Journal of Neuroscience Methods 200 (2011) 121–128

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

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

A novel method for producing mono-biotinylated, biologically active neurotrophic factors: An essential reagent for single molecule study of axonal transport

Kijung Sung[a], Michael T. Maloney[a], Jingkun Yang[b], Chengbiao Wu[c,*]

[a] Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA, United States
[b] Massachusetts Institute of Technology, Cambridge, MA, United States
[c] Department of Neurosciences, University of California, San Diego, CA, United States

ARTICLE INFO

Article history:
Received 3 March 2011
Received in revised form 14 June 2011
Accepted 20 June 2011

Keywords:
Nerve growth factor
Biotinylation
Quantum dots
Single molecule imaging
Axonal transport
Microfluidic nerve cell chamber
PC12 cells
Dorsal root ganglion
Signaling pathways
Receptors

ABSTRACT

In this report, we describe a novel method for producing mature and biologically active mono-biotinylated nerve growth factors (mBtNGF) that can be used for single molecule studies of real-time movement of neurotrophins within axons of neurons. We inserted an AviTag sequence into the C-terminal of the full length mouse preproNGF cDNA and cloned the fusion construct into a pcDNA3.1 mammalian expression vector. We also subcloned the *Escherichia coli* biotin ligase, BirA, into a pcDNA3.1 vector. These two plasmids were then transiently co-expressed in HEK293FT cells. As a result, the AviTag located in the C-terminal of preproNGF was selectively ligated to a single biotin by BirA. The prepro sequence of NGF was subsequently cleaved within the cell. Mature mono-biotinylated NGF (mBtNGF) was secreted into cell culture media and was purified using Ni resin. We carried out activity assays and our results showed that mBtNGF retained biological activities that were comparable to normal NGF purified from mouse sub maxillary glands. We further verified the biotinylation efficiency of mBtNGF and the level of non-biotinylated NGF was virtually undetectable in the final preparation. Finally, by conjugating to quantum-dot streptavidin, mBtNGF was successfully used for single molecule study of axonal NGF trafficking in neurons.

1. Introduction

Neurotrophic factors (NTs) are a family of small protein ligands that play an important role in regulating many aspects of neuronal function including survival, phenotypic maintenance, and synaptic plasticity (Chao, 2003; Glebova and Ginty, 2005; Huang and Reichardt, 2001, 2003; Sofroniew et al., 2001). The NT family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Chao, 2003; Glebova and Ginty, 2005; Sofroniew et al., 2001). Each NT exerts its biological function through a specific tyrosine receptor kinase (Trk): TrkA for NGF, TrkB for BDNF, TrkC for NT-3 and NT-4 (Chao, 2003; Huang and Reichardt, 2003). Mechanistically, NTs are released in vivo from the target tissues which neurons innervate. These secreted NTs bind to and activate their respective surface receptors to initiate neurotrophic signaling at the neuronal axon terminus. Once bound, the ligand/receptor signaling complex becomes rapidly endocytosed via receptor mediated internalization. The ligand/receptor complex is then retrogradely transported from the axonal terminus to the soma to effect nuclear gene expression (Ginty and Segal, 2002; Ibanez, 2007; Reichardt and Mobley, 2004; Sofroniew et al., 2001; Zweifel et al., 2005). Increasing evidence demonstrates that defect/deficiency in axonally transported NTs is linked to neuronal atrophy in a spectrum of neurodegenerative disorders including Alzheimer's disease (AD), Down syndrome (DS), prion disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) (Chao et al., 2006; Cooper et al., 2001; Megarbane et al., 2009; Wu et al., 2009). To gain further insight into the pathological mechanisms of these and other neurodegenerative diseases, it is imperative to develop tools/reagents that will afford the capability of detecting the molecular and cellular sequences of axonal transport of NTs with sensitive spatial and temporal resolutions.

Fluorescent single molecule detection methods offer a power tool to probe cellular processes such as axonal transport with spatial and temporal sensitivities that are unattainable using radioactive labels or conventional fluorescent proteins (Hohlbein

* Corresponding author at: Department of Neurosciences, School of Medicine, University of California, San Diego, CMM-W, Room 337 MC-0649, 9500 Gilman Drive, La Jolla, CA 92093-0649, United States. Tel.: +1 858 534 0996; fax: +1 858 534 4782.
E-mail address: chw049@ucsd.edu (C. Wu).

0165-0270/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2011.06.020

et al., 2010; Pons and Mattoussi, 2009; Raj and van Oudenaarden, 2009). Indeed, impressive advance has been made using the techniques in understanding the molecular details of NGF endocytosis and its ensuing retrograde transport within axons. These studies have demonstrated that under physiological conditions, a single NGF dimer is internalized at the growth cone of chicken dorsal root ganglion neurons (Tani et al., 2005) and that a single NGF dimer is carried in an endosome that is retrogradely transported within axons of rat E16 dorsal root ganglion neurons (Cui et al., 2007). To examine axonal transport of NTs using single molecule methods, it is critical to explore different ways to tag NTs with suitable fluorescent labels. Ideally, the labeling method will help to produce a molecule with the following characteristics: (1) it can be labeled at selective site(s) that will not interfere with NTs' biological activity; (2) each NT molecule can be tagged with only one label to facilitate single molecule studies; (3) the fluorescent label itself is ultra-bright so that one fluorescent molecule can be visualized under cellular backgrounds; and (4) the label must have excellent photo stability i.e. resistance to photo bleaching, a desired feature for tracking the movement of the same molecules over an extended time period within the long axons of neurons.

To date, both direct and indirect labeling methods have been developed to tag NTs. One direct labeling technique involves over expressing, in a mammalian cell line, of fluorescent chimeras consisting of the full length NT cDNA construct with its C-terminus fused to a fluorescent protein such as GFP or mCherry e.g. BDNF-GFP (Adachi et al., 2005; Hartmann et al., 2001; Haubensak et al., 1998), or BDNF-mCherry (Her and Goldstein, 2008). Since the chimera proteins, expressed within neurons, must undergo the process of intra-neuronal and intra-axonal targeting, axonal movement of these fluorescent chimeras may not faithfully reflect the transport behavior of neurotrophic signals derived from the axonal targets. Further, current technologies do not permit the detection of single GFP or mCherry molecules under cellular backgrounds, hence each fluorescent signal detected may represent many fluorescent protein molecules i.e. aggregates. We tried both NGF-GFP and NGF-Cy3, neither could be detected in axonal transport assays using cultured rat dorsal root ganglion neurons by live imaging (data not shown). These limitations will hinder efforts for performing studies like those showing a single NGF dimer is internalized at the growth cone (Tani et al., 2005) and transported within axon (Cui et al., 2007). To directly label NGF a fluorescent dye like Cy3 was crosslinked to purified NGF (Tani et al., 2005). Fluorescent dyes have two distinct advantages over fluorescent proteins in that they are much smaller, and have a higher quantum yield (i.e. brighter). Unfortunately, Cy3 and other fluorescent dyes, while useful for tracking NGF movement at the growth cone, are sensitive to photo bleaching and are thus not suitable for long term tracking of molecule movement within axons of neurons. Similarly, NGF was crosslinked to biotin and conjugated to Alexa-488 streptavidin (Bronfman et al., 2003) or quantum dot-streptavidin (Cui et al., 2007). These conjugates were used to study intracellular receptor trafficking (Bronfman et al., 2003) or axonal transport (Cui et al., 2007). Unfortunately, chemical crosslinking may occur at multiple sites and potentially result in inactivation of NT molecules. For instance, following crosslinking to biotin, each NGF monomer was found to carry 5–9 biotins (Bronfman et al., 2003; Cui et al., 2007). The presence of multiple biotin moieties spreading across the entire NGF molecule may yield a heterogeneously biotinylated NGF preparation. Although the preparation, as a whole, may retain bioactivity, the fundamental problem with such samples is that some NGF molecules, due to different numbers and sites of biotinylation, may have different binding, signaling and trafficking properties from other molecules. As such, it is impossible to compare these heterogeneously labeled individual NGF molecules for their binding, trafficking and signaling.

Here we report a novel approach to produce mono-biotinylated NGF molecules. We incorporated the biotin acceptor peptide, AP-tag (also known as AviTag, www.avidity.com) (Chen et al., 2005; Howarth et al., 2005; Howarth and Ting, 2008) into the 3′-end of a full length NGF cDNA to generate the NGF-Avi construct. AP is a 15–17 amino acid recognition sequence containing a lysine residue that can be specifically ligated to one biotin by the *Escherichia coli* biotin ligase BirA (Chen et al., 2005). By co-expressing NGF-Avi and BirA in HEK293 cells, NGF-Avi was efficiently biotinylated and we were able to purify mature mono-biotinylated NGF (mBt-NGF herein) to apparent homogeneity. We found the presence of the AviTag did not interfere with normal biological activities of NGF in activating TrkA-mediated signaling pathways. Finally, we mixed streptavidin-quantum dots (QD) with mBtNGF to produce ultra-bright and photo-bleaching resistant NGF conjugates that were used to track axonal movement of NGF by single molecule imaging in cultured rat dorsal root ganglion (DRG) neurons. The main advantages over previous approach are that the level of non-biotinylated NGF is less than 1% and that each protein has the same number of tags and at the same location, thus guaranteeing a homogenous preparation with uniform bioactivities and trafficking properties.

2. Materials and methods

2.1. Cloning

Mouse preproNGF was amplified by PCR from a NGF-GFP plasmid (a generous gift from Professor Lessmann, Mainz, Germany). The forward primer sequence was: 5′-acgaattccaccatg-tccatgttgttctacactctgatcactgcg-3′ and the reverse primer sequence was: 5′-gatggatccttcgtgccattcgattttctgagcctcgaagatg-tcgttcagaccgccaccgacctccacggcggtggc-3′. The reverse primer contains a sequence coding for the 17 amino acid AviTag: GGGLN-DIFEAQKIEWHE. The sequence was based on the #85 AviTag peptide sequence described previously (Schatz, 1993). One glutamic acid residue was added to the C-terminal AviTag based on a finding by Avidity (www.avidity.com) that it greatly enhanced the biotinylation rate of the AviTag (Beckett et al., 1999). Platinum pfx DNA polymerase (Invitrogen, Cat#11708021) was used following the manufacture's instruction. The 50 μl of reaction was denatured at 94 °C for 4 min, followed by 25 cycles of amplification (30 s at 94 °C; 30 s at 50 °C; 90 s at 68 °C). An additional extension was performed at 68 °C for 4 min. The PCR product was purified and digested with EcoRI (Fermentas, Cat# FD0274) and BamHI (Fermentas, Cat# FD0054). It was ligated in-frame into the pcDNA3.1-myc-His vector that was predigested with EcoRI/BamHI. The resulting construct was designated as pcDNA3.1-NGFavi. BirA was amplified by PCR from pET21a-BirA (purchased from www.Addgene.com; Plasmid# 20857) (Howarth et al., 2005) using primers (forward primer: 5′-gtgaac atg gctagcatgact-3′) and reverse primer (5′-ggtgctcgagtcatgcggccgcaagct-3′ (containing an XhoI site). The PCR was carried out using Pfx as described above. The PCR product was digested with XhoI (Fermentas, Cat# FD0694) and was subcloned into pcDNA3.1 myc.his (+) vector (Invitrogen) that was pre-cut with EcoRV (Fermentas, Cat# FD0303) and XhoI. The resulting plasmid was designated as pcDNA3.1-BirA. All primers were purchased from Elim Biopharmaceuticals, Inc (Hayward, CA). All constructs were verified by sequencing (Elim Biopharmaceuticals, Inc.).

2.2. Cell culture and transfection

PC12 cells or a subclone of PC12 cells, PC12M were cultured as described previously (Wu et al., 2001, 2007). To induce neurite outgrowth, the culture media was changed to serum-free, DMEM-