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Sp1 and Sp3 regulate transcription of the cyclin-dependent kinase 5 regulatory subunit 2 (p39) promoter in neuronal cells

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ABSTRACT

Cyclin-dependent kinase 5 (cdk5) activity is critical for development and function of the nervous system. Cdk5 activity is dependent on association with the regulators p35 and p39 whose expression is highly regulated in the developing nervous system. We have identified a small 200 bp fragment of the p39 promoter that is sufficient for cell type-specific expression in neuronal cells. Mutational analysis revealed that a cluster of predicted binding sites for Sp1, AP-1/CREB/ATF and E box-binding transcription factors is essential for full activity of the p39 promoter. Electrophoretic mobility shift assays revealed that Sp1 and Sp3 bound to sequences required for p39 promoter function and chromatin immunoprecipitation assays confirmed binding of these proteins to the endogenous p39 promoter. Furthermore, depletion of either Sp1 or Sp3 by siRNA reduced expression from the p39 promoter. Our data suggest that the ubiquitously expressed transcription factors Sp1 and Sp3 regulate transcription of the cdk5 regulator p39 in neuronal cells, possibly in cooperation with tissue-specific transcription factors.

1. Introduction

Regulated activity of cyclin-dependent kinase 5 (cdk5) is critical for the normal development and function of the central nervous system. Mice deficient in cdk5 die perinatally and show defects in neuronal migration in a number of brain compartments including the cerebral cortex, cerebellum and hippocampus [1]. Aberrant cdk5 activity has been implicated in neuronal cell death and the pathology of neurodegenerative diseases such as Alzheimer's and amyotrophic lateral sclerosis [2,3]. Cdk5 kinase activity is dependent on association with a regulatory partner, either p35 or p39, whose expression is highly regulated in the developing nervous system [4–6]. p35 expression is highest in post-mitotic neurons of the embryonic central nervous system, with expression peaking in migrating cells of the developing cerebral cortex [7]. In contrast, p39 expression is high in the spinal cord, peaks 1–3 weeks postnatally, and is maintained at high levels in the adult cerebellum [8–10]. The different patterns of expression of p35 and p39 may contribute to precise regulation of cdk5 activity.

It is not known whether expression of the cdk5 regulators p35 and p39 in neurons is determined by similar or different transcriptional mechanisms. We have previously identified a 17 bp GC-rich element in the p35 promoter that was both necessary and sufficient for neuron-specific expression [11]. Binding of the transcription factors Sp1, Sp3 and Sp4 to the critical GC box in the p35 promoter in vitro was found to correlate with promoter function in vivo. Sp4 expression is highest in the brain [12] and the transcriptional activities of the ubiquitously expressed Sp1 and Sp3 proteins were found to be higher in terminally differentiated neurons [11,13] suggesting that the regulated levels and activity of these GC box-binding proteins may contribute to the cell type-specific expression of p35 in post-mitotic neurons. Additional transcription factors contribute to the temporal, spatial and signal-dependent regulation of p35 in post-mitotic neurons. In cultured neurons, p35 mRNA levels are induced following activation of MAPK signalling pathways downstream of NGF and Fas, which may involve the Egr1 transcription factor [14,15]. The POU-domain transcription factors Brn-1 and Brn-2 have also been implicated in regulation of p35 and p39 transcription as loss of both Brn-1 and Brn-2 has been shown to reduce expression of p35 and p39 in some regions of the brain [16,17].

We have analyzed the promoter of the cdk5 regulator p39 in order to identify DNA elements and transcription factors that regulate expression of p39 in neurons. We have mapped the transcriptional start sites of the mouse p39 gene. Deletion analysis of the p39

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promoter identified a 200 bp GC-rich minimal promoter that is sufficient for high levels of expression in a neuroblastoma cell line (N2A), but not in a fibroblast cell line (NIH3T3). Mutational analysis of the minimal p39 promoter revealed that a tight cluster of predicted binding sites for Sp1, AP-1/CREB/ATF and E box-binding transcription factors was required for transcriptional activity in neuronal cells. Analysis of a double mutant suggests a coordinated action of transcription factors binding specific sites within the cluster. Predicted Sp1 binding sites required for promoter function were demonstrated to be required for high-affinity binding of the transcription factors Sp1 and Sp3 in vitro and these factors were shown to bind the endogenous p39 promoter in vivo. Knockdown analysis using siRNA specifically targeting either Sp1 or Sp3 revealed that both are required for full activity of the p39 promoter. These data suggest that Sp1 and Sp3 regulate expression of the neuron-specific Cdk5 regulator p39.

2. Materials and methods

2.1. Primer extension analysis

Total RNA was isolated using Trizol reagent (Invitrogen). For primer extensions, radiolabeled primers were hybridized at 60 °C for 90 min to 30 μg total RNA isolated from 15 day old mouse brain or liver and then reverse transcribed with Superscript II reverse transcriptase (Invitrogen). The primers used in primer extensions were Primer 1 5′GCGACAGCACCGTGCCCATCCT 3′ and Primer 2 5′GGAAGCAGGGGAAAGCGACAG 3′.

2.2. Northern blot analysis

For Northern analysis, 20 μg total RNA isolated from N2A and 3T3 cells was denatured and resolved by gel electrophoresis on 6% formaldehyde, 1% agarose gels and then transferred to Hybond N+ membrane (Amersham Biosciences). Hybridizations with ^{32}P-labeled p39 or GAPDH probes were carried out using Quickhyb (Statagene).

2.3. Cell culture and transfections

N2A and 3T3 cells were transiently transfected using LipofectAMINE (Invitrogen). 2×10^5 N2A or 3T3 cells were plated into 24-well tissue culture plates, and were co-transfected with 500 ng luciferase reporter construct and 25 ng of renilla reporter construct pRL-SV 24 h after plating. Cells were harvested 24 h after transfection, and luciferase and renilla assays were performed using the Dual Luciferase Assay kit (Promega). For knockdown assays with specific shRNA constructs, 10^5 cells were plated in 6 well plates and transfected with 1 μg of shRNA plasmid. For knockdown efficiency test, cells were co-transfected with 200 ng of pBABE puromycin resistant vector and selected 24 h after transfection with 1.5 μg/ml of puromycin for two more days before protein extraction. For luciferase assay cells were co-transfected with 1 μg of shRNA construct, 200 ng of reporter vector and 25 ng of pRL-SV. Luciferase and renilla activity were analyzed 48–72 h after transfection. All transfections were done in triplicate on multiple occasions.

2.4. Electrophoretic mobility shift assays

Nuclear extracts were prepared as described previously [18]. 3 μg nuclear extract was incubated with 100,000 cpm of PCR amplified (using ^{32}P-radiolabeled primers) p39−153/+46 or 4 pmol of ^{32}P-radiolabeled annealed oligonucleotide in a binding buffer containing 12.5 mM Hepes (pH 7.9), 6.25 mM $MgCl_2$, 0.5 μM $ZnSO_4$, 10% glycerol, 0.5 mM dithiothreitol, and 3 μg of salmon sperm DNA at room temperature for 20 min. Competitor oligonucleotides were incubated with the nuclear extract for 20 minutes at room temperature before addition of the radiolabeled probe. For supershift experiments, 2 μg of anti-Sp1 (Upstate Biotechnology), anti-Sp3 (Upstate Biotechnology), or anti-Sp4 (Santa Cruz Biotechnology) antibodies were incubated with the nuclear extract at room temperature for 20 min before addition of the radiolabeled probe. Free probe was separated from protein-DNA complexes by electrophoresis at 200 V on a 4% polyacrylamide gel in 0.5× TBE and 1% glycerol. The oligonucleotides used in the EMSA were gel-purified and annealed. The sequences of the top-strand oligonucleotides used in the EMSAs were:

WT: 5′GGGCCCAAGGGGTGGGGCTGACGCTGCAGCTGGCGCAGCTT 3′;
M2: 5′GGGCAACCTTTTGGGGGCTGACGCTGCAGCTGGCGCAGCTT 3′;
M3: 5′GGGCCCAAGGGGTGGGTAGTCATAGGCAGCTGGCGCAGCTT 3′;
M4: 5′GGGCCCAAGGGGTGGGGCTGACGCTGCATAGTTATACGCTT 3′;

2.5. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed following Upstate Biotechnology ChIP assay protocol with modifications. Neuro2A (N2A) cells near confluence were crosslinked with 1% formaldehyde for 15 min at 37 °C. After washes with ice cold phosphate buffer containing protease inhibitors, cells were resuspended in SDS lysis buffer. Chromatin was sonicated to between 500 and 1000 bp and immunoprecipitated with 3 μg of either anti-Sp1 (Upstate), anti-Sp3 (Santa Cruz), or rabbit anti-IgG (Promega) polyclonal antibodies. After phenol/chloroform extraction and ethanol precipitation, sheared DNA was amplified by real-time PCR using specific primers. The real-time PCR was performed as described by the manufacturer using the iQ SYBR Green Supermix (BioRad). 10 % of total starting material was precipitated and used as input.

p39-F 5′-GACCATTTTCACCTCACTTCAACC-3′
p39-R 5′-TCTACAGTCCCTTACCTTTTCCCAC-3′
actin-F 5′-TGAGAGGGAAATCGTGCGTGAC-3′
actin-R 5′-GCTCGTTGCCAATAGTGATGACC-3′

2.6. Plasmid constructs and DNA analysis

The indicated regions of the p39 promoter DNA were amplified by PCR and cloned upstream of luciferase in pGL2. Mutations of the p39 promoter were generated by PCR methods and verified by sequencing. Binding site predictions were carried out using web-based software at the Transfac web-site: http://transfac.gbf.de/TRANSFAC/. Short hairpin RNAs were cloned into pBS/U6 plasmid as described previously [19]. Sequences targeted in the Sp3 and Sp1 mRNAs as well as the negative control scrambled (scr) sequence are listed below:

Sp3-1: 5′GGGACCAACAACATCAAGAAG 3′
Sp3-2: 5′GGGAAGACCTCACATCTGGAG 3′
Sp1-1: 5′GGGTGCCAATGGCTGGCAGAT 3′
Sp1-2: 5′GGGAACATCACCTTGCTACCT 3′
scr: 5′GGGAATTAATATGCACAGGCC 3′.

3. Results

3.1. Identification of the mouse p39 transcription start site

As a first step to investigate the regulation of transcription of the cdk5 activator p39, the position of the p39 promoter was identified by mapping the transcription start site. Like many neuron-specific genes, the sequences upstream of the mouse p39 ORF are highly GC-rich and do not contain a consensus TATA-box. Primer extension analysis, using primers either spanning or 3′ to the p39 ATG (Primer 1 and Primer 2) and RNA isolated from 15 day old-mouse brain, indicated that there were at least 5 transcriptional start sites located between 63 and 95 bp upstream of the ATG (Fig. 1A). Transcripts of the same size were not detected using RNA isolated from 15 day old-mouse liver consistent