Biochemical and Biophysical Research Communications 400 (2010) 426–431

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Cyclin D1 blocks the anti-proliferative function of RUNX3 by interfering with RUNX3–p300 interaction

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ARTICLE INFO

Article history:
Received 18 August 2010
Available online 27 August 2010

Keywords:
Cyclin D1
RUNX3
p21
Cancer

ABSTRACT

Transcriptional function of cyclin D1, whose deregulation is frequently observed in human cancers, has been suggested to contribute to cancer formation. In the present study, we show that cyclin D1 protein inhibits RUNX3 activity by directly binding to it and interfering with its interaction with p300 interaction in lung cancer cells. Cyclin D1 inhibits p300-dependent RUNX3 acetylation and negatively regulates cyclin-dependent kinase (cdk) inhibitor *p21* expression. These transcriptional effects of cyclin D1 do not require cdk4/6 kinase activation. We propose that cyclin D1 provides a transcriptional switch that allows the tumor suppressor activity of RUNX3 to be repressed in cancer cells. Since RUNX3 plays tumor suppressive roles in a wide range of cancers, a non-canonical cyclin D1 function may be critical for neoplastic transformation of the epithelial cells in which RUNX3 regulates proliferation.

1. Introduction

D-type cyclins are implicated in controlling cellular progression through the G1 phase of the cell cycle. Among the D-type cyclins, cyclin D1 is the most strongly implicated in oncogenesis. Cyclin D1 is frequently overexpressed in a broad range of human cancers [1]. Further evidence that cyclin D1 can act in oncogenic fashion is its ability to cooperate with activated oncogenes in various transformation assays [2,3]. Moreover, transgenic mice overexpressing cyclin D1 in lymphoid progenitor or mammary epithelial cells are more susceptible to neoplastic growth of these cells [4,5].

D-type cyclins function through activation of cyclin-dependent kinase (cdk) 4/6, which then catalyzes the inactivating phosphorylation of pRb, leading to the derepression of a subset of proliferation-associated E2F target genes [6]. In addition, cyclin D1 also affects the activity of some cellular transcription factors without the participation of cdks [7–9]. There is strong genetic evidence that the transcriptional function of cyclin D1 is important for the development of mouse retinas [10]. Taken together, these observations suggest that the non-canonical transcriptional function of cyclin D1 may contribute to cancer formation.

In this context, the RUNX3 transcription factor poses a particular challenge as a target of cyclin D1 in cancer cells. The *RUNX3* gene is located on human chromosome 1p36, a region that has long been suggested to be a tumor suppressor locus for various cancers [11]. A tumor suppressive function of RUNX3 is indicated by the frequent inactivation of the *RUNX3* gene due to DNA hypermethylation in a broad range of cancers [12]. Furthermore, RUNX3 inhibits cancer cell growth by inducing the expression of cdk inhibitor *p21* [13]. Previous studies revealed that cdk6 or cyclin D3 interact with RUNX1, which negatively affects its activity [14,15]. These findings prompted us to question whether cyclin D1 contributes to cancer cell growth by interfering with RUNX3 activity. To examine this hypothesis, we focused on lung cancer cells in this study because both the inactivation of RUNX3 and the overexpression of cyclin D1 are frequently observed in this type of malignancy [12,16].

In the present study, we provide evidence that cyclin D1 blocks the tumor suppressor function of RUNX3 in cancer cells by directly interacting with it. The cyclin D1–RUNX3 interaction interferes with the RUNX3–p300 interaction. This leads to the inhibition of transcriptional activation by RUNX3, downregulation of *p21* expression, and the blocking of the RUNX3-dependent inhibition of cancer cell proliferation. Upregulation of cyclin D1 therefore provides a transcriptional switch that allows the tumor suppressor activity of RUNX3 to be repressed in cancer cells.

2. Material and methods

2.1. Cell culture

A549, Calu6, 293T, HeLa, and PLAT-A cells were grown in DMEM with 10% fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO_2 atmosphere.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.08.094

2.2. Cell cycle analysis

Cells (1×10^6) were centrifuged, resuspended in 1 mL of propidium iodide solution (0.05 mg/mL propidium iodide, 5 μg/mL DNase-free RNase A, 0.1% sodium citrate, and 0.1% Triton X-100), and then analyzed using flow cytometry.

2.3. Plasmids

The following expression plasmids were obtained from Dr. Motomi Osato and Dr. Kosei Ito: p21-promoter Luc, pcDNA3-Flag-RUNX3, and pEF-AS-RUNX3. The human RUNX3 and cyclin D1 cDNAs were subcloned into the pcDNA3 and pMSCV-Puro expression vectors, respectively.

2.4. Stable cell lines

Cells were transfected using FuGENE6 (Roche) according to the manufacturer's instructions. RUNX3-restored Calu6 cells were obtained by transfecting Calu6 cells with pcDNA3-Flag-RUNX3. G418-resistant clones were selected using G418 (1 mg/mL). Similarly, A549/AS-RUNX3 cells were obtained by transfecting A549 cells with pEF-AS-RUNX3. RUNX3-restored Calu6 clone 5 cells expressing cyclin D1 were obtained by retroviral transduction. RUNX3-restored Calu6 clone 5 cells were infected with retroviruses as described previously [14], and were selected by treating the cells with puromycin (2 μg/mL) for 5 days. As a control, we used an empty pMSCV-Puro retrovirus.

2.5. RNA isolation and RT-PCR analysis

Total RNA was extracted from cell lines using with a TRIZOL Kit (Invitrogen). The RT reaction was performed on a 2 μg sample of the total RNA with SuperScript II First-Strand Synthesis using the Oligo(dT) primer system (Invitrogen). Semi quantitative RT-PCR was carried out as described previously [14] with ExTaq DNA polymerase (Takara). Primer sequences and amplification conditions are available from the authors upon request. PCR products were electrophoresed on 1.5% agarose gels and visualized by means of ethidium-bromide staining.

2.6. Promoter assays

Luciferase activity-based promoter assays were performed in triplicate using the Dual-Luciferase Reporter Assay System (Promega).

2.7. DNA pull-down (DNAP) assay

Analysis of binding of transcription factor complexes to biotinylated probes was performed in 293T cells as described [14]. The sequence of the biotinylated probe was 5′-CATCTAACAGGAT GTGGTTTGACATTTA-3′.

2.8. Immunoprecipitation and Western blotting

Whole-cell lysates were extracted from cell lines using HKMG buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 0.5% of NP-40) supplemented with protease inhibitor cocktail (Nacalai Tesque). At 36 h after transfection, whole-cell lysates were incubated with appropriate antibodies and bound to protein A-sepharose beads (GE Healthcare) for 6 h on ice in HKMG buffer. The lysates or protein-complexes were separated on a SDS–polyacrylamide gel and transferred to PVDF membranes (Hybond-ECL, GE Healthcare). Proteins were detected using ECL (GE Healthcare). FLAG-tagged RUNX3 was detected using M2 anti-FLAG monoclonal antibody (Sigma). Furthermore proteins were detected using the following antibodies: p21, polyclonal C-19 anti-p21 (sc-397; Santa Cruz), cyclin-dependent kinase 4 (cdk4), polyclonal C-22 anti-cdk4 (sc-260; Santa Cruz), cyclin D1, polyclonal M-20 anti-cyclin D1 (sc-718; Santa Cruz), RUNX3, monoclonal R3-5G4 anti-RUNX3 (MBL), and β-actin, monoclonal AC-15 anti-β-actin (Sigma).

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed following the Upstate Biotechnology ChIP assay Kit protocol (catalog no. 17-295). Approximately 10^6 cells were used per ChIP reaction. Chromatin was immunoprecipitated using monoclonal M2 anti-FLAG (Sigma) or polyclonal N-15 anti-p300 antibodies (sc-584; Santa Cruz). The *p21* promoter region was amplified by PCR as described previously.

2.10. shRNA knockdown

An anti-human cyclin D1 shRNA retroviral vector was purchased from Openbiosystems. An empty pMKO.1 retrovirus was used as a control. RUNX3-restored Calu6 clone 5 cells were infected with retroviruses as described previously [14] and were selected by treatment with puromycin (2 μg/mL) for 5 days.

3. Results

3.1. RUNX3 upregulates p21 and inhibits cell proliferation in lung cancer cell lines

RUNX3 induces *p21* expression and inhibits cell proliferation in gastric and bile duct cancers [12,13]. Although RUNX3 has been suggested to be a tumor suppressor for lung cancer [17], the molecular mechanism that mediates this remains unclear. Therefore, we examined whether RUNX3 is involved in the induction of *p21* transcription in lung cancer cells. For this purpose, A549 lung cancer cells, which express endogenous RUNX3, were transfected with an antisense *RUNX3* construct, and a stably transfected cell line (AS-RUNX3) was isolated. It is important to note that the expression levels of p21 were reduced when RUNX3 expression was inhibited by antisense RUNX3 (Fig. 1A). This result shows that RUNX3 participates in the induction of *p21* transcription in lung cancer cells in vivo. Next we were interested in determining whether the restoration of RUNX3 expression induced *p21* transcription. Calu6 lung cancer cells, which lack endogenous RUNX3 expression, were transfected with a FLAG-tagged RUNX3 (FLAG-RUNX3) construct, and the stably transfected clones (clones 4 and 5) were isolated. The expression levels of *RUNX3* in clone 5 were estimated to be approximately five times higher than in clone 4 (Fig. 1B). We measured the levels of *p21, p15, p27*, and *p57* by RT-PCR in parental, mock vector-transfected Calu6 cells and RUNX3-restored clones. Among them, we found that *p21* was the most highly upregulated (Fig. 1B and data not shown). The expression levels of *p21* in clone 5 were estimated to be approximately four times higher than that in clone 4 (Fig. 1B). In contrast, relatively little change was observed in the expression of other CDK inhibitors (data not shown). The increased expression of p21 protein in RUNX3-restored Calu6 clone 5 was also confirmed by Western blotting (Fig. 1C). We did not observe the downregulation of cdk4 or cyclin D1 (Fig. 1C). These results strongly suggest that sustained expression of RUNX3 directly regulates *p21* gene expression in lung cancer cells. The RUNX3-dependent upregulation of p21 prompted us to examine whether RUNX3 can induce cell cycle arrest. To clarify this, cell cycle progression was measured using propidium iodide staining to determine cellular DNA content. As