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Cyclin C interacts with steroid receptor coactivator 2 and upregulates cell cycle genes in MCF-7 cells

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ABSTRACT

Steroid receptor coactivator 2 (SRC-2) is a coactivator that regulates nuclear receptor activity. We previously reported that SRC-2 protein is degraded through the action of cAMP-dependent protein kinase A (PKA) and cAMP response element binding protein (CREB). In the study presented here, we aimed to identify proteins that interact with and thereby regulate SRC-2. We isolated cyclin C (CCNC) as an interacting partner with the SRC-2 degradation domain aa 347-758 in a yeast two-hybrid assay and confirmed direct interaction in an *in vitro* assay. The protein level of SRC-2 was increased with CCNC overexpression in COS-1 cells and decreased with CCNC silencing in COS-1 and MCF-7 cells. In a pulse-chase assay, we further show that silencing of CCNC resulted in a different SRC-2 degradation pattern during the first 6 h after the pulse. Finally, we provide evidence that CCNC regulates expression of cell cycle genes upregulated by SRC-2. In conclusion, our results suggest that CCNC temporarily protects SRC-2 against degradation and this event is involved in the transcriptional regulation of SRC-2 cell cycle target genes.

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1. Introduction

The activity of nuclear receptors (NRs) depends not only on binding of their ligands (e.g. steroid hormones, metabolites and bile acids), but also on the presence of coactivators and corepressors. The steroid receptor coactivator (SRC) family consists of three members: SRC-1, SRC-2 and SRC-3 [1–3], which stimulate NR-mediated transcription. SRC-2 (also known as NCoA2, TIF2 or mouse homologue GRIP1) is involved in many important biological processes such as mammary morphogenesis and fertility [4] and bone metabolism [5]. Moreover, SRC-2 mediates hepatic glucose release during fasting and regulation of bile acid secretion [6,7], whereas absence of SRC-2 has been shown to protect against high-fat induced obesity [8]. It has also been shown that SRC-2 may have an oncogenic function in prostate cancer [9] as well as in breast cancer tissue, where its expression correlates with the expression of ERα [10]. During tumorigenesis, SRC-2 is initially increased in intraductal carcinomas compared to normal mammary glands. However, further tumor development is associated with a decrease in SRC-2 in invasive ductal carcinomas [11]. Moreover, cellular studies have shown that SRC-2 is functionally distinct from the other SRC members, as we have previously demonstrated stable knockdown of SRC-2 induced proliferation in ER-positive MCF-7 cells [12]. Additionally, unlike SRC-1 and SRC-3, SRC-2 can both coactivate and corepress ERα transcriptional activity [13].

The activity of SRC-2 depends closely on ubiquitin/proteasome-mediated protein turnover [14]. We previously found that SRC-2 coactivation of ERα-mediated transcription and SRC-2 protein level are tightly regulated by cAMP-dependent protein kinase (PKA) in two distinct ways. While short-term PKA activation increases the recruitment of SRC-2 to ERα and thereby promotes transcription

Abbreviations: 6His, hexahistidine tag; aa, amino acids; Bcl-2, apoptosis regulator B-cell CLL/lymphoma 2; cAMP, cyclic adenosine monophosphate; CCNC, cyclin C; CCPG1, cell cycle progression protein 1; CDK6, cyclin-dependent kinase 6; cDNA, coding DNA; CXCR4, chemokine (C-X-C motif) receptor 4; CHX, cycloheximide; E2, 17β-estradiol; EGR1, early growth response 1; ERα, estrogen receptor alpha; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRIP1, glucocorticoid receptor interacting protein 1; HA, hemagglutinin; KIF5C, kinesin family member 5C; LYN, v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; MCM7, minichromosome maintenance complex component 7; Myb, myeloblastosis viral oncogene homolog; NR2F2, COUP transcription factor 2; NSC, non-specific control siRNA; PKA, protein kinase A; Qn-RT-PCR, quantitative real-time PCR; RBBP8, retinoblastoma binding protein 8; SDS, sodium dodecyl sulfate; SEPT3, septin 3; siRNA, small interfering RNA; SRC, steroid receptor coactivator (NCoA); TBP, TATA box binding protein; WISP2, WNT1 inducible signaling pathway protein 2; Y2H, yeast two hybrid.

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initiation, long-term PKA activation induces SRC-2 protein degradation through the ubiquitin/proteasome pathway [15,16]. Furthermore, we have found that SRC-2 degradation involves cAMP response element binding protein (CREB) and two SRC-2 protein domains – aa 347-758 and aa 1121-1462 [17]. A scheme of SRC-2 protein structure and its domain aa 347-758 is shown in Fig. 1A.

Cyclins and cyclin-dependent kinases (CDKs) accumulate during tumorigenesis, thereby promoting the cell cycle, bypassing the control points for DNA integrity and diminishing apoptosis in mutated cells. Cyclins, in a large majority of cases, act through their associated CDKs and are often involved in transcriptional regulation. The function of cyclin C (CCNC), in contrast to most other cyclins, remains largely unknown. Hepatoma cell lines show CCNC upregulation, followed by increased proliferation, and a recent study points to CCNC as a main cell cycle effector of Wnt/β-catenin pathway in the early development of hepatoma [18,19]. Furthermore, CCNC is overexpressed in 88% colon cancers and 82.6% of breast cancers, and is found to correlate with increased proliferation in breast cancer, underlining its important function in tumorigenesis [20–22]. CCNC interacts with several cyclin-dependent kinases (CDKs), two of which confer its function in two different ways [23]. On one hand, CDK3 involves CCNC into cell cycle regulation, where CCNC stimulates re-entry into the cell cycle (G0/G1 transition), and it has been noted that this re-entry can occur directly into the G1/S transition point [24–26]. On the other hand, CCNC associates with CDK8 into a CDK module (SRB complex in yeast) as part of the Mediator and is involved in transcriptional regulation [27,28]. Although CDK module was first reported to repress transcription *in vitro* by phosphorylation of the CTD tail of RNApolII, further *in vivo* evidence shows the module has in fact a stimulatory effect on transcription in colon cancer [29,30]. CDK module is found in complex with ERα and can further activate transcription by modifying histones [31,32].

Unlike other cyclins, CCNC is highly expressed throughout the entire cell cycle and its protein level peaks merely two-fold just prior to G1/S transition, when it binds to CDK3 [33]. Endogenous CCNC is very stable, despite the fact that it is a cyclin (half-life of 4 h), and is stabilized by binding to and being phosphorylated by its CDKs [34]. CCNC protein structure is highly conserved and is comprised of two Cyclin repeats, characteristic for cyclins (Fig. 1B). In addition to each repeat having five helices, the N-terminus contains a highly mobile helix H_N and the protein lacks C-terminus helices [35]. CCNC exists in two protein isoforms (a and b) due to alternative splicing from a downstream transcriptional start codon. The two isoforms are differentially expressed in various tissues, but no functional distinction has been made between the two isoforms [36].

The aim of this study was to identify proteins which interact with the SRC-2 degradation domain aa 347-758 and study their involvement in regulation of SRC-2.

2. Materials and methods

2.1. Expression plasmid constructs, siRNA and chemicals

The expression of 6His-SRC-2, HA-SRC-2 [17,37] and Flag-CCNC [38] has been previously described. For knockdown experiments we tested several target-specific siRNA molecules in various combinations and concentrations and settled on a combination of two siRNAs targeting CCNC at starting mRNA positions 476 and 755. This combination produced no effect on mRNA and protein level of several tested genes. The target-specific and non-specific control (NSC) siRNA were custom-ordered (Sigma Aldrich, St. Louis, MO, USA) and are described elsewhere [39]. Cycloheximide (CHX) was purchased from Sigma (C7698) and resuspended in sterile water.

2.2. Yeast two-hybrid assay

The assay was performed by Hybrigenics, S.A, Paris, France (www.hybrigenics.com). Briefly, the cDNA coding for the aa 347-758 fragment of human SRC-2 was cloned as a C-terminal fusion to the DNA-binding domain of LexA in pB27 vector (N-LexA-SRC-2 347-758-C). The construct was introduced into yeast strains as bait to screen high-complexity random-primed cDNA library (prey library) of human embryonic stem cell, as previously described [40]. After selection on medium lacking leucine, tryptophane and histidine, positive clones were isolated and the corresponding prey fragments were identified using GenBank Database and bioinformatics tools (BLASTN). Predicted biological score (PBS) was calculated to assess the reliability of each interaction.

2.3. In vitro protein–protein interaction assay

Expression of recombinant 6His-SRC-2 protein in the insect cell line Sf21 and protein purification by affinity chromatography were previously described [17]. Two protein isoforms of CCNC were expressed *in vitro* from the same expression plasmid (pcDNA3.1-Flag-CCNC), using TnT® T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) in presence of [^{35}S]methionine (Perkin Elmer, Waltham, MA, USA). 1.6 μg of 6His-SRC-2, 6His-peptide (Covance, Princeton, NJ, USA), 6His-collagen COL4A3 and 6His-β-actin (Abcam, Cambridge, UK) were individually incubated with 15 μl [^{35}S]methionine-labeled CCNC protein as is described elsewhere [17,41].

2.4. Cell cultures, transfections and harvesting

COS-1 cells were transfected to overexpress proteins, using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA) as previously described [16]. For knockdown of CCNC, a total of 36 nM and

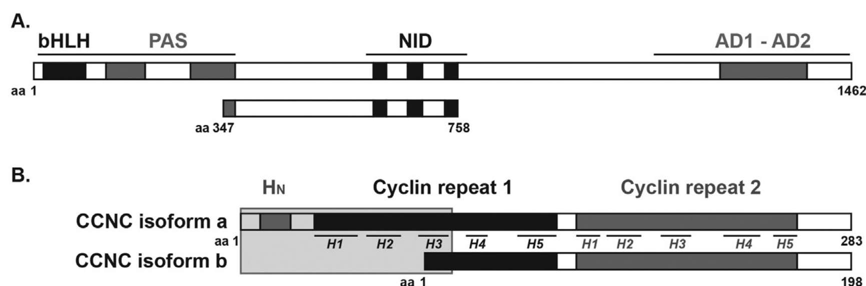

Fig. 1. Secondary structure of SRC-2 and CCNC. (A) Functional domains of full-length SRC-2, in addition to the SRC-2 domain aa 347-758, which was used as bait in the yeast two-hybrid assay. Basic helix-loop-helix domain (bHLH), Per-Arnt-Sim domain (PAS) and the nuclear receptor interaction domain (NID) are indicated, while AD denotes two activation domains (AD1 and AD2). (B) Secondary structure of CCNC comprises a highly mobile N-terminal helix (H_N), followed by two Cyclin repeats, each containing five helices (H1–H5). Two CCNC isoforms arise from an alternative transcriptional start site. Minimum element of CCNC, binding to SRC-2, is depicted as a grey frame. Adapted from Hoeppner et al. [35].

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