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Cell Cycle Regulatory Effects of Retinoic Acid and Forskolin Are Mediated by the *Cyclin C* Gene

Katri M. Makkonen[1]†, Marjo Malinen[1]†, Antti Ropponen[2], Sami Väisänen[1] and Carsten Carlberg[1,3]*

[1]*Department of Biosciences, University of Kuopio, FIN-70211 Kuopio, Finland*

[2]*Department of Clinical Microbiology, University of Kuopio, FIN-70211 Kuopio, Finland*

[3]*Life Sciences Research Unit, University of Luxembourg, L-1511 Luxembourg, Luxembourg*

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As a partner of cyclin-dependent kinase (CDK) 3, Cyclin C controls cellular proliferation and, together with CDK8, represses gene transcription. In this study, we showed that the highly expressed *Cyclin C* gene is a direct target of the nuclear hormone all-*trans* retinoic acid (RA) in HEK293 human embryonal kidney cells. The RA receptor (RAR) γ associates with a *Cyclin C* promoter region containing two RAR binding sites. The *Cyclin C* gene also directly responds to the cAMP activator Forskolin via the transcription factor CREB1 (cAMP response element-binding protein 1), for which we identified four binding sites within the first 2250 bp of its promoter. RARγ and CREB1 show functional convergence via the corepressor NCoR1, which controls in particular the Forskolin response of *Cyclin C*. The histone deacetylases 1, 5, 6, 7 and 11 are involved in the basal expression of *Cyclin C*, but in HEK293 and MCF-7 human breast carcinoma cells the antiproliferative effects of the histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) are not mediated by *Cyclin C*. However, cell cycle progressing effects of all-*trans* RA and Forskolin are dependent on *Cyclin C* expression levels. This suggests that the primary regulation of *Cyclin C* by all-*trans* RA and Forskolin mediates some of the cell cycle control actions of these compounds.

Keywords: chromatin; all-*trans* retinoic acid; CREB signaling; *Cyclin C*; nuclear receptor

Introduction

Cyclins associate with specific cyclin-dependent kinases (CDKs) and play central roles in cell cycle regulation and transcription.[1] While CDK7/cyclin H and CDK9/cyclin T are parts of the general transcription factor TFIIH[2] and the transcription elongation factor pTEF-β,[3] respectively, Cyclin C associates with CDK8, Mediator protein 12 (MED12) and MED13 to form a module of the Mediator complex.[4] Via DNA looping, the Mediator complex forms a bridge between transcription factors and the basal transcriptional machinery; without the Cyclin C/CDK8/MED12/MED13 module it displays strong coactivator function, but with the module it turns to a repressor.[5] In the latter case, the RNA polymerase II (pol II) carboxy-terminal domain is phosphorylated prematurely, which prevents the formation of a transcription initiation complex.[6] Another role of Cyclin C, in complex with CDK3, is the regulation of the G_0 to G_1 transition of the cell cycle through specific phosphorylation of the retinoblastoma protein pRb.[7] The fact that the *Cyclin*

**Corresponding author.* Life Sciences Research Unit, Université du Luxembourg, 162A, Avenue de la Faïencerie, L-1511 Luxembourg, Luxembourg. E-mail address: carsten.carlberg@uni.lu.

† K.M.K. and M.M. contributed equally to this work.

Abbreviations used: CBP, CREB-binding protein; CDK, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB1, cAMP response element-binding protein 1; DMSO, dimethyl sulfoxide; DR, direct repeat; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HDAC, histone deacetylase; MED1, Mediator protein 1; NCoR1, nuclear corepressor 1; pPol II, phosphorylated RNA polymerase II; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid response element; RE, response element; RXR, retinoid X receptor; SAHA, suberoylanilide hydroxamic acid; siRNA, small interfering RNA; TSS, transcription start site.

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C gene, being located in chromosome 6q21, is deleted in a subset of acute lymphoblastic leukemia further suggests its involvement in tumorigenesis.[8] Not much is known about the regulation of the *Cyclin C* gene regulation. In many tissues the gene is highly expressed, but in contrast to most other cyclins, Cyclin C protein amounts do not show very pronounced oscillations; the highest *Cyclin C* mRNA levels occur during G_0 exit.[8,9]

We have previously characterized the human *Cyclin C* gene as a primary target of the nuclear vitamin D receptor.[10,11] Subsequently, we screened the gene for further nuclear receptors and other transcription factors that may directly regulate the *Cyclin C* gene. The nuclear retinoic acid (RA) receptor (RAR) mediates the actions of retinoids derived from dietary vitamin A and from the provitamin β-carotene. RARs form heterodimers with another nuclear receptor, retinoid X receptor (RXR), and bind to a pair of hexameric DNA motifs arranged as a direct repeat (DR) with two or five intervening nucleotides, so called DR2- and DR5-type RA response elements (RAREs).[12,13] Ligand binding induces a conformational change within the ligand-binding domain of the receptor, resulting in the replacement of corepressor proteins, such as nuclear receptor corepressor 1 (NCoR1),[14] by coactivators, such as CREB-binding protein (CBP),[15] and members of the Mediator complex, such as MED1,[16,17] which finally leads to initiation of gene transcription.

The transcription factor cAMP response element-binding protein 1 (CREB1) is involved in long-term neuronal plasticity, cell survival, circadian rhythms, adaptation to drugs and hormonal regulation of metabolism[18] and has an impact in tumorigenesis.[19,20] CREB1 is activated by phosphorylation at residue S133 via protein kinase A, which can be initiated experimentally by the cAMP activator Forskolin.[21] Comparable to the ligand binding to RAR, the phosphorylation of CREB1 is essential for the exchange of corepressors by coactivators. CREB1 binds constitutively to cAMP response elements (CREs) of the consensus sequence TGACGTCA.[22] Mammalian genomes contain more than 10,000 copies of this sequence,[23] but their access is under cell-specific epigenetic control.[24]

Histone deacetylases (HDACs) indirectly interact with gene promoters and reduce transcription by removing acetyl groups from the side chain of specific lysine residue tails of core histones. Histone deacetylation results in compact chromatin structure and reduces the access of basal transcription factors to promoters.[25] HDACs can also inactivate directly nonhistone proteins, such as p53, E2F or α-tubulin, by deacetylation.[26–28] Therefore, HDACs have multiple influences on cellular processes. At present, 11 human HDACs are known.[29] HDACs 1, 2, 3 and 8 belong to class I, are ubiquitously expressed and seem to be involved more in general cellular processes. The class II HDACs 4, 5, 6, 7, 9 and 10 have more tissue-specific functions and distributions, while HDAC11 forms its own class.[30,31] HDAC inhibitors, such as suberoylanilide hydroxamic acid (SAHA), are known to influence preferentially proliferation, differentiation and death of cancer cells, and therefore they are promising anticancer compounds.[32–34]

In this study, we show that the human *Cyclin C* gene is a direct target of all-*trans* RA and that RARγ associates with a promoter region containing two RAREs. The gene also directly responds to Forskolin and we identified four CREs within the first 2250 bp of the promoter, to which CREB1 associates. RARγ and CREB1 show functional convergence via NCoR1, which controls in particular the Forskolin response of *Cyclin C*. HDACs 1, 5, 6, 7 and 11 influence the basal expression of *Cyclin C*, but antiproliferative effects of the HDAC inhibitor SAHA are not mediated by the gene. In contrast, the cell cycle progressing effects of all-*trans* RA and Forskolin in HEK293 human embryo renal cortical cells and in MCF-7 human breast carcinoma cells are dependent on *Cyclin C* expression levels. This suggests that all-*trans* RA and Forskolin control the cell cycle, at least in part, via their role as primary regulators of *Cyclin C* expression.

Results

Cyclin C is a primary target of all-*trans* RA and Forskolin

HEK293 cells are a model system[35] that we used in screening for potential direct activators of the *Cyclin C* gene. In this context, we tested the effects of the RAR ligand all-*trans* RA (0.1 μM in all experiments) and the CREB inducer Forskolin (10 μM in all experiments) in a time course of 1, 2, 4 and 24 h (Fig. 1). Quantitative real-time PCR showed that all-*trans* RA stimulation resulted after 4 h in a 2.1-fold induction of *Cyclin C* mRNA levels (Fig. 1a), while Forskolin treatment already induced a 1.6-fold induction after 1 h (Fig. 1b). After 24 h, Forskolin-treated samples showed a second peak of *Cyclin C* mRNA induction (2.3-fold), while at the same time the all-*trans* RA stimulated samples were slightly reduced (1.8-fold induction). As a control, we repeated the all-*trans* RA stimulation experiment in HaCaT human immortalized keratinocytes and observed significant inductions (1.6-fold after 2 h and 2.0-fold after 4 h) of the *Cyclin C* gene (Fig. S1). These induction factors are relatively low, but one has to take into account that *Cyclin C* is highly expressed in HEK293 cells (e.g., more than three times higher than the *Cyclin E1* gene; see Fig. S2); that is, large numbers of *Cyclin C* mRNA molecules have to be newly synthesized in order to measure a significant induction of the gene.

RAREs and CREs within the *Cyclin C* promoter

In order to study the molecular mechanism of the primary response of the *Cyclin C* gene to all-*trans* RA