Biochemical and Biophysical Research Communications 427 (2012) 41–46

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

CAC1 negatively regulates RARα activity through cooperation with HDAC

MinO Moon [a], Soo-Jong Um [b], Eun-Joo Kim [a,*]

[a] Department of Molecular Biology, BK21 Graduate Program, Dankook University, Yongin-si, Gyeonggi-do 448-701, Republic of Korea
[b] Department of Bioscience and Biotechnology/Institute of Bioscience, BK21 Graduate Program, Sejong University, Seoul 143-747, Republic of Korea

ARTICLE INFO

Article history:
Received 28 August 2012
Available online 11 September 2012

Keywords:
RARα
CAC1
HDAC
Transcriptional activity
Corepressor

ABSTRACT

Retinoic acid (RA) plays pleiotropic roles in cellular differentiation and animal development. RA responses are mediated by transcriptional activation by the retinoic acid receptor (RAR) and retinoid X receptor (RXR) in cooperation with various types of coregulators at RA-responsive gene promoters. Here, we identified CDK2-associated cullin (CAC1) as a novel type of RARα coregulator that interacts with RARα and inhibits its transcriptional activity. The CoRNR box of CAC1 is required for the binding to and inactivation of RARα. In addition, CAC1 cooperates with histone deacetylases (HDACs) to suppress RARα, probably by associating with HDAC. Finally, depletion of CAC1 increases RA-induced neuronal differentiation of P19 cells, a response accompanied by significant upregulation of the neuronal marker *nestin*. From these results, we suggest that CAC1 is a novel corepressor of RARα that cooperates with HDACs and is involved in the regulation of RA-induced cellular differentiation.

1. Introduction

The retinoic acid receptor (RAR) is a member of the nuclear hormone receptor (NR) superfamily that regulates various physiological processes, including differentiation, proliferation, and development [1–3]. NRs including RAR are ligand-inducible transcription factors that specifically regulate the expression of target genes. All NRs share a common modular structure consisting of an N-terminal transcriptional activation domain (AF-1), a conserved DNA-binding domain (DBD), a hinge region (D), and a C-terminal ligand-binding domain (LBD) that overlaps with the second transcriptional activation domain (AF-2) [1,2,4]. Retinoid signaling is generated by two families of nuclear receptors, the RAR and retinoid X receptor (RXR), which bind as RAR/RXR heterodimers to *cis*-acting RA response elements (RAREs) located in the regulatory sequences of target genes [5]. Upon binding to RAREs, RAR/RXR heterodimers activate target gene transcription through complex interactions with coactivators, chromatin remodeling factors, and components of the basal transcription machinery [6].

Transcriptional regulation mediated by RAR/RXR involves the binding and recruitment of various coactivators and corepressors to target gene promoters [7]. The first identified coactivators of retinoid receptors were members of the SRC/P160 family, which includes SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2/SRC-2, and pCIP/ACTR/AIB1/TRAM1/RAC3/SRC-3 [8,9]. Other coactivators are histone acetyltransferases (HATs) including CBP/p300 and pCAF, which acetylate lysine residues in the N-terminal tails of histones, and histone methyltransferases (HMTs) including CARM1 and PRMT1, which methylate arginine residues in histone H3 and H4 [10–14]. These coactivators modify the chromatin environment and thereby allow the recruitment of ATP-dependent chromatin remodeling factors to facilitate the initiation of transcription. In contrast, NR corepressors such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) bind to NRs in the absence of ligand and actively repress transcription of target genes by recruiting large repressor complexes including histone deacetylases (HDACs) and the nucleosome remodeling complex NURD [15,16].

Here, we identified CAC1 as a novel corepressor of RARα. In this study, we demonstrated that CAC1 binds directly to RARα through the conserved CoRNR motif and suppresses RARα-mediated transcriptional activity. CAC1 also interacts with HDAC and cooperates with it in the suppression of RAR activity. The suppressive function of CAC1 is associated with its inhibitory role in RA-dependent neuronal differentiation. These results suggest that CAC1 is a novel RARα corepressor that cooperates with HDAC and negatively modulates RA-induced cellular differentiation.

2. Materials and methods

2.1. Plasmids and cloning

All cDNAs were constructed according to standard methods and verified by sequencing. Full-length CAC1 cDNA and CAC1 cDNA

Abbreviations: CAC1, CDK2-associated cullin; RARα, retinoic acid receptor alpha; HDAC, histone deacetylase; AF-1, transcriptional activation function 1; LBD, ligand binding domain; DBD, DNA-binding domain; GST, glutathione *S*-transferase; β-gal, β-galactosidase; GFP, green fluorescent protein.

* Corresponding author. Fax: +82 31 8021 7201.
E-mail address: nbrejk@dankook.ac.kr (E.-J. Kim).

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http://dx.doi.org/10.1016/j.bbrc.2012.08.142

deletion mutants were amplified by PCR and subcloned into suitable vectors [Myc-tagged pcDNA3, pEGFP-C3 (BD Biosciences), and pGEX4T-1 (GE Life Sciences)].

2.2. Cell culture and differentiation

H1299 cells were grown and maintained in RPMI-1640 medium, and HeLa cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic–antimycotic mix (all from Gibco-BRL). All cells were grown and maintained in a 5% CO_2 atmosphere at 37 °C. For treatment with all-*trans* retinoid acid (AtRA) (Sigma), FBS was pretreated with charcoal. P19 cell differentiation was induced as described previously [17]. P19 cells were aggregated in bacterial Petri plates at a density of 10^5 cells/ml and treated with 1 mM RA for 96 h, with subculturing in fresh medium after treatment for 48 h. On day 4, the aggregates were transferred to cell culture plates and RA was eliminated from the medium. Cells were then seeded on 10 cm-diameter plates at a density of 3×10^5 cells/plate. After 12 h, the medium was replaced with medium containing 0.5% FBS, and the cells were allowed to differentiate for an additional 6 days.

2.3. Glutathione S-transferase pull-down assays

The experimental procedures were as described previously [18]. A GST fusion of CAC1 was expressed in *Escherichia coli* and purified on glutathione-Sepharose beads (GE Life Sciences) by standard methods. RARα protein was translated in vitro using the TNT rabbit reticulocyte system (Promega). Then 2 μg of GST or GST-CAC1 was mixed with 10 μl of in vitro-translated RARα protein.

2.4. Immunofluorescence microscopy

Twenty-four hours after GFP-RARα was co-transfected with Myc-tagged CAC1 on coverslips, H1299 cells were washed with phosphate-buffered saline (PBS), fixed through incubation with 4% paraformaldehyde in PBS for 1 min, and permeabilized via incubation in PBS containing 0.5% Triton X-100 for 4 min at 4 °C. After washing, the cells were incubated with anti-Myc antibody (Millipore) in blocking buffer (PBS containing 2% bovine serum albumin) for 1 h and then incubated with rhodamine-conjugated anti-mouse antibody (Millipore). After washing and mounting with 50 μl of Vecta Shield (Vector Laboratories), cells were visualized using an immunofluorescence microscope (Carl Zeiss).

2.5. Transient transfection and luciferase reporter assay

HeLa cells were seeded in a 6-well culture plate and transiently transfected with a RARE-luciferase reporter gene, and as an internal control, an SV40-driven-galactosidase (β-gal) expression vector. Depending on the experimental conditions, the RARα or CAC1 expression vector was co-transfected using Lipofectamine Plus reagent (Invitrogen). Luciferase activity was measured using an analytical luminescence luminometer according to the manufacturer's instructions (Promega), after the addition of 20 μl of luciferin to 20 μl of cell lysate. β-Gal activity was determined in 96-well plates by measuring absorbances at 405 nm using a microplate reader. Luciferase activity was normalized to β-gal activity.

2.6. Western Blotting (WB) and immunoprecipitation (IP)

IP and WB were performed as previously reported [19]. For WB, cells were lysed in lysis buffer supplemented with a protease

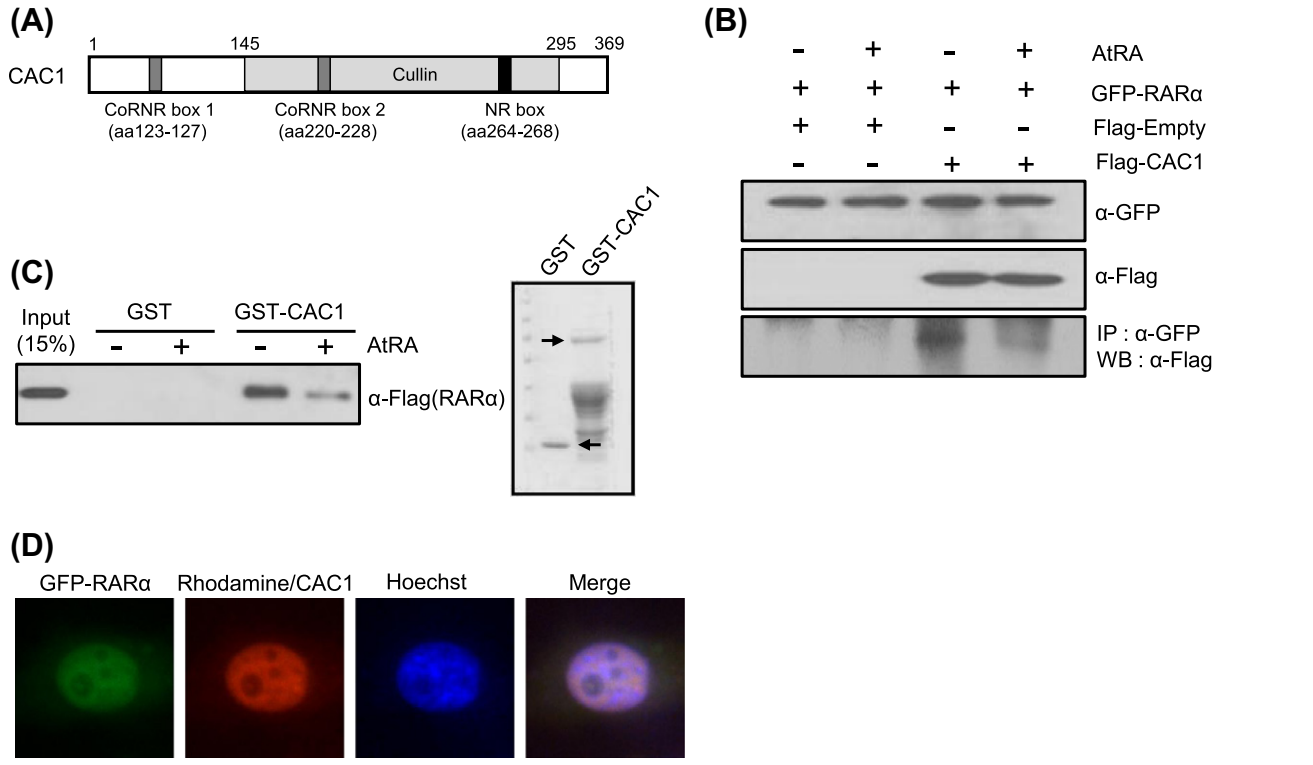

Fig. 1. CAC1 interacts with RARα. (A) Structural features of CAC1. CoRNR box, corepressor binding motif (L/IXXI/VI); NR box, coactivator binding motif (LXXLL); Cullin, cullin homology domain (amino acids 145–295). (B) IP assay. H1299 cells were co-transfected with GFP-fused RARα and Flag-empty or Flag-tagged CAC1 in the absence or presence of 2 μM AtRA. Lysates were subjected to IP using anti-GFP and bound protein was visualized by WB with anti-Flag antibody. (C) GST-pull down assay. In vitro translated Flag-tagged RARα was incubated with GST or GST-CAC1 in the absence or presence of 20 μM AtRA. The bound proteins were visualized by subsequent WB using anti-Flag antibody. (D) Immunofluorescence microscopy. H1299 cells were transfected with GFP-RARα and Myc-CAC1. The cellular location of CAC1 was visualized using rhodamine-conjugated anti-mouse IgG. The nuclei were visualized by Hoechst staining.