Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 116-122

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF BIOLOGICALLY ACTIVE RECOMBINANT HUMAN NERVE GROWTH FACTOR

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Received July 9, 1990

SUMMARY. The human NGF gene was isolated and inserted downstream from murine leukemia virus LTR in a plasmid having dihydrofolate reductase cDNA. The expression plasmid was introduced into CHO cells. Selection of the transformants for the resistance to methotrexate gave a CHO cell line which produced human NGF at a level of 4mg/L in the culture medium. The recombinant human NGF was purified to near homogeneity from the culture supernatant. The NH_2-terminal amino acid sequence, the COOH-terminal amino acid (Ala), and the amino acid composition of the human NGF were identical to those deduced from the nucleotide sequence of the human NGF gene. The recombinant human NGF was composed of 120 amino acid residues. Three disulfide likages were determined to be Cys^{15}-Cys^{80}, Cys^{58}-Cys^{108}, and Cys^{68}-Cys^{110}; the locations were identical to those in the mouse 2.5S NGF molecule. The specific biological activity of the recombinant human NGF was comparable with that of authentic mouse 2.5S NGF as determined by stimulation of neurite outgrowth from PC12 cells.

Nerve growth factor (NGF) is a protein essential for the development and maintenance of sympathetic and sensory neurons in the peripheral nervous system(1,2). Recent studies have shown that it may also be important for the development and maintenance of cholinergic neurons of the basal forebrain(3,4,5). NGF has been isolated from mouse submaxillary gland as β-subunit (β-NGF) which is composed of two identical polypeptides of 118 amino acid recidues.The amino acid sequence of β-NGF has been determined(6,7).

Little is known about human NGF (hNGF) presumably because of its low content in human tissues. The hNGF gene was cloned by Ullrich et al.(8). The amino acid sequence of hNGF deduced from the nucleotide sequence exhibits 90% similarity with that of mouse β-NGF(8). Although several papers have appeared so far describing the expression of the hNGF gene by E.coli, S.cerevisiae and COS cells(9,10,11), the productivity of these

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0006-291X/90 $1.50

organisms and the specific biological activity of the products were extremely low. Therefore, the physicochemical properties of these recombinant hNGF proteins have remained unclear. No papers have described the production of biologically active hNGF in an amount enough to investigate its protein-chemical or biological nature in detail.

In this paper, we describe the production of recombinant hNGF by Chinese hamster ovary (CHO) cells and its purification and characterization.

MATERIALS AND METHODS

Materials: Mouse 2.5S NGF was purchased from Wako Pure Chemical Industries, Ltd.. CHO cells were obtained through Dr.P.Berg. PC12 cells were purchased from the American Type Culture Collection.

Construction of expression plasmid: The hNGF gene was isolated from the Lambda EMBL3 human leukocyte genomic library(Clontech) using a 0.38kb synthetic DNA encoding hNGF(8) as a probe. A positive clone having a 15kb insert was obtained. The nucleotide sequence of an open reading frame found in the insert was identical to that of the open reading frame encoding the prepro-NGF (from Met^{-121} to Ala^{120}) reported by Ullrich et al.(8). A 0.8kb BclI-ApaI fragment encoding the COOH-terminal half of the pre-peptide,pro-peptide and mature NGF was isolated from the insert and ligated with a synthetic DNA encoding the NH_2-terminal half of the pre-peptide. The resulting fragment containing the intact hNGF gene was inserted downstream from the murine leukemia virus(MuLV) LTR in the plasmid pTB399(12) ,from which the interleukin-2 cDNA had been removed, to give plasmid pTB1054. A 2kb ClaI fragment containing the MuLV LTR and hNGF gene was isolated from pTB1054 and inserted into the ClaI site in the plasmid pTB348(12) having the dihydrofolate reductase(DHFR) cDNA to give an expression plasmid pTB1058 (Fig.1).

Culture conditions: Transformed CHO cells were grown in Dulbecco's modified Eagle's medium(DMEM) containing 5% fetal calf serum,35μg/ml proline,50IU/ml penicillin,and 50μg/ml streptomycin at 37°C in 5% CO_2.

Purification of recombinant hNGF: (p-Amidinophenyl)methanesulfonyl fluoride hydrochloride was added to the culture supernatant at a final concentration of 0.1mM. The culture supernatant was adjusted to pH6.0 with 0.2N acetic acid and centrifuged. The supernatant was applied to a S-Sepharose column (2.5X15cm) equilibrated with 0.1M phosphate buffer(pH6.0)-1mM EDTA. The column was washed with 0.1M phosphate buffer(pH6.0)-1mM EDTA-10% glycerol-0.15M NaCl, and then the recombinant hNGF was eluted with 20mM Tris-HCl (pH7.4)-1mM EDTA-10% glycerol-0.7M NaCl. Fractions containing hNGF were

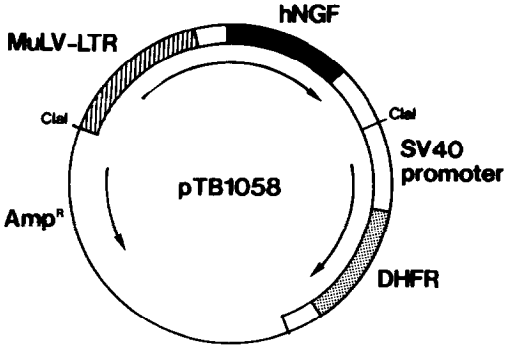

Fig.1. Structure of the expression plasmid for hNGF gene. Arrows indicate the direction of transcription.

combined and concentrated by ultrafiltration with an Amicon YM10 filter. The concentrate was applied to a Sephacryl S-100 HR (1.6X90cm) column equilibrated with 20mM Tris-HCl(pH7.4)-1mM EDTA-10% glycerol-0.7M NaCl, and the active entity was eluted with the same buffer. Fractions containing hNGF were combined and concentrated with Centriprep 10 (Amicon). The concentrate was subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on Asahipak ODP-50 (0.46X15cm) with a linear gradient of 0-90% acetonitrile containing 0.1% trifluoroacetic acid. The eluate containing hNGF was concentrated in vacuo to give purified hNGF as white powder.

Enzyme immunoassay(EIA): EIA was performed as described by Heinrich and Meyer(13).The purified hNGF obtained in this study was used as standard. The weight of the standard hNGF was determined by amino acid analysis.

Protein determination: Protein content was determined by BCA protein assay (Pierce) with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli(14).

Estimation of isoelectric point: The isoelectric point was estimated with an LKB 2117 multiphore II electrophoresis unit (Pharmacia-LKB) according to the directions of the supplier.

NH_2-terminal amino acid sequence, COOH-terminal amino acid, and amino acid composition: The NH_2-terminal amino acid sequence was determined with a gas phase protein sequencer (Model 470A,Applied Biosystems). The COOH-terminal amino acid was determined by hydrazinolysis(15). The amino acid composition was determined after hydrolysis for 24 hr with 5.7N HCl at 110°C in the presence of 4% thioglycolic acid. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer.

Determination of disulfide linkages: After digestion of purified hNGF with pepsin, the reaction mixture was loaded on a RP-HPLC column, TSK-gel ODS-120T (Tosoh)in the presence or absence of 20mM dithiothreitol, and the fragments were eluted by increasing acetonitrile concentration in the presence of trifluoroacetic acid. Peptide fragments containing disulfide linkages were determined by comparing the elution patterns. The fragments were further digested with thermolysin and the resulting smaller peptide frements were isolated by HPLC. The amino acid sequences and amino acid compositions of the cystine-containing peptides were determined.

Biological assay: Biological activity was assayed using PC12 cells as described by Stephani et al.(16) with some modifications.

RESULTS AND DISCUSSION

Establishment of the CHO cells producing hNGF

CHO $DHFR^-$ cells were transformed with pTB1058 by the calcium-phosphate coprecipitation method(17). $DHFR^+$ clones found to produce hNGF were then stepwise subcultured in the media containing 10nM, 100nM, 1μM, and 10μM methotrexate. A cell line, CHO-D31-10, obtained by this selection produced hNGF at a level of 4mg/L in the culture medium on the 7th day of growth.

Purification of recombinant hNGF

The cell line CHO-D31-10 was seeded at a density of $2.5X10^4$ cells/cm^2 and grown on a large scale. The recombinant hNGF was purified from the culture supernatant (2.2L) as described in MATERIALS AND METHODS. A summary of the purification is shown in Table 1. The amount of purified hNGF obtained was 1.6mg as determined by EIA. The value nearly accorded with that obtained from protein determination (1.3mg). SDS-polyacrylamide gel electrophoresis

Table 1. Summary of the purification of recombinant hNGF

Step	Volume (ml)	Total protein[1] (mg)	Total hNGF[2] (mg)	Yield (%)
Culture supernatant	2,200	11,000	5.3	100
S-Sepharose eluate	4.5	24	5.0	94
Sephacryl S-100 eluate	2.5	3.7	4.7	89
RP-HPLC eluate		1.3	1.6	30

1) Determined by BCA protein assay.
2) Determined by EIA.

(Fig.2) and RP-HPLC showed that the purity of the recombinant hNGF was more than 95%.

Properties of recombinant hNGF

The molecular weight of the recombinant hNGF was estimated to be 13,000 by SDS-polyacrylamide gel electrophoresis under reducing conditions. The hNGF migrated a little more slowly than did authentic mouse 2.5S NGF(Fig.2).

The isoelectric point of the recombinant hNGF was between 9 and 10; the mobility was nearly the same as that of the authentic mouse 2.5S NGF.

The NH_2-terminal amino acid sequence of the recombinant hNGF (NH_2-Ser-Ser-Ser-His-Pro-Ile-Phe-His-Arg-Gly-) was identical to that deduced from the nucleotide sequence of the hNGF gene(8). The COOH-terminal amino acid was determined to be Ala by hydrazinolysis, indicating that the recombinant hNGF has an additional two amino acid residues (Arg^{119}-Ala^{120}) at the COOH-terminus of the protein (118 amino acid residues) which Ullrich at al. proposed as mature hNGF based on the homology with mouse β-NGF (8). The amino acid composition of the purified hNGF was in fair agreement with that calculated from the deduced amino acid sequence consisting of 120 amino acid residues (Table 2).

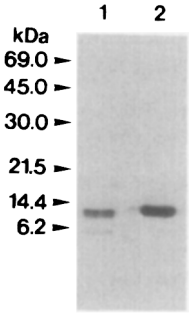

Fig.2. SDS-polyacrylamide gel electrophoresis under reducing conditions. Samples were (1) mouse 2.5S NGF and (2) recombinant hNGF(0.2 μg). Proteins were detected by silver staining. The sizes (kDa) of the standard proteins are shown on the left.

Table 2. Amino acid composition of hNGF

	Residues per molecule	
	Found	Calculated
Asx	13.0	13
Thr	9.7	10
Ser	9.2	11
Glx	6.6	6
Pro	2.9	3
Gly	7.3	7
Ala	6.9	7
Cys	ND	6
Val	12.8	13
Met	2.1	2
Ile	6.1	6
Leu	3.2	3
Tyr	2.3	2
Phe	7.3	7
Lys	9.1	9
His	3.9	4
Arg	7.3	8
Trp	3.0	3
Total		120

ND, Not determined.

On digestion with pepsin, a single peptide containing all the three disulfide linkages were isolated. On further digestion of this peptide with thermolysin, three fragments (fragments 1, 2, and 3) were obtained, each containing a disulfide linkage. The amino acid sequence of each fragment was determined. Three disulfide likages were found to be located at Cys^{15}-Cys^{80}, Cys^{58}-Cys^{108}, and Cys^{68}-Cys^{110} (Table 3). The presence of two cysteine residues in each fragment was confirmed by amino acid analysis employing the hydrolysis in thioglycolic acid (data not shown). The locations of the three disulside likages were identical to those in the

Table 3. Amino acid sequence of cystine-containing peptides

cycle	Fragment 1 residue (pmole)	Fragment 2 residue (pmole)	Fragment 3 residue (pmole)
1	Ser (60)	Phe (90) Ala (86)	Val (115)
2	Val (43) Tyr (43)	Glu (78)	Asp (70)
3	- -	Thr (45)	Ser (8)
4	Asp (43) Thr (28)	Lys (62)	Gly (47)
5	Ser (17)	-	-
6		Arg (78)	Arg (27)
7		Asp (49)	Gly (24)
8		Pro (33)	
9		Asn (31)	
10		Pro (11)	

S^{13}-V-C^{15}-D-S^{17} | S^{78}-Y-C^{80}-T^{81}

F^{54}-E-T-K-C^{58}-R-D-P-N-P^{63} | A^{107}-C^{108}

V^{64}-D-S-G-C^{68}-R-G^{70} | V^{109}-C^{110}

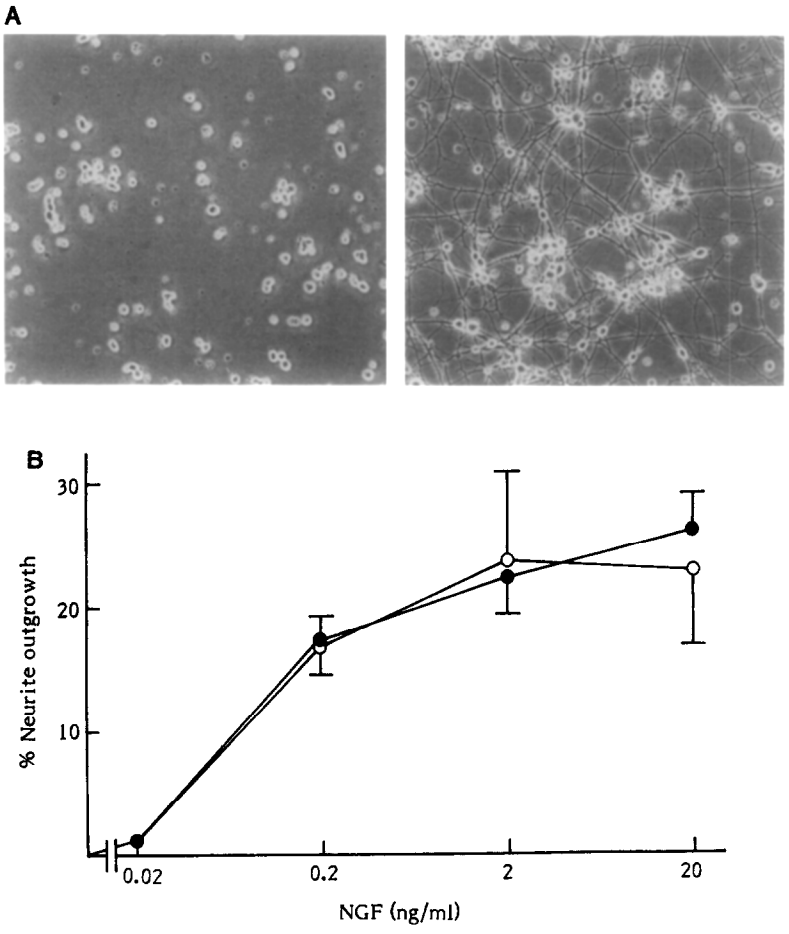

Fig.3. Biological activity of recombinant hNGF. (A) PC12 cells grown for 10 days in the absence(left) or presence(right) of hNGF(50ng/ml). (B) Dose-response curves for hNGF and mouse NGF. The primed PC12 cells were grown for 2 days in the presence of hNGF(closed circle) or mouse 2.5S NGF(open circle), and the numbers of cells with neurites were counted. The experiments were carried out in duplicate.

mouse NGF molecule (18,19), suggesting that the recombinant hNGF assumes the correct tertiary structure. The molecular weight of the recombinant hNGF was calculated to be 13,489.

Biological activity of recombinant hNGF

The recombinant hNGF stimulated neurite outgrowth from PC12 cells (Fig.3A). The activity was as high as that of the authentic mouse 2.5S NGF (Fig.3B). It also supported the survival of sensory neurons isolated from dorsal root ganglion of embryonic chicks (data not shown). The specific biological activity of recombinant mouse NGF and hNGF produced by E.coli (20) and S.cerevisiae (10),respectively, are reported to be 1/200-1/1000 of that of mouse NGF isolated from submaxillary gland(10,20). This may be due to incorrect folding of these recombinant proteins. In contrast, the

specific activity of the hNGF described here was comparable with that of authentic mouse 2.5S NGF. These results suggest that CHO cells produced the recombinant hNGF protein as a native form with a correct tertiary structure.

We have succeeded in producing substantial amounts of biologically active hNGF by the recombinant DNA technique using CHO cells. This will open the way for the basic and applied research on hNGF as a neurotrophic factor.

ACKNOWLEDGMENTS

We thank Dr.M.Nishikawa for his continued encouragement. Thanks are also due to Drs.K.Tsukamoto, M.Suno, K.Igarashi, T.Fukuda, K.Kato, and M.Takeuchi for helpful discussion and suggestions.

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