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Interaction of antibodies to synthetic peptides of proNGF with in vitro synthesized NGF precursors

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Sera raised against three synthetic peptides that reproduce sequences of the pro-nerve growth factor (proNGF) protein were tested in immunoprecipitation experiments using in vitro translation products of SP6-directed NGF mRNA in a rabbit reticulocyte lysate. The interaction of these antibodies with bacterially synthesized chimeric preproNGF was also examined. Digestion of the translation products by the γ-subunit generated the 22 and 18 kDa intermediates. A predominant 13 kDa intermediate was obtained after digestion of translation products in wheat germ extract. This is shown to be the N-terminal peptide by immunoprecipitation with an anti-peptide serum. These antibodies may be used to detect NGF precursor cleavage products in vivo.

Anti-peptide serum; Nerve growth factor, prepro-; Translation, in vitro; Immunoprecipitation; Processing

1. INTRODUCTION

The nucleotide sequence derived from the mouse submandibular gland (MSG) nerve growth factor (NGF) cDNA predicted an NGF precursor (prepro-NGF) of about 35 kDa which may be cleaved at dibasic residues to generate three additional peptides besides the NGF protein which is situated at the carboxyl terminus [1,2] (fig.1). Four different NGF mRNA species that diverge at their amino termini were identified, a result of alternative splicing and/or initiation from independent promotors [3]. They would generate, besides the 35 kDa precursor, another species of about 29 kDa.

Our antisera to NGF do not recognize the NGF precursor [4,5]. Therefore, in order to biochemically characterize this protein and to identify cell types that synthesize it, our strategy consisted of raising antibodies to synthetic peptides that reproduce sequences of this molecule. A protein of about 31 kDa, the putative NGF precursor, was immunoprecipitated by an anti-peptide serum in MSG and thyroid extracts [6]. Using affinity-purified immunoglobulins to these peptides, we localized proNGF-like immunoreactivity in the MSG [6,7], rat thyroid [6], and in neurons of the hippocampus, cortex and septum as well as in other regions of the rat brain [8].

In the present report we examined the interaction of these sera with in vitro synthesized NGF precursors from SP6-directed NGF mRNAs and with bacterially synthesized chimeric preproNGF.

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2. MATERIALS AND METHODS

Peptide N_2 corresponds to the sequence −163 to −139, N_4 to the sequence −71 to −45 and N_3 to the sequence −49 to −40 of the NGF precursor (see fig.3, [2]). Rabbits were immunized

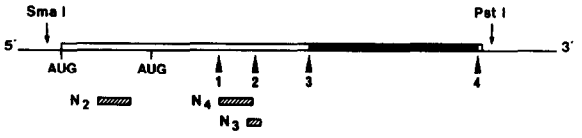

Fig.1. Schematic representation of preproNGF cDNA and protein. The box indicates the precursor protein, the solid box shows the βNGF and the thin line the untranslated regions. Arrows indicate possible cleavage sites. Hatched boxes correspond to epitopes reproduced by the synthetic peptides.

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with peptides coupled to keyhole limpet hemocyanin [7]. Titers of the sera were between 1×10^{-5} and 2×10^{-6} for their corresponding peptide as tested by ELISA techniques using preimmune sera for controls [7,9].

The *Sma*I-*Pst*I fragment of the mouse NGF cDNA (gift of Drs M. Selby and W. Rutter) was subcloned into the SP65 vector using standard techniques [10]. The recombinant DNA was linearized by *Pst*I and transcribed by SP6 polymerase (Promega/Biotec) according to the manufacturer's instructions. Capping of the NGF mRNA was done as in [11]. NGF mRNA was extracted with phenol/chloroform/isoamyl/alcohol, precipitated with ethanol and translated in a rabbit reticulocyte lysate or in a wheat germ extract (Promega/Biotec).

The bacterial clone expressing the chimeric preproNGF protein [9] was grown in M9 medium [10] with 1 mM IPTG and [^{35}S]sulfate (5 μCi/ml). Purification of the chimeric protein was done by APTG-Sepharose chromatography [9].

In vitro translation products of purified chimeric protein were incubated with 10 μl of serum in 300 μl of buffer A (0.1 M Tris, pH 8.2, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Na deoxycholate, 0.1% ovalbumin) and 100 μl of protein A-Sepharose with gentle agitation overnight at 4°C. The suspension was washed with 4 ml of buffer B (buffer A, 0.5 M LiCl). Elution of bound material, gel electrophoresis and autoradiography were done as before [4,6]. Digestion with the γ-subunit was done in 0.1 M Tris, pH 7.6, at 37°C for 1 h.

3. RESULTS AND DISCUSSION

In vitro translation of SP6-derived NGF mRNAs gave rise to two species of about 35 and 29 kDa which probably correspond to NGF precursors initiated from the first and the second AUG, respectively (fig.2, lane 2; fig.3, lane 4). Translational efficiency in the wheat germ extract was significantly reduced for uncapped NGF mRNA (fig.3, lane 3). These in vitro synthesized precursors constitute a convenient substrate to test the specificity of the sera to proNGF peptides. As shown in fig.2A, anti-N3 and anti-N4 sera immunoprecipitated both species (lanes 4 and 5) while the anti-N2 serum immunoprecipitated primarily the 35 kDa species (lane 6).

We previously described a bacterial clone synthesizing a β-galactosidase-fused preproNGF [9]. Purification by APTG-Sepharose from a bacterial extract yields the chimeric protein and a degradation product (fig.2B, lane 2). The anti-N2 serum

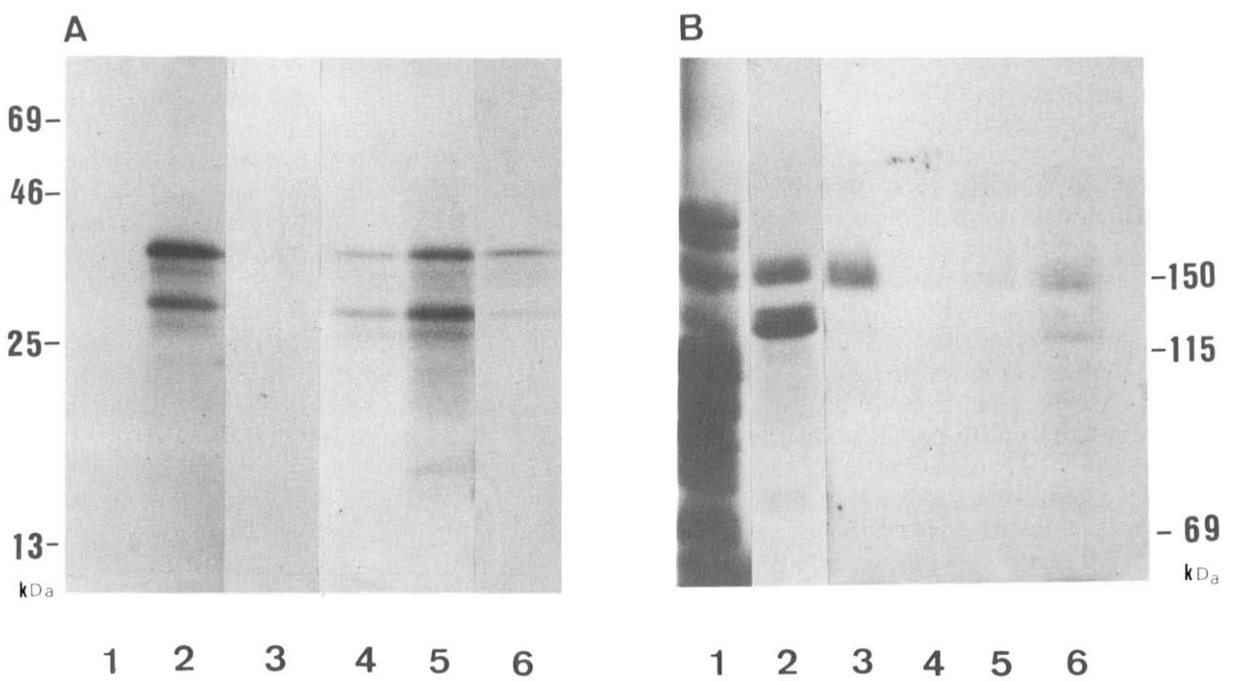

Fig.2. (A) Translation of SP6-directed NGF mRNAs in a rabbit reticulocyte lysate. Lanes: (1) no mRNA; (2) SP6-derived NGF mRNA; immunoprecipitation of translation products by: (3) preimmune serum; (4) anti-N3 serum; (5) anti-N4 serum; and (6) anti-N2 serum. (B) Lanes: (1) bacterial extract; (2) material purified by APTG-Sepharose chromatography; immunoprecipitation of 2 by: (3) anti-N2 serum; (4) preimmune serum; (5) anti-N4 serum; and (6) anti-N3 serum.

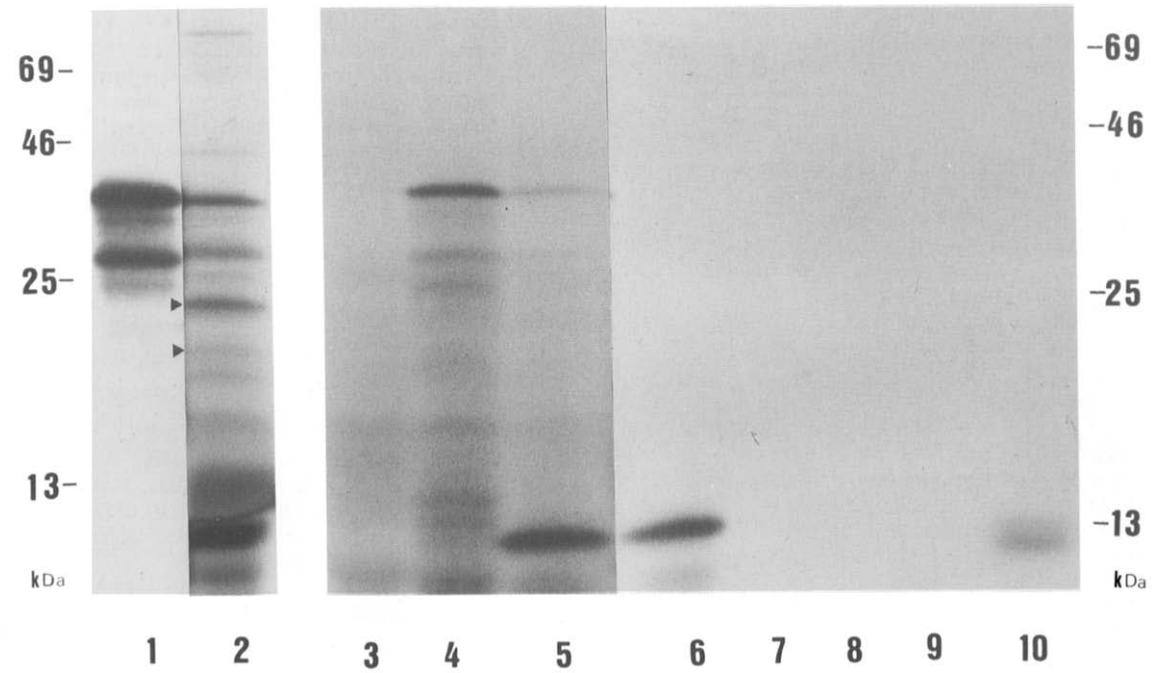

Fig.3. Processing of the in vitro translation products by the γ-subunit. Lanes: (1) translated SP6-derived NGF mRNA; (2) after digestion by the γ-subunit; (3) uncapped NGF mRNA translated in a wheat germ extract; (4) capped NGF mRNA translated as in 3; (5) after digestion by the γ-subunit; immunoprecipitation of the γ-subunit digests by: (6) anti-N2 serum; (7) anti-N4 serum; (8) anti-NGF serum; (9) preimmune serum; and (10) iodinated, purified βNGF.

(lane 3) and to a lesser degree the anti-N3 serum (lane 6) immunoprecipitated this protein, while the anti-N4 serum (lane 5) did not cross-react suggesting that the epitope reproduced by the N4 peptide is probably 'masked' in the chimeric protein. A comparable pattern of interaction was also observed when using ELISA techniques [9].

In the MSG, the NGF is associated with the γ-subunit [12,13], which is an arginine esteropeptidase and whose role in the maturation of proNGF is controversial. This is due to the fact that several cells or tissues which synthesize NGF do not synthesize the γ-subunit, e.g. mouse fibroblast L929 cells [14,15], mouse and rat iris [16] and the prostate of the guinea pig [17]. Most probably, in these systems a different protease than the γ-subunit is involved in the NGF processing. Digestion at cleavage sites 1, 2 and 3 would yield NGF-containing intermediates of 22, 18 and 13 kDa, respectively. Digestion of the NGF precursors synthesized in a reticulocyte lysate yielded the 22 and, to a lesser degree, the 18 kDa intermediates (fig.3, lane 2). Detection of the 13 kDa species is obscured by co-migration of lysate material in this area of the gel. Digestion of the NGF precursors synthesized in a wheat germ extract yielded primarily a 13 kDa peptide. This is not the βNGF but rather the N-terminal peptide cleaved at site 1, since it is immunoprecipitated by an anti-N2 serum (lane 6) but not by the anti-N4 or the anti-NGF sera (lanes 7,8). Digestion of the chimeric preproNGF with the γ-subunit did not liberate the βNGF protein either (results not shown). These results suggest that in vitro-synthesized or bacterially synthesized NGF precursors are abnormally folded and unspecifically degraded by the γ-subunit. Abnormal digestion of in vitro-synthesized NGF precursors was recently suggested by others [18,19]. However, in this report, using the anti-N2 serum, we provide direct evidence that the 13 kDa species generated by the γ-subunit is the N-terminal peptide and not the βNGF. These anti-peptide sera may therefore be useful to monitor NGF precursor-processed peptides in vivo.

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REFERENCES

[1] Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G.I. and Rutter, W.J. (1983) Nature 302, 538–540.
[2] Ullrich, A., Gray, A., Berman, C. and Dull, T.J. (1983) Nature 303, 821–825.
[3] Selby, M.J., Edwards, R., Sharp, F. and Rutter, W.J. (1987) Mol. Cell Biol. 7, 3057–3064.
[4] Dicou, E. and Brachet, P. (1984) Eur. J. Biochem. 143, 381–387.
[5] Wion, D., Dicou, E. and Brachet, P. (1984) FEBS Lett. 166, 104–108.
[6] Dicou, E., Lee, J. and Brachet, P. (1986) Proc. Natl. Acad. Sci. USA 83, 7084–7088.
[7] Dicou, E., Lee, J. and Brachet, P. (1988) Neurosci. Lett. 85, 19–23.
[8] Senut, M.-C., Lamour, Y., Lee, J., Brachet, P. and Dicou, E. (1989) Int. J. Dev. Neurosci., in press.
[9] Dicou, E., Houlgatte, R., Lee, J. and von Wilcken-Bergmann (1989) J. Neurosci. Res. 22, 13–19.
[10] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[11] Krieg, P.A. and Melton, D.A. (1984) Nucleic Acids Res. 12, 7057–7070.
[12] Greene, L.A., Shooter, E.M. and Varon, S. (1969) Biochemistry 8, 3735–3741.
[13] Bothwell, M.A. and Shooter, E.M. (1978) J. Biol. Chem. 253, 8458–8464.
[14] Dicou, E., Wion, D. and Brachet, P. (1983) C.R. Acad. Sci. Ser. C, 523–525.
[15] Pantazis, N.J. (1983) Biochemistry 22, 4264–4271.
[16] Murphy, R.A., Landis, S.C., Bernanke, J. and Siminoski, T. (1986) Dev. Biol. 114, 369–380.
[17] Rubin, J.S. and Bradshaw, R.A. (1981) J. Neurosci. Res. 6, 451–464.
[18] Edwards, R.M., Selby, M.J., Garcia, P.D. and Rutter, W.J. (1988) J. Biol. Chem. 263, 6810–6815.
[19] Jongstra-Bilen, J., Coblentz, L. and Shooter, E.M. (1989) Mol. Brain Res. 5, 159–169.