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Transcription of the mitochondrial citrate carrier gene: Identification of a silencer and its binding protein ZNF224

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ABSTRACT

In the last few years, we have been functionally characterizing the promoter of the human mitochondrial citrate carrier (CIC). In this study we show that CIC silencer activity extends over 26 bp (−595/−569), which specifically bind a protein present in HepG2 cell nuclear extracts. This transcription factor was purified by DNA affinity and identified as ZNF224. Overexpression of ZNF224 decreases LUC transgene activity in cells transfected with a construct containing the CIC silencer region, whereas ZNF224 silencing activates reporter transcription in cells transfected with the same construct. Moreover, overexpression and silencing of ZNF224 diminishes and enhances, respectively, CIC transcript and protein levels. Finally, ZNF224 is abundantly expressed in fetal tissues contrary to CIC. It is suggested that CIC transcriptional repression by ZNF224 explains, at least in part, the low expression of CIC in fetal tissues in which fatty acid synthesis is low.

Introduction

The mitochondrial citrate carrier (CIC) is a nuclear-encoded protein which is located in the inner mitochondrial membrane and belongs to the mitochondrial carrier protein family (see [1,2] for reviews). Its primary function is to catalyze the transport of citrate from the mitochondrial matrix to the cytosol in exchange for malate. In the cytosol, citrate produces acetyl-CoA and NADPH which are both necessary for fatty acid and sterol biosynthesis. Besides its fundamental role in lipogenesis, CIC is a key component of the isocitrate–oxoglutarate shuttle and the citrate–malate shuttle [2,3]. Moreover, it is involved in gluconeogenesis [2,3] and in the control of glucose-stimulated insulin secretion [4,5]. The human gene for CIC, named SLC25A1, is localized on chromosome 22q11.2 within a region implicated in DiGeorge syndrome [6]. CIC mRNA and/or CIC protein levels are high in liver, pancreas and kidney but low or absent in heart, skeletal muscle, placenta, brain and lungs [7]. CIC activity was found to be decreased in diabetic, hypothyroid and unfed rats [8–10] and in rats fed with a diet enriched with polyunsaturated fatty acids (PUFAs) [11].

Preceding studies from our laboratory, in which the human CIC gene promoter has been functionally analyzed, revealed that: (i) insulin upregulates and PUFAs downregulate CIC gene transcription through the SRE/SREBP-1 regulatory system [12]; (ii) demethylation of the proximal promoter and histone acetylation activate CIC gene expression by promoting binding of both Sp1 transcription factor and acetylated histone H3 to the CIC proximal promoter [13]; and (iii) FOXA acts as a strong enhancer of CIC gene expression by binding to a FOXA site [5]. Furthermore, LUC gene reporter and EMSA experiments led to the identification within the CIC gene promoter of an inhibitory domain (from −742 to −499 bp) [5], which is the object of the present study.

Herein we show that CIC silencer activity extends over 26 bp, from −595 to −569. The transcription factor that binds to the silencer region was purified from HepG2 cell nuclear extracts by DNA affinity and identified as ZNF224. Moreover, direct evidence is provided that overexpression of ZNF224 reduces LUC transgene expression activity as well as CIC transcript and protein levels, whereas ZNF224 silencing enhances LUC reporter activity and CIC gene expression.

Materials and methods

Construction of plasmids. The −1785/−20, −742/−20, −595/−20 and −568/−20 bp regions of the human CIC gene promoter were obtained by PCR and cloned into the pGL3 basic-LUC vector

Abbreviations: CIC, citrate carrier; Kap1, KRAB-associated protein 1; KRAB, Krüppel-associated box; LUC, luciferase; ZNF224, zinc finger protein 224.

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(Promega) upstream of the LUC gene coding sequence. The ZNF224 expression vector (pcDNA3–ZNF224) was obtained by cloning the human ZNF224 cDNA (Accession No. NM_013398.1) into the pcDNA3 vector (Invitrogen).

Cell culture, RNA interference and transient transfection. HepG2 and HEK293 cells (Sigma) were grown in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Transient transfection was performed as reported [12] using 0.5 μg of pGL3 basic-LUC vector containing the full-length −1785/−20 bp region of the CIC gene promoter, or deletion fragments of this region, and 10 ng of pRL-CMV (Promega) to normalize the extent of transfection. Transfected cells were assayed for LUC activity using the Dual-Luciferase® Reporter Assay System (Promega). For ZNF224 overexpression, HepG2 cells were transfected using 0.5 μg of pcDNA3–ZNF224 vector. In RNA interference experiments, the specific pre-designed small interfering RNA (siRNA) targeting human ZNF224 (s15303, Ambion) was transfected in HEK293 cells using the siPORT™ NeoFX™ Transfection Agent (Ambion). A siRNA (C6A-0126, Ambion) with no significant similarity to human, mouse, or rat gene sequences was used as negative control.

Protein purification. Protein purification was carried out using the DNA-binding protein purification kit (Roche), following the manufacturer's instructions. A long concatamer of the CIC gene −595/−569 bp region was obtained by self-primed PCR technique [14], phosphorylated at its 5′-end and ligated to streptavidin magnetic particles. The particles were incubated with HepG2 cell nuclear extracts, as previously described [15]. DNA-binding proteins were eluted with 0.1 and 0.5 M KCl.

Mass analysis and protein identification. Digestion of the protein bands excised from the gel, peptide mass "fingerprinting" and search of the tryptic fragment spectra against the NCBI protein databases were performed as previously described [15].

Other methods. Electrophoretic mobility shift assays (EMSA) were performed as described in Sambrook et al. [16]. Each double-stranded probe was 5′-end labeled using T4 polynucleotide kinase and [γ^{32}P]-ATP at 37 °C for 30 min. The gels were dried and images acquired by phosphoimager (Bio-Rad). The mutated sequences of the CIC gene −595/−569 bp region used herein are GGTGGGCCCCG GCAGGTGAGGCCCGGC (GM1), GGTGGGAAAAGGCAGGTGAGGCCC GGC (GM2), GGTGGGAACCTTCAGGTGAGGCCCGGC (GM3), GGTGGG AACCGGCATTTGAGGCCCGGC (GM4), GGTGGGAACCGGCAGGTTCTG CCCGGC (GM5) and GGTGGGAACCGGCATGTGAGGAACGGC (GM6), where the mutated nucleotides are underlined. Total RNA was extracted from 1×10^6 HepG2 or HEK293 cells or purchased by Clontech (human fetal liver RNA 636502, human liver RNA 636531, human fetal kidney RNA 636526 and human kidney RNA 636529) and reverse-transcripted [15]. Real-time PCR was performed as previously described [17]. Assays-on-demand for human CIC (Hs00761590_m1), human ZNF224 (Hs00273760_m1) and human β-actin (4326315E) were purchased from Applied Biosystems. All transcript levels were normalized against the β-actin expression levels. For Western blot analysis, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) subsequently treated with anti-CIC [13]) or anti-β-actin (BioLegend) antibodies. The immunoreaction was detected by the ECL plus system (Amersham).

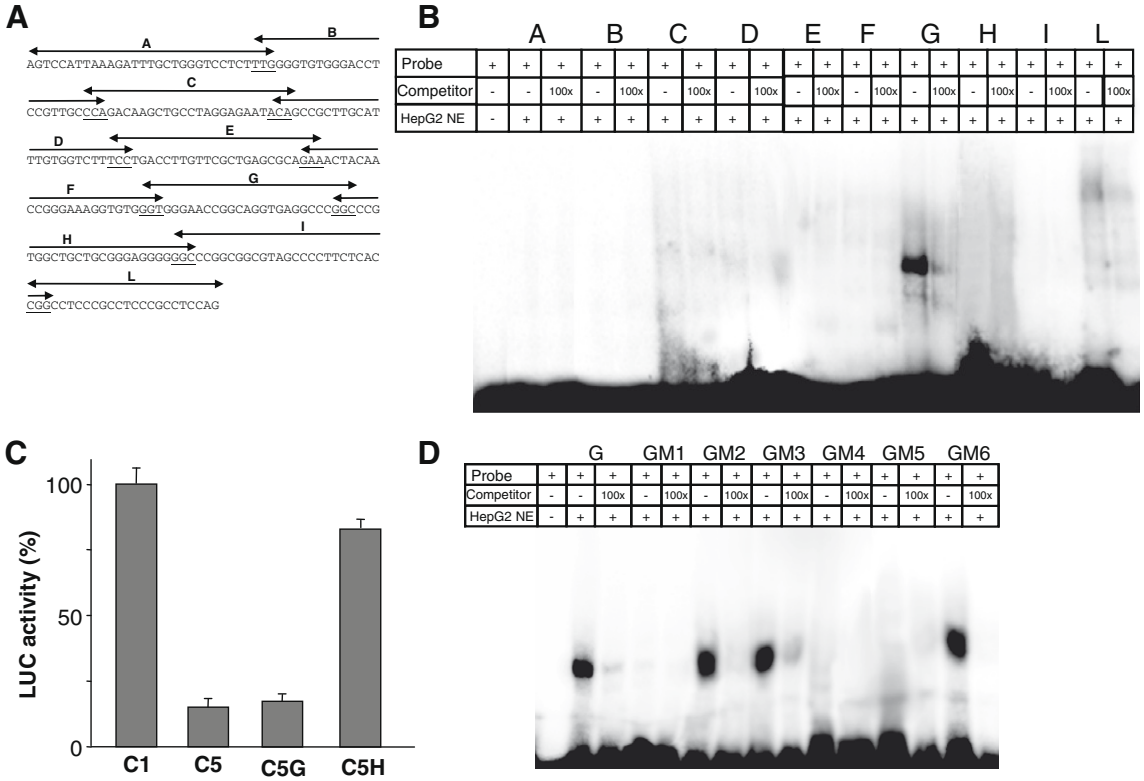

Fig. 1. Deletion analysis of the CIC gene promoter inhibitory domain. (A) Ten oligonucleotides of about 25 bp in length, encompassing the −742/−499 bp region of the CIC gene promoter and partially overlapping on both sides, are indicated with the letters from "A" to "L". (B) The 5′-end labeled DNA probes corresponding to oligonucleotides A–L were incubated with 10 μg protein of HepG2 nuclear extracts (HepG2 NE). Where indicated, unlabeled probe was added in 100-fold molar excess. (C) HepG2 cells, transfected with the pGL3 basic-LUC vector containing the −1785/−20 bp (C1), −742/−20 bp (C5), −595/−20 bp (C5G) and −568/−20 bp (C5H) regions of the CIC gene promoter, were assayed for LUC activity. Means ± SD of four duplicate independent experiments are shown. (D) The 5′-end labeled DNA probes, corresponding to the silencer region of the CIC promoter (probe G, −595/−569 bp) and mutated oligonucleotides of the same region (GM1–GM6), were incubated with 10 μg protein of HepG2 nuclear extracts (HepG2 NE). Where indicated, unlabeled probe was added in 100-fold molar excess.