Transcriptional Regulation of HMG-CoA Synthase and HMG-CoA Reductase Genes by Human ACBP

Christina Vock, Frank Döring and Inke Nitz

Molecular Nutrition, Christian-Albrechts-University of Kiel, Kiel

Key Words

Cholesterol • Gene regulation • HNF-4 α • Promoter analysis

Abstract

The acyl-CoA binding protein (ACBP) is an ubiquitarily expressed multi-functional protein which regulates basic cellular functions such as fatty acid and steroid metabolism. Since ACBP is described to interact with the transcription factor hepatocyte nuclear factor 4 α (HNF-4 α), we investigated the role of human ACBP on transcriptional regulation of the putative HNF-4 α target gene HMG-CoA synthase 1 (HMGCS1). As shown by promoter-reporter assays ACBP represses the HNF-4 α -induced activity of a 617bp HMGCS1 promoter fragment by approximately 80% in HepG2 cells as well as in non-endodermal HeLa cells devoid of HNF-4 α . Interestingly, reporter assays without co-transfection of HNF-4 α revealed that ACBP reduces the activity of the HMGCS1 promoter by about 60 to 80% in both cell lines. Activities of 417bp and 317bp HMGCS1 promoter fragments were 2.5 to 4 fold decreased by ACBP. Concordantly, the levels of HMGCS1-mRNA and -protein were diminished to 60% and 70% in ACBP-

expressing HeLa cells, respectively. Additionally, ACBP reduces the promoter activity and the mRNA levels of the cholesterologenic HMG-CoA reductase (HMGCR). In conclusion, we provide evidence that ACBP is a transcriptional regulator of the HMGCS1 and HMGCR genes encoding rate-limiting enzymes of cholesterol synthesis pathway.

Copyright © 2008 S. Karger AG, Basel

Introduction

Acyl-CoA binding protein (ACBP) is an essential member of lipid binding proteins that was originally isolated as diazepam binding inhibitor (DBI) from rat brain with its ability to displace diazepam from gamma aminobutyric acid receptors [1]. The ubiquitously expressed ACBP shows a high degree of structural and functional conservation [2] and exerts versatile cell and tissue type-specific functions as: regulation of glucose-induced insulin secretion from pancreatic beta cells [3, 4], modulation of cell proliferation in testicular Leydig cells [5], modulation of monocyte mediated inflammation [6, 7], antibacterial properties [8], release of cholecystokinin from intestine [9] or stimulation of steroidogenesis [10-12].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
1015-8987/08/0226-0515\$24.50/0

Accessible online at:
www.karger.com/cpb

Dr. Inke Nitz
Molecular Nutrition, Christian-Albrechts-University of Kiel
Heinrich-Hecht-Platz 10, 24118 Kiel (Germany)
Tel. +49 431 880 5662, Fax +49 431 880 5658
E-Mail initz@molnut.uni-kiel.de

The abundance of ACBP in energy-consuming cells and tissues, such as adrenal cortex or testis, and its high affinity towards medium- and long chain acyl-CoAs, support ACBPs crucial role in fatty acid metabolism [13-15]. Besides sequestering activated fatty acids, ACBP is particularly involved in the protection and pool formation of acyl-CoAs [16, 17] and their donation to cell organelles, membranes and special enzyme systems [16, 18-22]. Beside being intermediates and substrates in metabolism acyl-CoAs are regulators and signaling molecules, affecting a number of cellular functions including activity of ion channels, ion pumps or enzymes [reviewed in [23]]. Moreover, they are described to impact signal transduction pathways as well as gene expression [24]. The gene-regulatory function of acyl-CoAs is based on their ability to act as agonistic or even antagonistic ligands for a number of nuclear factors. This was in detail shown for the endodermal derived hepatocyte nuclear factor (HNF)-4 alpha [25], originally regarded as orphan nuclear factor, but also for peroxisome proliferator-activated receptors (PPARs) [26]. In this respect, ACBP impacts the availability and accessibility of acyl-CoAs not only for general metabolism but also for gene regulatory properties. Recently, expression profiling confirmed the importance of acyl-CoA ligand binding for the induction of transcriptional changes in *Acb1p*-depleted yeast [27]. A putative gene regulatory function of ACBP is moreover implicated, as ACBP directly interacted with HNF-4 α , thereby activating the transcription of genes involved in lipid and glucose metabolism [28]. In keeping with this, gene expression analysis revealed numerous genes of fatty acid, phospholipid as well as glycerol synthesis to be up-regulated in *Acb1p*-depleted yeast [27].

Until today data about the impact of ACBP on cholesterol metabolism are limited to steroidogenic cells and tissues. ACBP and its processing products tria kontatetraneuropeptide (TTN) and octadecaneuropeptide (ODN) are described to enhance steroidogenesis by activating the transport of cholesterol towards the inner mitochondrial membrane, a process thought to be mediated by the interaction with peripheral-type benzodiazepine receptor (PBR) [11, 29]. In the present study we analyzed the impact of ACBP on HMG-CoA synthase 1 (HMGCS1) and HMG-CoA reductase (HMGCR) in two different human cell lines by an ACBP overexpression approach. HMGCS1 and HMGCR encode the two key rate-limiting enzymes of the cholesterol biosynthesis pathway (Fig. 1). HMGCS1 catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA towards 3-hydroxy-3-methylglutaryl (HMG)-CoA. In a subsequent step HMGCR

Fig. 1. Localization of HMG-CoA synthase 1 and HMG-CoA reductase in the cholesterol biosynthesis pathway. HMG-CoA synthase 1 (HMGCS1) and HMG-CoA reductase (HMGCR) encode the two rate-limiting enzymes of the cholesterol biosynthesis pathway. HMGCS1 catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to HMG-CoA. In a second step HMG-CoA is converted by HMGCR to mevalonate. Subsequently, more than 25 enzymatic reactions are required to synthesize cholesterol.

uses HMG-CoA for mevalonate synthesis.

Materials and Methods

Cell culture and transient transfection

Human hepatocellular HepG2 and cervical HeLa cells were purchased from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). HepG2 cells were maintained in RPMI + 1% Glutamax (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS). HeLa cells obtained MEM media (Invitrogen, Carlsbad, USA) containing 10% FCS and 1% non-essential amino acids (PAA, Cölbe, Germany). Cells were grown until preconfluent density in a humidified 5% CO₂/95% air atmosphere at 37°C. Media was

Table 1. Sequence of primers used for cloning of SEAP reporter gene constructs. Forward primers contain the nucleotides CACC at 5' end for Gateway cloning.

SEAP reporter gene constructs	Sequence 5'→3'
HMGCS1 reporter gene constructs	
-617 bp forward	GTATTCTGCTCCAATTTGC
-417 bp forward	GGCCCGCATCTCCTCTCAC
-317 bp forward	TCTCGTGCCACCTCACGTC
-217 bp forward	CCTAGCGAGTCATCGCCTC
reverse primer	AAGGGAGTGAGCCACGAAAGG
HMGCR reporter gene construct	
-528 bp forward	AGTTACAGGTTTGAGGTG
reverse primer	TACGCACGCTCGGAGCTGGA

changed every 2 days and cells were passaged with 0.25% trypsin in PBS (HepG2) and 0.05% trypsin/0.02% EDTA solution.

Transient transfections were performed with FuGene6 transfection reagent according to the manufacturer's protocol (Roche, Basel, Switzerland). For qRT-PCR and immunodetection HepG2 (1.5×10^5) and HeLa (4×10^4) cells were plated in 24-well dishes. After 24 h, cells were transfected with 800 ng ACBP-*V5* expression plasmid in 6:1 ratio of FuGene6 reagent (μ l) to DNA (μ g). For ChIP analysis, 1×10^6 HeLa cells were seeded on 10 cm dishes (4 dishes per condition). Cells were transfected at 50% confluence with 15 μ g ACBP-*V5* expression plasmid in 3:1 ratio of FuGene6 reagent (μ l) to DNA (μ g) for 24 h.

Plasmids

All plasmids were produced using cloning based on PCR and Gateway technology (Invitrogen, Carlsbad, USA). Preparation of the human HNF-4 α expression plasmid is described by Klapper et al. [30]. Human ACBP isoform 1A (NM_001079862, NP_001073331) including stop-codon (ACBP) was cloned into the expression vector pcDEST40. To allow C-terminal fusion with V5 (ACBP-*V5*), site-directed mutagenesis (Stratagene, La Jolla, USA) was performed according to the manufacturer's protocol. Mutagenic primers were as follows (mutated sites underlined): for: 5'-AAA AAT ACG GGA TAG GAA AGG GTG GGC G-3' and rev: 5'-CGC CCA CCC TTT CCT ATC CCG TAT TTT T-3. Promoter fragments -617/-1 bp, -417/-1 bp, -317/-1 bp, and -217/-1 bp of human HMG-CoA synthase 1 (HMGCS1, NM_002130) and promoter fragment -528/-1 bp of human HMG-CoA reductase (HMGCR, NM_000859) were cloned into pSEAP-basic vector (Promega, Mannheim, Germany), containing cassette C of the Gateway vector conversion system. Positions are given relative to transcription start sites. PCR-primers for cloning of promoter fragments are depicted in Tab.1. All plasmids were approved by sequencing.

SEAP reporter gene assay

Promoter analyses were performed using secretory alkaline phosphatase (SEAP) as reporter gene. HepG2 (3×10^4) and HeLa (5×10^3) cells were seeded in 96-well plate format and cultured for 24 h. Cells were transfected using FuGene6 transfection reagent (Roche, Basel, Switzerland) after manufacturer's instruction in 6:1 ratio of FuGene6 to DNA with 60 ng of promoter

construct, 37.6 ng ACBP/ACBP-*V5* expression plasmid and 2.4 ng of luciferase-containing pGL3 control plasmid (Promega, Madison, USA) as transfection efficiency control. For transfection with human HNF-4 α , 50 ng promoter plasmid, 31.5 ng ACBP expression plasmid, 16 ng HNF-4 α and 2.0 ng pGL3 were accordingly transfected. The total amount of 100 ng plasmid per well was adjusted with empty pcDEST40 expression vector. Transfection of pSEAP2-basic without promoter fragment was used as negative control for background correction.

After 48 h media was removed and frozen at -20°C until measurement of SEAP activity with Great EscAPe SEAP chemiluminescence detection kit (BD Bioscience Clontech, Palo Alto, USA). Cells were washed with 1x PBS, lysed by the addition of Glo Lysis Buffer (1x) and kept at -20°C (Promega, Madison, USA). Luciferase activity was measured with commercial Bright-Glow Assay Reagent (Promega, Madison, USA) after manufacturer's protocol. Chemiluminescence measurement was performed on 96-well flat-bottom opaque microplate in GloMaxTM microplate luminometer (Turner Biosystems, Sunnyvale, USA). Each experiment was performed at least 3 times in triplicate.

Real-time RT-PCR (TaqMan®)

Quantification of HMGCS1 and HMGCR mRNA level was performed with quantitative RT-PCR based on TaqMan® principle. In brief, total RNA was isolated after 12, 24 and 48 h of transfection using RNeasy kit (Qiagen, Hilden, Germany). cDNA was prepared using iScript cDNA synthesis kit (BioRad Laboratories, Hercules, USA). QRT-PCR was done in triplicate in at least three independent cell culture experiments using ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, USA). HMGCS1 and HMGCR gene expression assays were commercially available from PE ABI. Quantification was based on comparative Δ Ct method using hypoxanthine phosphoribosyl-transferase 1 (HPRT1) as endogenous control.

Two negative controls (-RT/-RNA template) were included to detect possible contamination. PCR was performed according to the manufacturer's protocol (PE Applied Biosystems, Foster City, USA).

Immunodetection of ACBP and HMGCS1

Transient transfection of HeLa cells was performed as described. After 72 h cells were harvested and lysed in 1x reaction buffer (5 mmol/L colic acid, 0.1% Triton X-100 in PBS, pH7.4) incubated for 20 min at 4°C and subsequently sonicated. Protein concentration was measured with BCA kit (Pierce, Rockford, USA) according manufacturer's instruction. Reaction buffer (1x) was used as lysis buffer for subsequent cholesterol measurement [31].

Whole cell extracts were suspended in NuPage LDS sample buffer (Invitrogen, Carlsbad, USA) and denaturated at 70°C for 10 min. Samples were loaded on 12 % polyacrylamide gel and transferred by electroblotting to a PVDF membrane (Roth, Karlsruhe, Germany). ACBP specific polyclonal antibody (ACBP_{total}) was raised in rabbit by Eurogentec (Seraing, Belgium) after immunization of specific antigen (⁵⁸AWNELKGTSKEDAMK⁷²) mapping exon 3 and 4 of ACBP 1A isoform (NP_001073331).

For immunodetection membranes were probed with specific primary antibodies Ab ACBP_{total} (1:2000), HMG-CoA synthase 1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) or actin goat polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) followed by horseradish peroxidase-coupled secondary antibody (1:10.000). Immunoreactive bands were visualized using chemiluminescent substrate ChemiGlow and quantified with Alpha Ease FC software (AlphaImager HP, AlphaInnotech, San Leandro, USA).

Cholesterol measurement

Cellular cholesterol content was measured by Amplex® Red Cholesterol Assay (Molecular Probes/Invitrogen, Carlsbad, USA). Protein concentration of the samples was measured with BCA kit according manufacturers instructions (Pierce, Rockford, USA). Cellular cholesterol is oxidised to H₂O₂ and detected by fluorescent measurement using 10-acetyl-3, 7-dihydrophenoxazine (Amplex Red reagent) with Infinite F500 plate reader (λ_{ex} = 535nm; λ_{em} = 590 nm, Tecan Crailsheim, Germany). Results are expressed as cholesterol concentration ($\mu\text{g/ml}$) in corresponding samples of 10 μg protein absolute. Each sample was measured in duplicate.

Chromatin immunoprecipitation

ChIP assay was performed using ChIP-IT™ Express enzymatic kit (Active Motif, Carlsbad, USA) after manufacturer's protocol. Protein/DNA complexes were fixed with formaldehyde and chromatin was sheared enzymatically into uniform fragments of 200-1000 bp size. Specific ACBP/DNA complexes were precipitated overnight using 3 μg of ACBP_{total} primary antibody. To control efficiency of chromatin immunoprecipitation and to exclude non-specific DNA binding, 3 μg of rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, USA) was used in parallel. Chromatin was isolated according manufacturer's instructions and stored at -80°C until PCR analysis.

Chromatin was subjected to PCR analysis to amplify putative HMGCS1 promoter regions subjected to ACBP interaction. Primers for amplification of 207 bp HMGCS1 promoter fragment were as follows: forward 5'-TGG CCC GCA TCT CCT CTC AC-3' and reverse 5'-GCT AGG ATT TTC CCT

Fig. 2. HNF-4 α induced HMGCS1 promoter activity was suppressed by ACBP in HepG2 (A) and HeLa (B) cells. HepG2 and HeLa cells were transfected with HNF-4 α alone or together with ACBP-V5 expression plasmid producing the 14 kDa V5-tagged ACBP. SEAP activity was measured 48 h post-transfection and normalized to luciferase containing pGL3. Data are presented as mean $\pm$ SD (n=3; Student's t-test).

CGTG -3. Amplification was carried out under following cycling conditions: 3 min at 94°C, 5 cycles with 30s at 94°C, 30s at 64°C, 30 s at 72°C, 5 cycles with 30s at 94°C, 30s at 62°C, 30 s at 72°C, followed by 30 cycles of 30s at 94°C, 30s at 60°C, 30 s at 72°C and final extension at 72°C for 10 min. As negative control genomic primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used (F: 5'-TCC ATG ACA ACT TTG GTA TCG TGG-3', R: 5'-GAC GCC TGC TT-CAC CAC CTT CT-3'). Amplification of chromatin prior to immunoprecipitation was used as an input control. ChIP was performed in two independent experiments comprising independent cell passages. Chromatin of each experiment was precipitated twice with appropriate antibody.

Statistical analysis

Statistical analysis was performed with GraphPad Prism4 (Graphpad software, Inc., San Diego, USA) using 2-tailed Student's paired t-test. All values were expressed as mean $\pm$ SD. Significant differences were considered for P values less than 0.05.

Results

ACBP represses the HNF-4 α -induced promoter activity of HMGCS1

ACBP is described to interact with HNF-4 α [28], a transcription factor that controls glucose, fatty acid and cholesterol metabolism [32]. Because ACBP is also described as putative regulator of cholesterol metabolism

Fig. 3. Expression of ACBP suppressed the activity of basal 617bp HMGCS1 promoter (3A) and of 417bp and 317bp HMGCS1 promoter deletion fragments (3B) in HepG2 and HeLa cells. (3A) Activity of basal 617bp HMGCS1 promoter was assessed in HepG2 (A) and HeLa (B) cells transiently transfected with both ACBP expression plasmids producing either the 14 kDa ACBP-V5 fusion protein or the native 10 kDa ACBP. (3B) Activity of truncated -417/-1 and -317/-1 HMGCS1 promoter fragments was analyzed in ACBP-V5 expressing HepG2 (A) and HeLa (B) cells. SEAP activity was measured after 48 h and normalised to luciferase activity. Each experiment was performed in three independent cell passages in triplicate for each sample. Data are presented as mean $\pm$ SD. Student's paired t-test (*** p < 0.0001, ** p < 0.001, * p < 0.01).

[11, 29], we investigated the impact of HNF-4 α and ACBP on the promoter activity of HMGCS1. This gene encodes a rate-limiting enzyme of cholesterol synthesis and contains a putative HNF-4 α binding site within its promoter region at position -274 to -249. We performed promoter-reporter assays with a functional 617bp segment of the HMGCS1 promoter. This HMGCS1-SEAP construct showed an about 60-fold higher activity in comparison to the negative control plasmid pSEAP-basic

Fig. 4. Expression of ACBP decreased HMGCS1-mRNA and protein levels in HeLa cells. (4A) HepG2 (A) and HeLa (B) cells were transiently transfected with ACBP-V5 expression plasmid for 24 h and 48 h. HMGCS1 transcript levels were measured by real-time RT-PCR. Data are presented as x-fold expression in comparison to control cells incubated only with FuGene6 (mean $\pm$ SD; n =3-4). The p-value is indicated. (4B) A representative Western blot shows 48 h post-transfection immunodetection of ACBP, HMGCS1 and β -actin (loading control) in ACBP-overexpressing (ov) and in control (co) HeLa cells (1) indicates the detection of V5-tagged ACBP and (2) the level of HMGCS1 in the transfectant.

(data not shown) and could therefore be classified as moderate promoter. As shown in Fig. 2, HNF-4 α led to 2-fold activation of basal HMGCS1 promoter activity in human liver HepG2 cells. Interestingly, co-transfection of a V5-tagged human ACBP cDNA (ACBP-V5) together with HNF-4 α strongly decreased the HNF-4 α effect by a factor of 5 (Fig. 2). In comparison to HepG2 cells which contain endogenous HNF-4 α , HeLa cells show no HNF-4 α expression [33]. Here, the HNF-4 α -

Fig. 5. Expression of ACBP suppressed HMGCR in HeLa cells at the transcriptional level. HepG2 (A) and HeLa (B) cells were transiently transfected with ACBP-*V5* expression plasmid (5A) HMGCR SEAP activity was measured after 48 h and normalized to luciferase activity. The results are presented as mean $\pm$ SD ($n=4$; *** $p<0.0001$). (5B) HMGCR transcript levels were assessed by real-time RT-PCR 24 h and 48 h after ACBP-*V5* expression. Data are presented as x-fold expression in comparison to control cells incubated only with FuGene6 (mean $\pm$ SD, $n=3-4$). The p-value is indicated.

induced activity of the HMGCS1 promoter is decreased by ACBP-*V5* to nearly 25% (Fig. 2). Thus, we found evidence in two different cell lines that ACBP represses the HNF-4 α -induced promoter activity of the HMGCS1 gene.

ACBP is a negative transcriptional regulator of HMGCS1

Based on our findings we speculate that ACBP may function as a negative transcriptional regulator of HMGCS1. To test this, ACBP-mediated activity of the HMGCS1 promoter was determined without co-transfection of HNF-4 α . As depicted in Fig. 3A, the ACBP-*V5* fusion protein significantly repressed the activity of the 617bp HMGCS1 promoter fragment by

	48 h	72 h
ACBP- <i>V5</i>	1.61 $\pm$ 0.42 μ g/ml	3.17 $\pm$ 1.12 μ g/ml
control	1.47 $\pm$ 0.92 μ g/ml	2.85 $\pm$ 1.3 μ g/ml

Table 2. Total cholesterol levels did not change in HeLa cells transfected for 48 h and 72 h with ACBP-*V5*.

63% and 83% in HepG2 and HeLa cells, respectively. In order to exclude possible artifacts of the V5-tag, a plasmid encoding native ACBP was also used. As shown in Fig. 3A, promoter activity of HMGCS1 was strongly repressed by native ACBP to 20% in HepG2 and to 25% in HeLa cells.

In order to provide further evidence for repressive effect of ACBP on HMGCS1 promoter activity, we performed reporter gene assays using -417/-1, -317/-1, and -217/-1 bp fragments of the HMGCS1 promoter. Since the shortest 217bp fragment showed only poor activity in HepG2 and HeLa cells, an ACBP-mediated repressive effect would hardly be detectable. Therefore this fragment was excluded from ongoing experiments. In comparison to the -617/-1 HMGCS1 promoter fragment, -417/-1 and -317/-1 constructs showed 50-70% activity in both cell lines. As shown in Fig. 3B, these shorter HMGCS1 promoter fragments respond strongly to ACBP-*V5*. For the -417/-1 construct, a repressive effect to 27% and 21% could be observed in HepG2 and HeLa cells, respectively. ACBP-*V5* mediated decrease of HMGCS1 -317/-1 promoter activity was 62% in HepG2 cells and 47% in HeLa cells. Together, these findings show that ACBP represses the activity of different HMGCS1 promoter constructs.

ACBP decreases the levels of HMGCS1-mRNA and -protein

To analyze if the repressive effect of ACBP on HMGCS1 promoter could also be observed at the transcript level, we quantified HMGCS1 mRNA levels in HepG2 and HeLa cells 24 h and 48 h after transfection of ACBP-*V5*. As shown in Fig. 4A, transcript levels of HMGCS1 did not change significantly in dependence of ACBP expression and time in HepG2 cells. In HeLa cells mRNA levels of HMGCS1 were considerably diminished to 64% ($p=0.064$) and 70% ($p=0.057$) 24 h and 48 h after ACBP-*V5* transfection, respectively.

We further investigated if ACBP decreases

HMGCS1 at protein level. For this purpose, we analyzed HMGCS1 protein levels in HeLa cells expressing ACBP-V5. Expression rate of the 14 kDa V5-tagged ACBP was also assessed and quantification was based on densitometric measurement. Based on a 6-fold enrichment of V5-tagged ACBP compared to endogenous ACBP levels (Fig. 4B, upper panel) we observed reduced HMGCS1 protein levels to 70% compared to control cells after 48 h (Fig. 4B, middle panel). Together, mRNA as well as protein level of HMGCS1 were negatively regulated by ACBP.

The activity of a HMGR promoter and the HMGR mRNA is reduced by ACBP

In order to extend our analysis regarding ACBP as a transcriptional regulator, we investigated the HMGR gene, which encodes the second rate-limiting enzyme in cholesterol synthesis. As shown in Fig. 5, ACBP-V5 reduces the activity of a 528bp promoter fragment of HMGR by 68% and 84% in HepG2 as well as in HeLa cells. The HMGR-mRNA is not substantially affected by ACBP-V5 in HepG2 cells. However, in HeLa cells ACBP decreases HMGR mRNA levels significantly to 56% after 24 h. Distinct HMGR mRNA repression was not observed 48 h post-transfection. Thus, ACBP is also a negative transcriptional regulator of HMGR.

No impact of ACBP on cellular cholesterol level

Given that ACBP-V5 represses HMGCS1 and HMGR at transcriptional level, we investigated the impact of ACBP on cholesterol levels in HeLa cells. As shown in Tab. 2, 48 h and 72 h after ACBP-V5 transfection we observed no difference in total cholesterol levels between control cells and cells expressing ACBP-V5.

Chromatin immunoprecipitation revealed no direct interaction of ACBP with HMGCS1 promoter

To identify a possible direct binding of ACBP to HMGCS1 promoter we performed chromatin immunoprecipitation in HeLa cells transfected with ACBP-V5 for 24 h. Precipitated DNA was subjected to PCR analyses using primers to amplify a 207 bp fragment corresponding to the HMGCS1 promoter sequence -418/-212. As depicted in Fig. 6, specific amplification of -418/-212 bp HMGCS1 promoter fragment was obtained in the presence of ACBP antibody. Amplified 207 bp HMGCS1 promoter fragment was less abundant when precipitated with non-specific IgG antibody. PCR analysis with human GAPDH primer set showed enrichment of

Fig. 6. Chromatin Immunoprecipitation revealed no direct binding of ACBP to HMGCS1 regulatory DNA-sequences. Formaldehyde cross-linked chromatin was prepared from HeLa cells and immunoprecipitated with antibodies to ACBP and immunoglobulin G (IgG) as negative control. PCR was performed with specific primers for the HMGCS1 promoter -418/-212 and for GAPDH as a negative control. A sample representative of the total input chromatin (input DNA) was included in the PCR analysis. Lane 5 shows a PCR negative control. Transfection experiment was performed in duplicate each with 2 independent immunoprecipitations. M, 100 bp DNA marker.

306 bp GAPDH amplicon with ACBP antibody precipitated chromatin when compared with IgG precipitated chromatin. Enrichment of control GAPDH PCR product in ACBP precipitated chromatin samples indicates unspecific binding of chromatin towards polyclonal ACBP antibody.

Discussion

ACBP is a multi-functional protein which is involved in basic cell metabolism such as fatty acid metabolism and steroidogenesis. ACBP functions also in highly specialized and regulated processes such as insulin [3, 4] and cholecystokinin secretion [9]. The best established role of ACBP is its ability to bind long-chain fatty acid-coenzyme A esters (acyl-CoAs). ACBP acts as a cellular acyl-CoA transporter and pool former. Beside being intermediates of fatty acid biosynthesis and consumption, acyl-CoAs are described as regulatory and signaling molecules involved in gene expression and cell metabolism [23]. Therefore, ACBP is an important regulator protein for cell signaling and gene regulation. It is also known

that ACBP is involved in steroid biosynthesis by increasing cholesterol transport to mitochondria [34]. Here we show by promoter-reporter assays in two different cell lines that ACBP decreases the transcription of the HMGCS1 and HMGCR gene encoding the initial enzymes of cholesterol biosynthesis. The increased ACBP expression was highly comparable to liver ACBP level achieved in transgenic rats [35]. The HMGCS1 catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA towards 3-hydroxy-3-methyl-glutaryl (HMG)-CoA. HMGCR catalyzes subsequently the conversion of HMG-CoA to mevalonate. In comparison to significantly decreased promoter activities and mRNA levels of HMGCS1, western analyses revealed only a slight reduction in protein expression level to 70%. Analogous to this weak down-regulation no changes in cholesterol levels were observed under the applied experimental conditions. This was also observed by others [36] and might reflect the complex regulation of the cholesterol biosynthesis, a pathway that comprises numerous stages with counteracting regulatory mechanisms. In general, cholesterol synthesis is tightly controlled in order to meet cellular cholesterol demands [37]. Thus, versatile transcriptional and post-transcriptional mechanisms are employed. These include not only feed-back control of target gene transcription and translation but also allosteric control as well as cholesterol uptake [38]. Especially, HMGCR is regulated by sterols and mevalonate derived non-sterols at post-transcriptional and post-translational level [39]. Whereas sterol level mainly determine HMGCR mRNA transcription, non-sterol derivatives control the above described post-transcriptional mechanisms [38]. Regarding cholesterol levels, those regulatory mechanisms might compensate the repressing effect of ACBP on the transcription of HMGCS1 and HMGCR. Thus, it would be of interest to measure metabolites, for example HMG-CoA or mevalonate, in closer vicinity to the repressed HMGCS1 and HMGCR. Branch-points of this pathway lead also to the synthesis of non-sterol isoprenoids which are required for the synthesis of dolichol, tRNA ubiquinone and also for prenylated proteins such as Ras and Rabs [38]. The impact of ACBP on those pathways was so far not in focus.

To get insight into the precise function of ACBP as a transcriptional regulator we performed ChIP analyses using the promoter fragment which showed the strongest suppressive effect of ACBP on HMGCS1. This approach revealed no genomic sites that were directly bound by ACBP. Therefore, ACBP seems not to be a transcription factor. A way in which ACBP could regulate the

expression of HMGCS1 and HMGCR is physical interaction with HNF-4 α . Petrescu et al. (2003) reported direct interaction of ACBP with HNF-4 α as well as a stimulatory effect of ACBP on HNF-4 α mediated transactivation of an apolipoprotein B (ApoB) promoter construct [28]. Contrarily to this stimulatory effect, ACBP lowered the HNF-4 α induced activity of the HMGCS1 promoter. Further, ACBP repressed the activity of the HMGCS1 and HMGCR promoter fragments in HeLa cells. This non-endodermal cell line does not express HNF-4 α [33]. Thus, a profound function of HNF-4 α on ACBP mediated transcriptional repression of HMGCS1 and HMGCR is not presumable. A study by Misawa et al. (2003) confirmed interaction of sterol-responsive element binding protein (SREBP)-2 with HNF-4 α to effectively activate cholesterol genes, including low density lipoprotein receptor (LDLR), sterol isomerase (SI) and HMGCS1 [40]. Here we show that HNF-4 α alone transactivates HMGCS1 promoter under sterol-independent conditions. This may provide further insights into the complex regulation of cholesterol synthesis pathway.

In principal, SREBP-2 is another transcription factor which may interact with ACBP. Supportively, our HMGCS1 promoter fragments contain two functional sterol regulatory element (SRE)-like motifs within the region -290 to -281 and -308 to -299, which are also conserved in the hamster HMGCS1 gene [41, 42]. SREBPs require co-regulatory proteins such as CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) or the universal transcription factor SP-1 for their gene regulatory function. In preliminary experiments, we tested possible interaction between SREBP-2 and ACBP using HMGCS1 promoter constructs in HeLa cells. SREBP-2 could not abolish ACBP repressive effect on basal HMGCS1 promoter activity suggesting no functional interaction.

It has been shown that overexpression of ACBP increases cellular acyl-CoA content in yeast and mouse liver [17, 43, 44]. Acyl-CoAs are important ligands for several enzymes and are implicated in regulation of gene transcription [45, 46]. In this context, the well known transcription factor PPAR α , which interacts with the retinoid X receptor (RXR), is described to directly interact with acyl-CoAs [26] and may regulate HMGCS1. Competition of acyl-CoA binding by ACBP and PPAR α might be one possible mechanism of the observed ACBP effect on HMGCS1. Interestingly, ACBP repressed 317 bp HMGCS1 promoter fragment less strongly in comparison to 417 bp promoter construct. Thus, ACBP

might interfere with HMGCS1 promoter activity within the region -417 and -317. Promoter analysis applying Genomatix *MatInspector* (www.genomatix.de) revealed binding sites for various transcriptional regulators. A putative recognition site for vitamin D receptor/RXR heterodimers (VDR/RXR) was identified. Conditions of heterodimeric complex formation include the presence of the cholesterol-derived 1,25-dihydroxyvitamin D3 [47]. Taken together, the contribution of PPAR α /RXR and/or VDR/RXR to the suppression of HMGCS1 by ACBP needs to be investigated in further studies.

In conclusion, our findings indicate for the first time that ACBP regulates HMGCS1 and HMGCR, two enzymes in the initial steps of cholesterol biosynthesis. Further work is necessary to delineate the precise mechanisms by which ACBP represses HMGCS1 and HMGCR gene expression.

Abbreviations

ChIP (chromatin immunoprecipitation); HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA); HNF-4 α (hepatocyte nuclear factor 4 alpha); LDL (low density lipoprotein); PPAR (peroxisome proliferator-activated receptor); RXR (retinoid X receptor); SI (sterol isomerase); VDR (vitamin D receptor).

Acknowledgements

This work was financially supported by the BMBF-Project "Fat and Metabolism - Genevariation, Generegulation and Genefunction" (AZ 0312823A, B, C). We also thank Y. Signal and D. Hallack for excellent technical assistance.

References

- Guidotti A, Forchetti CM, Corda MG, Konkell D, Bennett CD, Costa E: Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. *Proc Natl Acad Sci U S A* 1983;80:3531-3535.
- Kragelund BB, Knudsen J, Poulsen FM: Acyl-coenzyme A binding protein (ACBP). *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* 1999;1441:150-161.
- Chen ZW, Agerberth B, Gell K, Andersson M, Mutt V, Ostenson CG, Efendic S, Barrossoderling J, Persson B, Jornvall H: Isolation and characterization of porcine diazepam-binding inhibitor, apolipoprotein not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin resistance. *European Journal of Biochemistry* 1988;174:239-245.
- Borboni P, Condorelli L, De Stefanis P, Sesti G, Lauro R: Modulation of insulin secretion by diazepam binding inhibitor and its processing products. *Neuropharmacology* 1991;30:1399-1403.
- Garnier M, Boujrad N, Oke BO, Brown AS, Riond J, Ferrara P, Shoyab M, Suarez-Quian CA, Papadopoulos V: Diazepam binding inhibitor is a paracrine/autocrine regulator of Leydig cell proliferation and steroidogenesis: action via peripheral-type benzodiazepine receptor and independent mechanisms. *Endocrinology* 1993;132:444-458.
- Taupin V, Gogusev J, Descamps-Latscha B, Zavala F: Modulation of tumor necrosis factor- α , interleukin-1 β , interleukin-6, interleukin-8, and granulocyte/macrophage colony-stimulating factor expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetra-neuropeptide: evidence for a role of prostaglandins. *Mol Pharmacol* 1993;43:64-69.
- Taupin V, Herbelin A, Descamps-Latscha B, Zavala F: Endogenous anxiogenic peptide, ODN-diazepam-binding inhibitor, and benzodiazepines enhance the production of interleukin-1 and tumor necrosis factor by human monocytes. *Lymphokine Cytokine Res* 1991;10:7-13.
- Agerberth B, Boman A, Andersson M, Jornvall H, Mutt V, Boman HG: Isolation of three antibacterial peptides from pig intestine: gastric inhibitory polypeptide (7-42), diazepam-binding inhibitor (32-86) and a novel factor, peptide 3910. *Eur J Biochem* 1993;216:623-629.
- Herzig KH, Schon I, Tatemoto K, Ohe Y, Li Y, Folsch UR, Owyang C: Diazepam binding inhibitor is a potent cholecystokinin releasing peptide in the intestine. *Proc Natl Acad Sci U S A* 1996;93:7927-7932.
- Boujrad N, Hudson JR, Jr., Papadopoulos V: Inhibition of hormone-stimulated steroidogenesis in cultured Leydig tumor cells by a cholesterol-linked phosphorothioate oligodeoxynucleotide antisense to diazepam-binding inhibitor. *Proc Natl Acad Sci U S A* 1993;90:5728-5731.
- Papadopoulos V, Brown AS: Role of the peripheral-type benzodiazepine receptor and the polypeptide diazepam binding inhibitor in steroidogenesis. *J Steroid Biochem Mol Biol* 1995;53:103-110.
- Besman MJ, Yanagibashi K, Lee TD, Kawamura M, Hall PF, Shively JE: Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc Natl Acad Sci U S A* 1989;86:4897-4901.
- Knudsen J, Hojrup P, Hansen HO, Hansen HF, Roepstorff P: Acyl-CoA-binding protein in the rat. Purification, binding characteristics, tissue concentrations and amino acid sequence. *Biochem J* 1989;262:513-519.
- Mogensen IB, Schulenberg H, Hansen HO, Spener F, Knudsen J: A novel acyl-CoA-binding protein from bovine liver. Effect on fatty acid synthesis. *Biochem J* 1987;241:189-192.
- Mikkelsen J, Knudsen J: Acyl-CoA-binding protein from cow. Binding characteristics and cellular and tissue distribution. *Biochem J* 1987;248:709-714.
- Rasmussen JT, Rosendal J, Knudsen J: Interaction of acyl-CoA-binding protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor. *Biochemical Journal* 1993;292:907-913.

- 17 Mandrup S, Jepsen R, Skott H, Rosendal J, Hojrup P, Kristiansen K, Knudsen J: Effect of heterologous expression of acyl-CoA-binding protein on acyl-CoA level and composition in yeast. *Biochemical Journal* 1993;290:369-374.
- 18 Chao H, Martin GG, Russell WK, Waghela SD, Russell DH, Schroeder F, Kier AB: Membrane charge and curvature determine interaction with acyl-CoA binding protein (ACBP) and fatty acyl-CoA targeting. *Biochemistry* 2002;41:10540-10553.
- 19 Rasmussen JT, Faergeman NJ, Kristiansen K, Knudsen J: Acyl-CoA binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for beta-oxidation and glycerolipid synthesis. *Biochemical Journal* 1994;299:165-170.
- 20 Jolly CA, Wilton DC, Schroeder F: Microsomal fatty acyl-CoA transacylation and hydrolysis: fatty acyl-CoA species dependent modulation by liver fatty acyl-CoA binding proteins. *Biochim Biophys Acta* 2000;1483:185-197.
- 21 Gossett RE, Edmondson RD, Jolly CA, Cho TH, Russell DH, Knudsen J, Kier AB, Schroeder F: Structure and function of normal and transformed murine acyl-CoA binding proteins. *Arch Biochem Biophys* 1998;350:201-213.
- 22 Abo-Hashema KAH, Cake MH, Lukas MA, Knudsen J: The interaction of acyl-CoA with acyl-CoA binding protein and carnitine palmitoyltransferase I. *International Journal of Biochemistry & Cell Biology* 2001;33:807-815.
- 23 Faergeman NJ, Knudsen J: Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochemical Journal* 1997;323:1-12.
- 24 DiRusso CC, Heimert TL, Metzger AK: Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in *Escherichia coli*. Interaction with the fadB promoter is prevented by long chain fatty acyl coenzyme A. *J Biol Chem* 1992;267:8685-8691.
- 25 Hertz R, Magenheimer J, Berman I, Bar-Tana J: Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4alpha. *Nature* 1998;392:512-516.
- 26 Elholm M, Dam I, Jorgensen C, Krogsdam AM, Holst D, Kratchmarova I, Gottlicher M, Gustafsson JA, Berge R, Flatmark T, Knudsen J, Mandrup S, Kristiansen K: Acyl-CoA esters antagonize the effects of ligands on peroxisome proliferator-activated receptor alpha conformation, DNA binding, and interaction with Co-factors. *J Biol Chem* 2001;276:21410-21416.
- 27 Feddersen S, Neergaard TB, Knudsen J, Faergeman NJ: Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA binding protein dependent mechanism in *Saccharomyces cerevisiae*. *Biochem J* 2007;407:219-230.
- 28 Petrescu AD, Payne HR, Boedecker A, Chao H, Hertz R, Bar-Tana J, Schroeder F, Kier AB: Physical and functional interaction of acyl-CoA-binding protein with hepatocyte nuclear factor-4 alpha. *Journal of Biological Chemistry* 2003;278:51813-51824.
- 29 Yanagibashi K, Ohno Y, Kawamura M, Hall PF: The regulation of intracellular transport of cholesterol in bovine adrenal cells: purification of a novel protein. *Endocrinology* 1988;123:2075-2082.
- 30 Klapper M, Bohme M, Nitz I, Doring F: The human intestinal fatty acid binding protein (hFABP2) gene is regulated by HNF-4alpha. *Biochem Biophys Res Commun* 2007;356:147-152.
- 31 Butchbach ME, Tian G, Guo H, Lin CL: Association of excitatory amino acid transporters, especially EAAT2, with cholesterol-rich lipid raft microdomains: importance for excitatory amino acid transporter localization and function. *J Biol Chem* 2004;279:34388-34396.
- 32 Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ: Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 2001;21:1393-1403.
- 33 Olsen L, Bressendorff S, Troelsen JT, Olsen J: Differentiation-dependent activation of the human intestinal alkaline phosphatase promoter by HNF-4 in intestinal cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G220-226.
- 34 Lacapere JJ, Papadopoulos V: Peripheral-type benzodiazepine receptor: structure and function of a cholesterol-binding protein in steroid and bile acid biosynthesis. *Steroids* 2003;68:569-585.
- 35 Oikari S, Ahtialansaari T, Heinonen MV, Mauriala T, Auriola S, Kiehne K, Folsch UR, Janne J, Alhonen L, Herzig KB: Downregulation of PPARs and SREBP by acyl-CoA-binding protein overexpression in transgenic rats. *Pflugers Arch* 2008;456:369-377.
- 36 Dolt KS, Karar J, Mishra MK, Salim J, Kumar R, Grover SK, Qadar Pasha MA: Transcriptional downregulation of sterol metabolism genes in murine liver exposed to acute hypobaric hypoxia. *Biochem Biophys Res Commun* 2007;354:148-153.
- 37 Brown MS, Goldstein JL: Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. *J Lipid Res* 1980;21:505-517.
- 38 Goldstein JL, Brown MS: Regulation of the mevalonate pathway. *Nature* 1990;343:425-430.
- 39 Nakanishi M, Goldstein JL, Brown MS: Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J Biol Chem* 1988;263:8929-8937.
- 40 Misawa K, Horiba T, Arimura N, Hirano Y, Inoue J, Emoto N, Shimano H, Shimizu M, Sato R: Sterol regulatory element-binding protein-2 interacts with hepatocyte nuclear factor-4 to enhance sterol isomerase gene expression in hepatocytes. *J Biol Chem* 2003;278:36176-36182.
- 41 Inoue J, Sato R, Maeda M: Multiple DNA elements for sterol regulatory element-binding protein and NF-Y are responsible for sterol-regulated transcription of the genes for human 3-hydroxy-3-methylglutaryl coenzyme A synthase and squalene synthase. *J Biochem* 1998;123:1191-1198.
- 42 Dooley KA, Millinder S, Osborne TF: Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y. *J Biol Chem* 1998;273:1349-1356.
- 43 Knudsen J, Faergeman NJ, Skott H, Hummel R, Borsting C, Rose TM, Andersen JS, Hojrup P, Roepstorff P, Kristiansen K: Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size. *Biochem J* 1994;302 (Pt 2):479-485.
- 44 Huang H, Atshaves BP, Frolov A, Kier AB, Schroeder F: Acyl-coenzyme A binding protein expression alters liver fatty acyl-coenzyme A metabolism. *Biochemistry* 2005;44:10282-10297.
- 45 Choi JY, Stukely J, Hwang SY, Martin CE: Regulatory elements that control transcription activation and unsaturated fatty acid-mediated repression of the *Saccharomyces cerevisiae* OLE1 gene. *J Biol Chem* 1996;271:3581-3589.
- 46 Kamiryo T, Parthasarathy S, Numa S: Evidence that acyl coenzyme A synthetase activity is required for repression of yeast acetyl coenzyme A carboxylase by exogenous fatty acids. *Proc Natl Acad Sci U S A* 1976;73:386-390.
- 47 Cheskis B, Freedman LP: Ligand modulates the conversion of DNA-bound vitamin D3 receptor (VDR) homodimers into VDR-retinoid X receptor heterodimers. *Mol Cell Biol* 1994;14:3329-3338.