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Pro-region of neurotrophins determines the processing efficiency

Hiroshi Nomoto *, Masatoshi Takaiwa, Akihiro Mouri, Shoei Furukawa

Laboratory of Molecular Biology, Gifu Pharmaceutical University, Gifu 502-8585, Japan

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Abstract

Neurotrophins are synthesized as precursors called pro-neurotrophins and then mature neurotrophins are formed proteolytically from them. Recent findings revealed that pro- and mature neurotrophins elicit opposite functional effects on cell survival, highlighting the importance of this processing step. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) belong to the neurotrophin family and are mutually homologous, but BDNF is less efficiently processed. In order to find the reason for this, we examined some possibilities by using PC12 cells, and found that the pro-region, especially the last half of it, affected very much the processing efficiency of these neurotrophins.
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Keywords: Nerve growth factor (NGF); Brain-derived neurotrophic factor (BDNF); Protein processing; Propeptide; PC12 cells

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) belong to the neurotrophin family, the member of which function to promote neuronal survival and differentiation or to control synaptic functions [1]. These two molecules are mutually homologous, especially in their mature regions (about 50% identity) [2]. The mature proteins emerge by the cleavage at the center of the precursors (pro-neurotrophins) in either trans-Golgi or secretory granules by pro-protein convertases [3–6]. NGF is then released mainly through the constitutive secretory pathway from cells in peripheral tissues and nerves [5,7], or partly by regulated secretion pathways [8–12]. In contrast, BDNF is primarily secreted in an activity-dependent manner [8,13]. Further, proNGF, but not proBDNF, was shown to be efficiently cleaved by endoproteases in hipocampal neurons and AtT 20 cells transfected with neurotrophin DNA [8]. Therefore, neurotrophins are sorted into either the constitutive or regulated secretory pathways depending on their structures, and the sorting may be linked closely to the efficiency of their proteolytic cleavage.

The processing step to produce mature neurotrophins is essential for the function of neurotrophins, since proNGF was recently reported to preferentially bind to the p75 neurotrophin receptor (p75NTR), and this interaction induced apoptosis [14]. ProBDNF was also reported to be an apoptotic ligand, inducing neuronal death in a p75NTR-dependent manner [15]. Thus, pro- and mature neurotrophins elicit opposite effects on cell survival. Previously published data indicate that a significant amount of pro-neurotrophins are actually secreted extracellularly, particularly in the case of CNS neurons [16–19]. Moreover, increased levels of proNGF have been reported in the brains of patients with Alzheimer's disease [20,21], in oligodendrocytes following spinal cord injury [22], or in corticospinal neurons after CNS injury [23].

It is intriguing that the two homologous neurotrophins are different in their processing and secretion manners, which should be closely linked to their functions. Such differences should be derived from their structures, especially from those of their pro-regions, because the homology of the pro-regions is low compared with that of their mature regions. Furthermore, the pro-region of neurotrophins is attracting much attention since a single amino acid change (V66M) in the pro-region of human BDNF was found to

* Corresponding author. Fax: +81 58 237 5979.
E-mail address: hnomoto@gifu-pu.ac.jp (H. Nomoto).

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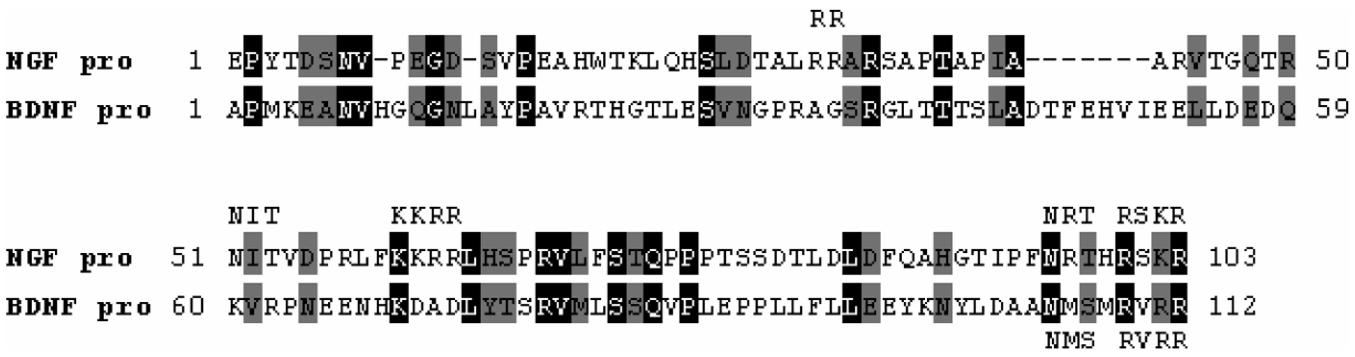

Fig. 1. Aligned propeptide sequences of mouse NGF and rat BDNF. The same amino acids between the neurotrophins are expressed as white characters on a black background, and similar amino acids are shadowed. The processing site (RSKR and RVRR), the basic amino acid clusters of NGF (RR and KKRR), and the N-linked oligosaccharide binding sites (NIT and NRT in NGF and NMS in BDNF) are indicated outside the sequences.

affect synaptic targeting and activity-dependent secretion of the protein, which was associated with abnormal hippocampal neuronal function as well as impaired episodic memory in human subjects [24]. The pro-regions of NGF and BDNF are aligned in Fig. 1. Apparent differences between the two peptides are basic amino acid clusters in the pro-regions, amino acid sequence at the cleavage site, the numbers of the N-linked carbohydrate chains, a non-aligned gap of NGF between its 42nd and 43rd amino acid, and so on. NGF has two basic amino acid clusters, RR (−73 to −72, 31st–32nd in Fig. 1) and KKRR (−44 to −41, 60th–63rd), which are conserved among mouse, rat, and human NGFs, but do not exist in BDNF of any species. We have recently examined their role in processing, and found that these basic amino acid clusters contributed little to the production of mature NGF, although they were actually cleaved in cells by a pro-protein convertase, furin [25]. Concerning the N-linked carbohydrate chains, NGF has two chains in its pro-region, whereas BDNF has only one.

In the present study, we used PC12 cells to examine what causes the difference in the processing and secretion of NGF and BDNF, focusing on the amino acid sequence at the cleavage sites and in the whole pro-region, and found a part of the pro-regions to be especially influential.

Materials and methods

Plasmid construction. The expression plasmid of mouse NGF was constructed as already reported [12]. The expression plasmid of rat BDNF was prepared by inserting the PCR product of BDNF into the mammalian expression vector pGW1 [12]. Rat and mouse BDNF are almost the same except for 2 amino acids; i.e. −98 Ala (6th in Fig. 1) of the rat is Val in the mouse and −86 Ala (18th in Fig. 1) of the rat is Gly in the mouse. The full-length BDNF sequence (corresponding to 249 amino acids) was amplified from the cDNA obtained from rat hippocampus by using the following primers: B1, 5′-CGGAATTCGAGTGATGACCATCCTTTTCCTTAC-3′ (sense, *Eco*RI site at 5′-end) and B2, 5′-CGGATCCCTATCTTCCC CTTTTAATGGTCAGTG-3′ (antisense, *Bam*HI site at 5′-end). The PCR product was cut with *Eco*RI and *Bam*HI and ligated into pGW1, which had been cut with the same enzymes.

The expression plasmid of BDNF with the cleavage site of NGF (designated as BDNFpN) was made as follows: BDNF pro-region before the cleavage site was amplified from the BDNF expression plasmid above by using primers B1 and BP3Nc: 5′-GTGCGTACGGTTTGCGG CATCCAGGTAAT-3′ (antisense, *Spl*I site at 5′-end), and the BDNF mature region with the cleavage site of NGF was amplified from the BDNF expression plasmid by using primers, BM5Nc (5′-AACCG TACGCACCGGAGCAAGCGCTCATCCGACCCCGCCCGCCGT-3′, sense, *Spl*I site and NGF cleavage site at 5′-end) and B2. The PCR product was cut with *Spl*I and *Eco*RI or *Bam*HI and ligated into pGW1, which had been cut with *Eco*RI and *Bam*HI. The resultant construct (BDNFpN) has a part of NGF sequence, RTHRSKRS, instead of the original MSMRVRRH (−7 to +1). The expression plasmid of NGF with the cleavage site of BDNF (designated as NGFpB) was made by site-directed mutagenesis described earlier [26]. Briefly, the NGF expression plasmid was subjected to thermal cycle reaction of DNA polymerase with a primer NBcN: 5′-AGGACTCACCGGGTCCGGAGATCATCCACC CAC-3′ (containing *Bsp*EI site), and then the product was digested with *Dpn*I and double stranded DNA was re-synthesized with DNA polymerase. The resultant construct (NGFpB) has a part of BDNF sequence, RVRR, instead of original RSKR (−4 to −1).

The exchange of the pro-region was accomplished as follows: the pro- and mature-regions of NGF and BDNF were amplified separately, and were ligated with the expression vector pGW1 with a combination of the pro- and mature-region of different neurotrophins. The pro-region of NGF was amplified from the NGF expression plasmid by using primers N1 (sense, *Eco*RI site at 5′-end), which was already used to construct NGF expression plasmid [12], and NP3 (5′-GAAGGATCCGAGTGG CGCTTGCTCCGGTGAGTC-3′, antisense, *Bam*HI site at 5′-end). The pro-region of BDNF was amplified from the BDNF expression plasmid by using primers B1 and BP3 (5′-TCTAGATCTCCGAACCCTCATAGA CAT-3′, antisense, *Bgl*II site at 5′-end). The mature-region of NGF was amplified from the NGF expression plasmid by using primers NM5 (5′-AGTCTAGATCTTCCACCCACCCAGTCTTCC-3′, sense, *Bgl*II site at 5′-end) and RK3 (5′-AACCATTATAAGCTGCAA-3′, antisense, the down stream sequence from the multicloning site of the vector, and the PCR products are labile to some restriction enzymes of the multicloning site). The mature-region of BDNF was amplified from BDNF expression plasmid by using primers BM5 (5′-TCCGGATCCCGCCCGCCGT GGGGAGCTGA-3′, sense, *Bam*HI site at 5′-end) and B2. The expression plasmid for a chimera neurotrophin with the NGF pro-region and BDNF mature region (designated as N-BDNF) was made by ligating the NGF pro-region cleaved by *Eco*RI and *Bam*HI with the BDNF mature region cleaved by *Bam*HI and *Hin*dIII, and inserting the combination into pGW1 cleaved by *Eco*RI and *Hin*dIII. In a similar way, the expression plasmid for a chimera neurotrophin with BDNF pro-region and NGF mature region (designated as B-NGF) was made by ligating the BDNF pro-region cleaved by *Eco*RI and *Bgl*II with the NGF mature region cleaved by *Bgl*II and *Bam*HI, and inserting the combination into pGW1 cleaved by *Eco*RI and *Bam*HI.

Further exchange of a part of the NGF pro-region with the corresponding part of BDNF was conducted by utilizing *Xma*I site in the BDNF pro-region, which site is located at position #281 from the start codon corresponding to −37 Ser (76th in Fig. 1). The expression plasmid for a chimera neurotrophin with the N-terminal part of pro-region of

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