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Identification and characterization of a truncated isoform of NELL2

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ABSTRACT

NELL2 is a neuron-specific secreted glycoprotein containing an N-terminal thrombospondin I-like domain (TSP-N). In this study, we describe NELL2-Tsp, a novel alternative splice variant of rat NELL2. NELL2-Tsp uses an alternate stop codon resulting in a C-terminal truncated form of NELL2, containing a signal peptide and a TSP-N domain. NELL2-Tsp is a glycosylated protein specifically expressed in brain tissue. NELL2-Tsp and NELL2 are secreted, likely due to the putative signal peptide. However, due to the truncation, the secreted portion of NELL2-Tsp is smaller than that of NELL2. Immunoprecipitation analysis confirmed that NELL2-Tsp was able to associate with NELL2 and with itself. In addition, expression of NELL2-Tsp notably reduced secretion of NELL2 and inhibited NELL2-mediated neurite outgrowth. These results suggest that NELL2-Tsp may act as a negative regulator of wild-type NELL2.

Introduction

Nel, a protein strongly expressed in *n*eural tissues and containing *E*GF-*l*ike domains, was first identified in a chicken embryo-derived cDNA library [1]. Expression of the Nel gene is restricted to neural tissues after hatching, while the mRNA of this gene is found in all tissues during fetal development [1]. Subsequent studies identified a mammalian counterpart of chicken Nel, NELL2, in human and rat tissues [2,3]. NELL2 is strongly expressed in the mammalian brain [3,4] and is neuron-specific within the nervous system [4,5].

Previous studies have reported that different sizes of NELL2 mRNAs were present in developing mouse embryos [3], adult human tissues [2], and the human neuroblastoma IMR32 cell line [6] by Northern blot analysis. The differences in mRNA sizes may be due to alternative splicing. Recently, we reported a novel cytosolic form of rat NELL2 (cNELL2) that is generated by alternative splicing [7]. cNELL2 mRNA has a single deletion of 129 bp, equivalent to exon 3. However, the difference in length of cNELL2 and NELL2 mRNAs are not great enough to be detected by Northern blot analysis. We hypothesized that additional NELL2 splice variants might exist.

In this study, we used 3′ RACE PCR to identify a novel splice variant of NELL2 (NELL2-Tsp). NELL2-Tsp is generated by alternative splicing between exon 9 and exon 21, resulting in a C-terminal truncated variant of NELL2 containing a signal peptide and a TSP-N domain. In addition, Western blot analysis confirmed NELL2-Tsp is a secreted glycoprotein and can self-oligomerize as does wild-type NELL2. Moreover, NELL2-Tsp bound wild-type NELL2 and inhibited the secretion and function of wild-type NELL2. Based on these results, we propose that NELL2-Tsp is a negative regulator of wild-type NELL2.

Materials and methods

Antibodies. Monoclonal antibodies specific for GFP (B-2) and HA (F-7) were obtained from Santa Cruz Biotechnology. A monoclonal antibody specific for Flag (F6531-5MG) and a polyclonal antibody specific for actin (A2066) were purchased from Sigma. An anti-neurofilament monoclonal antibody (SMI-31) was purchased from Sternberger.

3′ Rapid amplification of cDNA ends (3′ RACE). NELL2-Tsp cDNA was obtained using a 3′-RACE System (Takara). Briefly, 1 μg of total RNA from adult male Sprague–Dawley rat (SD, 14-weeks-old) brain was reverse-transcribed using the supplied adapter primer. A supplied universal amplification primer and a specific internal sense primer (5′-TGCCAAGTGGCACAAGCTCTCCTTAGCC-3′) were used for PCR amplification. The PCR products were cloned into a pGEM-T Easy vector (Promega) and sequenced.

RNA extraction and RT-PCR. Total RNA was isolated from tissues of adult male Sprague–Dawley rats (SD, 14-weeks-old) using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol.

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Total RNA samples (1 μg) was reverse-transcribed using 50 pmol random primers, SuperScript™ II Reverse Transcriptase (Invitrogen), and 1 mM dNTPs at 42 °C for 1 h. Forward primer A (5′-TGTCTAGAGCCTGAGCCC-3′) and reverse primer B (5′-CAA TAGCTTGTTTCAGGGTT-3′) were designed for specific detection of NELL2-Tsp cDNA. Rat GAPDH primers were used as an internal control and amplified a 111-bp fragment (5′-AACCTGCCAAGTAT-GATGAC-3′ and 5′-TGTTGAAGTCACAGGAGACA-3′). NELL2-Tsp fragments were amplified by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s (Fig. 1C). GAPDH fragments were amplified under the same conditions except that only 25 cycles were performed and the extension time was 30 s. PCR products were analyzed by agarose gel (1.5%) electrophoresis, purified, ligated into a pGEM-T Easy vector (Promega), and sequenced.

Plasmid construction. Full-length NELL2-Tsp (Accession No.: GQ376510) was cloned into several destination vectors (non-tagged, C-terminal EGFP, C-terminal HA, and C-terminal Flag tagged vectors) using the Gateway Cloning System (Invitrogen). The amino-terminal primer 5′-GGGGACAAGTTTGTACAAAA AAGCAGGCT**CCACC**ATGGAATCCCGGGTATTACTGAGA-3′ was used to introduce an *att*B1 sequence (underlined) followed by a Kozak sequence (bold) upstream of the coding sequences of NELL2-Tsp. Similarly, the carboxy-terminal primer (5′-GGGGACCACTTTGTA CAAGAAAGCTGGGTTGGCTCTAGACAACTTGGCT-3′) was used to introduce an *att*B2 sequence immediately after the last amino acid of the gene. After amplification of full-length NELL2-Tsp, cDNA was cloned into pDONR207 vectors and converted into destination vectors, including pDS_XB-HA, pDs_XB-Flag, pDS_XB-EGFP, and pDS_XB-RFP expression vectors. Full-length NELL2 (Accession No.: AY089719) was cloned as previously described [7].

Cell culture and transfection. COS-7 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin under a humidifying atmosphere containing 5% CO_2 at 37 °C. Cells were seeded onto poly-L-lysine-coated coverslips for imaging analysis or 60-mm dishes for Western blot analysis. Transfection with expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were cultured for an additional 24 h in growth medium before further analyses. When necessary, 5 μg/

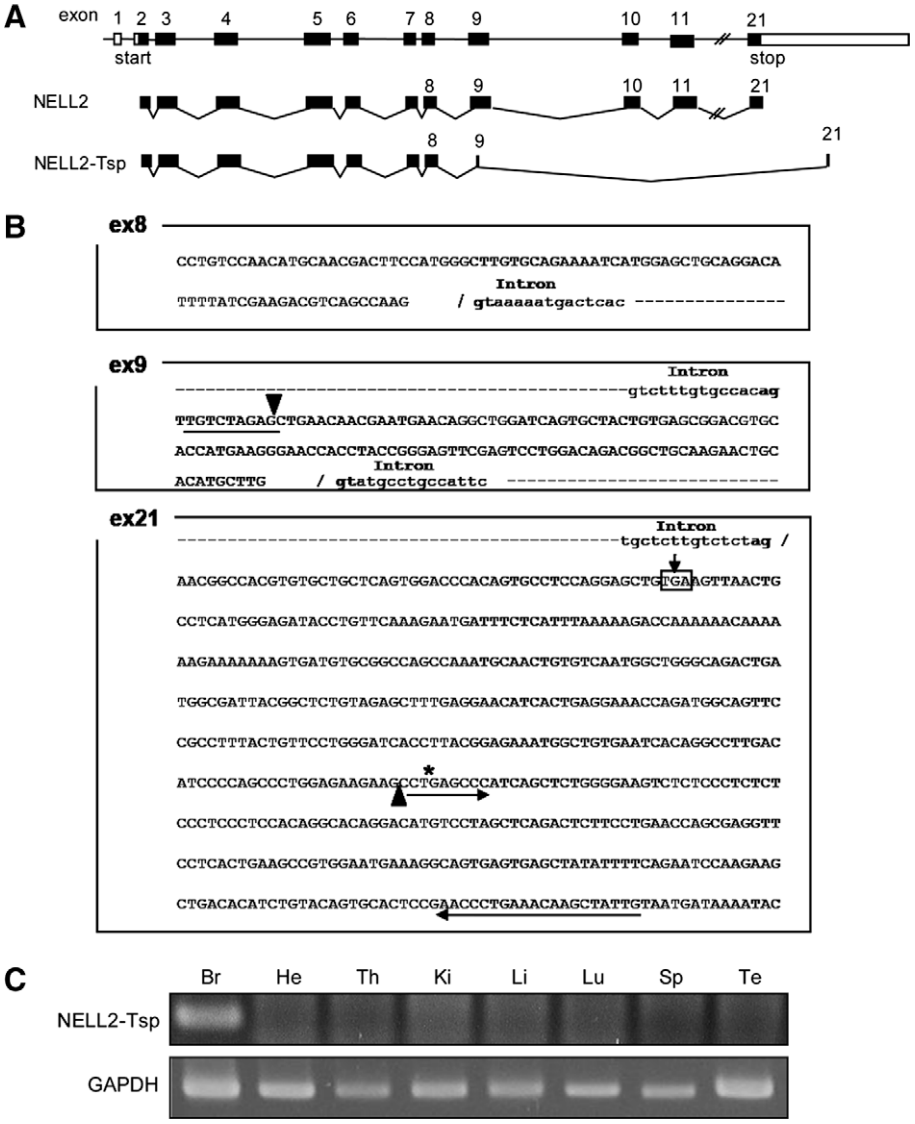

Fig. 1. Identification of NELL2-Tsp. (A) Schematic representation of wild-type NELL2 and the splice variant NELL2-Tsp. NELL2-Tsp is generated by alternative splicing between intermediate sites of exon 9 and exon 21, resulting in a C-terminal truncated form of the NELL2 protein. (B) Sequences of exon 8, exon 9, and exon 21 of NELL2. Capital letters and small letters represent exon and intron sequences, respectively. Black arrowheads indicate the splicing sites of NELL2-Tsp between exon 9 and exon 21. The box and asterisk in exon 21 indicate the stop codons for NELL2 and NELL2-Tsp, respectively. (C) Expression analysis of NELL2-Tsp in various tissues. Specific RT-PCR primers for NELL2-Tsp are indicated by arrows in (B). Br, brain; He, heart; Th, thymus; Ki, kidney; Li, liver; Lu, lung; Sp, spleen; Te, testis.