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TRANSCRIPTOM-BASED IDENTIFICATION OF A PUTATIVE ROLE FOR THE HUMAN ACYL-COA-BINDING-PROTEIN (ACBP) IN VESICULAR TRAFFICKING

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Abstract – In the present study we performed a transcriptom-based analysis of human Acyl-CoA-Binding-Protein (ACBP) target genes. By applying Genomatix *BiblioSphere* expert level based co-citation filter 4 (GFG level 4) ras homolog gene family member B (RhoB) and its interacting rhophilin-2 (Rhpn2) were refined from 64 ACBP sensitive genes. TaqMan-based qRT-PCR confirmed the accuracy of the array-derived expression data. Based on Gene Ontology (GO) classification RhoB and Rhnp2 were allocated to endosomal transport and signaling processes. Thus, we suggest RhoB and Rhnp2 as ACBP target genes contributing to the proposed and evolutionary conserved function of ACBP in vesicular transport processes.

Key words: Acyl-CoA-Binding-Protein, vesicular transport, target genes

INTRODUCTION

The human acyl-CoA binding protein (ACBP) is a highly conserved 10 kDa protein which can be found in nearly all eukaryotes (6). ACBP, also named DBI (diazepam binding inhibitor) was firstly identified as a neuropeptide inhibiting diazepam binding to the GABA (γ-aminobutyric acid) receptor (14). Subsequent studies have shown that ACBP possesses various highly specialized functions such as inhibition of glucose-stimulated insulin secretion (5, 7), modification of cholecystokinin release (17), stimulation of steroidogenesis (4) as well as modulation of cell proliferation (13). A putative gene-regulatory (22) as well as pro-apoptotic function (26) is also described for ACBP.

Abbrevations: ACBP, Acyl-CoA-Binding-Protein; **BSPE,** BiblioSphere Pathway Edition; **DBI,** diazepam binding inhibitor; **EGR1,** early growth response 1; **FC,** fold change; **FDR,** false discovery rate; **GABA,** γ-aminobutyric acid; **GO,** Gene Ontology; **GTP,** Guanosintriphosphat; **HepG2,** Human hepatocellular liver carcinoma cell line; **HPRT1,** hypoxanthine phosphoribosyl-transferase 1; **PIP2,** phosphatidylinositol 4,5 bisphosphate; **RhoB,** ras homolog gene family member B; **Rhpn2,** rhophilin-2; **THBS1,** thrombospondin 1.

Based on its structural properties ACBP binds long-chain fatty acyl-CoA esters (LCFA-CoAs) with very high affinity and specificity (25). Thereby intracellular acyl-CoAs are transported towards metabolizing enzymes and transport systems via ACBP (19). Moreover sequestering detergenic acyl-CoAs by ACBP is a prerequisite to sustain cellular function (10). Disruption of the ACBP gene in *Saccharomyces cervisiae* results in modified acyl-CoA composition and strongly diminished levels of the very-long chain acyl-CoA C26:0 (12). In addition, ACBP-knock-out yeast cells show impaired sphingolipid synthesis, aberrant membrane structure and vesicle accumulation. Since a similar phenotype was also found in cells with a disturbed Golgi function, an involvement of ACBP in vesicular trafficking was hypothesized (12). Accordingly, ongoing studies demonstrated a perturbed assembly of vesicle fusion proteins in ACBP disrupted yeast cells (9). In the nematode *Caenorhabditis elegans* the membrane-associated ACBP MAA-1 is also involved in endosomal vesicle transport (18).

To analyze a putative role of human ACBP in vesicular transport we subjected expression

profiling data obtained from siRNA mediated ACBP-knockdown in human liver HepG2 cells to further *in-silico* text-mining analysis. This approach reveals transcriptional down-regulation of genes associated with endosomal transport and signal transduction in ACBP-depleted HepG2 cells.

MATERIALS AND METHODS

The experimental setup was comprehensively described in a manuscript currently under revision. The human hepatocellular carcinoma cell line HepG2 was cultured in RPMI-Medium + 1% Glutamax (Invitrogen, Carlsbad, USA) containing 10% fetal calf serum in a 5% CO_2/95% air atmosphere at 37°C. HepG2 cells were transfected with an ACBP specific siRNA (5'-GAAAAAATACGGGATATGA -3') (Dharmacon Research, Lafayette, USA), in order to deplete cellular ACBP level. A scrambled non-targeting siRNA was used as negative control (Dharmacon Research, Lafayette, USA). In brief, $4.5x10^5$ cells were seeded in 6-well plates (Sarstedt, Newton, USA). Cells were transfected at 30-40% confluency in a final siRNA concentration of 100nmol/L with Dharmafect4 transfection reagent (Dharmacon Research, Lafayette, USA) according to the manufacturer's instructions. 24 h post-transfection medium was changed. 72-h after transfection of ACBP specific and control siRNAs HepG2 cells were prepared for protein isolation and subsequent immunoblotting as previously described (28).

72-h post transfection total RNA was isolated with RNeasy kit (Qiagen, Hilden, Germany) for subsequent gene expression profiling. RNA amount and integrity was assessed spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) and by using the Bioanalyzer (Agilent, Palo Alto, USA). Experiments were done in triplicate and each approach consisted 3 pooled wells. Microarray experiments were performed on the human Genome U133 Plus 2.0 Genechip array according to the manufacturer's instructions (Affymetrix, Santa Clara, USA). Comparison of expression profiles from the scrambled siRNA control (set as baseline) and the ACBP specific siRNA transfected sample (designated as the experiment) was done with Genomatix ChipInspector software (Munich, Germany), which includes a single sided permutation T-test. For determination of differentially regulated genes the fold change (FC) was set 1.5, means 50% change, and false discovery rate (FDR) rests with 1%. Beside that, only significant detected transcripts after MAS5.0, as a single-rank call algorithm, were taken for further analysis.

We utilized the text-mining system Genomatix *BiblioSphere Pathway Editio*n (BSPE) (www.genomatix.de) to identify the putative functional connection between the regulated target genes. Gene Ontology (GO) functional classification was performed by using Affymetrix NetAffyx Analysis Center (www. Affymetrix.com).

For real-time reverse transcriptase PCR (RT-PCR) cDNA was prepared with iScript cDNA synthesis kit (BioRad Laboratories, Hercules, USA). cDNA was diluted 1:5 with RNase-free H_2O before PCR analysis. RT-PCR was performed with the following assays: Rhophilin-2, (Hs00369111_m1); ras homolog gene family member B, (Hs00269660_s1); early growth response, (Hs_00152928_m) obtained from ABI (PE Applied Biosystems, Foster City, USA. Quantities of target mRNA were determined via comparative Ct method (*delta delta Ct*) using hypoxanthine phosphoribosyl-transferase 1 (HPRT1) as endogenous control.

RESULTS AND DISCUSSION

Recently, we identified and analyzed ACBP sensitive genes which are involved in cholesterol and fatty acid metabolism. In this former study, cellular ACBP level were depleted via the transfection of an ACBP specific siRNA in HepG2 cells. At protein level, ACBP-siRNA caused an approximately 80% reduction of ACBP 72 h post- transfection in HepG2 cells (Figure 1). Afterwards microarray analysis was performed using the human Genome U133 Plus 2.0 Genechip (Affymetrix). The resulting ACBP-regulon contains 64 significantly differential expressed genes with a minimal fold change of 1.5 (single-sided Permutation T-test, $p \leq 0.05$). In order to assess putative functional connections between these ACBP regulated genes, appropriate GeneIDs were uploaded to the Genomatix *BSPE* software. This text-mining tool uses literature based co-citations of gene names and synonyms. To reach high data specificity and validity, we employed the expert-level based co-citation filter 4 (GFG level 4). Based on these stringent criteria, we refined four ACBP-sensitive genes which are consistently down-regulated (Tab. 1) and are functional connected (Figure 2). These genes encode ras homolog gene family member B (RhoB), Rhophilin-2 (Rhpn2), early growth response 1 (EGR1) and thrombospondin 1 (THBS1). As shown in Tab. 1, the accuracy of the original array-derived expression data was confirmed by TaqMan-based qRT-PCR.

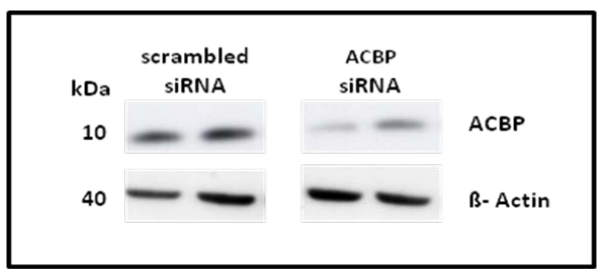

Figure 1. Repression of ACBP-protein after siRNA transfection. HepG2 cells were transfected with 100 nmol/L of ACBP specific siRNA and scrambled siRNA as negative control. Samples for western blot analyses were generated 72 h post-transfection. Beta-actin served as loading control. A representative immunoblot is depicted.

Table 1. ACBP regulated genes in the identified network

Gene ID[a]	Gene symbol	Description	Mean FC microarray	Mean FC qRT-PCR	GO biological process	GO molecular function
85414	Rhpn2	Rhophilin-2, Rho GTPase	-1.53 ± 0.10	-1.63 ± 0.30	signal transduction	protein binding
388	RhoB	Ras homolog gene family, member B	-1.52 ± 0.15	-1.49 ± 0.21	endosomes to lysosome transport Rho protein signal transduction, regulation of angiogenesis	GTPase activity
1958	EGR1	early growth response 1	-1.67 ± 0.33	-1.62 ± 0.09	regulation of transcription	transcription factor activity
7057	THBS1	thrombospondin 1	-1.53 ± 0.12	n.a.	cell adhesion	signal transducer activity structural molecule activity

Data compromise 3 independent experiments and are presented as mean fold change (FC) ± SD
[a] GeneID is based on NCBI GenBank database ; n.a., not analyzed

In order to allocate the refined genes according their involvement in *biological processes* and their *molecular functions* the GO classification tool was applied (Tab. 1). This approach classified THBS1 as a cell adhesion molecule with signal transducer activity. A role of ACBP in this process was not described. The EGR1 gene was classified as a regulator of transcription providing supporting evidence for an involvement of ACBP in gene expression (22). Concordant to former results proposing a role for ACBP in vesicular trafficking (9, 12, 18), RhoB and Rhpn2 were identified to be functional in endosomal transport and signaling. RhoB belongs to the Rho GTPase family compromising also Cdc42, Rac, RhoA and RhoC. Rho GTPases are critical regulators of actin dynamics and have been involved in the control of endocytosis (11). RhoB localizes to the cytoplasmic face of endosomal membranes and is associated with the multivesicular body, a prelysosomal compartment (1, 24). Consistent with its localization RhoB controls the traffic of several receptors through the endocytic compartment (8). One putative mechanism for the function of RhoB in vesicular transport processes is its endosomal interactions with actin (11). The actin cytoskeleton itself represents a major component of the endocytic pathway shuttling vesicles towards microtubules (2). RhoB itself can regulate the synthesis of phosphatidylinositol 4,5 bisphosphate (PIP2), inducing fusion competence of secretory vesicles (16). Taken together, RhoB possesses pleiotrophic functions in vesicular trafficking. Thus, a down-regulation of the RhoB gene by ACBP may perturbate intracellular transport of vesicles and proteins. In accordance to our hypothesis, ACBP down-regulates the RhoB effector protein Rhpn2 to a similar extent (Table 1). Rhpn2 was originally isolated upon stimulation of the cAMP-signalling pathway in thyroid (29). Former studies have shown phenotypic changes characterized by a loss and disassembly of actin stress fibers in Rhpn2 expressing HeLa cells (21). Cell culture experiments have specifically shown Rhpn2 recruitment to vesicular structures by GTP-bound form of RhoB. These structures might belong probably to late endosomes (20, 27) which play a role in cellular sorting, like the sorting of internalized membrane constituents tagged for degradation (3).

As a summary, we propose that the human ACBP is involved in vesicular trafficking. This hypothesis is based on the following observations. Firstly, RhoB and Rhpn2 are interacting and central proteins involved in vesicular trafficking via actin cytoskeleton assembly. Secondly, our transcriptom-based analyses revealed that the RhoB and Rhpn2 genes are targets of ACBP. Thirdly, ACBP functions in fatty acid and cholesterol metabolism which affects lipid composition and thus the assembly of the fusion machinery (9). Acyl-CoAs for example serve as cofactors during the budding process (15) and are required for the attachment and uncoating of membrane vesicles (23). Finally, former results already suggested abrogation of vesicular disturbances in ACBP knock-out yeast complemented by human ACBP (9). However, comprehensive functional studies will be required to elucidate the role of RhoB and Rhpn2 in ACBP mediated vesicular transport.

Figure 2. BiblioSphere text-mining analysis of ACBP regulated genes. Sixty-four ACBP regulated genes with a minimal FC of 1.5 were up-loaded in BSPE. The co-citation level GFG level 4 ("expert level") was applied and thus four down-regulated genes were identified. Thereby, EGR1 and THBS1 are functionally connected as well as RHOB and RHPN2. IN, input gene; TF, transcription factor; M, gene product is part of a metabolic pathway. Abbreviations and descriptions are listed in Table 1.
→ indicates activation
connection annotated by Genomatix experts
transcription factor binding site match in the target promoter

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