Biochemical and Biophysical Research Communications 442 (2013) 189–194

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Zinc finger protein 219-like (ZNF219L) and Sox9a regulate *synuclein-γ2* (*sncgb*) expression in the developing notochord of zebrafish ☆

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ARTICLE INFO

Article history:
Received 6 November 2013
Available online 20 November 2013

Keywords:
Synuclein-γ2 (*sncgb*)
ZNF219-like (ZNF219L)
Sox9a
Notochord
Zebrafish

ABSTRACT

Zebrafish *synuclein-γ2* (*sncgb*) has been reported to be expressed specifically in the notochord. However, the mechanism by which the *sncgb* gene promoter is regulated has not been described. In this paper, we demonstrate that Zinc finger protein 219-like (ZNF219L) and sox9a are involved in the regulation of *sncgb* gene expression. Furthermore, we observed that over-expression of both ZNF219L and Sox9a resulted in increased *sncgb* expression. In addition, ZNF219L is physically associated with Sox9a, and simultaneous morpholino knockdown of *znf219L* and *sox9a* caused a synergistic decrease of *sncgb* expression in the notochord. Taken together, our results reveal that coordination of ZNF219L with Sox9a is involved in the regulation of notochord-specific expression of *sncgb*.

1. Introduction

The first member of the synuclein family was isolated from the purified cholinergic synaptic vesicles of the Pacific electric ray, *Torpedo californica* [1]. In humans, the synuclein family consists of α-, β-, and γ-synuclein genes (*SNCA*, *SNCB*, and *SNCG*, respectively). Misfolded α-synuclein protein has been reported to be involved in the pathological accumulation of intraneuronal protein aggregates (Lewy bodies and Lewy neurites), thereby leading to cell dysfunction and cell death in the pathogenesis of Parkinson's disease (PD) [2,3]. Furthermore, β-synuclein acts as a regulator of α-synuclein-induced neurotoxicity [4]. Although there is no clear evidence that γ-synuclein is involved in neurodegenerative diseases, γ-synuclein has been reported to be overexpressed in breast carcinomas and ovarian cancer [5].

In contrast to humans, the synuclein family in zebrafish consists of β-, γ1-, and γ2-synuclein genes (*sncb*, *sncga*, and *sncgb*, respectively). Early expression of zebrafish *sncb* begins at the trigeminal placode, before extending to the ventral diencephalon, olfactory placode, ventral tegmentum, and spinal cord neurons. *Sncga*, on the other hand, is expressed in cells of the nervous system, including hindbrain neurons, cranial ganglia, and retinal cells; simultaneous knockdown of *sncb* and *sncga* was reported to decrease spontaneous motor activity [6]. Expression of *sncgb* is significantly different from that of *sncga*; *sncgb* transcription is restricted to the notochord throughout embryogenesis, from the 13 somite-stage to 2 days post-fertilization (dpf) [7]. However, like human *SNCG*, the function and regulatory processes of *sncgb* are still unknown.

Zinc finger protein 219 (ZNF219) is a transcription factor partner of SOX9, required for the regulation of mammalian chondrocyte differentiation [8–10]. We previously reported that the *zinc finger 219-like* (*znf219L*) gene is required for regulating the expression of the collagen type 2 alpha 1a (*col2a1a*) gene in the zebrafish notochord [11]. We cloned the zebrafish ZNF219L gene based on mammalian ZNF219, which contains nine C2H2-type zinc finger domains. We further demonstrated that zebrafish ZNF219 recognizes the GGGGG motifs in the *col2a1a* promoter through its sixth and ninth zinc finger domains, thereby up-regulating promoter activity in a luciferase assay. In addition, morpholino knockdown of *znf219L* decreased endogenous expression of *col2a1a* in the notochord [11]. Based on these findings, we hypothesized that ZNF219L may regulate the expression of notochord-related genes during early developmental stages.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.042

In this study, we demonstrate that *sncgb* is also a notochord-specific target gene of zebrafish ZNF219L. We confirm that ZNF219L activates *sncgb* expression in the notochord via binding to a GGGGG motif in the *sncgb* promoter. In addition, we demonstrate that ZNF219L forms a complex with Sox9a, and is required for the regulation of *sncgb* expression in the notochord. Knockdown of *znf219L* and *sox9a* resulted in a synergistic decrease of endogenous *sncgb* expression in the notochord. Collectively, these results indicate that coordination of ZNF219L and Sox9a regulates *sncgb* gene expression in the notochord.

2. Materials and methods

2.1. Zebrafish care

Zebrafish embryos were raised at 28.5 °C, and different developmental stages were determined based on criteria described in the *Zebrafish Book* [12]. All animal procedures were approved by Academia Sinica Institutional Animal Care and Utilization Committee (ASIACUC) (protocol #10-12-114).

2.2. Morpholino oligonucleotide (MO) injection

Antisense MOs were designed and obtained from Gene Tools (Philomath, OR, USA). MO sequences were as follows: zebrafish *znf219L*-MO, 5′-GTC TAT GCC ATG CTT CAC TTC CTT G-3′; *sox9a*-MO, 5′-AAT GAA TTA CTC ACC TCC AAA GTT T-3′; control-MO, 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. The MOs were diluted to produce 24 μg/μl injection stocks, which were stored at −20 °C prior to use.

2.3. Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes (Roche, Penzberg, Germany) were generated by *in vitro* transcription of linearized pGEM-T-easy plasmid (Promega) carrying the 3′-UTR of the appropriate zebrafish gene. To synthesize digoxigenin-labeled (Roche, Penzberg, Germany) antisense RNA probes, pGEM-T easy-*sncgb* 3′-UTR was linearized with *Pst* I and transcribed with T7 RNA polymerase. Whole-mount *in situ* hybridization was performed as previously described [13,14].

2.4. Cell culture

Carp fin (CF) epithelioid cells [15] were maintained at 27 °C in Leibovitz's L-15 media supplemented with 10% fetal bovine serum. CF cells were transfected as previously described [16]. NIH/3T3 cells (ATCC CRL-1658; Manassas, VA, USA) were cultured in a humidified atmosphere of 5% CO_2 at 37 °C, in high-glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA).

2.5. Luciferase reporter assay

Luciferase reporter gene assays were performed using pGL3 Luciferase Reporter Vectors (Promega) containing a modified coding region for firefly (*Photinus pyralis*) luciferase driven by the zebrafish *sncgb* 2.0-kb proximal promoter or 2.0-kb mut (in which the ZNF219L binding site was changed from GGGGG to GAAAG by PCR mutagenesis). The following primers were used to amplify the zebrafish *sncgb* 2.0-kb proximal and 2.0-kb mut promoter: forward primer: 5′-GGC ATA AAT CCA TGT ATG CGT CAA AAC GGC-3′; reverse primer: 5′-CTG GAT CTG GTG CTC GTC TAT AGC TGG-3′. The luciferase reporter constructs were transfected into CF epithelioid cells. One microgram of promoter DNA and 0.5 mg of pSV-β-galactosidase were co-transfected into CF epithelioid cells using the PolyJet *In Vitro* DNA Transfection Reagent (SignaGen Laboratories, Ijamsville, MD, USA). Transfections were performed using 12-well plates, and approximately 1×10^5 cells were seeded 1 day before transfection. Cells were lysed 2 days after transfection (by which time cells had grown to 90% confluency), and luciferase activity was determined using a luminometer (Promega), according to the manufacturer's protocol. Transfection efficiency was normalized by determining the activity of β-galactosidase activity in the cell lysates. All presented data are the results of three independent experiments, and were statistically analyzed by one-way ANOVA. Data represent means ± s.d.

2.6. Western blot

Western blot was performed by incubating membranes with anti-HA monoclonal antibody (1:3000; Santa Cruz, CA) at 4 °C overnight. Signals were detected using an enhanced chemiluminescence (ECL) kit (NEN Life Science Products, MA).

2.7. Oligonucleotide precipitation assay

NIH 3T3 cells were lysed in lysis buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 10 mg/ml leupeptin, 1 mM PMSF, 0.2 mM sodium orthovanadate] for use in the *in vitro* binding assay. The lysates were pre-incubated with streptavidin-agarose beads for 3 h, and then incubated for 16 h with 1 mg of poly(dI-dC) and 1 mg of a biotinylated double-stranded oligonucleotide probe. The biotinylated double-stranded oligonucleotide probe contained the ZNF219L binding element present in the *sncgb* gene promoter, and was generated using the following primer pair: BS sense primer: 5′-GGC AAA AAG GGG AGG GGG TGG ATG GGT TTG-3′; anti-sense primer: 5′-CAA ACC CAT CCA CCC CCT CCC CTT TTT GCC-3′.

Table 1
Primers used for PCR amplification of cDNA encoding full-length and deletion mutations of zebrafish *znf219L*.

Gene	Sequences of forward (F) and reverse (R) primers
znf219L	HindIII-F, 5′-AAA AGC TTA TGG ATT CCC CAC CAG AAT GTA TGC TG-3′
	KpnI-R, 5′-GGG GTA CCC TAT TCC CCC AAG ACA CCT TCC TCC TC-3′
znf219L-Δ1	HindIII-F, 5′-AAA AGC TTCCA GAA ACT CCC GTC CCA CAA ATG TCT-3′
	KpnI-R, 5′-GGG GTA CCC TAT TCC CCC AAG ACA CCT TCC TCC TC-3′
znf219L-Δ2	HindIII-F, 5′-AAA AGC TTCAT CAG CAT CCT TCA TCA C-3′
	KpnI-R, 5′-GGG GTA CCC TAT TCC CCC AAG ACA CCT TCC TCC TC-3′
znf219L-Δ3	HindIII-F, 5′-AAA AGC TTA TGG ATT CCC CAC CAG AAT GTA TGC TG-3′
	KpnI-R, 5′-GGG GTA CCT GGA CAT GCC CAC TCT GGC AC-3′
znf219L-Δ4	HindIII-F, 5′-AAA AGC TTC GTG AGC AAA GGA ATG CTA TGG CA-3′
	KpnI-R, 5′-GGG GTA CCC TAT TCC CCC AAG ACA CCT TCC TCC TC-3′
znf219L-Δ5	HindIII-F, 5′-AAA AGC TTA TGG ATT CCC CAC CAG AAT GTA TGC TG-3′
	KpnI-R, 5′-GGG GTA CCT GCC ATA GCA TTC CTT TGC TCA CG-3′
znf219L-Δ6	HindIII-F, 5′-AAA AGC TTA TGG ATT CCC CAC CAG AAT GTA TGC TG-3′
	KpnI-R, 5′-GGG GTA CCG TTA CCC ATT TCT GCA ACC TG-3′