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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).

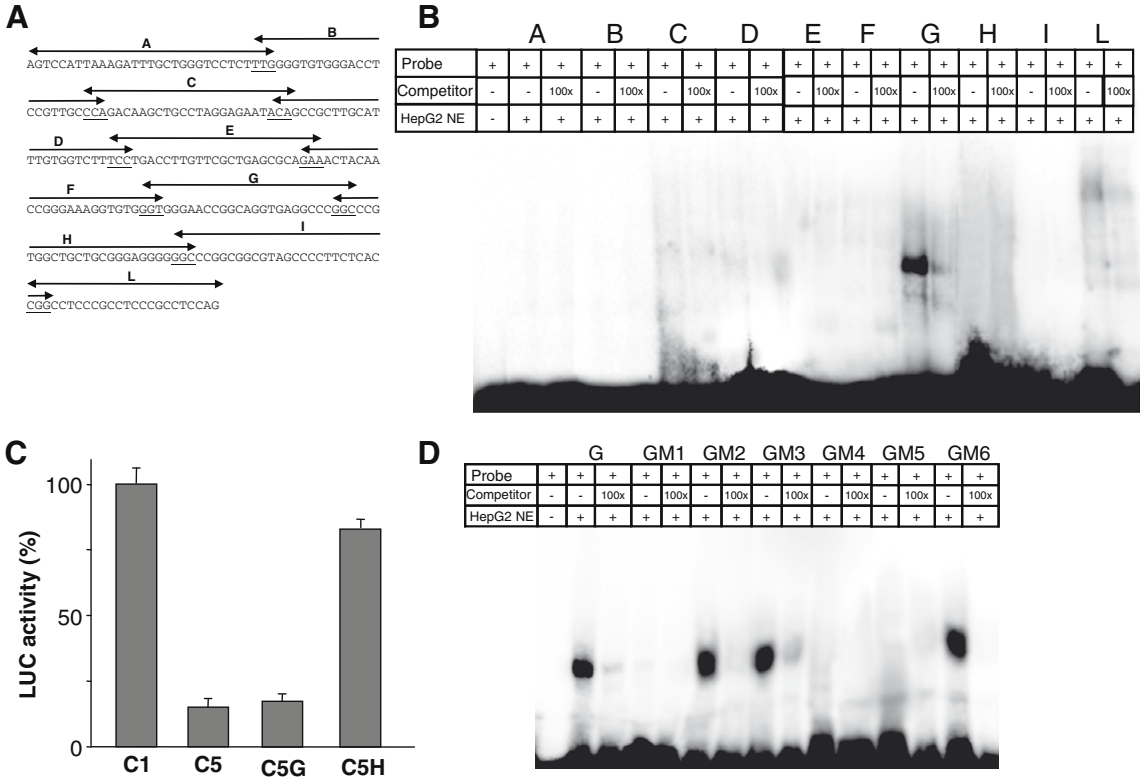

Fig. 1. Deletion analysis of the CIC gene promoter inhibitory domain. (A) Ten oligonucleotides of about 25 bp in length, encompassing the −742/−499 bp region of the CIC gene promoter and partially overlapping on both sides, are indicated with the letters from "A" to "L". (B) The 5′-end labeled DNA probes 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.

Results

Identification and characterization of the silencer element of the CIC gene promoter

In a recent study, we have identified an inhibitory domain between −742 and −499 bp through deletion analysis of the −1785/−335 bp region of the human CIC promoter [5]. A computer search of a vertebrate transfactor database (http://www.cbrc.jp/research/db/TFSEARCH.html) with the −742/−499 bp region of the CIC gene failed to reveal a transfactor that recognizes the DNA sequence of this region. In order to map the CIC gene silencer with greater accuracy, 10 probes of about 25 bp in length, encompassing the region from −742 to −499 bp and partially overlapping on both sides (Fig. 1A), were labeled and used in EMSA experiments with HepG2 cell nuclear extracts (Fig. 1B). No protein binding occurred with probes A–F and H–L. A band shift was observed only with probe G corresponding to the sequence 5′-GGTGGGAACCGGCAG GTGAGGCCCGGC-3′ (from −595 to −569 bp). This band disappeared by competition with 100-fold excess of unlabeled probe (Fig. 1B).

The presence of a negative regulatory element from −595 to −569 bp of the CIC gene was confirmed by measuring LUC transgene expression activity in HepG2 cells transfected with the following four constructs containing the respective bp region: C1, −1785/−20 of the CIC gene promoter; C5 and C5G, promoter fragments −742/−20 and −595/−20, respectively; and C5H, promoter fragment starting immediately downstream of probe G (see Fig. 1B), i.e. from −568 to −20 (Fig. 1C). The gene reporter activity of C5G was nearly as low as that of C5 harboring the entire 5′-flanking region of the CIC gene previously found to confer silencer activity [5]. By contrast construct C5H, harboring the −568/−20 bp region and differing from construct C5G only for lack of the −595/−569 bp region, showed substantial promoter activity (>80% of C1 activity). These results clearly show that protein binding and gene silencing activity of the CIC gene promoter inhibitory domain resides in the −595/−569 bp region.

The specificity of the protein-binding activity displayed by the silencer region (probe G from −595 to −569 bp) was demonstrated not only by competition with unlabeled probe but also by site-directed mutagenesis (Fig. 1D). In fact, no binding was observed when the dinucleotide AA at positions −589/−588 was mutated into CC (GM1), the dinucleotide GG at positions −581/−580 was changed into TT (GM4) and the trinucleotide GAG at positions −578/−576 into TCT (GM5). By contrast, the oligonucleotides GM2, GM3 and GM6, in which CC were mutated into AA at positions −587/−586, GG into TT at −585/−584 and CC into AA at −574/−573, respectively, retained protein-binding activity (Fig. 1D). These findings indicate that specific nucleotides of the −595/−569 bp CIC silencer region are indispensable for binding one or more proteins of HepG2 nuclear extracts.

Purification and identification of the CIC silencer-binding protein

Proteins that bind the CIC silencer were purified from HepG2 cell nuclear extracts by DNA affinity using a long concatameric oligonucleotide of the region −595/−569 bp of the CIC gene as affinity ligand. The purified fraction eluted at 0.5 M KCl contained two bands with apparent molecular masses of about 100 and 60 kDa (Fig. 2A). Mass spectrometry analysis of the tryptic digests obtained from the two protein bands excised from the gel, together with database search, identified the protein band at 100 kDa as ZNF224. In fact, 15 tryptic peptides generated from the protein band at 100 kDa matched the ZNF224 sequence and overlapped

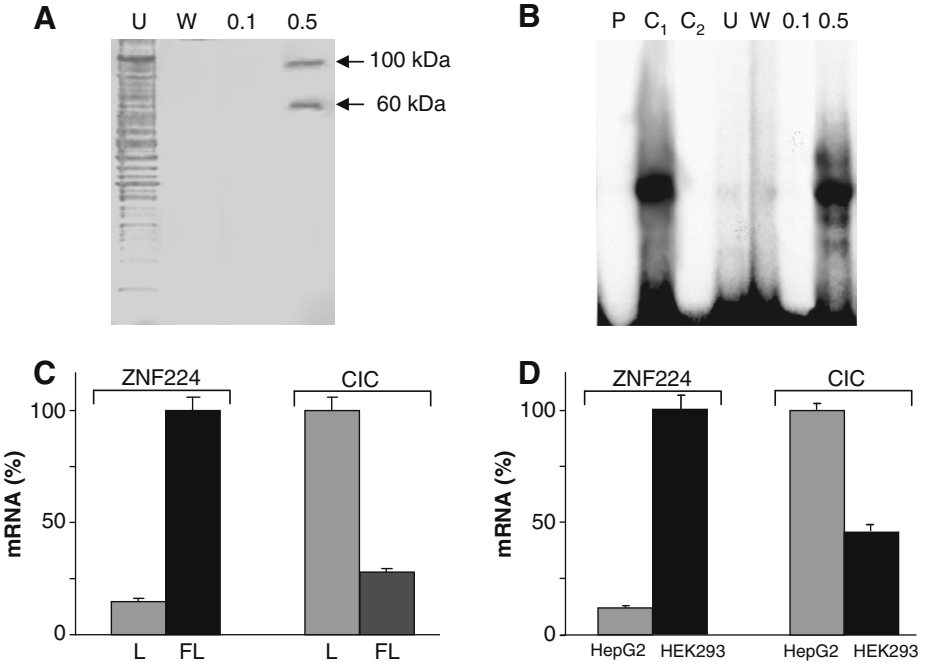

Fig. 2. (A) Affinity purification of the silencer-binding proteins. HepG2 nuclear extracts were incubated with a concatamer of the CIC gene −595/−569 bp region which had been ligated to streptavidin. The supernatant was removed and the bound proteins were eluted with four washing steps and with a buffer containing 0.1 and then 0.5 M KCl. Protein fractions, pooled and concentrated from five replicates, were analyzed by SDS–PAGE and stained with Coomassie Blue. Lane U, supernatant; lane W, last washing step; lanes 0.1 and 0.5, fractions eluted with 0.1 and 0.5 M KCl, respectively. (B) The 5′-end labeled DNA probe corresponding to the −595/−569 bp region of the CIC gene was incubated with 10 μg protein of HepG2 nuclear extracts (lane C_1), with the protein fractions of Fig. 2A (lanes U, W, 0.1 and 0.5) or none (lane P). Lane C_2, as lane C_1 in the presence of a 100-fold molar excess of unlabeled probe. (C) Total RNA from fetal liver (FL) and adult liver (L) was used to quantify ZNF224 and CIC mRNAs by real-time PCR. (D) Total RNA from HepG2 and HEK293 cells was used to quantify ZNF224 and CIC mRNAs by real-time PCR. In (C) and (D) means ± SD of three duplicate independent experiments are shown; differences between samples and relative controls (set at 100%) were significant ($P << 0.05$, one-way ANOVA).

29% of the ZNF224 sequence with a mowse score of 152 ($P << 0.05$). The protein at 60 kDa, identified as keratin, was a contaminant. The functional integrity of the DNA affinity-purified protein was verified by EMSA. The fraction eluted at 0.5 M KCl showed a strong shifted band (Fig. 2B), which was similar to that obtained with HepG2 cell nuclear extracts (lane C_1). In contrast, no binding activity was exhibited by the proteins that had not been bound by the DNA ligand (lane U).

mRNA levels of ZNF224 and CIC in fetal and adult tissues

Since ZNF224 belongs to the KRAB-containing zinc finger protein subfamily, which is known to be involved in the regulation of gene expression during embryogenesis and fetal life [18,19], the transcript levels of both ZNF224 and CIC were determined in fetal and adult liver tissues (Fig. 2C). Real-time PCR showed that the amount of ZNF224 mRNA was ~7-fold greater in fetal than in adult liver. Vice versa, the amount of CIC mRNA was ~4-fold higher in adult than in fetal liver (Fig. 2C). Similar results for both ZNF224 and CIC were obtained when comparing fetal and adult kidney (data not shown), although the absolute amount of ZNF224 mRNA was greater in fetal kidney than in fetal liver, and vice versa for CIC mRNA which was greater in adult liver than in adult kidney. An opposite pattern of ZNF224 and CIC expression was also found in HepG2 and HEK293 cell lines, i.e. a greater level of CIC mRNA in HepG2, and vice versa a higher level of ZNF224 mRNA in HEK293 cells (Fig. 2D).

Effect of ZNF224 overexpression and silencing on CIC gene expression

To investigate the role of ZNF224 in the regulation of CIC gene expression, we measured the LUC gene reporter activity in HepG2 cells transfected with the pGL3 basic-LUC vector containing the −595/−20 bp or −568/−20 bp region of the CIC gene in the presence of the pcDNA3–ZNF224 vector or of the empty vector. Forty-eight hours after transfection, LUC activity was markedly diminished in cells transfected with the LUC vector containing the silencer (−595/−569 bp) region (C5G) and overexpressing ZNF224 (Fig. 3A). Of note, in the control experiment, when the silencer region was lacking in the LUC plasmid (C5H) no repression

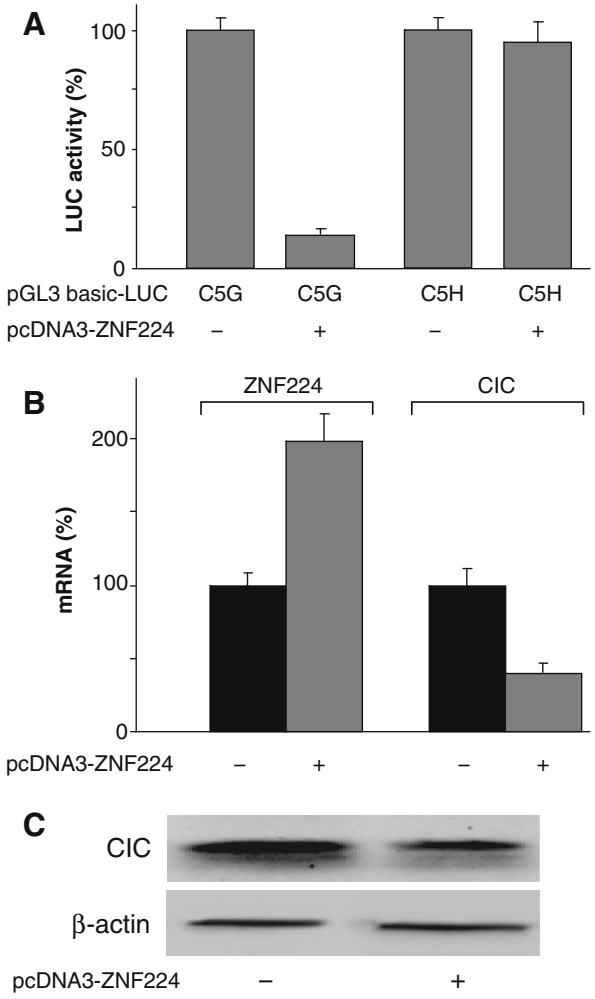

Fig. 3. Effect of ZNF224 overexpression on CIC gene expression. (A) HepG2 cells, co-transfected with the pcDNA3–ZNF224 (+) or empty vector (−) and with the pGL3 basic-LUC vector containing the −595/−20 (C5G) or −568/−20 bp region (C5H) of the CIC gene promoter, were assayed for LUC activity. (B) Total RNA extracted from HepG2 cells transfected with the pcDNA3–ZNF224 (+) or empty vector (−) was used to quantify ZNF224 and CIC mRNAs by real-time PCR. (C) CIC and β-actin of HepG2 cells transfected with the pcDNA3–ZNF224 (+) or empty vector (−) were immunodecorated with specific antibodies. In (A) and (B) means ± SD of three duplicate independent experiments are shown; differences between samples and relative controls (set at 100%) were significant ($P << 0.05$, one-way ANOVA).

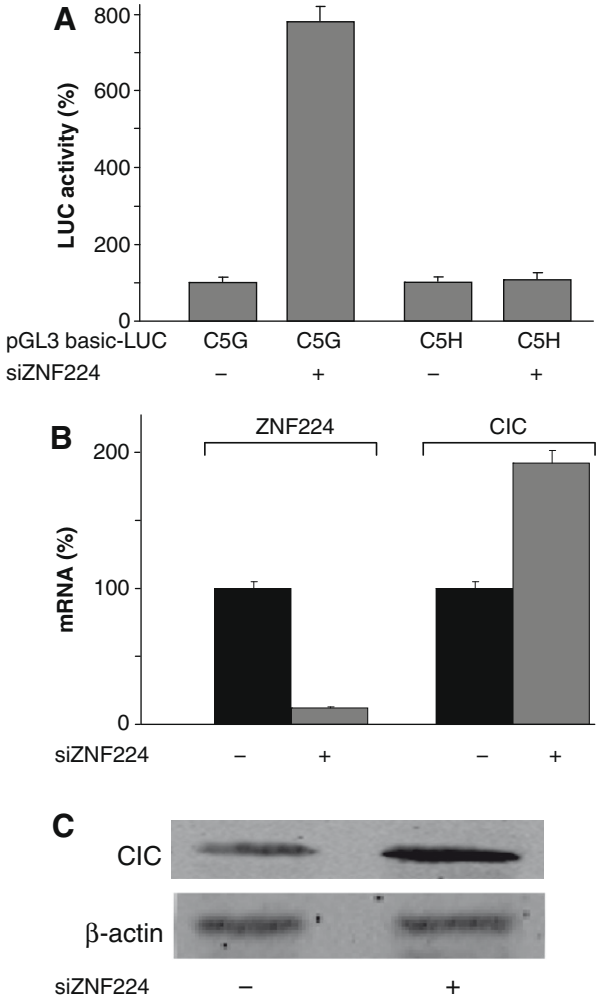

Fig. 4. Effect of ZNF224 silencing on CIC gene expression. (A) HEK293 cells, co-transfected with the siRNA targeting human ZNF224 (+) or control siRNA (−) and with the pGL3 basic-LUC vector containing the −595/−20 (C5G) or −568/−20 bp region (C5H) of the CIC gene promoter, were assayed for LUC activity. (B) ZNF224 and CIC mRNAs of HEK293 cells, transfected with siRNA targeting human ZNF224 (+) or control siRNA (−), were quantified by real-time PCR. (C) CIC and β-actin of HEK293 cells, transfected with siRNA targeting human ZNF224 (+) or control siRNA (−), were immunodecorated with specific antibodies. In (A) and (B) means ± SD of three duplicate independent experiments are shown; differences between samples and relative controls (set at 100%) were significant ($P << 0.05$, one-way ANOVA).

of LUC activity was observed upon ZNF224 overexpression (Fig. 3A). In agreement with the above-repressed LUC activity exhibited by C5G in ZNF224-overexpressing cells, ZNF224 overexpression also induced a decrease of both CIC transcript and protein levels (Fig. 3B and C). In another set of experiments, HEK293 cells, which expressed more ZNF224 than HepG2 cells (Fig. 2D), were co-transfected with siRNA targeting human ZNF224 (or control siRNA) and with the pGL3 basic-LUC vector containing the −595/−20 bp or −568/−20 bp region (Fig. 4A). Seventy-two hours after transfection, LUC activity was considerably enhanced by ZNF224 silencing in cells transfected with the C5G but not the C5H construct, which lacks the silencer region (Fig. 4A). Consistently, both CIC transcript and protein levels were significantly increased by ZNF224 silencing as compared to those in control cells (Fig. 4B and C). All together, the above-reported results provide clear evidence for direct involvement of ZNF224 in the regulation of CIC gene expression.

Discussion

The results presented above show that a well-defined region of the CIC gene promoter consisting of 26 bp (from −595 to −569 bp) acts as a silencer. This CIC promoter element displays a pronounced repression of transcription, as it strongly decreases LUC transgene expression activity in transfected cells. Moreover, it exhibits highly specific protein-binding activity in the presence of HepG2 cell nuclear extracts, as shown by site-directed mutagenesis of 13 bp within the −595/−569 bp sequence. The identification of the CIC promoter silencer has allowed us to purify a DNA-binding protein, identified as ZNF224, also known as BMZF2 (bone marrow zinc finger 2) [18,19]. ZNF224 belongs to the subfamily of zinc finger proteins that contain the KRAB domain at their amino terminus [20]. KRAB behaves as a transcriptional repressor domain by binding to corepressor proteins, among which Kap1 is absolutely required to mediate transcriptional repression [20,21].

ZNF224 was first identified in the hematopoietic system [18]. It is expressed in abundant amounts in fetal tissues and at low levels in adult tissues and is localized to the nucleus [18,19,22]. Little is known about the function of ZNF224. However, it has been shown to interact with WT1, another zinc finger protein encoded by the Wilms' tumor suppressor gene, and this interaction inhibits transcriptional activation by WT1 [19]. Moreover, ZNF224 has been shown to bind a negative regulatory element of the aldolase A gene and to repress its transcription [22].

In this study the ZNF224-mediated negative regulation of CIC gene transcription is directly demonstrated by the observations that (a) ZNF224 overexpression markedly suppresses LUC reporter activity in HepG2 cells transfected with the LUC vector containing the −595/−569 bp negative element of CIC promoter, and (b) ZNF224 silencing markedly activates LUC reporter activity in HEK293 cells transfected with the LUC vector harboring the −595/−569 bp region. Furthermore, the role of ZNF224 in CIC gene transcriptional repression is demonstrated by the decrease in the levels of both CIC transcript and protein induced by ZNF224 overexpression and by their increase caused by ZNF224 silencing.

Several observations point to the need for effective regulation of CIC gene expression in different tissues and under different physiological conditions. CIC is present in very minute amounts in the mitochondrial membrane [23]; it is essential in intermediary metabolism and, in particular, in fatty acid and sterol biosynthesis [1,2]; and CIC transcript and protein levels vary in different tissues [7] and between fetal and adult tissues (this work). The functional analysis of CIC gene promoter reported in previous studies [5,12,13] and herein has led to identify several mechanisms regulating the expression of this gene, which involve activation by SREBP-1, Sp1 (binding unmethylated elements) and FOXA, and repression by ZNF224. Remarkably, ZNF224 and CIC display an opposite pattern of expression in fetal tissues. It is likely that the negative regulation of CIC gene transcription exerted by ZNF224 explains, at least in part, the low expression of CIC in fetal tissues. This conclusion is consistent with the finding that in humans the placenta is relatively permeable to free fatty acids and in the fetus *de novo* fatty acid synthesis is low [24,25].

Acknowledgments

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