Available online at www.sciencedirect.com
SCIENCE DIRECT
Neuroscience Letters 398 (2006) 161–166
Neuroscience Letters
www.elsevier.com/locate/neulet

Characterization of the nuclear targeting signal of REST/NRSF

Masahito Shimojo *

Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Biomedical Biological Sciences Research Building, 741 Limestone Street, Lexington, KY 40536-0509, USA

Received 21 October 2005; received in revised form 22 November 2005; accepted 29 December 2005

Abstract

RE-1 silencer transcription factor (REST), also known as neuron-restrictive silencer factor (NRSF), contains nine Cys_2-His_2 type zinc finger domains (ZFDs). REST/NRSF is localized to the nucleus, where it represses the transcriptional activity of a large number of neuronal genes in non-neuronal cells. It has been suggested that REST/NRSF contains a nuclear localization signal (NLS) corresponding to amino acids (512–522). However, our studies showed that REST4, a REST/NRSF splicing isoform, which contains the N-terminal 5 of 9 ZFDs, efficiently localized to the nucleus. On the other hand REST1, another REST/NRSF splicing isoform, which contains 4 of the 9 ZFDs, localized to the cytosol. In this study REST-ΔC, which contains 8 ZFDs with the NLS (512–522) deleted, was found to localize to the nucleus in HeLa, COS and PC12 cells. Complete deletion or mutation of NLS (512–522) still permitted REST/NRSF to be localized to the nucleus in HeLa, COS and PC12 cells. In contrast REST/NRSF constructs which contain a deletion of ZFD-5 mislocalized to the cytosol. A point mutation in the zinc finger structure that disrupts its conformation remains nuclear. These data suggest that REST/NRSF contains a NLS around ZFD-5, while the putative NLS at residues 512–522 is non-functional.

Keywords: Transcription; Repressor; Nuclear translocation; Nuclear localization signal; Gene regulation

The RE-1 silencer transcription factor (REST) [3] or neuron-restrictive silencer factor (NRSF) [13] is a transcriptional repressor, which contains 9 Krüppel-like zinc finger domains. REST/NRSF blocks transcription of its target genes such as ChAT [7], SCG10 [8] and type II sodium channel [6] by binding to a specific consensus 21 bp cis-element which is present in the regulatory regions of its target genes [14]. Although REST/NRSF was originally thought to only function in non-neuronal cells, a number of recent reports suggest that REST/NRSF is expressed in some neurons [9]. The mechanism controlling not only neuronal gene expression but also neuronal cell differentiation is complex and not understood. REST/NRSF is downregulated during the induction and maintenance of neuronal differentiation [1] while overexpression of REST/NRSF in differentiating neurons disrupts neuronal gene expression and causes axon guidance errors [11].

A number of splicing isoforms of REST/NRSF have been identified [9,10]. One of these truncated forms, REST4 contains only 5 of the 9 zinc finger domains of REST/NRSF. Expressed recombinant FLAG-REST4 was localized to the nucleus, while REST1, another of the REST/NRSF isoforms containing only the first 4 zinc finger domains, localized to the cytosol. Thus, it was suggested that sequences in the vicinity of zinc finger domain 5 contains a nuclear localization signal [16]. However, Grimmes et al. [5] reported that deletion of a putative NLS located within residues 512–522 of the larger REST/NRSF molecule caused it to localize to the cytosol in COS cells. In order to determine whether there are two nuclear localization signals in REST/NRSF, one near zinc finger 5 and the other at the more C-terminal residues 512–522, we studied the nuclear targeting of REST/NRSF deletion and point mutations of the putative nuclear localization signals. The results of this study show that the region around zinc finger domain 5 is the sole functional nuclear localization signal in both REST4 and REST/NRSF.

Deoxyribonucleotides, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Oligonucleotides were purchased from MWG Biotech (Charlotte, NC). The QuickChange^R site-directed mutagenesis kit and PfuUltra^R Hotstart DNA polymerase were purchased from Stratagene (La Jolla, CA). The ECL+Plus Western blotting detection system and Hybond-P membrane were purchased from Amersham Life Science Inc.

* Tel.: +1 859 323 6128; fax: +1 859 257 2283.
E-mail address: mshim1@uky.edu.

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2005.12.080

(Arlington Heights, IL). Effectene transfection reagent and plasmids purification kits were obtained from Qiagen Inc. (Valencia, CA). AlexaFluor^{R}488 linked IgG was from Molecular probes (Eugene, OR), while vectashield H-1000 was from Vector Laboratories, Inc (Burlingame, CA). The NE-PER-extraction kit was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest quality available.

N-Terminal FLAG epitope-tagged human REST/NRSF and REST-ΔNLS$^{(512–522)BamHI}$, in which nucleotides 1535-1566 were removed and replaced with a *Bam*HI site were generously provided by Dr. Gail Mandel (SUNY at Stony Brook). For mutagenesis, the specific primers used with the QuickChange^{R} site-directed mutagenesis kit were as follows.

5′-ATA CAG GAA GCA ATT CAG AAC TGG AAG TTG ACA GCC AT-3′; 5′-ATG GCT GTC AAC TTC CAG TTC TGA ATT GCT TCC TGT AT-3′ (for NLS deletion); 5′-GGA AGC AAT TCA GAA GCT TTC AGT AAA ACT GCT GAT AGT ATG GAC GCT CTG GAA GTT GAC AGC-3′; 5′-GCT GTC AAC TTC CAG AGC GTC CAT ACT ATC AGC AGT TTT ACT GAA AGC TTC TGA ATT GCT TCC-3′ (for NLS mutation); 5′-ACA TTT AAA TGG CTT CTC ACC TCC TGT ATG AGT TCT AAC ATG-3′; 5′-CAT GTT AGA ACT CAT ACA GGA GGT GAG AAG CCA TTT AAA TGT-3′ (for zinc finger domain 5 deletion). 5′-ACA TTT AAA TGG CTT CTC ACC TCC TGT ATG AGT TCT AAC ATG-3′; 5′-CAT GTT AGA ACT CAT ACA GGA GGT GAG AAG CCA TTT AAA TGT-3′ (for zinc finger domain 5 mutation).

Nuclear and cytoplasmic fractions from HeLa and COS cells were prepared by using the NE-PER-extraction kit (Pierce) according to the manufacture's instructions. The protein content of each sample was determined using the BCA protein assay system (Bio-Rad). Cell extracts were separated by SDS-polyacrylamide gel electrophoresis, and transferred to Hybond^{TM}-P membranes (Amersham), followed by detection with the ECL+Plus system (Amersham) according to the manufacture's instructions. Anti-REST/NRSF monoclonal antibody (a generous gift from Dr. D.J. Anderson (Caltech)) and purified anti-RILP polyclonal antibody [17] were used.

HeLa, COS and PC12 cells were cultured in Dulbecco's modified Eagles medium with 10% fetal bovine serum at 37 °C in 5% CO_2. For transfection, cells were plated on glass coverslips (22 mm × 22 mm) in 6 well plates and transfected on the next day. The transfected cells were then grown for 24 h, fixed in dry ice-methanol for 5 min, washed three times with PBS, and blocked in TBS-T-M (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, 5% skim milk). Fixed cells were incubated with the appropriate antibody (anti-RILP antibody at 10 μg/ml or anti-FLAG antibody at 35 μg/ml) in TBS-T-M at room temperature for 30 min. Cells were washed three times in TBS-T-M, followed by incubating with AlexaFluor^{R}488 linked IgG (Molecular probes) in TBS-T-M. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in PBS (1:1000) for 5 min and then washed with PBS. Coverslips were mounted in Vectashield H-1000 (Vector Laboratories, Inc.). Fluorescence was observed using a Nikon E600 epifluorescent microscope (Melville, NY).

It was previously reported that in COS cells wild type REST/NRSF localized to the nucleus, but that the REST mutant REST-ΔNLS$^{(512–522)BamHI}$ showed a cytosolic localization [5], suggesting that the sequence KFSKTKKSKRK is the nuclear localization signal of REST/NRSF. This is illustrated in Fig. 1. On the other hand we found that the region around zinc finger 5 of REST4, which is conserved in REST/NRSF also acts as a NLS. In order to resolve this apparent discrepancy we determined the intracellular localization of FLAG-tagged REST/NRSF, FLAG-tagged REST4, and FLAG-tagged mutants. This analysis included the NLS deletion mutant REST-ΔNLS$^{(512–522)BamHI}$ studied by Grimes et al. [5] in which a *Bam*HI site was inserted into the REST/NRSF gene producing a deletion of the critical residues 512 to 522 and an insertion of Gly-Ser in this region. Other constructs we studied include a deletion construct in which residues 512 to 522 were deleted with no insertion (REST-ΔNLS$^{(512–522)}$), and a mutant in which the NLS sequence was scrambled (REST-ΔNLS^{mut}), these are shown in Fig. 2A and B. These FLAG-tagged human REST/NRSF cDNAs were transfected and expressed in HeLa, COS and PC12 cells. After 24 h post-transfection, cells were incubated with anti-FLAG monoclonal antibody followed by AlexaFluor^{R}488 linked Ig and analyzed by immunofluorescense. As shown in Fig. 2C, wild-type human REST/NRSF was exclusively localized to the nucleus. REST-ΔNLS$^{(512–522)BamHI}$ also localized to the nucleus in HeLa, COS and PC12 cells, however, with COS cells a nearly equal amount was in the cytosol. With HeLa, a very small but detectable amount of the mutant was seen in the cytosol. As expected REST-ΔC showed an exclusive nuclear localization, confirming it contains an NLS (Fig. 2).

To determine whether the cytosolic REST-ΔNLS$^{(512–522)BamHI}$ mutant arises due to the introduction of a cryptic export signal, HeLa, COS and PC12 cells were treated with leptomycin B to inhibit nuclear export. As seen in Fig. 2C, leptomycin B treatment increased the nuclear localized REST-ΔNLS$^{(512–522)BamHI}$ in HeLa, COS and PC12 cells, suggesting that in fact this mutant enters, but also exits the nucleus. REST-ΔNLS$^{(512–522)}$, in which amino acids 512–522 were removed completely, localized mainly to the nucleus with a small amount of the protein seen in the cytosol, while the mutant in which amino acids 512–522 were scrambled

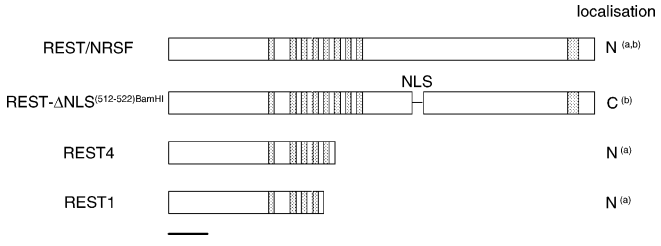

Fig. 1. Schematic of human REST/NRSF constructs. Schematic representation of the REST/NRSF protein. Human REST/NRSF contains 9 zinc finger domains. The dotted boxes indicate zinc finger domains. The ΔNLS$^{(512–522)BamHI}$ mutant is a REST/NRSF mutant in which amino acids 512–522 were deleted [5]. REST4 and REST1 are splicing isoforms of REST/NRSF, and contain 5 and 4 zinc finger domains, respectively. Localisation of each construct is shown as nuclear (N) or cytosol (C). The size bar indicates 100 amino acids. (a) Reference [16], (b) reference [5].