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The PcG protein HPC2 inhibits RBP-J-mediated transcription by interacting with LIM protein KyoT2

Hongyan Qin[a], Dewei Du[b], Yangting Zhu[a], Junfeng Li[a], Lei Feng[a], Yingmin Liang[c], Hua Han[a,c,*]

[a] *State Key Laboratory of GI Cancer Biology, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Chang-Le Xi Street, Xi'an 710032, Shaanxi Province, PR China*
[b] *Department of Nephrology, Tangdu Hospital, Fourth Military Medical University, Chang-Le Xi Street, Xi'an 710032, Shaanxi Province, PR China*
[c] *Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Chang-Le Xi Street, Xi'an 710032, Shaanxi Province, PR China*

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Abstract The DNA-binding protein recombination signal-binding protein-Jk (RBP-J) plays a key role in transcriptional regulation by targeting the intracellular domain of Notch (NIC) and the Epstein–Barr virus nuclear antigen 2 (EBNA2) to specific promoters. In the absence of the Notch signaling, RBP-J acts as a transcriptional suppressor through recruiting co-suppressors such as histone deacetylase (HDAC). KyoT2 is a LIM domain protein that suppresses the RBP-J-mediated transcriptional activation. In the current study, we show that the polycomb group (PcG) protein HPC2, which functions as a transcriptional suppressor, is a candidate of KyoT2-binding proteins. To confirm the physical and functional interaction between KyoT2 and HPC2, we carried out yeast two-hybrid, GST-pull down, co-immunoprecipitation, as well as mammalian two-hybrid assays. Our results showed HPC2 and KyoT2 interacted both in vitro and in vivo, probably through the C-terminal fragment of HPC2 and LIM domains of KyoT2. In addition, we also found that overexpression of HPC2, not only inhibited transactivation of a RBP-J-dependent promoter by NIC, but also transactivation by RBP-J–VP16, a constitutively active form of RBP-J. Taken together, our results suggested that KyoT2 might inhibit the RBP-J-mediated transactivation through NIC by recruiting co-suppressors such as HPC2.

Keywords: Notch; RBP-J; KyoT2; HPC2; Transcription

1. Introduction

The Notch signaling pathway is an evolutionarily conserved pathway participating in cell fate determination steps in development (reviewed in [1]). Notch was identified in *Drosophila* decades ago and encodes a type I transmembrane protein receptor that contains multiple structural motifs. The extracellular domain of Notch contains 36 EGF-like repeats and 3 LIN-12/Notch repeats. The intracellular domain consists of a RAM (recombination signal-binding protein-Jk (RBP-J)-associating molecule) domain, 6 ankyrin/CDC10 repeats, nuclear localization sequences, a transactivation domain, and a C-terminal PEST domain. Genetic and biochemical analyses have demonstrated that when Notch is triggered by association with its ligands, a proteolysis reaction occurs within the transmembrane domain of the receptor, and the intracellular domain of Notch (NIC) is released. The released NIC translocates into the nuclear and serves as a transcriptional activator of downstream genes. However, because NIC does not possess a DNA-binding activity, it requires a DNA-binding protein, Su (H) (Suppressor of Hairless) in *Drosophila* and RBP-J in mammals, to mediate its transcriptional activation activity [1,2]. In mammals, although multiple members of Notch family (Notch 1–4 in human and mouse) and their ligands (Delta1 and Jagged1 and 2 in mouse) have been identified, evidence has shown that RBP-J is the intranuclear target of all four types of the Notch receptors [3,4].

RBP-J recognizes a consensus sequence C/TGTGGGAA that exists in multiple differentiation-regulating genes such as members of the mouse Hes (Hairy and enhancer of split) family [5]. NIC activates promoters recognized by RBP-J through replacement of transcriptional suppressors by the CDC10/ankyrin repeats, and through recruitment of two conserved histone acetyltransferases, PCAF (p300/CBP-associated factor) and GCN5, by the internal transactivation domain located downstream to the CDC10/ankyrin repeats [6,7]. In addition to NIC, RBP-J also mediates transactivation of the Epstein–Barr (EB) virus nuclear antigen 2 (EBNA2), a crucial molecule involved in cell immortalization and transformation by the EB virus [8,9].

On the other hand, in the absence of transactivators like NIC or EBNA2, RBP-J functions as a transcriptional suppressor [10]. Multiple molecules have been proposed to participate in the transcriptional suppression by RBP-J, such as histone deacetylase (HDAC), SMRT/N-CoR (silencing mediator for retinoid and thyroid receptor/nuclear receptor co-repressor), CIR (CBF1 interacting co-repressor), SAP30, and MINT (MSX2-interacting nuclear target protein) [11–16]. Although, these molecules have been identified to negatively regulate Notch signaling, however, the molecular mechanism of the RBP-J-mediated transcriptional suppression is still elusive.

KyoT2 is a LIM domain protein and interacts with RBP-J through a binding motif on its C-terminus generated by alternative mRNA splicing [17]. Previous studies had shown that

*Corresponding author. Fax: +86 29 83246270.
E-mail address: huahan@fmmu.edu.cn (H. Han).

Abbreviations: RBP-J, recombination signal-binding protein-Jk; NIC, intracellular domain of Notch; EBNA2, Epstein–Barr virus nuclear antigen 2; PcG, polycomb group; HDAC, histone deacetylase; cDNA, complementary DNA; RT-PCR, reverse transcription polymerase chain reaction

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KyoT2 inhibits transactivation of promoters containing the RBP-J recognition sites by blocking interaction between RBP-J and transactivators such as NIC and EBNA2. However, our recent results have suggested that in addition to act as a competitor for binding sites, KyoT2 may regulate RBP-J through its LIM domains, which have been shown to function as a protein–protein interaction interface [18–20]. Thus, through interaction with LIM domains of KyoT2, RING1, a member of the polycomb group (PcG) proteins, might suppress transactivation of RBP-J and regulate Notch signaling during mammalian development [21].

HPC2, another member of the PcG proteins, was also identified as a candidate of KyoT-interacting molecule in the yeast two-hybrid screening [21]. In this study, we investigated the physical and functional interaction of KyoT2 with HPC2. We showed that HPC2 physically interacts with KyoT2 both in vitro and in vivo. Overexpression of HPC2 suppressed transactivation of an RBP-J-dependent promoter by NIC, as well as transcription activity of a constitutively active RBP-J, the RBP-J–VP16 fusion protein. Our data further suggested that KyoT2 might inhibit the RBP-J-mediated transactivation by NIC through recruiting HPC2, in addition to RING1.

2. Materials and methods

2.1. RT-PCR

The coding sequence of human HPC2 complementary DNA (cDNA) was amplified by reverse transcription polymerase chain reaction (RT-PCR) from total RNA of Hela cells using the Trizol reagent according to the manufacturer's instruction (Invitrogen). The primers used for PCR were 5′-CCATGGAGCTGCCAGCTGTTGGCGAG-3′ and 5′-CCTCCGGCTACACCGTCACGTACTCC-3′. The amplified fragment was cloned into a T-vector (Promega, Germany) and confirmed by DNA sequencing.

2.2. Yeast two-hybrids assay

All bait plasmids, including pGBKT7–KyoT1, pGBKT7–KyoT2, pGBKT7–KyoT2–LIM1, and pGBKT7–KyoT2–LIM2, for the yeast two-hybrid assay were described previously [21]. The full-length HPC2 cDNA was inserted in frame into pGADT7 to construct a prey plasmid (pGADT7–HPC2). Prey plasmids with truncated HPC2 (pGADT7–HPC2–N with amino acids 1–374, and pGADT7–HPC2–C with amino acids 375–588) were generated by restriction digestion and ligation, and confirmed by sequencing. Plasmids were used to transform the yeast strain AH109 in combinations as described in the results by the LiAc method, and grown clones were tested for nutritional phenotypes. Single clone was tested by liquid β-galactosidase assay for β-galactosidase activity.

2.3. Cell culture and transfection

HEK293 and 293T cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (Gibco BRL). Cells were plated onto 6 well plates at a density of 5×10^5 cells per well and cultured at 37 °C with 5% CO_2 overnight for transfection. The transfection was performed at 90% cell confluence with a total amount of 2 μg DNA (for details, see Section 3) per well using Lipofectamine™ 2000 (Invitrogen), following the manufacturer's protocol.

2.4. GST-pull down assay

The GST–KyoT2 fusion protein was produced as described previously [21]. The coding region of full-length HPC2 cDNA was inserted into pCMV2-Flag, and the generated plasmid (pCMV2-Flag-HPC2) was used to transfect 293T cells. Harvested cell extracts were incubated with the purified GST–KyoT2 or GST (used as a negative control) protein, respectively, and the protein–protein interaction was assayed by pulling down with the glutathione–Sepherose beads (Sigma), followed by immunoblotting with an anti-Flag antibody (M2, Sigma).

2.5. Co-immunoprecipitation

Plasmids pCMV-KyoT2-Myc, pCMV2-Flag-RING1, and pCMV2-Flag-RBP-J were described previously [21]. Plasmids were transfected into 293T cells using Lipofectamine™ 2000 as shown in Section 3. Sixty hours after transfection, cells were collected and lysed using the phospho-lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP40, 1 mg/ml BSA, and 0.1 mM PMSF). Immunopreciptation was carried out with an anti-Myc (9E10, Santa Cruz) antibody. After washing extensively with the phospho-lysis buffer, co-precipitated proteins were analyzed by SDS–PAGE followed by Western blotting using the anti-Flag antibody, or antibodies against RBP-J (K0043) [17] (provided by T. Honjo), HPC2 (a gift from BQ Jin, unpublished), or RING1 (C-20) (Santa Cruz). Expression of the Myc-tagged proteins was detected by Western blotting of the cell lysates using the anti-Myc antibody.

2.6. Reporter assays

For mammalian two-hybrid experiments, the full-length KyoT2 cDNA and HPC2 cDNA were inserted in frame into multiple cloning sites of pCMX–GAL4–DBD and pCMX–VP16(NLS) (generously provided by T Honjo), to generate plasmids pCMX–GAL4–DBD–KyoT2 and pCMX–VP16–HPC2, respectively. The plasmids were co-transfected with a reporter construct (TK MH100 × 4 luc), in which the luciferase gene was under control of a promoter containing multiple recognition sites of the GAL4 DNA-binding domain. Cells were lysed 48 h after transfection with a hypotonic buffer (91 mM K_2HPO_4, 9 mM KH_2PO_4, 10% glycerol, 1 mM DTT, 10% Triton X-100), and the level of the luciferase in cell lysates was examined as described. Transfection efficiency was calibrated by co-transfection with pSV-β-gal, followed by examining the β-galactosidase activity in cell lysates. Each experiment was repeated at least three times and data were analyzed with the Student's t test.

Transactivation of the RBP-J-responsive promoter was detected using reporter assay with the reporter construct pGa981-6, which contains a hexamerized 50 bp EBNA2 response element of the TP-1 promoter and is strictly dependent on RBP-J [3]. Expression vectors for KyoT2 (pEFBOS-KyoT2), RING1 (pEFBOS-RING1), HPC2 (pEFBOS-HPC2) and NIC (pEFBOS-NIC) were constructed by insertion of the full-length cDNA of KyoT2, RING1, HPC2, as well as NIC into pEFBOS-neo vector, respectively. Cells were collected 48 h after transfection with different plasmids and the luciferase activity was examined as above. pSV-β-gal was included in each transfection as an internal control of the transfection efficiency.

2.7. CHIP assays

Chromatin immunoprecipitation (CHIP) assay was carried out using a kit from Upstate (Milton Keynes, UK) according to the manual provided by the supplier. Briefly, NIH3T3 cells were transfected with expression vectors for Myc-NIC or Myc-KyoT2 plus Flag-HPC2, together with pGa981-6. Forty-eight hours after transfection, cells were crosslinked with 1% formaldehyde, disrupted and ultra-sonicated. The cell lysates were immunoprecipitated with anti-Myc, anti-Flag, or anti-RBP-J, with a preimmune serum as a control. The crosslinking of the immunoprecipitates were reversed by heating up, and bound DNA was amplified using primers to the promoter of pGa981-6 containing RBP-J-recognizing sequence. The sequence of the primers was 5′-gtagatcccgactcgtgg-3′ and 5′-tttcccacggtgcccttc-3′. The amplified fragments were analyzed using 3% agarose gel electrophoresis.

3. Results

3.1. KyoT2 interacts with HPC2 through LIM domains in yeast

In a screening of KyoT2-interacting proteins using the yeast two-hybrid system with KyoT2 as bait [21], we identified HPC2 as another candidate of KyoT2-binding proteins. To confirm the interaction between KyoT2 and HPC2, and to identify potential domains of KyoT2 and HPC2 responsible