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Injury-induced CRMP4 expression in adult sensory neurons; a possible target gene for ciliary neurotrophic factor

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ABSTRACT

Neurotrophic cytokines, such as ciliary neurotrophic factor (CNTF) play an important role in the development and regeneration of the nervous system. In the present study, we screened gene expression induced by CNTF in adult dorsal root ganglion (DRG) neurons using the Illumina microarray. We found that the expression of both short and long forms of collapsin response-mediator protein 4 (CRMP4) was increased in cultured primary sensory neurons by CNTF. In addition, sciatic nerve injury induced the expression of CRMP4 mRNA and protein in DRG neurons. Finally, the increased CRMP4 protein was transported into peripheral axons following nerve injury. These findings indicate that CRMP4 may be a target gene for CNTF in the regenerative axon growth of DRG neurons after injury.

Peripheral nerve injury induces the expression of many genes in dorsal root ganglion (DRG) neurons that are ultimately implicated in successful regeneration [2]. The axotomy-induced gene expression involves activation of several transcription factors. For example, the induction of the immediate early gene, activating transcription factor 3 enhances regenerative axon growth [20]. Cyclic AMP responsive element binding protein is also involved in the transcription of many regeneration-related genes in adult sensory neurons [4]. Several evidence suggest that signal transducer and activator of transcription 3 (STAT3) plays an important role in the injury-induced regenerative axon growth of DRG neurons. First, STAT3 is activated and transported into DRG neurons after peripheral nerve injury [9]. Second, the inhibition of STAT3 activation after injury suppressed regenerative axon growth *in vitro* and *in vivo* [5,6]. These findings indicate that ligands activating those transcription factors may be essential for the successful regeneration of peripheral nerves following injury.

The gp130-related cytokines such as interleukin-6, leukemia inhibitor factor (LIF) and ciliary neurotrophic factor (CNTF) activate STAT3 in a variety of cells [7,15]. We previously reported that CNTF might be one of the endogenous ligands for the activation of STAT3 in sensory neurons after peripheral nerve injury [24]. However, the molecular mechanism of CNTF-induced regenerative axon growth is largely unknown at present. In the present study, in order to investigate the molecular mechanism of the CNTF-mediated regenerative axon growth, we tried to determine possible target genes for CNTF in the adult DRG neurons by using an Illumina gene chip array. We screened ~20,000 genes in the adult primary sensory neurons after CNTF treatment and found that 9 genes were elevated more than two folds by CNTF. Among them, we analyzed injury-induced collapsin response-mediator protein 4 (CRMP4) expression in detail.

Dissociated DRG neuronal cultures were performed according to our previous study [24]. All procedures were performed according to protocols approved by the Dong-A University Committee on animal research, which follows the guideline for animal experiments established by the Korean Academy of Medical Sciences. Briefly, DRGs from adult Sprague–Dawley rats were enzymatically digested with 0.125% collagenase type I (Sigma, St. Louis, MO, USA) and 0.25% trypsin for 30 min at 37 °C. After enzymatic digestion, the cells were dissociated by mechanical trituration through a fire-polished glass pipette. The cells were plated onto 35 mm dishes and cultured for 36–48 h in the presence of cytosine arabinoside (10 μM). The cells were washed and cultured in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum and N_2 supplement for another one day and treated with CNTF (50 ng/ml) for 3 and 14 h.

Total RNA isolated from cells by using Trizol reagent were prepared from three independent experiments ($n = 3$). RNA samples were processed for hybridization to microarrays by the Macrogen Biochip corporation (Macrogen, Seoul, Korea) as previously described [11]. Briefly, 100 ng of biotin-labeled cRNA samples were

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Table 1
PCR primers and annealing temperature.

Gene name	Forward primer 5′–3′	Reverse primer 5′–3′	Product size
Ab2-143	CCATCCCATTTGAGATGCTT	GATTGGTGGGGCATTTTCTA	219 bp (59)
Ac1147	CACACTTGCTGCTGTGGTTC	ACGGAAGGAGCCTTTTAACC	197 bp (59)
CRMP4a	GAAGAACATTCCTCGGATCAC	CTCCATTCTCGGCATGAACT	590 bp (57)
CRMP4b	GGATTTTGATGCCCTGAGTG	TCTGCAAGACCTCCTTGCCAG	201 bp (57)
CRMP4C	CTTTGCATGTGGACATCACC	CTCCATTCTCGGCATGAACT	201 bp (57)
LRRG	CCTCAGAGTGAAAGGCTGGA	CCCTTGTATTTGGGGCATAG	203 bp (59)
Ednrb	CTGTGGGGATCACAGTGTTG	GGGGCTTTCCTTTGTAGTCC	204 bp (59)
MARCKS	GTCGCCTTCCAAAGCAAAT	CGGCTGCCTCATCCTTATC	192 bp (59)
Paladin	ATCCAGCAGGTGAACACTCC	AAGGAGAGGTGGTGGTTCCT	201 bp (59)
Pdpk1	AACGAAGACATGCACATCCA	AAGTCCTGCCACAAGCTGAT	198 bp (59)
RCK	ACTTCGGATGTGACCTCCAC	CCGTTCAAGTAAGGGGATGA	214 bp (59)
GAPDH	TGCCGCCTGGAGAAACCTGC	TGAGAGCAATGCCAGCCCCA	172 bp (57)

The numbers in the parenthesis indicate the annealing temperature.

hybridized to Sentrix Rat-Ref-12-v1 Expression Bead Chips containing gene-specific oligonucleotides (~22,000 genes, Illumina, Inc., San Diego, CA, USA). Hybridization was detected with 1 μg/mL of Cy3-streptavidine (Amersham Biosciences, Piscataway, NJ, USA), and the chips were scanned with an Illumina Beadarray Reader. After image scan, the BeadStudio program (Macrogen) was used to generate microarray data for the genes that were differentially expressed. For RT-PCR analysis, cDNA was synthesized with 500 ng of total RNA using Ready-to-go-your-prime first strand beads (Amersham Biosciences) as indicated by the manufacturer. The primer sequence and annealing temperature are summarized in Table 1.

For sciatic nerve lesion, adult Sprague–Dawley rats were anesthetized with a mixture of ketamine and rumpun (2:1) as previously described [10]. Briefly, the sciatic nerve was exposed at the mid-thigh level and 2 mm-piece of the nerve was resected and discarded. The regeneration was inhibited by deflection of the proximal stump. Skin incision was closed with sutures, and animals were housed in plastic cages for various times after injury. At two days after the nerve transection injury, animals were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH, 7.2). Lumbar DRGs were taken out, cryoprotected and frozen in a block of OCT compound as previously described. Serial cross-sections (16 μm) were cut in a cryocut (Frigocut 2800N, Leica, Germany) and stored at −70 °C. The frozen sections were subjected to immunohistochemical staining or *in situ* hybridization histochemistry as previously described [10]. For immunofluorescent staining, the slides were blocked with PBS containing 0.2% Triton X-100 and 2% bovine serum albumin for 1 h. The sections were then incubated with primary antibody (anti-CRMP4, Chemicon, 1:1000) for 16 h at 4 °C and washed three times with PBS. Next, the slides were incubated with Cy3- or Alexa 488-conjugated secondary antibody (1:800, Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 3 h at room temperature. The sections were then washed three times with PBS, and coverslips were adhered to glass slides with a mounting medium and viewed using a laser confocal microscope (LSM510, Carl Zeiss, Germany).

For making rat CRMP4 cRNA probes, a 201 bp cDNA fragment (407–608 bp) of rat CRMP4a (Fig. 1B) was PCR amplified and subcloned into a pGEM-T vector (Promega, Madison, WI, USA). The recombinant clones were verified by sequencing. The plasmid containing the fragment of CRMP4 cDNA was linearized at NcoI or SalI sites located in the polylinker for antisense and sense probes, respectively. Labeling of RNA probes with digoxigenin-11-UTP (DIG-11-UTP) was performed using an *in vitro* transcription kit (Roche Diagnostics, IN, USA). The amount and size of the transcribed cRNA were estimated with RNA gel electrophoresis. For *in situ* hybridization, frozen sections were fixed again in 4% PFA for 10 min, washed three times with phosphate-buffered saline (PBS), and finally acetylated for 10 min. After prehybridization, the sections were incubated with the hybridization buffer (50% formamide, 4× SSC, 0.1% CHAPS, 5 mM EDTA, 0.1% Tween-20, 1.25× Denhartdt's, 125 μg/ml yeast tRNA, 50 μg/ml heparin, 200 ng of digoxigenin-labeled probes) for 18 h at 57 °C. Non-specific hybridization was removed by washing in 2× SSC for 10 min at room temperature and the treatment with RNase A (10 μg/ml), followed by final washing with 0.1× SSC at 57 °C for 15 min. For immunological detection of digoxigenin-labeled hybrids, the sections were incubated with anti-digoxigenin alkaline phosphatase (Roche Diagnostics, 1:1500) for 1 h, and the color reaction was done with 4-nitroblue tetrazolium chloride (330 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml). Sections were dehydrated and mounted with Crystalmount.

For Western blot analysis, sciatic nerves were harvested and homogenized with a polytron homogenizer in modified radioimmune precipitation assay lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.5% deoxycholic acid, 2 μg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM sodium orthovanadate, and 1× protease inhibitor cocktail). Protein (25–35 μg) was separated by SDS-polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking with 0.1% Tween-20 and 5% nonfat dry milk in Tris-buffered saline (TBS, 25 mM Tris–HCl pH 7.5, 140 mM NaCl) at room temperature for 1 h, the membrane was incubated with primary antibodies (1:500–1000) in TBST containing 2% nonfat dry milk at 4 °C overnight. After three washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000) for 1 h at room temperature. The signals were detected using the Enhanced Chemiluminescence System (Amersham Biosciences).

After the Illumina gene chip array was performed, a total of 9 genes were increased more than twofold at 3 and 14 h after CNTF treatment (Table 2). In order to confirm the microarray results, we performed RT-PCR analysis. As shown in Fig. 1A, the mRNA expression levels of Ac1147, CRMP4, paladin, PFTAIRE protein kinase 1 and RCK were increased by CNTF treatment. However, the mRNA expressions of Ab2-143, endothelin receptor b, LRRG00135 and myristoylated alanine-rich protein kinase C substrate (MARCKS) showed minimal changes in expression after CNTF treatment.

CRMP is a family of phosphoproteins (CRMP1–5) that participates in axonal growth [1,16,22,23]. We thus focused on the CNTF-induced expression profile of CRMP4 in DRG neurons. It is known that CRMP genes each produce short and long forms of their transcript by using different promoters [16]. CRMP4a is the originally identified isoform [23], and the long form, CRMP4b, has a longer amino-terminal that is encoded by another exon [16] (Fig. 1B). Using specific PCR primers, we examined which isoforms of CRMP4 are induced in primary DRG neurons by CNTF. The RT-PCR experiments revealed that the expression of both forms of CRMP4