Synthesis and partial maturation of the α- and γ-subunits of the mouse submaxillary gland nerve growth factor in *Xenopus laevis* oocytes

Didier Wion, Eleni Dicou and Philippe Brachet

Unité de Différenciation Cellulaire, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cédex 15, France

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Sera raised against the α-, β- and γ-subunits of the mouse 7 S NGF were used to characterize translation products coded by submaxillary gland mRNAs microinjected into *Xenopus* oocytes. Anti-βNGF sera did not cross-react with any material. In contrast, the precursors of the α- and γ-subunits, as well as that of renin were identified. Use of tunicamycin, and a comparison of the translation products obtained in oocytes or in the reticulocyte lysate indicated that oocytes achieved the cleavage of signal sequences, the glycosylation of the α- and γ-precursors, and the subsequent secretion of the 3 proteins. In the submaxillary gland, however, the mature forms of αNGF, γNGF and renin are composed of peptides of smaller size than those produced by the oocytes. These latter appear to lack specific proteases involved in the terminal processing of the submaxillary gland proteins.

Nerve growth factor *Renin* *Translation* *Precursor* *Submaxillary gland* Xenopus *oocyte*

1. INTRODUCTION

NGF is synthesized in the male mouse submaxillary gland. It is found as a complex of 3 proteins with a stoichiometry $\alpha 2:\beta:\gamma 2$ [1,2]. The β-subunit contains the neurotrophic activity. It is formed by a dimer of identical chains of 118 amino acids [3]. The α-subunit has no known enzymatic activity. The γ-subunit is a protease with sequence analogies to the kallikrein-like enzymes contained in the gland [4,5]. The 3 subunits undergo post-translational modifications. The βNGF monomer is thought to derive from a precursor of M_r about 30000 [6]. The γNGF is possibly involved in its maturation [7]. Additional limited proteolysis of βNGF generates the 2.5 S NGF [3,8]. γNGF is a glycoprotein. It has an M_r of about 26000 and is a heterogeneous mixture of 2 or 3 chains with M_r ranging from 6000 to 17000 [9,10]. αNGF has an M_r of about 27000 and is composed of two chains of M_r of 17000 and 9000 [11].

Here, *Xenopus* oocytes and reticulocyte lysates were programmed with poly(A)-RNAs isolated from the mouse submaxillary gland. The synthesis and processing of material cross-reacting with sera raised against the α-, β- and γ-subunits was investigated. The processing of another protein of the submaxillary gland, renin, was studied in parallel.

Abbreviations: αNGF, βNGF, γNGF, α-, β- and γ-subunit of the mouse 7 S nerve growth factor; SDS, sodium dodecyl sulfate; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Preparation of mouse submaxillary mRNA*

Submaxillary glands were excised from adult male 129 mice. RNA was extracted using the urea-lithium chloride procedure [12]. Poly(A)-RNAs were isolated by two passages on oligo(dT)cellulose.

2.2. *Protein synthesis*

Reaction mixtures containing 8 μl of nuclease-treated reticulocyte lysate (Amersham N90), 1 μl

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of [^{35}S]methionine (10 mCi/ml) and 1 μl of poly(A)-RNAs (250 μg/ml), were incubated for 1 h at 30°C.

Batches of 10 *Xenopus laevis* oocytes were microinjected with 50 nl of mRNA (1 mg/ml) with or without tunicamycin [13] at 40 μg/ml. Injected oocytes were incubated for 24 h at 19°C in 100 μl of Barth's saline containing 10 μl of [^{35}S]methionine (10 mCi/ml). Incubation media were removed and oocytes were homogenized in 1 ml of buffer A consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Na deoxycholate, 0.1% methionine and ovalbumin 0.1%, as in [14].

2.3. *Immunoadsorption of polypeptides*

Five μl reticulocyte lysate or 500 μl oocyte homogenate or 50 μl oocyte incubation medium were adjusted with buffer A to 1 ml and were supplemented with 2 μl of specific antiserum and 0.01 g protein A–Sepharose Cl 4 B (Pharmacia). After incubation overnight at 4°C under gentle agitation, protein A–Sepharose was washed with 15 ml of 0.1 M Tris (pH 7.9), 0.5 M LiCl and 0.05% DTT. Immunoadsorbed material was eluted with electrophoresis sample buffer [15], subjected to electrophoresis in SDS-18% polyacrylamide slab gel, and revealed by autoradiography [16]. In competition experiments, 10 μg of the corresponding purified protein was added together with the antiserum.

2.4. *Preparation of the purified α-, β- and γ-subunits and of their antisera*

The α- and γNGF were purified as in [2]. Although the protein samples which served for inoculation of rabbits appeared pure after coloration of polyacrylamide gels with silver stain, antisera showed cross-reactivity in reconstruction experiments performed with the purified radioiodinated proteins. For competition experiments, α- and γNGF were further purified by affinity chromatography. The protease inhibitor aprotinin was coupled to CNBr-activated Sepharose (Pharmacia) following the manufacturer's instruction. The column was equilibrated with 0.05 M Tris (pH 8) and 0.5 M KCl. The α-subunit passed through the column, while the γ-subunit was retained, and eluted in 0.05 M glycine (pH 3) and 0.5 M KCl as in [17]. The mixture of β and of 2.5 S NGF was purified as in [8]. Serum raised against this material appeared specific. The β/2.5 S NGF was also denatured by treatment with iodoacetic acid [7] and a serum was raised against this material. It immunoprecipitated both the native and the denatured βNGF. Renin and renin-antiserum were gifts of P. Corvol.

3. RESULTS

3.1. *Identification of the precursors of αNGF, γNGF and renin*

Translation of submaxillary gland mRNAs in *Xenopus* oocytes yielded 3 peptides which were recognized to different extents by the anti-α- and the anti-γNGF sera. Their M_r were of about 43000, 32000 and 26000; they will be referred to as 43-kDa, 32-kDa and 26-kDa species hereafter. Radioactivity associated with the 26-kDa species

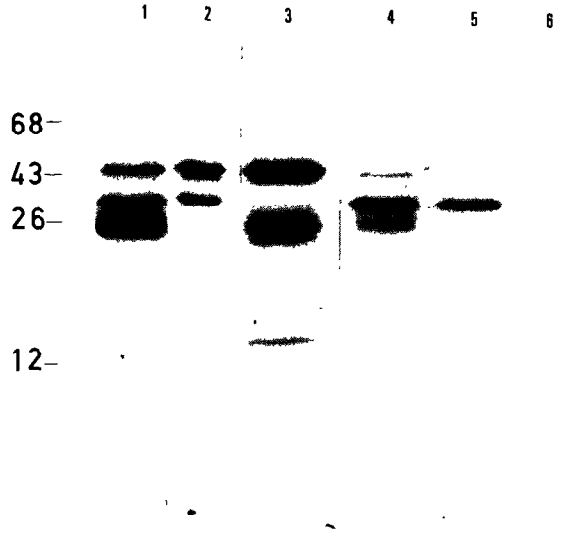

Fig.1. Synthesis of the αNGF and γNGF precursors in *Xenopus* oocytes programmed with male mouse submaxillary gland poly(A)-RNAs. Translation products immunoprecipitated by appropriate antisera were fractionated in SDS/18% acrylamide gels. Slot 1, immunoprecipitation with the anti-γNGF serum; slot 2, the same in the presence of 10 μg of purified γNGF; slot 3, the same in the presence of 10 μg of αNGF; slot 4, immunoprecipitation with the anti-αNGF serum; slot 5, the same in the presence of γNGF; slot 6, the same in the presence of αNGF. The exposure time was 18 h, except for slot 3 which was exposed during 42 h. $M_r \times 10^{-3}$ are given.

was relatively more abundant when immunoprecipitations were performed with the anti-γNGF serum (fig.1, slot 1). In contrast, the 32-kDa peptide was largely prominent with the antiserum to αNGF (slot 4). Sera raised against the native or the denatured form of βNGF failed to immunoprecipitate any labelled material (not shown). The 3 peptides cross-reacting with the anti-γNGF and anti-αNGF sera were characterized further in competition experiments with the purified submaxillary gland proteins. As shown in fig.1, addition of 10 μg purified γNGF resulted in the loss of the 26-kDa peptide (slots 2,5) while purified αNGF eliminated the 32-kDa species and reduced the radioactivity associated with the 26-kDa band (slots 3,6). βNGF had no effect (not shown). Data reported in fig.2 show that the 43-kDa peptide immunoprecipitated by the anti-γNGF serum and the renin precursor retained by an antiserum to renin had the same electrophoretic mobility. Moreover, renin specifically competed with the 43-kDa species (slots 2,3). Taken together, these data provide strong evidence that the 26-kDa, 32-kDa and 43-kDa peptides represent precursors of the γ- and α-subunits, and of renin, respectively. Contamination of the α sample with γNGF or some other protein of the kallikrein family showing cross-reacting activity [4,5] may explain the relative competition exerted by this material on the 26-kDa peptide. Contamination of NGF preparations by trace amounts of renin was already reported [18] and should account for antirenin activity in sera raised against the α- and γ-subunits.

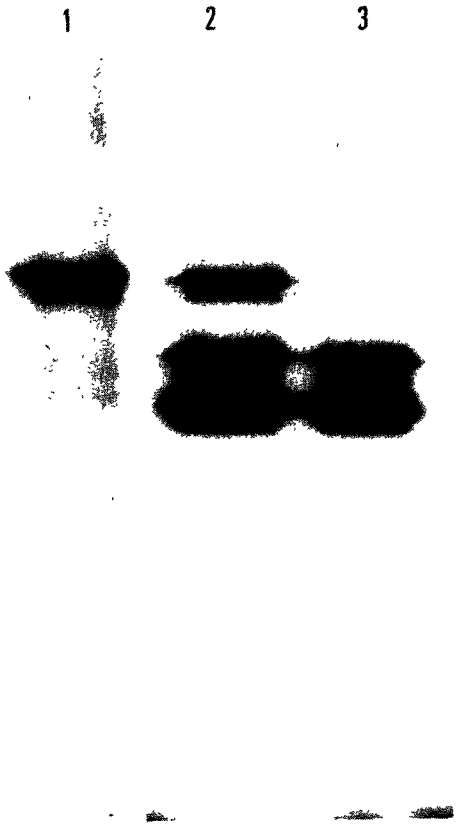

Fig.2. Identification of the renin precursor. Slot 1, translation product synthesized in *Xenopus* oocytes were immunoprecipitated with the renin-antiserum; slot 2, immunoprecipitation with anti-γNGF serum; slot 3, the same in the presence of purified renin.

3.2. *Processing of the precursors of αNGF, γNGF and renin*

In oocytes treated with an inhibitor of glycosylation, tunicamycin [13,19], the M_r of the αNGF and γNGF precursors decreased to values of 30000 and 24000 correspondingly. There was no change in the M_r of the renin precursor (fig.3,4). The 3 precursors were also found in the oocyte incubation medium. Excretion was not accompanied by any significant change of their electrophoretic mobilities (fig.3,4).

Immunoadsorption and competition experiments were performed in parallel with translation products synthesized in reticulocyte lysate. In this system, αNGF, γNGF and renin precursors displayed M_r values of about 26000, 31000 and 45000, respectively (fig.3,4, slots 5). This latter value agrees with published data [12,20].

4. CONCLUSION

Evidence is presented concerning the identification of the precursors of renin, and of the α- and γ-subunits of the mouse submaxillary gland NGF, among the translation products of the gland poly(A)-RNAs in *Xenopus* oocytes and in reticulocyte lysate. Precursors found in this latter system displayed the larger M_r and are likely to represent the primary translation products. M_r values of precursors synthesized in tunicamycin-treated oocytes were smaller. The shift of 1000–2000 Da presumably results from the

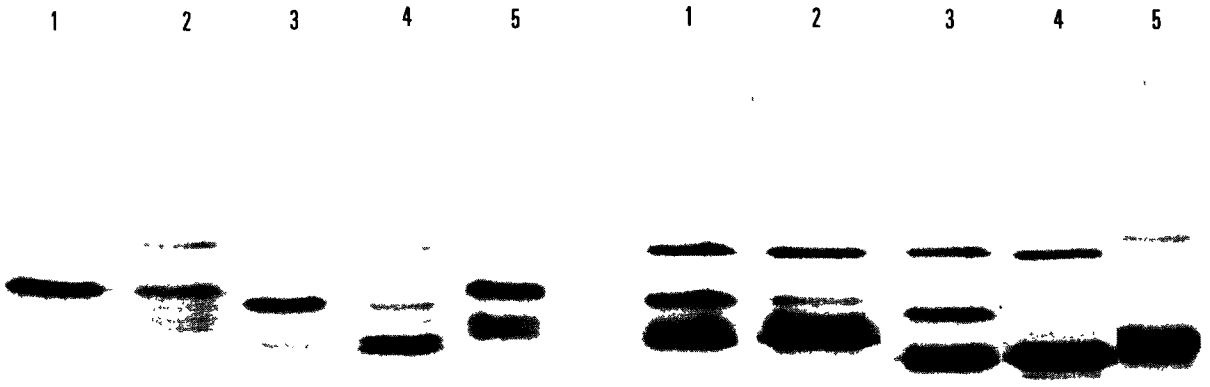

Fig.3. Processing of the αNGF precursor. Translation products synthesized in *Xenopus* oocytes or in a reticulocyte lysate were immunoprecipitated with the αNGF-antiserum. Slot 1, peptides synthesized in microinjected oocytes; slot 2, peptides secreted by the oocytes; slot 3, peptides synthesized in tunicamycin-treated oocytes; slot 4, peptides secreted by tunicamycin-treated oocytes; slot 5, translation products synthesized in a reticulocyte lysate. All samples were run in the same gel.

Fig.4. Processing of the γNGF precursor. Translation products synthesized in *Xenopus* oocytes or in a reticulocyte lysate were immunoprecipitated with the γNGF-antiserum. Slot 1, peptides synthesized in microinjected oocytes; slot 2, peptides secreted by the oocytes; slot 3, peptides synthesized in tunicamycin-treated oocytes; slot 4, peptides secreted by tunicamycin-treated oocytes; slot 5, translation products synthesized in a reticulocyte lysate. All samples were run in the same gel.

cleavage of a signal peptide which accompanies the synthesis of secretory proteins in oocytes [21,22]. Comparison with values obtained in the absence of tunicamycin indicates that α- and γNGF precursors were glycosylated while renin was not (or poorly). This resembles the processing events that take place in the gland. However, oocytes fail to generate the low-M_r peptides which constitute in the gland the mature forms of αNGF, γNGF and of renin [9–11,23]. Oocytes appear to lack the processing machinery which characterizes the mouse submaxillary gland. Absence of this machinery may possibly account for the failure to detect the precursor of the βNGF in this system.

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