RESEARCH COMMUNICATION

Acyl-CoA binding protein is an essential protein in mammalian cell lines

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In the present work, small interference RNA was used to knock-down acyl-CoA binding protein (ACBP) in HeLa, HepG2 and Chang cells. Transfection with ACBP-specific siRNA stopped growth, detached cells from the growth surface and blocked thymidine and acetate incorporation. The results show that depletion of ACBP in mammalian cells results in lethality, suggesting that ACBP is an essential protein.

Key words: ACBP, acyl-CoA esters, lipid metabolism, small interference RNA (siRNA).

INTRODUCTION

Living cells encounter a number of problems in handling hydrophobic metabolites such as fatty acids, fatty acid derivatives and phospholipids. Such molecules are poorly soluble, partition into biomembranes and bind unspecifically to cellular proteins and enzymes, having adverse effects on their activities. Despite this, it is well recognized that these biomolecules also have important signalling properties. Fatty acyl-CoA esters, which are both important intermediates in lipid metabolism and signal molecules [1], are strong detergents, suggesting that their intracellular concentration must be tightly controlled.

The majority of the cellular long-chain acyl-CoA esters are presumed to be sequestered with acyl-CoA binding protein (ACBP) *in vivo* (reviewed in [1]). Intracellular ACBPs are members of a multigene family (reviewed in [2]). The basal isoform is highly conserved and is found in all eukaryotic species examined, ranging from yeast and plants, to reptiles, birds and mammals [3]. ACBP binds long-chain acyl-CoA esters specifically, with an affinity of approximately 1 nM [4–6]. Its ability to mediate intermembrane acyl-CoA transport *in vitro* [7], to protect acyl-CoA esters from acyl-CoA hydrolases [8], to protect acetyl-CoA carboxylase from the inhibitory effects of acyl-CoA esters [8] and to create an intracellular acyl-CoA pool [9,10] indicate that ACBP is involved in intracellular handling of acyl-CoA esters. Regardless of the known *in vitro* functions, only limited information has been obtained about the function of ACBP *in vivo*. ACBP expression is significantly up-regulated during adipocyte differentiation, and expression of high levels of ACBP antisense RNA in 3T3-L1 pre-adipocytes decreased endogenous ACBP levels and accumulation of triacylglycerols [11]. Depletion of Acb1p (the yeast homologue of ACBP) in *Saccharomyces cerevisiae* results in severe pertubations in membrane assembly, organization and organelle structures [12], suggesting that it may be involved in vesicular trafficking and in the maintenance of the integrity of cellular compartments. Recently, it was reported that targeted disruption of ACBP in *Trypanosoma brucei* results in lethality [13], suggesting that ACBP plays an essential role in this organism. In the present work, we show for the first time that knock-down of ACBP in human cell lines by small interference RNA (siRNA) results in metabolically inactive and non-viable cells, strongly implying that ACBP plays a pivotal role in human cells.

EXPERIMENTAL

Cell cultures, siRNA preparation and transfection

HeLa, HepG2 and Chang cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum and penicillin and streptomycin. Cells were regularly passaged to maintain exponential growth, but did not exceed 6 passages. Prior to transfection (24 h), cells were trypsinized, diluted in fresh media without antibiotics and transferred to six-well plates. On the day of transfection, cells were at 50–70 % confluency. Transfections of siRNA were carried out using OLIGOFECTAMINE™ according to the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). Target-specific siRNA duplexes were designed as described previously [14] and purchased from Dharmacon Research (Lafayette, CO, U.S.A.). The human ACBP (NCBI accession no. M15887)-specific siRNA was positioned at 199–219 relative to the start codon and was compared with sequences in the human EST (expressed sequence tag) database to confirm that no other genes were targeted. A sequence targeting the *Photinus pyralis* firefly luciferase gene (NCBI accession no. X65324) over DNA bases 153–175 was used as an unspecific siRNA control [15]. Unless otherwise stated, all experiments were carried out at least three times.

Western blotting

For Western blotting, cells attached to the plate were scraped off with a rubber policeman and combined with cells in the medium. The entire population of cells were then harvested by centrifugation for 5 min at 800 ***g***, washed in ice-cold PBS, solubilized in SDS-sample buffer (150 μl) and denatured by heating to 95 °C for 5 min. Equal amounts of protein were separated on 10 or 15 % (w/v) polyacrylamide gels and transferred on to a

Abbreviations used: ACBP, acyl-CoA binding protein; Acb1p, the yeast homologue of ACBP; EST, expressed sequence tag; PARP, poly(ADP-ribose) polymerase; siRNA, small interference RNA.

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nitrocellulose membrane. Immunostaining with specific antibodies and peroxidase-conjugated secondary antibodies (Promega) was carried out using enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Uppsala, Sweden).

Thymidine incorporation, acetate incorporation and DNA fragmentation

Thymidine incorporation was determined as described previously [16], using [*methyl*-^{3}H]thymidine (Amersham Pharmacia Biotech). Briefly, HeLa, HepG2 or Chang cells were transfected with 10 nM siRNA against either ACBP or firefly luciferase (LUC GL2), and 16 h prior to analysis, 1 μCi of [^{3}H]thymidine was added to each well. The entire cell population was harvested 72 h post-transfection and ^{3}H-thymidine-labelled DNA was extracted and analysed by scintillation counting. DNA fragmentation was examined using an apoptotic DNA ladder kit, according to the manufacturer's instructions (Roche, Mannheim, Germany). Acetate incorporation was carried out by incubating transfected HepG2 cells with [1-^{14}C]acetate (2 μCi/well) (Amersham Pharmacia Biotech) for 4 h. Cells were harvested as described above and washed twice with ice-cold PBS. Lipids were extracted as described previously [17], and acetate incorporation was determined by scintillation counting.

RESULTS

To assess the role of the basal form of ACBP in human cell lines, we designed siRNA duplexes directed against ACBP (Figure 1A). In parallel with the target-specific siRNA duplex, we used a non-specific duplex of firefly luciferase from *P. pyralis* (LUC GL2) at the same final concentration as the specific siRNAs tested. The ACBP-specific siRNA was used to search the human EST database to confirm that the chosen sequence did not share any significant identity with any other known genes. When Hela cells were transfected with increasing concentrations of siRNA duplex directed against ACBP we observed that the level of ACBP was significantly reduced, as detected by Western blotting (Figure 1B). Transfection with increasing concentrations of the LUC GL2 siRNA duplex did not lead to a decrease in the level of ACBP. The amount of loaded protein was equal, as judged from the level of β-actin. These observations show that we are able to reduce the level of ACBP in HeLa cells by RNA interference in a specific manner.

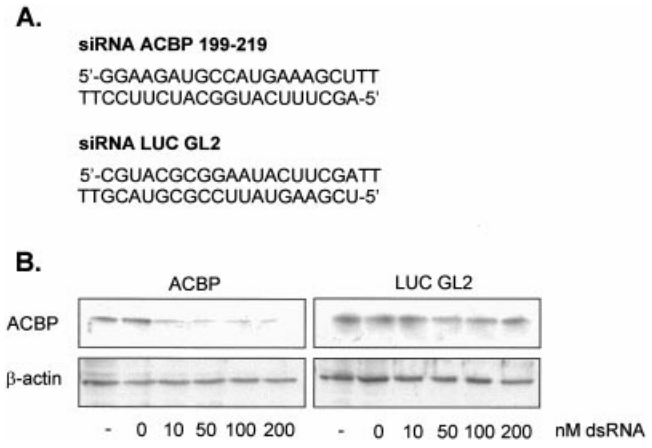

Figure 1 Design and use of siRNA duplexes

(**A**) The siRNA sequence targeting human ACBP (NM_020548) was from position 199 to 219 relative to the start codon. The siRNA sequence targeting firefly luciferase from *P. pyralis* has been described previously [15]. The ACBP-specific siRNA was compared with sequences in the human EST database to confirm that no other genes were targeted. (**B**) HeLa cells were grown and transfected in six-well plates with siRNA duplexes (final concentration indicated along the bottom) as described in the Experimental section. Cells, either floating in the medium or attached to the bottom of the well, were harvested 72 h post-transfection and the silencing effect of ACBP was analysed by Western blotting. Equal loading was evaluated by Western blotting against β-actin. The final concentration of siRNA duplexes is indicated. The dash (–) indicates untransfected cells, and 0 nM denotes the addition of buffer instead of siRNA to the culture.

Depletion of Acb1p in *S. cerevisiae* results in severe pertubations in membrane assembly, organization and organelle structures [12], and targeted disruption of ACBP in *T. brucei* results in lethality [13], suggesting that ACBP plays an essential role in this organism. Consistent with these reports, we observed severe alterations in cell morphology upon ACBP silencing (Figure 2A). Targeted knock-down of ACBP resulted in observable growth arrest and detachment of cells from the growth surface, resulting in cells floating in the media (Figure 2A). At the lowest concentration of siRNA ACBP, a few cells that were still attached to the growth surface could be observed; however, as the concentration of siRNA ACBP was increased, only a minute fraction of attached cells were observed. The detached cells were stained blue when Trypan Blue was added directly to the well, a strong indication of cell death (Figure 2B). In a similar fashion, silencing of ACBP in HepG2 and Chang cells also resulted in growth arrest and in detached cells with severe changes in their morphology (Figure 2C).

That the cells are metabolically inactive is further supported by the observation that ACBP-silenced cells are unable to incorporate thymidine into cellular DNA. ACBP-silenced cells only incorporated approx. 5% of the added thymidine into DNA compared with LUC 2GL control-transfected HeLa and HepG2 cells (Table 1), while approx. 15% was incorporated into ACBP-silenced Chang cells. These data strongly imply that ACBP-silenced cells are unable to divide. That ACBP-silenced cells are metabolically inactive is further supported by the observation that acetate incorporation is reduced to 12% of control transfected cells (Table 1).

In an effort to determine how cell death occurs in ACBP-silenced cells, we looked at parameters typically associated with either apoptotic cells or necrotic cells. Fragmentation of DNA is a typical measure of cells undergoing apoptosis. When DNA was extracted from HeLa cells transfected with LUC GL2, control siRNA or ACBP siRNA, and analysed by agarose electrophoresis, we did not observe fragmentation of the DNA as in U937 apoptotic control cells (Figure 3). The DNA extracted from ACBP-silenced cells only appeared mildly smeared and showed no fragmentation, which implied that the cells were necrotic and not apoptotic. Analysis of DNA at 24 and 48 h post-transfection showed no sign of fragmentation (results not shown). As DNA fragmentation is a marker for late apoptosis, we hypothesized that early apoptosis could be induced prior to cell death. In order to address this question we examined whether activation of caspases occured in ACBP-silenced cells. Certain caspases, e.g. caspase 3 and 7, are activated during early stages of apoptosis, and are found to cleave the 113 kDa poly(ADP-ribose) polymerase (PARP) to fragments of 89 and 24 kDa. Detection of the 89 kDa PARP fragment by Western blotting thus serves as an early marker of apoptosis. Interestingly, ACBP silencing resulted in activation of caspases, as the majority of the detectable PARP corresponded to a mobility of 89 kDa. Thus these results indicate that ACBP silencing in HeLa cells results in induction of early apoptosis, but prior to induction of late apoptosis, cell death emerges.

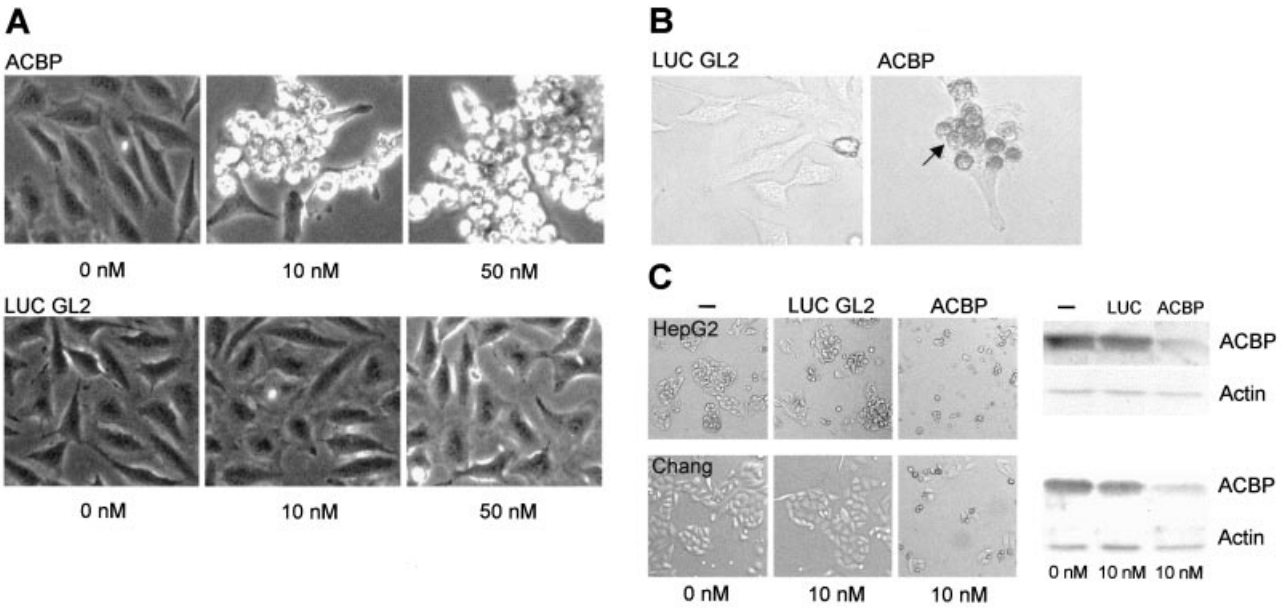

Figure 2 Silencing of ACBP results in profound changes in cell morphology and non-viable cells

(**A**) Phase-contrast microscopy of HeLa cells transfected with siRNA against ACBP (upper panel) or firefly luciferase (LUC GL2; lower panel). The final concentration of siRNA duplexes is indicated. (**B**) Microscopic image of Trypan Blue-stained HeLa cells after transfection with 10 nM siRNA against either ACBP or firefly luciferase (LUC GL2). Trypan Blue was added directly to wells containing transfected cells and analysed by optical microscopy. The arrow marks Trypan Blue-stained cells. (**C**) Phase microscopy of HepG2 cells (upper panel) and Chang cells (lower panel) transfected with siRNA against firefly luciferase (LUC GL2) or ACBP. Cells were harvested and the level of ACBP and β-actin was determined by Western blotting. The final concentration of siRNA duplexes is indicated. Controls (–) received buffer instead of siRNA.

Table 1 [^{3}H]Thymidine and [^{14}C]acetate incorporation into ACBP-silenced cells

Mean ± standard error of the mean of two independent experiments is shown.

	Radioactivity incorporated/well (d.p.m.)	
	siRNA LUC GL2	siRNA ACBP
[^{3}H]Thymidine		
HeLa	79377.8 ± 10041.3	3613.2 ± 345.7
HepG2	520603 ± 3687.6	26553.8 ± 4716.7
Chang	216174 ± 1697.8	31520.3 ± 104.3
[^{14}C]Acetate		
HepG2	12376.0 ± 329.3	1519.7 ± 92.3

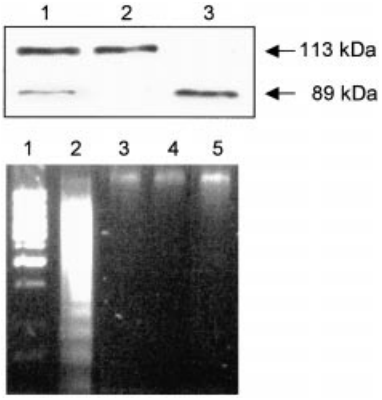

Figure 3 Determination of early and late apoptosis events

Upper panel: HeLa cells were transfected with 10 nM siRNA against either firefly luciferase (LUC GL2, lane 2) or ACBP (lane 3), and analysed by Western blotting 72 h post-transfection for PARP cleavage, a measure for caspase activation during early apoptosis. Control transfection (lane 1) received buffer instead of siRNA. Lower panel: late apoptosis was determined by DNA fragmentation. DNA was extracted 72 h post-transfection and analysed by agarose electrophoresis. Lane 1, 1 kb DNA ladder; lane 2, fragmented DNA from apoptotic U937 cells provided in the kit; lane 3, control transfected HeLa cells; lane 4, cells transfected with 10 nM siRNA targeted against firefly luciferase (LUC GL2); and lane 5, cells transfected with 10 nM siRNA targeted against ACBP.

DISCUSSION

The fact that ACBP is highly conserved in all eukaryotes from yeast to man and plants to reptiles, and that it is ubiquitously expressed, suggests that ACBP serves a basic function in all cells. In the present study we have been able to reduce the level of ACBP in HeLa, HepG2 and Chang cells by siRNA, and show for the first time that ACBP silencing leads to growth arrest and cell death, strongly suggesting that ACBP plays an essential role in the human cell lines studied. These observations are highly consistent with the fact that targeted disruption of ACBP in *T. brucei* results in lethality [13]. Depletion of Acb1p in *S. cerevisiae* leads to a significant retardation of growth, which can be complemented by ectopic expression of both mouse and human ACBP (N. J. Færgeman and J. Knudsen, unpublished work) indicating that ACBP is functionally identical in yeast and man, making yeast a potential model system for studying the molecular mechanism underlying the function of ACBP.

Depletion of Acb1p in yeast does not affect the acyl-CoA pool size, but changes the acyl-CoA pool composition dramatically by

increasing stearoyl-CoA approx. 5-fold [12]. This observation suggests that Acb1p does not control the overall acyl-CoA level, but rather is required for creating a specific pool of acyl-CoA esters. This view is supported by the observation that the overall phospholipid composition and turnover is unaffected by Acb1p depletion, indicating that general glycerolipid synthesis does not depend on Acb1p. However, depletion of Acb1p in yeast leads to profound alterations in the morphology of subcellular organelles and to the accumulation of a large number of vesicles and aberrant membrane structures in the cytosol [12], indicating that the Acb1p-bound acyl-CoA pool is required for membrane trafficking. MS analysis of phospholipids extracted from isolated plasma membranes revealed an increased level of sphingolipids and lysophospholipids in Acb1p-depleted cells compared with wild-type cells [12], supporting the notion that ACBP plays a specific and pivotal role in intracellular trafficking of acyl-CoA esters required for membrane remodelling or synthesis of specific lipids required for vesicular trafficking and membrane turnover.

It has been suggested that the intracellular concentration of unbound acyl-CoA esters is tightly controlled by the presence of specific ACBP and acyl-CoA thioesterases [1]. In such a cellular environment, transport by diffusion of unbound acyl-CoA esters is very unlikely, and suggests that supply of substrates to acyl-CoA-consuming enzymes depends on direct transfer from acyl-CoA synthetases, or relies on ACBPs or carrier proteins. Thus the fact that *S. cerevisiae*, in contrast with mammalian cells, does not express a cytosolic acyl-CoA hydrolase may explain why yeast, but not human, cell lines are viable in the absence of ACBP.

Acyl-CoA esters have also been shown to have regulatory properties that are similar to those of other celluar components, e.g. protein kinases, ion channels and transcription factors (reviewed in [1,18]). Hence, depletion of the ACBP could have multiple detrimental effects on essential cellular signal pathways. Thus it is conceivable that cellular depletion of ACBP leads to pertubation of cellular acyl-CoA trafficking, resulting in hampering of essential pathways or functions in which long-chain acyl-CoA esters participate [18]. This may also explain the inability of pre-adipocytes to differentiate upon expression of ACBP antisense [11]. Although it would have been interesting to know how ACBP silencing affects the acyl-CoA level, we believe that attempts to measure the acyl-CoA level in the present study would be meaningless, since ATP depletion and cell death in itself would deplete cells of acyl-CoA esters [19]. Cell death may also explain why ACBP-silenced cells cannot complete the apoptotic program.

The present work also shows that ACBPs carry out specific functions in the cell that cannot be replaced by any of the proteins which harbour an intrinsic ACBP domain. Besides the basal ACBP, four other ABCP gene products have been identified within the human genome, containing 281, 358, 407 and 527 amino acids respectively [2]. In addition to the ACBP domain, the 281-amino-acid and the 358-amino-acid protein contain two ankyrin repeats and a Δ^3,Δ^2-enoyl-CoA isomerase domain respectively. The observation that depletion of ACBP in human cell lines is lethal makes these cell lines unsuitable for studying ACBP function *in vivo*. However, *S. cerevisiae* only expresses one basal ACBP isoform, the function of which can be complemented by human or mouse ACBP (see above). Thus yeast may therefore be an excellent model system to study the *in vivo* function of ACBP.

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