Biochimica et Biophysica Acta 1801 (2010) 537–546

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Specific interaction between E2F1 and Sp1 regulates the expression of murine CTP:phosphocholine cytidylyltransferase alpha during the S phase

Claudia Elena, Claudia Banchio *

IBR (Instituto de Biología Molecular y Celular de Rosario), Consejo Nacional de Investigaciones Científicas y Técnicas, Área Biología, Departamento de Ciencias Biológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario, Argentina

ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form 6 January 2010
Accepted 13 January 2010
Available online 21 January 2010

Keywords:
Pcyt1a gene promoter
Cytidylyltransferase
Phosphatidylcholine
E2F1
Sp1
S phase

ABSTRACT

CTP:phosphocholine cytidylyltransferase alpha (CCTα) is a key enzyme for phosphatidylcholine biosynthesis in mammalian cells. This enzyme plays an essential role in all processes that require membrane biosynthesis such as cell proliferation and viability. Thus, CCTα activity and expression fluctuate during the cell cycle to achieve PtdCho requirements. We demonstrated, for the first time, that CCTα is localized in the nucleus in cells transiting the S phase, whereas it is localized in the cytoplasm of G_0-arrested cells, suggesting a specific role of nuclear CCTα during the S phase. We also investigated how E2F1 influences the regulation of the CCTα-promoter during the S phase; we demonstrated that E2F1 is necessary, but not sufficient, to activate CCTα expression when this factor is over-expressed. However, when E2F1 and Sp1 were over-expressed, the transcription from the CCTα-promoter reporter construct was super-activated. Transient transfection studies demonstrated that E2F1 could super-activate Sp1-dependent transcription in a promoter containing only the Sp1 binding sites "*B*" or "*C*", and that Sp1 could activate Sp1-dependent transcription in a promoter containing the E2F site, thus, further demonstrating a functional interaction of these factors. In conclusion, the present results allowed us to portray the clearest picture of the CCTα-gene expression in proliferating cells, and understand the mechanism by which cells coordinate cell cycle progression with the requirement for phosphatidylcholine.

1. Introduction

The G_1 to S phase transition requires the periodic expression of several genes necessary for doubling cell components prior to mitosis. Among these, the expression and regulation of genes involved in nucleotide biosynthesis and DNA replication have been well described [1–3]. However, since phosphatidylcholine (PtdCho) is the major cellular component of biological membranes in eukaryotes, its role in cell cycle progression is also highly important. In fact, cell viability and cell cycle progression are sensitive to membrane PtdCho content; Chinese hamster ovary cells harboring a temperature-sensitive mutation in CTP:phosphocholine cytidylyltransferase (CCT) do not synthesize PtdCho at 40 °C and undergo apoptosis unless rescued by the addition of PtdCho or lyso-PtdCho [4]. Moreover, a net phospholipids accumulation during the S phase was defined among a number of cell types including fibroblasts, HeLa cells [5,6], macrophages [7] and mast cells [8].

The cytidine-diphosphocholine (CDP-choline) pathway is the predominant route for PtdCho synthesis in most tissues [9]. The rate of PtdCho biosynthesis is governed in many instances by the rate of conversion of phosphocholine to CDP-choline in a reaction catalyzed by the CCT [9]. In mammals, this enzyme is encoded by two genes: *Pcyt1a* located on murine chromosome 16, that encodes the CCTα protein from alternative transcripts termed CCTα1 and CCTα2 [10], and *Pcyt1b* located on the X chromosome which encodes the CCTβ2 and CCTβ3 proteins from differentially spliced transcripts [11–15]. A distinguishing feature of CCTα is the presence of an N-terminal nuclear localization signal (NLS) that directs the enzyme to the nucleus in many cultured cells [16]. CCTβ isoforms lack an NLS and consequently are found associated with the endoplasmic reticulum (ER). CCTα protein is ubiquitously expressed in nucleated cells [17], and its expression is tightly regulated at both the transcriptional and post-transcriptional levels to supply the demands of PtdCho according to different physiological conditions. *Pcyt1*a gene transcription is enhanced during the S phase of the cell cycle [18] in line with the long-held view that PtdCho synthesis can be rapidly modulated by reversible binding of CCTα to cellular membranes without an increased amount of CCTα protein [19,20]. Thus, cells can respond to the demand for PtdCho immediately. In this sense, by using different cell lines, different physiological conditions and different approaches, several authors demonstrated that CCTα binds to the endoplasmic reticulum, Golgi apparatus and nuclear envelope membranes [21–23]. However, no information has been reported demonstrating CCTα localization specifically during the S phase of the cell cycle.

Previous works [24–27] described the mechanisms that regulate CCTα expression according to cell cycle progression. It was demonstrated that CCTα mRNA increased when cells reach the S phase due,

* Corresponding author. Tel.: +54 341 4350661; fax: +54 341 4804601.
E-mail address: banchio@ibr.gov.ar (C. Banchio).

1388-1981/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbalip.2010.01.005

in part, to the binding of phosphorylated Sp1 to the Sp1 binding site, named *B*, located at position (−70/−58) of the CCTα proximal promoter. Moreover, it was shown that Sp1 phosphorylation depends on the action of CyclinA/E-Cdk2 [24,25].

The E2F1 protein regulates the cell cycle by controlling the coordinate transcription of a large number of targets, including genes involved in DNA replication and cell cycle progression, thus integrating cellular signals and coordinating cell proliferation [28,29]. These genes are expressed at low levels during quiescence and are induced as cells traverse the G_1/S phase transition and enter the cell cycle. An E2F binding site [26] located in the CCTα promoter that together with Sp1 binding site "*C*" is involved in the recruitment of histone deacetylase (HDAC) and retinoblastoma (Rb), thus, repressing CCT expression during G_0 phase when the requirement for PtdCho decreases [7,30].

The present work provides data demonstrating an additional mechanism that regulates CCTα activity and expression during the S phase of the cell cycle. First, we demonstrated, for the first time, that CCTα localizes both in the nucleus and cytoplasm during the S phase and only in the cytoplasm in quiescent mouse embryo fibroblasts. These results could support a previous hypothesis about the role of the enzyme as regulator of the cell cycle [7,31,32]. Second, we provide additional evidence involving E2F1 as a transcription factor that activates CCTα expression during the S phase of the cell cycle and demonstrated that E2F1 mediated superactivation requires Sp1 to achieve growth dependent regulation. Similar machinery regulates the expression of other genes required for DNA synthesis, cell cycle progression, DNA damage repair, apoptosis, cell differentiation and development [33–35]. Therefore, these results contribute to understand mechanisms that coordinate macromolecular and bulk membrane phospholipid production with cell cycle progression.

2. Materials and methods

2.1. Tissue culture

C3H10T1/2 mouse embryo fibroblast cell line (ATCC CCL-226) was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Media was supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml) and cells were maintained in a 5% CO_2, humidified incubator at 37 °C. C3H10T1/2 fibroblasts were arrested in G_0 by incubation in culture medium with low serum (0.5%) for 72 h and released by addition of fresh medium containing 10% FBS. Schneider SL2 cells (kindly provided by Dr. P. Wappner) were cultured in Schneider's medium supplemented with 10% heat-inactivated fetal calf serum at room temperature. Transient transfections with the CCTα promoter-luciferase reporter plasmid (1 μg) and CCTα promoter-luciferase reporter plasmid containing mutations that alter E2F and/or Sp1 transcription factor binding sites, Luc.C7 (−1268/+38), Luc. C7delE2F, Luc.C7delAB, Luc.C7delAC, and Luc.D2 (−130/+38) [25,36], respectively, were performed using a cationic liposome method [37]. All dishes received 0.5 μg of CMV-β-galactosidase (PROMEGA) as a control for transfection efficiency. Luciferase and β-galactosidase assays were performed using the PROMEGA dual assay system, as recommended by the manufacturer and luminometric measurements were made using Fluskan Ascent FL Type 374 (Thermolabsystems). Luciferase activity was normalized to β-galactosidase activity and expressed as a ratio luciferase/β-galactosidase. Vectors designed to generate siRNA-Sp1 and to over-expressed Sp1 were previously used in [25], and vectors enabling the expression of siRNA-E2F1 and the over-expression of E2F1 were kindly provided by Dr. D. Cress and Dr. C. Scherr, respectively.

2.2. Preparation of nuclear extracts and electromobility-shift assay (EMSA)

Total nuclear extracts of C3H10T1/2 cells were prepared as described previously [38]. Protein concentrations were determined by the Coomassie brilliant blue method [39].

Two sets of probes were used for EMSA assays. The DNA probes of 400 bp (CCTα-E2F and CCTα-E2F-mut) were obtained by PCR using the ChIPCT1 (5-TTgCCCTCgCCTCTACTCCTgCTC) and ChIPCT2 (5-CTCCCgCCCgCCCTCTTgTC) primers (see Fig. 2), and Luc.C7 and Luc.C7delE2F plasmids as starting material. The PCR products were purified using Qiaex II (Gel Purification Kit). Five picomoles of double stranded DNA probes were 5′-labelled using [γ^{32}P]-ATP and T4 polynucleotide kinase (Fermentas). For each binding reaction (20 μl): 10 μl of 2× binding buffer (50 mM Tris–HCl, pH 7.5, 40% glycerol, 100 mM KCl, 100 mM Dithiothreitol, 2 mg/ml BSA, 0.2% Triton X-100 and 0.5 μg of poly-dI-dC), 3–5 μg of nuclear extract and labelled probe (25,000 cpm) were incubated for 30 min at room temperature. The other pair of probes were oligonucleotides carrying the E2F elements (wt-E2F): E2FWTF (5′-ggATgTgTggCgCCTCCTg-CCgCC-3′) and E2FWTR (5′-ggCggCAggAggCgCCACACATCC-3′), or harboring mutations that disrupt E2F binding site (del-E2F): E2FdelF (5′-ggATgTgCTCC-TgCCgCCggTCTC-3′) and E2FdelR (5′-gAgACCggCgg-CAgg-AgCACATCC-3′); sequences were synthesized by Invitrogen. Complementary oligonucleotides (10 μM of each) were heated at 90 °C for 5 min and then slowly cooled at room temperature and labelled as was previously described. For each binding reaction (20 μl), 10 μl of 2× binding buffer (10 mM Tris–HCl pH: 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM Dithiothreitol, 10% glycerol and 150 μg/ml of poly-dI-dC), 4 μg of nuclear extract and labelled probe (30,000 cpm) were incubated for 30 min at room temperature. In both experiments for supershift analysis, 1 μg of antibodies specific for E2F1 and Sp1 (Santa Cruz Biotechnology) were added for 20 min after incubation of the probe with the nuclear extract. Binding reactions were terminated by the addition of gel loading buffer (30% v/v glycerol, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol). The complexes were separated on a non-denaturing 6% or 4% (w/v) polyacrylamide gel and visualized by autoradiography of the dried gel.

2.3. Chromatin immunoprecipitation (C h I P) assay

C3H10T1/2 fibroblast cells were grown for 24 h in DMEM containing 10% FBS. After 72 h of serum deprivation, cells were released by the addition of 10% FBS. Cells synchronized at S phase of the cell cycle were incubated with 1% formaldehyde for 5 min at 37 °C. Cells were collected, lysed, and sonicated three times for 10 s each at 2.5% with an ultrasonic processor XL from Heat Systems and treated for ChIP as recommended by the manufacturer (Upstate). An aliquot of lystates (20 μl) was taken out as input control, and each of the immunoprecipitating antibodies were added to the supernatant fraction and incubated 2 h at 4 °C with rotation. Anti-E2F1 antibody was purchased from Santa Cruz Biotechnology and anti-rabbit IgG Horseradish peroxidase (GE-Healthcare) was used as control. For PCR analysis, we used the following set of primers: ChIPCT1 and ChIPCT2. PCR was performed using 5 μl of template DNA, 2.5 mM $MgCl_2$, and 20 pmol of each primer for 30 cycles at 94 °C for 1 min, 64 °C for 1 min, and 72 °C 1 min.

2.4. Immunohistochemistry and confocal microscopy

C3H10T1/2 fibroblasts were grown on coverslips, fixed for 5 min with 4% (v/v) formaldehyde at room temperature and washed 3 times with PBS according to conventional protocols. Cells were then incubated with primary antibodies against CCTα (F-17 Santa Cruz Biotechnology), CCTβ (kindly provided by Dr. S. Jackowski), cyclin D and cyclin E (Santa Cruz Biotechnology) at room temperature for 90 min in an appropriate dilution in PBS and 0.05% saponin. After removing the antibody, cells were washed three times with PBS, and incubated during 60 min at room temperature with anti-rabbit IgG conjugated with Alexa 488 and anti-goat IgG conjugated to Cy3, respectively. Prolong Gold with DAPI (Invitrogen) was used to dye DNA material and as an antifade reagent. Immunostained cells were visualized with a laser scanning confocal microscope Nikon, Model