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A comparative analysis of the structural, functional and biological differences between Mouse and Human Nerve Growth Factor

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ABSTRACT

NGF is the prototype member of the neurotrophin family of proteins that promote the survival and growth of selected neurons in the central and peripheral nervous systems. As for all neurotrophins, NGF is translated as a pre-pro-protein. Over the years, NGF and proNGF of either human or mouse origin, given their high degree of homology, have been exploited for numerous applications in biomedical sciences.

The mouse NGF has been considered the golden-standard for bioactivity. Indeed, due to evolutionary relatedness to human NGF and to its ready availability and by assuming identical properties to its human counterpart, the mouse NGF, isolated and purified from sub-maxillary glands, has been tested not only in laboratory practice and in preclinical models, but it has also been evaluated in several human clinical trials.

Aiming to validate this assumption, widely believed, we performed a comparative study of the biochemical and biophysical properties of the mouse and human counterparts of NGF and proNGF. The mature and the precursor proteins of either species strikingly differ in their biophysical profiles and, when tested for ligand binding to their receptors, in their *in vitro* biological activities. We provide a structural rationale that accounts for their different functional behaviors.

Despite being highly conserved during evolution, NGF and proNGF of mouse and human origins show distinct properties and therefore special care must be taken in performing experiments with cross-species systems in the laboratory practice, in developing immunoassays, in clinical trials and in pharmacological treatments.

1. Introduction

The Nerve Growth Factor (NGF) is the prototype of the neurotrophins protein family [1]. It is involved in the survival and growth of sensory neurons, as well as in the maintenance of neurons in the central nervous system [1–4]. In addition to its neuronal targets, NGF has been shown to act on a number of non-neuronal targets, including other cells of the brain such as astrocytes and microglia, cells of the immune system such as mast cells and basophils, keratinocytes, blood vessel endothelial cells and many others [1,5–8]. It exerts its action through the TrkA receptor (tyrosine kinases superfamily) and the $p75^{NTR}$ receptor (tumor necrosis factor receptors superfamily) [9].

NGF is translated as a precursor protein (pre-proNGF), secreted and processed by furin in the Trans Golgi Network, and by other proteases in the extracellular space [10,11]. ProNGF shows different receptors binding affinities from those of the mature NGF. It preferentially binds to and activates $p75^{NTR}$ instead of TrkA, and induces apoptosis in different cellular systems with a mechanism involving both $p75^{NTR}$ and sortilin, a specific receptor for the pro-peptide domain of proNGF [12–14].

After its discovery and in the following studies, it became clear that NGF, due to its pleiotropic properties, has great pharmacological potentialities for the treatment of neurodegenerative diseases, of peripheral neuropathies, and in processes in which tissue regeneration is involved [15–17].

In view of the high levels and easy purification of biologically active NGF from male mouse submandibular glands [18], mouse NGF has been used over the years not only in the laboratory practice, but also in some pre-clinical and clinical studies [19–30]. Indeed, the high level of amino acid sequence identity (85.06% proNGF; 89.17% NGF; 80.99% propeptide) of the mouse and human proteins led to the *a priori* assumption, with no direct experimental validation, of mouse NGF being fully superimposable to human NGF with respect to their biochemical, biophysical and biological properties.

In our lab, human and mouse NGF are currently produced as purified recombinant protein in *E. coli* [31,32]. Surprisingly, during the experimental handling of the proteins, we observed that both recombinant

Abbreviations: NGF, Nerve Growth Factor; CD, circular dichroism; TrKA, tyrosine kinase A, SPR, Surface Plasmon Resonance; PBS, phosphate buffer saline; MAb, monoclonal antibody.

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proNGF and NGF from mouse and human exhibited very different behaviors (as an example, during the limited proteolysis of proNGF with trypsin in order to obtain mature NGF, mouse and human proNGF showed very different kinetics of proteolytic cleavage).

In order to examine in greater depth these experimental observations, we carried out, a comparative study of biochemical, biophysical and biological properties of the mouse and human NGF and proNGF.

We report that the proteins from the two species are indeed quite different from one another, in terms of stability and of biological activities in cross-receptor studies. A structural interpretation of the observed differences between mouse and human NGF and proNGF is presented. The present study clearly pinpoints that much attention should be paid to the results of *in vitro* and *in vivo* experiments as well as preclinical and clinical practice when using NGF proteins of different species.

2. Materials and methods

2.1. Heterologous E. coli expression and purification of wild-type NGF

All the hereafter reported characterizations were performed on the short forms of the recombinant mouse and human proNGF, namely m- or hproNGF25, respectively (according to the nomenclature reported in [32]). For the sake of brevity, throughout the manuscript the proteins are simply named as mproNGF or hproNGF. Their respective mature proteins are named as mNGF and hNGF.

mproNGF and mNGF were produced and purified following the protocols described in [32].

The hproNGF cDNA was subcloned in the prokaryotic expression vector pET11a and the protein expression was carried out in the strain Rosetta (DE3).

hproNGF was expressed as recombinant protein in *E. coli*, refolded from inclusion bodies and purified by using a protocol modified from [32]. Mature hNGF was obtained from its respective hproNGF, by *in vitro* proteolytic cleavage with trypsin, as described below.

2.2. Kinetics of proteolytic cleavage

The kinetics of proteolytic cleavage of hproNGF or mproNGF was compared by using 3 different proteolytic enzymes: trypsin (Promega), furin (Sigma-Aldrich) and plasmin (Sigma-Aldrich). For all the digestions, 100 μg of hproNGF or mproNGF, at the concentration of 0.6 μg/ml, was processed.

For the trypsin digestion, the reaction was carried out in Sodium Phosphate 50 mM pH 7 at 4 °C, at the ratio of 1:250 enzyme:substrate. A sample of 15 μl of the reaction mixture was taken at time 0, and after 15, 30, 45, 60, and 90 min and 2, 4, and 22 h.

The proteolytic cleavage with furin was done in Hepes 25 mM pH 7 at 4 °C, with 6 U of enzyme. A sample of 15 μl of the reaction mixture was taken at time 0, and after 15, 30, 45, 60, and 90 min and 2, 4, and 22 h.

For the proteolysis with plasmin, the reaction was carried out in Sodium Phosphate 50 mM pH 7 at 37 °C. 0.005 U of plasmin were added to the reaction mixture. A sample of 15 μl of the reaction mixture was taken at time 0, and after 5, 10, 15, 20, 25, 30, 45, and 60 min.

For each sample, the reaction was blocked by addition of Laemmli sample buffer and boiling (10 min). Samples were analyzed by SDS-PAGE.

2.3. Circular dichroism (CD) measurements

CD measurements were carried out with a JASCO J-810 circular dichroism spectrometer at 20 °C. Far-UV CD (185–250 nm) spectra (averaged over 8 accumulations, acquisition time 1 s) were recorded at protein concentrations of 0.5–1.0 mg/ml in Sodium Phosphate 50 mM pH 7, with a 0.02 cm demountable quartz cuvette cell. Spectra were buffer corrected. Mean ellipticity values were calculated as previously reported [31]. The web based server DICHROWEB was used to estimate the secondary structure content, using the CDSSTR algorithm (Set 3) [33].

2.4. Chemical denaturation

The dependence of the denaturation on the denaturant concentrations was evaluated by incubating the proteins in serial dilutions of 8 M Guanidinium Chloride (GdmCl) in Sodium Phosphate 50 mM pH 7. The samples were incubated at room temperature for 20 h. The protein concentration was 20 μg/ml for NGFs, and 40 μg/ml for proNGFs. The protein solution was prepared both in the native buffer (50 mM Sodium Phosphate pH 7) and in the different concentrations of denaturing buffer (0.5 to 6 M GdmCl in 50 mM Sodium Phosphate, pH 7).

Fluorescence measurements were performed with the EnSpire® Multimode Plate Reader Spectrometer Perkin Elmer. The measurements were done in Optiplate 96 well (Perkin Elmer), containing 100 μl of protein solution per well (duplicates). The fluorescence emission spectra were recorded from 300 to 500 nm, at a speed of 1 nm/s, with 100 flashes, using an excitation wavelength of 280 nm. The experiment was carried out at least twice for each protein.

All spectra were corrected against the corresponding buffer. The data were normalized, in order to compare the different NGFs. The relative fraction of native NGF molecules, were obtained according to the following formula:

$$\alpha = (I - I_D)/(I_N - I_D)$$

where:

I = fluorescence signal at a certain GdmCl concentration.
I_D or I_N = signal of the denatured or native component at the same GdmCl concentration, respectively. I_D or I_N were calculated from the linear dependence of the fluorescence of the denatured or native protein from the concentration of the denaturing medium.

The data were fitted using a phenomenological equation to calculate the Guanidinium concentration inducing the 50% effect on the peak shift. The following equations were used

1) mNGF and hNGF

$$y = [a/(1 + \exp(-(x-b)/c))]$$

where y is the fraction of folded protein as by the normalization, x is the Guanidinium concentration and a, b, and c are the fitting parameters.

2) mproNGF and hproNGF

$$y^{(-1)} = a + bx^3 + ce^x$$

where y is the fraction of folded protein, x is the Guanidinium concentration and a, b, and c are the fitting parameters.

2.5. Thermal denaturation

Solutions composed of 7.5 μl of 300 × Sypro Orange (Molecular Probes) and 17.5 μl of proteins (NGFs or proNGFs) at the concentration of 0.7 mg/ml in Sodium Phosphate 50 mM pH 7, were added to the wells of a 96-well thin-wall PCR plate (Bio-Rad). Buffer and water were added as blank and control samples, respectively. The plates were sealed with Optical-Quality Sealing Tape (Bio-Rad) and heated in an iCycler iQ Real Time PCR Detection System (Bio-Rad) from 20 to 90 °C in increments of 0.2 °C/20 s. Fluorescence changes in the wells of the plate were recorded