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Short communication

The human neurokinin B gene, TAC3, and its promoter are regulated by Neuron Restrictive Silencing Factor (NRSF) transcription factor family

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ABSTRACT

We have previously shown that one of the major determinants directing the expression of the preprotachykinin-A (TAC1) gene, which encodes the neuropeptide substance P, is the transcription factor Neuronal Restrictive Silencer Factor (NSRF), which is also termed Repressor Element-1 Silencing Factor (REST). In rodent models of epilepsy, NRSF and its truncated isoform short NRSF (sNRSF), also termed REST4, are increased as an immediate response to seizure. In similar models the neurokinin B (NKB) gene (TAC3) is also induced and NKB has also been shown to be proconvulsant. In this communication we have demonstrated that both the TAC3 endogenous gene and its promoter are regulated, directly or indirectly, by the NRSF transcription factors resulting in both the increased expression of the endogenous gene and increased reporter gene activity. We demonstrate by chromatin immunoprecipitation analysis that NRSF and sNRSF will bind to the NKB promoter *in vivo*. Consistent with a model in which NRSF modulation of TAC3 gene expression is a mechanism that operates during epilepsy, the observed increases in both the level of the endogenous gene and the activity of the NKB promoter by these NRSF variants, were diminished by the action of the anticonvulsant drug, carbamazepine.

1. Introduction

Neurokinin B (NKB) is a member of the tachykinin family, and is encoded by the preprotachykinin-B gene, termed TAC3 (human) or TAC2 (rodent). The human TAC3 gene consists of seven exons, with exon 5 encoding the NKB peptide. NKB, similar to the TAC1 encoded substance P (SP), is proconvulsant in rodent epilepsy models, with elevated NKB immunoreactivity and mRNA levels observed in the rat hippocampus following kainic acid administration (Marksteiner et al., 1992; Wasterlain et al., 2002; Chen et al., 2007). Furthermore, NKB peptide agonist administration has been shown to exacerbate kainic acid induced neuronal death in mice (Chen et al., 2008). We have previously shown that the Neuron Restrictive Silencing Factor (NRSF) also known as Repressor Element-1 Silencing Transcription Factor (REST) and its truncated isoform sNRSF (also termed REST4) regulate the SP encoding TAC1 gene (Quinn et al., 2002; Spencer et al., 2006). Recently, rodent neurokinin B (TAC2) has been proposed to be a NRSF target based on the presence of a putative NRSF binding site, the NRSE (neuron restrictive silencing element), which was identified bioinformatically, over 20 kb upstream from the gene (Otto et al., 2007). We have identified a putative NRSE spanning +50 to +71 of the human NKB promoter, based on the consensus sequence (T C/T AG A/C A/G CC NN A/G G A/C G/C AG) (Wu and Xie, 2006) and incorporating the core sequence CAGCACC (Bruce et al., 2004). In this communication, we have attempted to validate if NRSF could regulate the NKB gene and its proximal promoter and whether that activity could be modulated by the action of an anticonvulsant.

2. Materials and methods

2.1. Generation of human NKB promoter reporter gene constructs

Two human NKB promoter reporter gene constructs were generated by PCR amplification of the NKB proximal promoter region spanning either −757 to +181 (pNKB-757) or −289 to +181 (pNKB-289). These regions contained the putative NRSE, also termed Re-1 (Repressor Element-1), based on the consensus sequence (T C/T AG A/C A/G CC NN A/G G A/C G/C AG) (Wu and Xie, 2006) and incorporated the core sequence CAGCACC (Bruce et al., 2004), spanning +50 to +71 of the human NKB promoter. The PCR products were subsequently cloned into the pGL3basic (Promega) luciferase reporter gene vector. Primers used to amplify each fragment were designed with flanking MluI (forward primer) and XhoI (reverse primer) restriction enzyme sites for directional cloning into the corresponding MluI and XhoI sites in pGL3basic. Furthermore, an additional AA sequence was added 5′ of each primer to enhance cleavage efficiency. The primers used to clone the −757 to +181 NKB construct (pNKB-757) were: For 5′-AAACGCGTCTCGTGAAACTCCACAACGA-3′, Rev 5′-AACTCGAGCATCCAGCATTCTCCCACTT-3′. The primers used for the −289 to +181 NKB construct (pNKB-289) were:

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For 5′-AAACGCGTCTTCTGAGGCGGCTATTGAG-3′, Rev 5′-AACTCG-AGCATCCAGCATTCTCCCACTT-3′.

2.2. Cell culture

The human neuroblastoma cell lines SK-N-AS and SH-SY5Y were obtained from American Type Culture Collection. SK-N-AS cells were routinely maintained in Dulbecco's modified eagle's medium (DMEMs) (Sigma), 10% Foetal bovine serum (FBS) (Perbio, Hyclone), 1% penicillin + streptomycin, 1% non-essential amino acids (Autogen Bioclear), 1% 200 mM L-glutamine, at 37 °C/5% CO_2. SH-SY5Y were routinely maintained in a 1:1 ratio of Earle's modified eagle's medium (EMEMs) (Sigma) and HAM's F12 (Sigma), 10% Foetal bovine serum (FBS) (Perbio, Hyclone), 1% penicillin + streptomycin, 1% non-essential amino acids (Autogen Bioclear) and 1% 200 mM L-glutamine, at 37 °C/5% CO_2. Cells were plated out onto 24-well plates for reporter gene transfections, 6-well plates for endogenous gene assays, or T175 flasks for chromatin immunoprecipitation (ChIP) assays.

2.3. Cell transfection

SK-N-AS cells were plated out onto sterile 24-well plates, and maintained until 70% confluent. Immediately prior to transfection, cells were washed with PBS and fresh medium was added. Cells were transfected with ExGen 500 (Fermentas) according to manufacturer's guidelines. Briefly, 1 μg reporter gene construct (pNKB-757 or pNKB-289) was added together with 1 μg expression construct (REEX1 or HZ4) to 100 μl 150 mM NaCl. Twenty nanograms of TK renillin (Promega) was also added to act as an internal control. This was combined with 3.3 μl of ExGen 500/1 μg DNA and incubated for 20 min at room temperature. The resulting ExGen-DNA mix was added to each well of cells and incubated for 48 h before being processed for the luciferase assay. For endogenous gene assays, cells were plated out onto sterile 6-well plate, and maintained until 70% confluence. Cells were transfected using ExGen 500 as above, with 3 μg expression construct (REEX1 or HZ4) in 100 μl 150 mM NaCl. Transfected cells were maintained for either 24 h or 48 h before being processed for RNA extraction. Baseline time points were obtained from transfected cells, immediately processed at 0 h. REEX1 contains the full length human NRSF cDNA cloned into pCMV vector and was a kind gift from Prof G. Mandel (Howard Hughes Medical Institute, Stony Brook, NY). HZ4 expression construct contains 2 kb of human NRSF sequence comprising the entire N-terminal domain, DNA binding domain and lacking the C-terminal repression domain, analogous to sNRSF, under the control of a CMV promoter, and was a gift from Dr. D.J. Anderson (Howard Hughes Medical Institute, Pasadena, CA).

2.4. Luciferase reporter gene assay

Cells were washed twice in sterile PBS, lysed with 100 μl 1× passive lysis buffer (Promega) and incubated with gently rocking for 20 min. Lysates were transferred to 1.5 ml Eppendorf tubes and briefly centrifuge at 1500 rpm for 1 minute to remove cellular debris. Dual luciferase assay was performed by a Glomax™ 96 Microplate Luminometer (Promega) according to manufacturer's instructions. The ratio of Firefly luciferase to renillin luciferase units were calculated and compared to reporter gene construct alone (no expression constructs transfected).

2.5. RNA extraction and reverse transcription

Total RNA was extracted from SK-N-AS and SH-SY5Y cells using TRIzol (Invitrogen), according to manufacturer's guidelines. The resulting RNA pellet was resuspensed in RNase free water. The RNA concentration was determined using a spectrophotometer (Genoway). One microgram of total RNA was converted into cDNA using the reverse transcriptase system (Promega). Briefly, 1 μg of RNA was combined with 1 μg of oligo dT primers, 2 μl reverse transcriptase buffer, 2 μl 10 mM dNTPs, 0.5 μl RNAsin ribonuclease inhibitor, 4 μl 25 mM $MgCl_2$, 0.75 μl reverse transcriptase plus dH_2O to give a total volume of 20 μl, for each RT-PCR reaction. The reverse transcription reaction was carried out at 42 °C for 15 min, followed by 95 °C for 5 min to inactivate the reverse transcriptase.

2.6. Polymerase chain reaction (PCR)

For expression profiling, 10 ng cDNA was used in each PCR reaction. Briefly, 10 ng cDNA was added to 4 μl 25 mM $MgCl_2$, 5 μl Go-Taq reaction buffer (Promega), 0.5 μl 20 pmol forward and 0.5 μl 20 pmol reverse primers, 1 μl 10 mM dNTPs and 0.2 μl (1 unit) Go-Taq polymerase (Promega). Thermal cycling conditions were as follows; 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and a final 72 °C for 2 min. Primers used to amplify NKB were Forward: 5′-TGCTGCTATTCACAGCCATC-3′ and Reverse: 5′-AGCAATCCCTCCAGAGATGA-3′, giving a product of 174 bp. Primers used to amplify NRSF were Forward: 5′-TAT-GCGTACTCATTCAGGTGA-3′ and Reverse: 5′-TTTGAAGTTGCTTC-TATCTGCTGT-3′, giving a product of 166 bp. Primers used to amplify sNRSF were Forward: 5′-GGATACCATTTGGTAATATTTAC-3′ and Reverse: 5′-TTTGAAGTTGCTTCTATCTGCTGT-3′, giving a product of 191 bp. Primers used to amplify RNA polymerase II (Pol II) were Forward: 5′-GCACCACGTCCAATGACAT-3′ and Reverse: 5′-GTGCGGCTGCTTCCATAA-3′, giving a product of 267 bp. Endogenous mRNA band intensities where measured using the Scion Image software (Scion Corporation, NIH, Baltimore, USA). Band intensities for NKB where normalized against the house keeping gene, RNA polymerase II (Pol II).

2.7. Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation assays, cells were cultured in sterile T175 flasks, and maintained until 70% confluence. DNA and protein from human SK-N-AS and SH-SY5Y neuroblastoma cells were cross-linked with 1% formaldehyde and mixed thoroughly at room temperature for 10 min. Chromatin immunoprecipitation (ChIP) was performed using ChIP-IT express (Active Motif, Belgium) according to the manufacturers′ instructions. The antibodies used were polyclonal NRSF and sNRSF antibodies, raised against the epitopes CYFLEEAAEEQE (R2174) and SGCDLAG (Belyaev et al., 2004) respectively, and where a kind gift from Prof. Noel Buckley (University College London, UK). In addition, we used the ChIP-IT™ human control kit negative control mouse IgG antibody. Briefly, cross-linking was quenched by addition of Glycine "Stop-Fix" buffer and nuclei were prepared and lysed in lysis buffer supplemented with protease inhibitor cocktail (PIC) and PMSF to ensure that the protein/DNA interactions were preserved during chromatin purification and immunoprecipitation. Chromatin was sheared by sonication, 20× 30 s pulses at 50% power using a Microson sonicator (Misonix). Efficiency of sonication was determined by agarose gel electrophoresis. Following phenol-chloroform extraction, DNA concentration was determined. 6.3 μg of sheared chromatin, 3 μg of antibody and 25 μl of protein G magnetic beads were mixed for immunoprecipitation with ChIP buffer supplemented with PIC. The samples were incubated at 4 °C overnight on a rotating wheel. Following this step the samples were transferred to clean siliconised tubes to reduce background contamination. DNA was eluted, cross-links were reversed and the DNA was purified before subsequent PCR analysis. The PCR conditions for amplification of the NKB proximal promoter were 36 cycles consisting of 95 °C denaturing step (30 s), 64 °C annealing step (30 s) and 72 °C extension step