Biochimica et Biophysica Acta 1779 (2008) 805–810

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

KyoT3, an isoform of murine FHL1, associates with the transcription factor RBP-J and represses the RBP-J-mediated transactivation

Liang Liang [a,1], Hong-Wei Zhang [a,1], Jie Liang [a,1], Xiao-Li Niu [b], Su-Zhen Zhang [a], Lei Feng [a], Ying-Min Liang [b,*], Hua Han [a,b,*]

[a] State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an 710032, PR China
[b] Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710032, PR China

ARTICLE INFO

Article history:
Received 21 October 2007
Received in revised form 31 July 2008
Accepted 1 August 2008
Available online 9 August 2008

Keywords:
RBP-J
KyoT/FHL
LIM domain
Transcription
Transcription factor

ABSTRACT

Previously, we have shown that KyoT2, an isoform of the four and a half LIM domain protein 1 (FHL1), modulates Notch signaling via repressing RBP-J-mediated transactivation. In this study, we investigated the effect of another isoform of FHL1, KyoT3, on transactivation of a RBP-J-dependent promoter. We found that KyoT3 was expressed widely in a variety of tissues. By constructing EGFP fusion proteins, we showed that KyoT3 locates preferentially in nucleus. KyoT3 interacted with RBP-J, as shown by co-immunoprecipitation assays. Moreover, we demonstrated by a reporter assay that KyoT3 repressed transactivation of a RBP-J-dependent promoter, which was activated by both the Notch intracellular domain and Epstein–Barr virus nuclear antigen 2, an EB virus-encoded oncoprotein. These results suggest a multi-elemental control of the Notch signaling pathway, which is critical for cell differentiation in development.

1. Introduction

Notch signaling is an evolutionarily conserved pathway that is used by metazoans to control a broad range of developmental processes, including cell fate determination, differentiation, proliferation and apoptosis through local cell–cell interactions [1]. Triggering of Notch receptors by ligands induces cleavage of the receptors and the release of the Notch intracellular domain (NIC) [2]. The latter then translocates into the nucleus and binds to a DNA-binding protein recombination signal-binding protein-Jκ (RBP-J) to transactivate transcription of target genes [3,4]. In mammals, four Notch genes have been identified (Notch1–4) [1], and all four Notch receptors bind to RBP-J to exert their transactivation activities [5–9]. In addition to NIC, RBP-J also mediates transactivation of the Epstein–Barr (EB) virus nuclear antigen (EBNA) 2 and therefore may play a role during the immortalization of cells infected by the EB virus [10,11]. On the contrary, in the absence of transactivators such as NIC or EBNA2, RBP-J represses transcription of downstream promoters [12]. This is mostly attributable to the participation of multiple co-suppressors and/or adaptor molecules recruited by RBP-J [13]. To date many co-suppressors have been identified, such as histone deacetylases (HDACs) [14], MSX2-interacting nuclear target protein (MINT) [15,16], and KyoT2 [17].

KyoT2 is a LIM domain protein. The LIM domain was named by the first letter of LIN-11, Isl1, and MEC-3, with a consensus sequence of $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_2$(C/H/D) (X denotes any amino acid) forming a two-tandem-adjoined zinc-finger motif. The most widely recognized function of the LIM domain is to provide a protein-binding interface to mediate protein–protein interactions, but recent studies have revealed its regulative functions in cytoskeleton architecture, gene expression, and signal transduction as well [18]. The four and a half LIM (FHL) proteins, also known as skeletal muscle LIM proteins (SLIM), are a family of LIM-only proteins, characterized by four complete LIM domains preceded by an N-terminal half LIM domain [19]. Five members have been identified in this protein family. FHL1 is also named as KyoT1 and is mainly related to skeletal muscle differentiation and dynamics [17,20–22], while FHL2 and 3 interact with various transcription factors bearing multiple functions [23–25]. FHL4 is expressed in the seminiferous epithelium and might be involved in spermatogenesis [26]. The last member of the FHL family, ACT, was found in human tumor cell lines and was suggested to play a role in oncogenesis [27]. KyoT2/FHL1C is a product of alternative splicing of the primary transcript of KyoT1/FHL1 mRNA, which maintains the N-terminal two and a half LIM domains of KyoT1 and generates a C-terminal 27 amino acid residue motif that contains a WW sequence recognizing RBP-J [17,28]. Our previous results have suggested that KyoT2 could interact with RBP-J and repress RBP-J-mediated Notch signaling by competitive binding with RBP-J and by

* Corresponding authors. H. Han is to be contacted at Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Chang-Le Xi Street #17, Xi'an 710032, PR China. Tel.: +86 29 83374488; fax: +86 29 83246270. Y.-M. Liang, Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, PR China. Tel.: +86 29 84777426.
E-mail addresses: liangym@fmmu.edu.cn (Y.-M. Liang), huahan@fmmu.edu.cn (H. Han).
[1] These authors contributed equally to this study.

1874-9399/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagrm.2008.08.001

recruiting co-repressors such as RING1 and/or HPC2 [29,30]. We have also shown that KyoT2 is a substrate of small ubiquitin-like modification (SUMO) [31].

KyoT3/FHL1B (also named as SLIMMER) is also generated by alternative splicing of primary KyoT1 transcript. Bioinformatical analysis suggests that KyoT3 may possess the N-terminal three and a half LIM domains of KyoT1, followed by a stretch of novel sequence encoding three tandem nuclear localization signals (NLS), one nuclear export signal (NES), and the same RBP-J-binding motif as in KyoT2 at the C-terminus [32,33]. But, whether KyoT3 interacts with RBP-J and regulates Notch signaling via RBP-J has not been shown yet. We report here that KyoT3 was mainly localized in the nucleus, where it associated with RBP-J and could repress transactivation of RBP-J by NIC.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) and DNA recombination

Two fragments of the full length cDNA of KyoT3 were amplified from a murine embryonic cDNA library by PCR using primers 5′-GGCACCATGTCGGAGAAGTTCGAC, 5′-CTCGAGGAGCTGCTAAGCTTC-GAT, 5′-ATCGAAGCTTAGCAGCTCCTCGAG, and 5′-CGGAGCATTTTTTG-CAGTGGAAG. An XhoI site was introduced to facilitate joining of the two fragments to generate the full length KyoT3 gene, without changing the amino acid sequence. The amplified fragments were inserted into pMD18T vector (Takara Dalian Biotechnology, Dalian, China), followed by sequencing. The NLS fragment of KyoT3 was amplified by PCR using primers 5′-ATGAAGAAGTGTGCTGGATGCAA-GAAC and 5′-GCGTTTCCCGTGGCACACTG, with the full length KyoT3 cDNA as a template, and was also confirmed by DNA sequencing.

The KyoT3 mutant (KyoT3*N*) without NLS was constructed by PCR using primers 5′-GGCACCATGTCGGAGAAGTTCGAC, 5′-TTAGATCTGGC-CACAAAGTTCTTGTAGC, 5′-TTAGATCTTTGCCTCTCACCCTGTTTC, and 5′–5′-CGGAGCATTTTTTGCAGTGGAAG, with the full length KyoT3 as a template. A Bgl II site was introduced to facilitate sub-cloning.

RT-PCR was performed using total RNA extracted from mouse tissues using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA was reverse-transcribed by random priming using a cDNA synthesis kit (TOYOBO Shanghai Biotech., Shanghai, China), and was then subjected to PCR amplification using the primers 5′-AAGAAGTGTGCTGGATGCAAGAACC and 5′-CTCGAGGAGCTGCTAAGC-TTCGAT. The position of the primers is indicated in Fig. 1A.

pEGFP-N1-KyoT3, pEGFP-C2-KyoT3, pEGFP-N1-KyoT3-NLS, pEGFP-C2-KyoT3-NLS, pEGFP-N1-KyoT3*N*, and pEGFP-C2-KyoT3*N* were constructed by insertion of the full length KyoT3, its NLS fragment, or KyoT3*N* into pEGFP-N1 and pEGFP-C2 vectors (Clontech, Polo Alto, CA) in-frame. Myc-tagged KyoT3 (pCMV-KyoT3-myc) was constructed by inserting full length KyoT3 cDNA into pCMV-myc (Clontech) in-frame. pCMV-RBP-J-Flag, pEF-BOS-myc-NIC, pGa981-6, pCMV-KyoT2-myc, and pCMV-EBNA2 were described before [29,34]. All of the new constructs were confirmed by DNA sequencing.

2.2. Cell culture and transfection

Hek293 and HeLa cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Invitrogen). Cells were plated in 6- or 24-well plates at a density of 4×10^5or 1×10^5cells per well. Transfection was performed at a confluence of about 80% on the next day using Lipofectamine™ 2000 (Invitrogen). After being cultured further in medium for indicated periods of time, the cells were harvested for further experiments. For the observation of intracellular EGFP, HeLa cells were transfected with plasmids as described above and were examined under a fluorescence microscope (Olympus, Japan) 24 h after the transfection. Images were collected using a digital camera (Olympus).

2.3. Co-immunoprecipitation and western blot

HeLa cells were transfected using Lipofectamine™ 2000 with plasmids in combinations as indicated in the Results and discussion. Cell lysates were prepared 60 h after transfection by lysing cells with the phospho-lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1% NP-40, 1 mg/ml chick ovary albumin, 0.1 mM PMSF). Cell lysates with equal amount of total proteins were incubated with an anti-Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) antibody or an anti-Flag (M2, Sigma-Aldrich Biotechnology, St. Louis, MI) antibody,

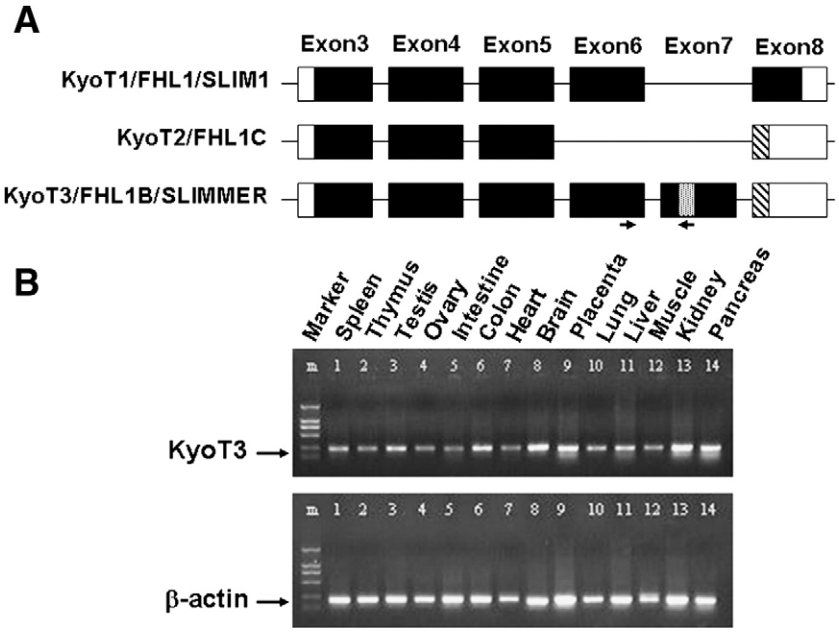

Fig. 1. Expression of KyoT3 in mouse tissues. (A) Schematic representation of the structure of mouse KyoT1, KyoT2 and KyoT3. Open and filled boxes are untranslated and translated regions of exons, while lines are introns. Boxes with lines and dashes indicate the RBP-J-binding motif and NLS, respectively. Arrows represent the position and direction of PCR primers used in (B). (B) RT-PCR. Total RNA was prepared from different tissues and reverse-transcribed, and cDNA from equal amount of RNA was used to amplify mouse KyoT3 (230 bp). Mouse β-actin in lower penal was used as an internal control.