Neurochemistry International 60 (2012) 852–863

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Pro-domain in precursor nerve growth factor mediates cell death

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ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form 5 March 2012
Accepted 8 March 2012
Available online 28 March 2012

Keywords:
Nerve growth factor
Pro-domain
Neurotrophic
Sortilin
Caspase
$p75^{NTR}$
Naja sputatrix
$pro^{(R/G)}NGF$
trkA

ABSTRACT

Nerve growth factor (NGF) is synthesized as a precursor, proNGF that undergoes post-translational processing to generate the biologically active mature NGF. While the neurotrophic function of NGF is well established, the activity of the proNGF precursor is still unclear. In this study, we have cloned the pro-domain of the precursor NGF molecule and have elucidated its function. We have used both mature and the furin resistant $pro^{(R/G)}NGF$ as controls in our experiments. Both $pro^{(R/G)}NGF$ and mature NGF (NGF) exhibited neurotrophic activity on PC12 cells while the pro-domain itself promoted cell death. The pro-domain, has been found to mediate apoptosis possibly by promoting the formation of a signaling complex comprising of endogenous $p75^{NTR}$ receptor, Bim/Bcl2 group of proteins and JNK and MEK1/2 signaling pathways.

1. Introduction

Nerve growth factors belong to the family of neurotrophins (Bibel and Barde, 2000) and their pleiotropic actions are mediated by two structurally unrelated classes of receptors, the 140-kDa tropomyosin-related kinase (Trk) receptor tyrosine kinase (RTK) and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor (TNF) receptor superfamily. Interaction of neurotrophin to Trk receptors promotes cell growth, survival or differentiation while binding of neurotrophin to $p75^{NTR}$ have been widely known to be involved in cell death mechanism mainly apoptosis (Bibel and Barde, 2000; Chao, 2003). The association between neurotrophin and $p75^{NTR}$/Trk heterodimer interactions could result in either cell survival or cell death (Eide et al., 1993; Friedman and Green, 1999).

Although NGF was first discovered in snake venoms (Cohen and Levi-Montalcini, 1956), the NGFs isolated from the mouse submaxillary glands (mouse NGF; Lipps, 1998) and human placental tissues and body fluids have been studied extensively (Goldstein et al., 1978 and Lipps, 2000). The NGF from cobras has been reported to be most efficient in inducing neurite outgrowth on PC12 cells as compared to NGFs derived from other types of snakes (Lipps, 2000) or even the mammalian derived NGF. Naturally (biologically) all NGFs are produced first as a proNGF molecule with 241 amino acids which is later processed to form 13 kDa mature NGF comprising 118–120 amino acids and 3 disulphide bonds (Guo et al., 1999; Koh et al., 2004). *In vivo*, NGF exists as parallely arranged noncovalent dimer of 26.5 kDa (McDonald et al., 1991). The pro-domain of proNGF molecule was initially thought to have a role in assisting folding of the mature neurotrophin (Rattenholl et al., 2001; Seidah et al., 1996). However, in recent years, proNGF has been reported to promote apoptosis via its interaction to $p75^{NTR}$ and sortilin receptors (Jansen et al., 2007; Nakamura et al., 2007; Teng et al., 2005; Nykjaer et al., 2004). In human, increased expression of proNGF has been associated with slowed neurodegeneration in Alzheimer's patient (Sobottka et al., 2008). The proNGF molecule is usually cleaved within the trans-Golgi network by furin-like enzymes (Hosaka et al., 1991; Dubois et al., 1995) to release an active mature NGF. Recently, extracellular cleavage of proNGF by both plasmin and matrix metalloprotease-1 (MMP) has also been reported (Lee et al., 2001). Plasmin cleavage of proNGF produces the mature form (~14 kDa), whereas MMP-7 results in a 17 kDa intermediate (Darling et al., 1983). The mature NGF is cleaved from the proNGF molecule at the −1 and −2 amino acid (KR) positions (relative to the amino acid sequence of mature NGF). Thus, studies on the functional properties of proNGF have included the cleavage resistant mutation at this site (Fahnestock et al., 2004; Heymach et al., 1996; Pagadala et al., 2006).

We have earlier shown that the NGF derived from *Naja sputatrix* venom is functionally more active compared to the mouse NGF in

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http://dx.doi.org/10.1016/j.neuint.2012.03.006

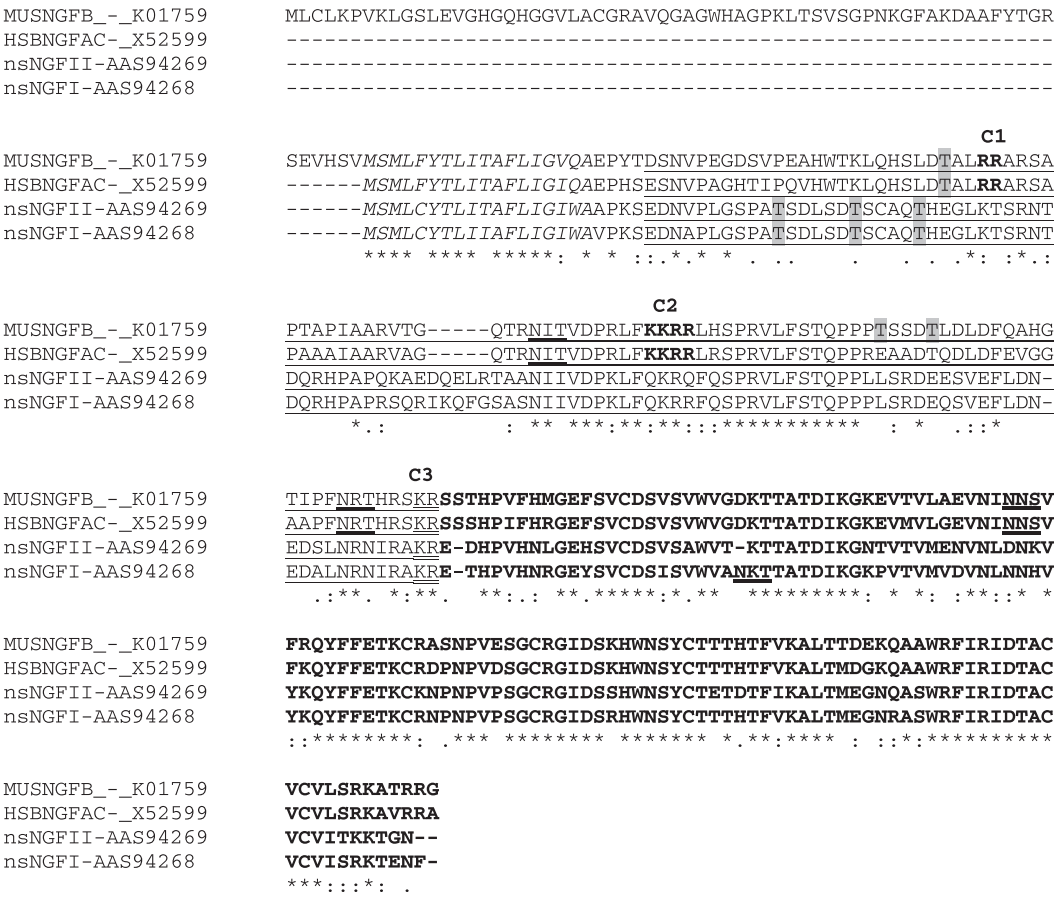

Fig. 1. Clustal alignment of NGFs. NGF amino acid sequences from *Naja sputatrix* (nsNGFI & II), mouse NGF (MUSNGFB) and human (HSBNGFAC) were compared by multiple sequence alignment with the ClustalW program (http://align.genome.jp/sit-bin/clustalw). The signal peptide region is in *italic fonts*, N-glycosylation site is underlined and O-glycosylation is in grey boxes. The pro-domain region is underlined (pro-domain construct) and the mature peptides are in **bold** fonts. The proteolytic cleavage site (KR) for the mature peptide is indicated by underlined fonts. This site is mutated [Arginine (R) to Glycine (G)] in the pro$^{(R/G)}$NGF construct. Identical amino acids among the three sequences are shown by (*). The 3 furin cleavage sites as reported by Pagadala et al. (2006) are indicated by C1, C2 and C3.

Table 1
Amino acid sequence similarity for NGFs from human, mouse and *Naja sputatrix*. Multiple clustal alignment was carried out using ClustalW software from http://align.genome.jp/sit-bin/clustalw.

NGF domains (% similarity)	nsNGFI (AAS94268)	nsNGFII (AAS94269)	hsaNGF (X52599)	mmuNGF (K01759)	
Precursor	100	82.6	56.4	53.91	nsNGFI (AAS94268)
Prodomain	100	84.1	33.98	32	
Mature	100	89	75.4	67.2	
Precursor	82.6	100	51.5	48.9	nsNGFII (AAS94269)
Prodomain	84.1	100	32	29.1	
Mature	89	100	73	65	

inducing neurite outgrowth in PC12 cells (Koh et al., 2004). In this study, the properties of the proNGF and pro-domain derived from the venom gland of *Naja sputatrix* (Koh et al., 2004) were investigated and compared with that of mature NGF. Functional studies showed that pro$^{(R/G)}$NGF behaved similar to the NGF, by providing neuroprotection under both serum-starved and ischemic conditions, but to a lesser extent, whereas the pro-domain itself, has been found to be apoptotic and likely to activate the caspase-mediated cell death pathway via Bim, p75^{NTR} and JNK and MEK1/2 signaling pathways.

2. Materials and methods

2.1. Cell lines

CHO and PC12 cells were obtained from A.T.C.C. (Manassas, VA, USA.) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin (Koike, 1983). The ability of NGF to elicit neurite outgrowth on PC12 cells was determined as described by Greene and Tishler (Greene and Tishler, 1976).

2.2. Plasmid constructions and production of stable transfectants

Oligonucleotide primers were synthesized based on the sputa NGF cDNA sequence. The cDNA encoding full length (mature) NGF, pro$^{(R/G)}$NGF and pro-domain (together with the leader sequence) were amplified by PCR using gene specific primers that contained restriction sites for *KpnI* and *XhoI*. The PCR products were cloned into pT7Blue$^{(R)}$ vector (Novagen, Madison, WI, USA) and later subcloned into pcDNA4. All constructs were sequenced to verify the correct mutations and the reading frames. Plasmids with the mutated cDNAs were amplified in *Escherichia coli* and later purified for transfection into CHO cells. Stable transfectants of