen containing solutions were incubated with 6 µl rabbit anti-NGF serum (100 mg lyophilized serum/ml HOH) first at 37° for 30 min and then at 4° overnight. After the addition of 30 µl goat anti-rabbit IgG serum a further incubation was performed at 37° for 30 min and in ice for 4 h. The resulting immunoprecipitates were washed 3 x with 0.15 M NaCl, 10 mM phosphate buffer, pH 7.0, and subsequently dissolved in 200 µl of 2% SDS, 50 mM dithiothreitol, 10 mM sodium phosphate buffer, pH 7.0. The samples were then incubated at room temperature for 3 h in the dark under reducing atmosphere, subsequently treated with 100 µl 1 M iodoacetic acid, pH 7.0, at room temperature for 5 min and finally supplemented with 70 µl ß-mercaptoethanol. They were then dialyzed against water, lyophilized and the dried residues dissolved in 2% SDS, 5% ß-mercaptoethanol, 10 mM phosphate buffer, pH 7.0. Prior to electrophoresis the samples were heated at 90° for 5 min. Electrophoresis was performed on SDS-10%-polyacrylamide gels according to the method of Weber and Osborn (17). Gels were scanned at A_{280}, sliced, and counted for radioactivity after Protosol treatment (15).

RESULTS AND DISCUSSION

The mRNA of rat glioma C_6 cell polyribosomal preparations was translated in a highly efficient heterologous cell free system that was originally developed (15) for translating neuroblastoma polyribosomal mRNA. The synthesis of NGF related products was tested by treating 100,000 x g supernatants of reaction mixtures with rabbit anti-NGF serum, subsequent quantitative precipitation of antigen- antibody complexes with goat anti-rabbit IgG serum and final analysis of the precipitates by SDS polyacryamide gel electrophoresis.

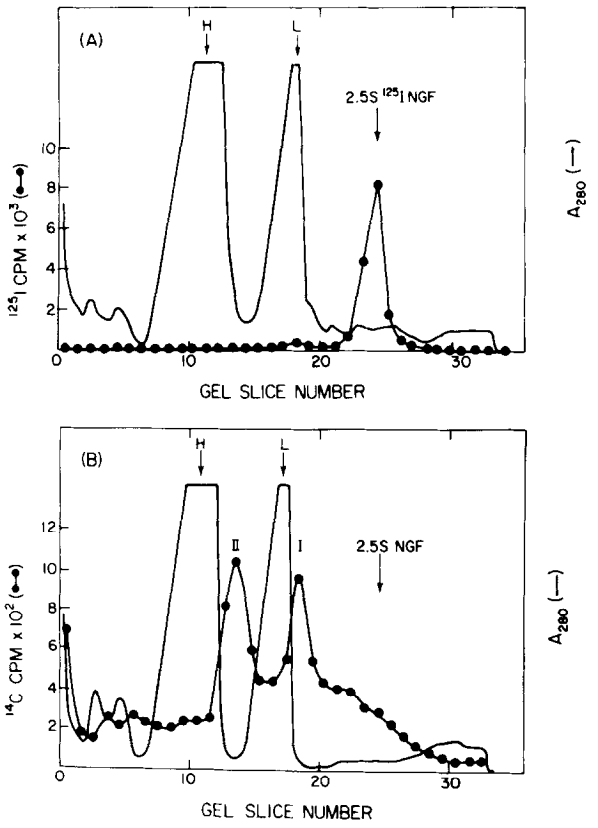

Fig. 1: Electrophoresis of immunoprecipitated *in vitro* synthesized glioma C6 cell proteins and 2.5S NGF.

300 µl-Aliquots of 100,000 x g supernatant fractions of *in vitro* synthesis reaction mixtures or solutions containing 5 x 10^4 dpm ^{125}J-NGF (13) were incubated with anti-2.5S NGF serum. Immunoprecipitates were isolated and analysed by SDS polyacrylamide gel electrophoresis as described in the text. A) ^{125}J-2.5S NGF, B) *in vitro* synthesized proteins. L and H indicate the position of light and heavy immunoglobulin chains.

Fig. 1B shows immunoprecipitated *in vitro* synthesized glioma C_6 cell proteins. Neither one of the two principal peaks observed migrated at the position of submaxillary gland 2.5S NGF (Fig. 1A). At that position only a shoulder of *in vitro* synthesized protein material was observed. As judged by their relative position to the heavy (MW ~53,000) and light (MW ~23,000) immunoglobulin, and the 2.5S NGF polypeptide chains, the material of peak I had an apparent MW of ~21,000, that of peak II ~43,000.

As revealed by control experiments (data not shown), about 30% of the peak II material aggregated without the addition of antiserum. This material probably consisted of actin and tubulin because these proteins have a tendency to form aggregates under the protein synthesis conditions used (15), and furthermore show similar electrophoretic mobilities as the peak II material.

Extracts of submaxillary gland cells can be considered as likely sources of NGF specific proteolytic cleavage activities. In order to test whether the anti-NGF crossreacting *in vitro* synthesized C_6 cell proteins could be proteolytically converted to the size of 2.5S NGF, *in vitro* protein synthesis reaction mixtures were therefore incubated with submaxillary gland extracts prior to immunoprecipitation. As shown in Fig. 2, this treatment in fact caused the disappearance of all the peak II and most of the peak I material. A new peak which became apparent comigrated with the polypeptide chains of submaxillary gland 2.5S NGF as well as immunoprecipitating material extracted from radioactively labeled C_6 cells (Fig. 2, lower curve).

These results provided preliminary evidence that the two *in vitro* synthesized polypeptides with MW of ~21,000 and ~43,000 respectively were indeed precursers of a smaller glioma C_6 cell NGF of MW ~13,000. The smaller one of these putative precursers corresponded in size to a NGF precurser found in submaxillary glands by Berger and Shooter (6). A correlate to the larger precurser has not been shown thus far in any other system, although some indirect evidence suggested the occurrence of a second precurser in submaxillary glands (6). The large size (about twice that of the smaller precurser) makes this second precurser distinct from "pre-pro" precurser forms of other secretory proteins, which were found to exceed "pro"

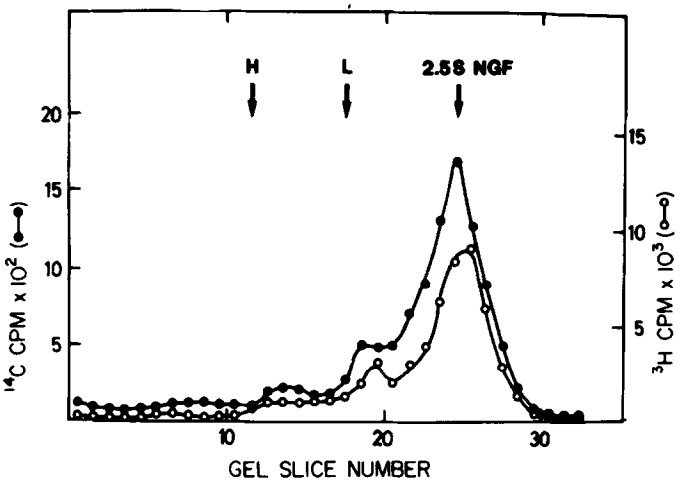

Fig. 2: Electrophoresis of immunoprecipitated in vitro and in vivo synthesized C_6 cell proteins after incubation with submaxillary gland extract.

For in vivo labeling with radioactive precursers, cells were grown in medium supplemented with 5 μCi/ml of ^{3}H-aminoacid mixture for 5 days. They were then harvested, homogenized and a a soluble extract was prepared by centrifugation at 17,000 x g for 30 min. 300 μl-Aliquots of this extract and of a 100,000 x g supernatant fraction of an in vitro protein synthesis reaction mixture were incubated separately with 100 μl of a submaxillary gland extract at 37° for 30 min. Mixtures were then spun at 15,000 x g for 30 min and supernatants incubated with anti-2.5S NGF serum as described above. The positions of light and heavy IgG chains and that of 2.5S NGF, run in parallel, are indicated by arrows. Proteins synthesized in vivo (o--o), in vitro (●--●).

forms in size by only 15 - 30 aminoacids (18,19). The in vitro protein synthesis system described above should be useful for studying the processing of these NGF-related glioma C_6 cell polypeptides.

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