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In vitro assay of neurofilament light chain self-assembly using truncated mutants

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Abstract

Neurofilaments (NFs) are heteropolymers composed of light (NF-L), middle (NF-M), and heavy (NF-H) subunits, present in most neurons. NF-L polymerizes on its own to provide a scaffold on which regular NFs form via the cross-bridging of NF-M or NF-H. To clarify the mechanism of regulation of NF-L self-assembly, we developed an assay using truncated mutant NF-L fused to glutathione-*S* transferase (GST). Western immunoblotting data show that the GST-fused head–rod domains of NF-L are necessary and sufficient for detecting assembled NF-L. The levels of self-assembled NF-L subunits detected using GST fusion proteins were consistent with those detected by electron microscopy and turbidity assay. Our results collectively imply that GST-fused head–rod domains of NF-L are critical tools for analyzing NF-L self-assembly *in vitro*.

Keywords: Neurofilament light chain; Self-assembly assay; GST pull-down; Turbidity; Electron microscopy; Western blotting; PC12 cells; SW13 cells

1. Introduction

Most eukaryotic cells have three cytoskeletal structures composed of actin microfilaments, microtubule, and intermediate filaments. Neurofilaments (NFs) are intermediate filaments in large myelinated axons of neurons. NFs are obligate heteropolymers capable of assembly with either NF-L/NF-M or NF-L/NF-H (Ching and Liem, 1993; Lee et al., 1993; Nakagawa et al., 1995). Each NF contains three domains, specifically, an N-terminal head domain, an α-helix rich rod domain, and a C-terminal tail domain (Fuchs and Weber, 1994; Grant and Pant, 2000; Al-Chalabi and Miller, 2003). Newly synthesized NF subunits must be assembled and transported from the cell body to axonal neurites in neurons. NF accumulation in the cell body is associated with neurodegenerative diseases, such as Charcot-Marie-Tooth disease (Watson et al., 1994; Fabrizi et al., 2004; Petzold, 2005), Alzheimer's disease (Shepherd et al., 2002; Norgren et al., 2003), and amyotrophic lateral sclerosis (Hirano et al., 1984; Collard et al., 1995; Grant and Pant, 2000). However, the factors and mechanisms that regulate NF assembly remain to be elucidated. A number of studies demonstrate that phosphorylation of the head domain inhibits self-assembly or induces disassembly of polymerized NF-L (Sihag and Nixon, 1991; Hisanaga et al., 1994; Mukai et al., 1996). In contrast, others show that phosphorylation of bovine NF-L by cdc2 kinase does not induce NF-L disassembly (Guan et al., 1992) or preclude assembly (Gibb et al., 1996). Thus, to clarify the mechanisms of NF assembly and accumulation, it is important to identify and characterize the supplementary factors that participate in the regulation of cytoplasmic assembly, axonal transport and organization of NFs. In this study, we attempt to establish an assay system for NF-L self-assembly. We demonstrate for the first time that the GST-fused NF-L-head–rod domain pull-down assay followed by Western immunoblotting is effective in the detection of NF-L self-assembly *in vitro*.

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2. Materials and methods

2.1. Reagents

Monoclonal (mAb 1615) and polyclonal anti-NF-L were purchased from Chemicon (Temecula, CA). Monoclonal anti-NF-M (mAb RNF403) for immunocytochemistry was obtained from MP Biochemicals (Aurora, OH). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies were acquired from Upstate Inc. (Lake Placid, NY), and fluorescein-conjugated Affinipure goat anti-rabbit IgG and rhodamine-conjugated Affinipure goat anti-mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

2.2. Cell culture

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 5% horse serum and 100 U penicillin-streptomycin at 37 °C. A human adrenal carcinoma cell line, SW13 (Vim$^-$), was cultured in DMEM supplemented with 10% FBS.

2.3. Vector constructs

Full-length cDNA of mouse NF-L in a pET-3d vector was generated as described previously (Kim and Kang, 2003). For GST fusion constructs, amplified rat cDNAs encoding NF-L (purchased from American Type Culture Collection, ATCC) were ligated into the *Eco*RI/*Sal*I restriction site of pGEX-5X-1. For the expression of domain-specific NF-L fusion proteins, amplified cDNA for the NF-L head domain [NF-L (H), amino acid residues 1–93], rod domain [NF-L (R), residues 93–397], head/rod domain [NF-L (H/R), residues 1–397], and tail domain [NF-L (T), residues 398–542] were separately inserted into the *Eco*RI/*Sal*I sites of pGEX-5X-1 (Kim et al., 2006). To examine intermediate filament formation in mammalian cells, rat NF-L (ATCC) and NF-M cDNA (kindly provided by Dr. Liem R. K.H., Columbia University) were ligated into pEGFP and pREFA vectors, respectively. All constructs were prepared using the Qiagen Plasmid Maxi Kit (Quiagen Inc., Santa Clarita, CA), and confirmed by direct sequencing of the ligation sites.

2.4. Transfection and protein expression

SW13 cells (Vim$^-$) were transiently co-transfected with pEGFP-NF-L and pREFA-NF-M using a ProFection kit (Promega, Madison, WI). PC12 cells were transfected with either pEGFP, pEGFP-NF-L or pEGFP-NF-L (H/R).

Mouse cDNA encoding full-length NF-L (pET-NF-L) was expressed in *E. coli*, and homogenously purified, as described previously (Kim and Kang, 2003). GST-NF-L fusion proteins were expressed in *E. coli*, and lysates incubated with glutathione sepharose (GSH) beads to collect GST-fused proteins. Bound beads were washed extensively with Igepal buffer (20 mM Tris–Cl, pH 7.5, 1% Igepal CA-630, 300 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium orthovanadate) for further experiments, including NF-L self-assembly and protein–protein interactions.

2.5. In vitro assay of NF-L self-assembly

To assess NF-L polymerization, mouse NF-L purified from *E. coli* in urea buffer (25 mM Na-phosphate, pH 7.5, 6 M urea, 1 mM EGTA and 1 mM dithiothreitol) were dialyzed against 1000× NF-L polymerizing buffer (20 mM Tris–Cl, pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin) on ice (12 h with two buffer changes). Proteins were cleared by centrifugation (100,000 × *g*, 1 h). Assembly was initiated by the addition of glutathione sepharose 4B-bound GST-NF-L (H/R) fusion proteins [final reaction volume of 200 μl containing 2.5 μg NF-L and 0.5 μg GST-NF-L (H/R)] with gentle shaking at 35 °C. $CaCl_2$ was added to the assembly reaction at the required concentrations. The resulting proteins bound to sepharose beads were washed three times with Igepal buffer. Bound samples were resolved by 10% SDS-PAGE, and blotted onto PVDF membranes for Western immunoblotting with monoclonal anti-NF-L antibodies.

2.6. Immunofluorescent microscopy

SW13 cells were seeded on glass coverslips in six-well plates, and co-transfected with a combination of pREFA-NF-M and either pEGFP-NF-L or pEGFP-NF-L (H/R), using a calcium phosphate co-precipitation kit (Promega, Madison, WI). Following transfection, cells were grown for 2 days in DMEM. Next, cells were fixed at 37 °C for 10 min in 4% paraformaldehyde, and incubated with monoclonal anti-NF-M for 1 h at room temperature in a humidity chamber. Following complete washing with PBS, cells were incubated with rhodamine-conjugated Affinipure goat anti-mouse IgG. Immunostained cells were observed with a confocal laser beam microscope.

PC12 cells transfected with pEGFP, pEGFP-NF-L or pEGFP-NF-L (H/R) were grown for 2 days in DMEM. Cells were serum-starved for 1 day, and treated with nerve growth factor (NGF) for a further 2 days. Next, cells were fixed at 37 °C for 10 min in 4% paraformaldehyde, and washed twice with PBS. Specimens were observed with a confocal laser beam microscope.

2.7. Electron microscopy

For negative staining of the NF-L filament, a 5 μl aliquot of the reaction mixture was mixed with an equivalent volume of 2% uranyl acetate for 1 min, and the mixture adsorbed for 1 min to a carbon-coated copper grid (200-mesh). The grid was washed with a drop of assembly buffer, and air-dried after removal of excess liquid by suction with a sharp-cut filter paper. Specimens were examined on a transmission electron microscope (TEM) at normal magnification (either 20,000× or 30,000×).