Lipid-binding proteins modulate ligand-dependent *trans*-activation by peroxisome proliferator-activated receptors and localize to the nucleus as well as the cytoplasm

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Abstract Peroxisome proliferator-activated receptors (PPARs) are activated by a variety of fatty acids, eicosanoids, and hypolipidemic and insulin-sensitizing drugs. Many of these compounds bind avidly to members of a family of small lipid-binding proteins, the fatty acid-binding proteins (FABPs). Fatty acids are activated to CoA esters, which bind with high affinity to the acyl-CoA-binding protein (ACBP). Thus, the availability of known and potential PPAR ligands may be regulated by lipid-binding proteins. In this report we show by transient transfection of CV-1 cells that coexpression of ACBP and adipocyte lipid-binding protein (ALBP) exerts a ligand- and PPAR subtype-specific attenuation of PPAR-mediated *trans*-activation, suggesting that lipid-binding proteins, when expressed at high levels, may function as negative regulators of PPAR activation by certain ligands. Expression of ACBP, ALBP, and keratinocyte lipid-binding protein (KLBP) is induced during adipocyte differentiation, a process during which PPARγ plays a prominent role. We present evidence that endogenous ACBP, ALBP, and KLBP not only localize to the cytoplasm but also exhibit a prominent nuclear localization in 3T3-L1 adipocytes. In addition, forced expression of ACBP, ALBP, and KLBP in CV-1 cells resulted in a substantial accumulation of all three proteins in the nucleus. These results suggest that lipid-binding proteins, contrary to the general assumption, may exert their action in the nucleus as well as in the cytoplasm.—Helledie, T., M. Antonius, R. V. Sørensen, A. V. Hertzel, D. A. Bernlohr, S. Kølvraa, K. Kristiansen, and S. Mandrup. **Lipid-binding proteins modulate ligand-dependent *trans*-activation by peroxisome proliferator-activated receptors and localize to the nucleus as well as the cytoplasm. *J. Lipid Res.* 2000.** 41: **1740–1751.**

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Fatty acids and fatty acid-derived metabolites exert profound effects on cellular proliferation, differentiation, transformation, and homeostasis. Alterations in lipid homeostasis are associated with common and serious diseases such as atherosclerosis and noninsulin-dependent diabetes, and the nutritional intake of specific types of unsaturated fatty acid may contribute to tumor development and progression (1–5). Cellular uptake of fatty acids may proceed by diffusion through the lipid bilayer of the cell membrane, but findings clearly point to the involvement of protein-facilitated uptake systems exhibiting saturation kinetics (6). Fatty acid uptake has been shown to be influenced by intracellular fatty acid-activating enzymes and lipid-binding proteins/fatty acid-binding proteins (FABPs). Amongst these, acyl-CoA synthetase has been suggested to function in concert with the fatty acid transporter protein (FATP) (7), and a possible interaction between the intracellular domain of the fatty acid transporter (FAT) and heart FABP (H-FABP) has also been reported (8). The FABPs constitute a family of proteins that now encompasses more than 20 related proteins. Even though the overall identity of the amino acid sequences may be as low as 20%, the FABPs share a common three-dimensional structure comprising a so-called β barrel formed by 10 antiparallel β strands organized in two five-stranded β sheets almost perpendicular to each other, and two α helices that have been hypothesized to function as a portal lid controlling entry and release of ligands from the ligand-binding

Abbreviations: ACBP, acyl-CoA-binding protein; A-FABP, adipocyte-FABP/aP2; ALBP, adipocyte lipid-binding protein; ANOVA, analysis of variance; BSA, bovine serum albumin; C/EBP, CCAAT/enhancer-binding protein; DC-Chol, 3-β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol; DMSO, dimethyl sulfoxide; E-FABP, epidermis-FABP/MAL1; FABP, fatty acid-binding protein; FAT, fatty acid transporter; FATP, fatty acid transporter protein; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; H-FABP, heart FABP; KLBP, keratinocyte lipid-binding protein; LBD, ligand-binding domain; L-FABP, liver FABP; LIC, ligand-induced complex; MEM, minimal essential medium; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; SDS, sodium dodecyl sulfate; TTA, tetradecylthioacetic acid.

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pocket created by the two β sheets. Apart from fatty acids, the FABPs bind a large variety of hydrophobic compounds including eicosanoids, retinoids, and peroxisome proliferators. Binding specificity varies among the different members of the FABP family, with liver FABP (L-FABP) being the most promiscuous (9).

FABPs are generally considered as cytoplasmic proteins playing a role in the uptake and intracellular transport of fatty acids. Numerous experiments have demonstrated a correlation between expression of FABPs and the uptake and intracellular utilization of fatty acids (9), and direct evidence of such a role has been presented (10–12). In addition, the work by Sorof and co-workers (13–15) has implicated L-FABP in linoleic acid and peroxisome proliferator-dependent regulation of proliferation of hepatoma cells.

Activation of fatty acids to acyl-CoA esters is necessary for further metabolic conversions. In addition, work has clearly established acyl-CoA esters as important regulatory molecules modulating a large variety of cellular processes including gene expression (16). FABPs can bind acyl-CoA esters (17) and were originally assumed to function also as intracellular transporters of acyl-CoA esters. However, two high affinity acyl-CoA-binding proteins, the acyl-CoA-binding protein (ACBP) and the sterol carrier protein 2, have since been characterized (18, 19). ACBP has been extensively characterized, and has by a combination of in vitro and in vivo analyses been shown to function as an acyl-CoA pool former and transporter (16). The structure and binding properties of ACBP are completely different from those of other lipid-binding proteins. In contrast to the β-sheet secondary structure of FABPs, ACBP is composed of four α helices. Furthermore, by binding of acyl-CoA esters to ACBP, the CoA moiety is used as a lid and the hydrocarbon chain is totally buried in the hydrophobic binding pocket (20), whereas the hydrocarbon chain is partly exposed to the solvent in FABPs (21). This explains the high affinity and specificity of ACBP for acyl-CoA esters.

Members of the peroxisome proliferator-activated receptors (PPARs) are involved in fatty acid-dependent regulation of gene expression. It is evident that other factors participate in fatty acid-dependent transcriptional regulation (22), but several lines of evidence underscore the pivotal roles played by the PPARs. A diverse group of peroxisome proliferators, thiazolidinedione antidiabetic drugs, fatty acids, and fatty acid metabolites has been shown to induce PPAR-dependent *trans*-activation, and several of these were shown to be bona fide ligands (23–26). Binding of these to the PPARs results in recruitment of coactivators and transcriptional activation (27–30). Whereas several fatty acids have been demonstrated to be ligands and activators of the PPARs, little is known about the role of their CoA esters. Hertz, Berman, and Bar (31) showed that cotransfection with an expression vector expressing the long-chain acyl-CoA synthetase inhibited PPARα-mediated *trans*-activation, suggesting that the fatty acids rather than their CoA esters are the activators of PPARα.

Because FABPs and ACBP are implicated in intracellular trafficking of fatty acids and acyl-CoA esters, it would be expected that these proteins also affected the transduction of lipid-mediated signaling to the PPARs. However, this assumption has never been thoroughly investigated. ALBP and keratinocyte lipid-binding protein (KLBP) are able to bind several of the identified PPAR ligands (K_d in the upper nanomolar to low micromolar range) (32). In contrast, ACBP binds only medium- to long-chain CoA esters with high affinity (33) and, consequently, any effect of ACBP on the PPAR-mediated *trans*-activation would be expected to be mediated via effects on sequestering or handling of intracellular acyl-CoA esters. The effect of the lipid-binding proteins on PPAR-mediated *trans*-activation may depend on their intracellular localization. By using cellular fractionation it has been reported that H-FABP and L-FABP may be present in the nuclear fraction (34–36). However, a comprehensive analysis of the intracellular localization of FABPs has not been undertaken.

Fat is one of the major tissues involved in lipid homeostasis, and fatty acids exert at least partly via PPARs a profound effect on the development and function of fat tissue (37–41). At least three different lipid-binding proteins are induced during adipocyte differentiation: the adipocyte lipid-binding protein ALBP/aP2 (or A-FABP) (42, 43), the KLBP/MAL1 or E-FABP (44), and ACBP (45). In the present report we examine how coexpression of ACBP, ALBP, and KLBP affects PPAR-mediated *trans*-activation. Our results indicate that expression of high levels of lipid-binding proteins attenuates PPAR-mediated *trans*-activation in CV-1 cells. In addition, we present evidence that these proteins, apart from being present in the cytoplasm, also localize to the nucleus in different cell types, and we show that the intracellular distribution of ACBP and ALBP changes during the process of adipocyte differentiation of 3T3-L1 cells.

MATERIALS AND METHODS

Plasmids

For transient transfections, the plasmids pUAS$_{Gal}$-LUC (46) and PPRE$_3$-TK-LUC (47) were used as reporters and pSV-β-Galactosidase-control (Promega, Madison, WI) was used for normalization. The plasmids pcDNA1-GAL4-PPARα(LBD), pcDNA1-GAL4-PPARδ(LBD), pcDNA1-GAL4-PPARγ(LBD), and pcDNA3-GAL4-VP16 contain the GAL4 DNA-binding domain and PPARα ligand-binding domain [LBD; amino acids (aa) 166–468], PPARδ LBD (aa 137–440), PPARγ LBD (aa 203–505), and the herpes simplex VP16 *trans*-activation domain (aa 416–487), respectively. The vectors pcDNA1-ACBP, pcDNA3-ALBP, and pcDNA3-KLBP contain the ACBP, ALBP, and KLBP cDNA, respectively. In addition, the following plasmid were used: pCMX-mRXRα (48), pSG5-PPARα (49), pSG5-PPARδ (50), pSPORT-PPARγ2 (51), and pOb1 (52).

Cell culture

3T3-L1 cells were maintained and differentiated by the 3-isobutyl-1-methylxanthine/dexamethasone/insulin procedure as previously described (53). Accumulation of lipid droplets was determined by oil-red O staining as described (54).

CV-1 cells were grown in Eagle's minimal essential medium

(MEM) with Earle's balanced saline solution (GIBCO, Grand Island, NY) supplemented with streptomycin (100 μg/ml), penicillin (62.5 μg/ml), and fetal calf serum [FCS, 10% (v/v)]. The medium was changed every other day.

To strip serum, AG-1X-8 resin (25 g/500 ml serum; Bio-Rad, Hercules, CA) was added, and the serum was incubated at room temperature for 12–16 h with constant stirring. Serum was decanted and fresh AG-1X-8 resin (25 g/500 ml serum) and activated charcoal powder (5 g/500 ml serum; Bie & Berntsen, Rødovre, Norway) were added, and stirring was continued at room temperature for 4 h. Resin and charcoal were removed by repeated centrifugations, and serum was filtered through a 0.4-μm pore size filter followed by sterile filtration through a 0.2-μm pore size filter.

Western blot analysis

3T3-L1 cells from days 0, 2, 4, and 10 were lysed in 0.5 ml of 2.5% sodium dodecyl sulfate (SDS) sample buffer per 10-cm dish. Lysates were subjected to SDS-polyacrylamide gel electrophoresis [16% gels for detection of lipid-binding proteins and 12% gels for detection of CCAAT/enhancer-binding protein (C/EBPα)]. Approximately 20 μg of cellular protein was loaded per lane. The separated proteins were transferred to a polyvinylidene difluoride membrane and stained with Ponceau S for control of equal load. The membranes were blocked in 5% (w/v) nonfat dry milk and incubated with the appropriate primary antibodies (affinity-purified rabbit antimouse ACBP, rabbit antimouse KLBP, rabbit antimouse ALBP, and rabbit antimouse C/EBPα) for 1 h and with horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA) for another hour. Immunoreactive protein bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Transient transfections

CV-1 cells were grown to 50–70% confluency in 60-mm dishes and transfected with a total of 6 μg of DNA per dish, using the 3-β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol) lipofection procedure (55). The amounts of vectors used in each individual transfection are indicated in the appropriate figure legends. When necessary pcDNA3.1 was added to ensure equal DNA/promoter load. All transfections were performed in triplicate. The DNA was left on the cells for 5–7 h before the medium was changed to MEM containing resin/charcoal-stripped complete serum (10%, v/v) supplemented with either activator [tetradecylthioacetic acid (TTA) or BRL49653] in dimethyl sulfoxide (DMSO) or DMSO alone. After 48 h, the CV-1 cells were washed in phosphate-buffered saline (PBS) and scraped off the plate in lysis buffer [100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM dithioerythiol; Tropix, Bedford, MA]. Lysates were stored at −80°C. Luciferase and β-galactosidase (Tropix) activities were determined with a Berthold (Bad Wildbad, Germany) MicroLumat LB96B luminometer, using 96-well microtiter plates and 25 and 10 μl of lysate for the luciferase and β-galactosidase assays, respectively. Results were tested by single classification analysis of variance (ANOVA).

Immunostaining and confocal microscopy

CV-1 cells transiently transfected with ACBP, KLBP, or ALBP expression vectors (pcDNA1-mACBP, pcDNA3-mALBP, or pcDNA3-mKLBP) were grown in MEM containing FCS (10%, v/v). Twenty-four hours after transfection the cells were fixed for 1 min in paraformaldehyde (3%, w/v) in PBS at room temperature, washed in paraformaldehyde (3%, w/v) in PBS–methanol 1:1 (v/v), permeabilized in methanol at 4°C for 20 min, and incubated for 15 min in goat serum (10%, v/v) in PBS at room temperature (M. Westergaard and S. Junker, unpublished). The cells were incubated at 37°C for 30 min with the relevant rabbit antibodies in PBS containing bovine serum albumin (BSA; 1%, w/v) followed by incubation for 30 min at 37°C with fluorescein isothiocyanate (FITC)-conjugated swine antirabbit IgG antibodies (F0205; Dako) in PBS containing 1% BSA. For counterstaining of nuclei, cells were incubated for 2–4 min in Hoechst 33258 (1 μg/ml) in PBS. The cells were covered with 10% antifade (phenylenediamine at 10 mg/ml in PBS, pH 8.0) diluted in glycerol (87%, v/v), and analyzed by fluorescence microscopy.

3T3-L1 cells were fixed on day 0, 2, 4, 10, and 16 of differentiation in paraformaldehyde (4%, w/v) in PBS at 4°C for 5 min, and permeabilized in 70% ethanol for at least 5 min at −20°C. The permeabilized cells were incubated at room temperature for 1 h with the relevant affinity-purified rabbit antibody followed by incubation for 1 h with FITC-conjugated antirabbit IgG antibodies in PBS supplemented with BSA (1%, w/v). 3T3-L1 cells were counterstained in the same manner as CV-1 cells and analyzed by confocal laser scanning microscopy (TCS; Leica, Bensheim, Germany). The primary antibodies were those used for Western blotting.

RESULTS

The effect of ACBP, ALBP, and KLBP coexpression on PPAR-mediated *trans*-activation

To investigate the possible effects of lipid-binding proteins on ligand-dependent PPAR-mediated *trans*-activation, CV-1 cells were transiently transfected with the reporter construct $PPRE_3$-TK-LUC and vectors expressing the different PPARs. The cells were cotransfected or not with vectors expressing one of the lipid-binding proteins, and after transfection cells were treated or not with ligands/activators of the PPARs.

The PPAR activator TTA was chosen for several reasons. First, results from our laboratory have demonstrated that TTA activates all PPAR subtypes although the activation of PPARα is by far the most robust. In keeping with this, ligand-induced complex formation assays indicate that TTA is a true ligand of PPARδ as well as PPARα (23). Thus, all receptors could be stimulated with the same ligand. Second, TTA is readily converted to a CoA ester but is unable to undergo β-oxidation. Thus, the concentration of TTA as well as its CoA ester is likely to build up in the cells after addition to the medium. Finally, whereas TTA would be expected to bind to ALBP and KLBP, its CoA ester but not TTA itself would be expected to bind to ACBP, that is, FABPs as well as ACBP could potentially influence activation of PPAR by this ligand.

As shown in **Fig. 1**, the TTA-stimulated *trans*-activation by all three PPAR subtypes was moderately decreased by coexpression of ACBP and ALBP. TTA-independent *trans*-activation by PPARδ and PPARγ tended to be slightly reduced as well, indicating that the effect of these lipid-binding proteins is not entirely dependent on the addition of an exogenous ligand. Coexpression of KLBP resulted in a more pronounced decrease (approximately 50% reduction) in *trans*-activation by all three PPAR subtypes, and this effect appeared to be TTA independent. The absence of any significant effect of cotransfection with a vector (pOb1) expressing high levels of leptin indicates that the inhibitory effect of the lipid-binding pro-

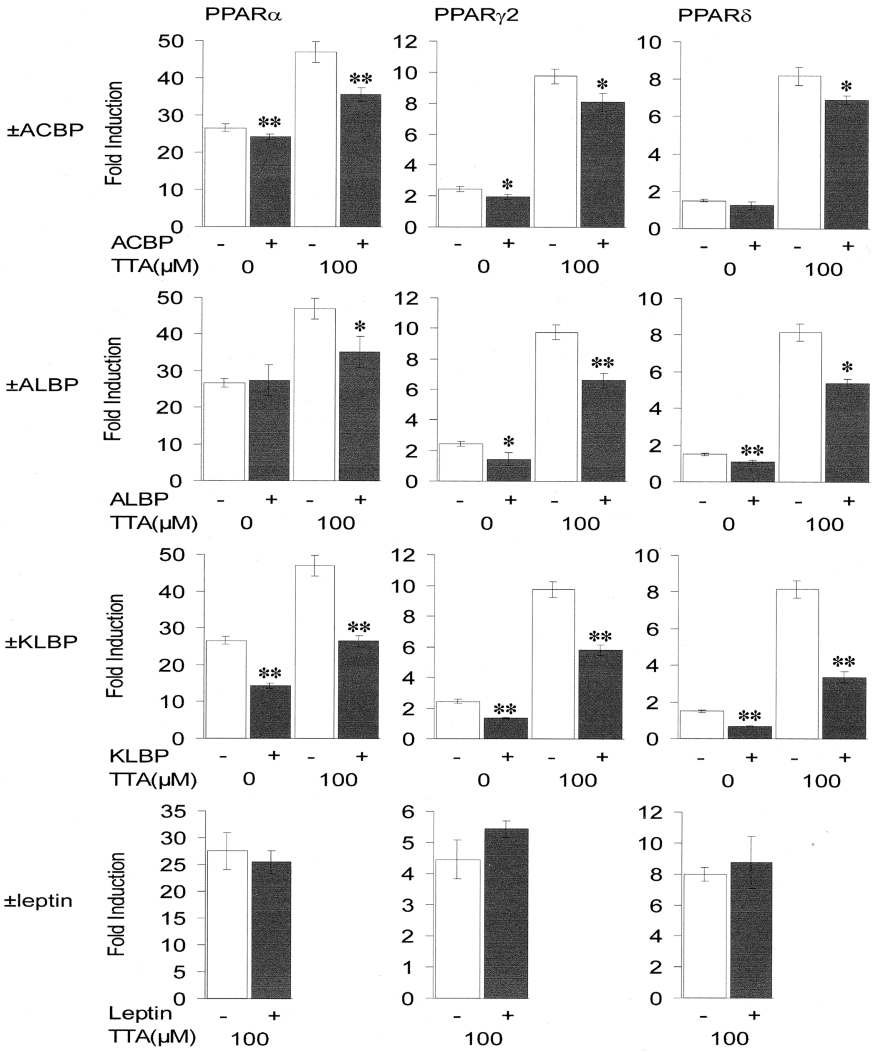

Fig. 1. The effect of lipid-binding proteins on full-length PPAR-mediated *trans*-activation. CV-1 cells were transiently transfected with the reporter plasmid $PPRE_3$-TK-LUC (1 μg), pSV-β-galactosidase-control (0.5 μg), pCMX-mRXRα (0.5 μg), and either pSG5-PPARα (0.5 μg), pSG5-PPARδ (0.5 μg), or pSPORT-PPARγ2 (0.5 μg) expressing the full-length PPAR subtypes. Cells were treated or not with 100 μM TTA and cotransfected or not with pcDNA1-ACBP (1 μg), pcDNA3-ALBP (1 μg), pcDNA3-KLBP (1 μg), or pOb1 (1 μg) as indicated. Equal DNA/promoter load was obtained by the addition of the empty pcDNA 3.1 vector. Luciferase activities were normalized to β-galactosidase activities. The normalized luciferase activities are shown as fold induction relative to that of transfection with the reporter plasmid alone. Each column and error bar represents the average and the standard deviation, respectively, of three independent transfections. Results were tested by single classification ANOVA. * $P < 0.05$; ** $P < 0.01$. The results are representative of three or more experiments.

teins is specific and is not merely due to the expression of high levels of an exogenous protein. Leptin expression from the pOb1 plasmid is driven by the strong cytomegalovirus promoter, which is also used to drive the expression of the lipid-binding proteins. When BRL49653 was used as an activator of PPARγ, ACBP and ALBP had no significant effect on ligand-dependent *trans*-activation whereas cotransfection with KLBP resulted in a decrease in *trans*-activation potential similar to the decrease observed for TTA-induced *trans*-activation (**Fig. 2**).

To investigate whether the lipid-binding proteins exerted their effects through the LBD of the PPARs, CV-1 cells were transiently transfected with the reporter construct $pUAS_{Gal}$-LUC and vectors expressing fusions of the GAL4 DNA-binding domain and the LBD of the different PPARs. The cells were cotransfected or not with vectors expressing one of the lipid-binding proteins or leptin (**Fig. 3**). Coexpression of ALBP resulted in a significant reduction in TTA-dependent *trans*-activation by all the PPARs, whereas coexpression of ACBP consistently reduced the *trans*-activation by PPARγ and PPARδ, but not that by PPARα. In a few experiments coexpression of ACBP resulted in attenuation of PPARα-mediated *trans*-activation (results not shown); however, the magnitude of the attenuation was variable and always smaller than that observed by coexpression of ALBP. A minor reduction in the TTA-independent *trans*-activation by cotransfection with ALBP or ACBP was also observed in some transfec-

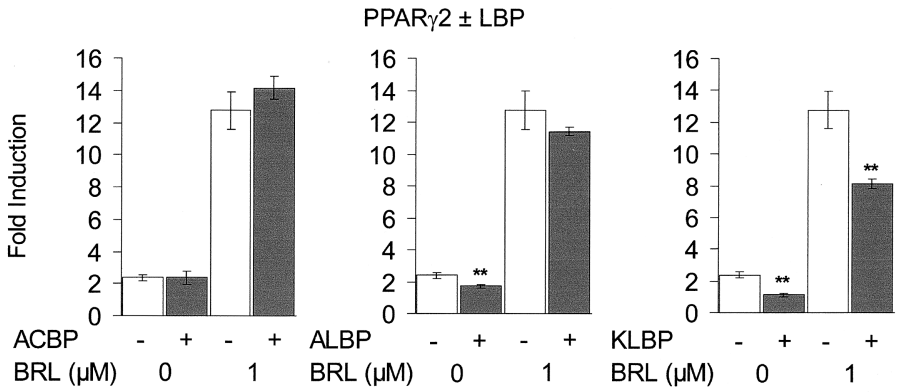

Fig. 2. The effect of lipid-binding proteins on PPARγ-mediated *trans*-activation, using BRL49653 as a ligand. CV-1 cells were transfected as indicated in Fig. 1, except that TTA was replaced by BRL49653. Each column and error bar represents the average and the standard deviation, respectively, of three independent transfections. Results were tested by single classification ANOVA. ** $P < 0.01$. The results are representative of three experiments.

tions (results not shown), indicating that lipid-binding proteins may also interfere with endogenous ligands. The effect of ALBP and ACBP on PPAR-mediated *trans*-activation was clearly dose dependent. Coexpression of KLBP resulted in a pronounced dose-dependent repression of the TTA-dependent as well as TTA-independent *trans*-activation by all three PPAR subtypes. Cotransfection with the leptin expression vector had no significant effect. Thus, the results obtained with the GAL4-PPAR(LBD) constructs reflected those obtained with constructs expressing full-length PPAR, indicating that the lipid-binding proteins exert their inhibition through the LBD of the PPARs.

To investigate whether the inhibitory effect of the lipid-binding proteins was specific for PPAR-mediated *trans*-activation, the effect of lipid-binding proteins on GAL4-VP16-mediated *trans*-activation was determined. As shown in **Fig. 4**, coexpression of ACBP and ALBP had no effect on VP16-mediated *trans*-activation. Surprisingly, we found that KLBP attenuated VP16-mediated *trans*-activation. However, the dose-dependent effect of KLBP on VP16-mediated *trans*-activation was less pronounced than that observed on PPAR-mediated *trans*-activation, suggesting that KLBP apart from a general inhibitory action exerts a more specific effect on PPAR-dependent *trans*-activation.

Finally, the effect of coexpression of ALBP and ACBP on the dose-response curves for TTA activation of PPARs was investigated. As seen in **Fig. 5**, PPARα is activated by TTA at a concentration as low as 5 μM, whereas activation of PPARδ needed concentrations of about 20 μM and activation of PPARγ concentrations between 20 and 40 μM. As noted earlier, cotransfection of lipid-binding proteins had little effect on TTA-independent *trans*-activation. Coexpression of ALBP resulted in a shift to the right of the dose-response curve for TTA activation of all the PPARs. Similarly, coexpression of ACBP resulted in a shift to the right of the dose-response curve for TTA activation of PPARδ and PPARγ. However, ACBP had little effect on TTA-induced PPARα *trans*-activation. An attenuation of PPARα *trans*-activation by coexpression of ACBP was observed only at low concentrations, that is, 5 μM.

Ectopically expressed ACBP, ALBP, and KLBP localize to the nucleus in CV-1 cells

The results of the transient transfection experiments showed that forced expression of lipid-binding proteins downregulated PPAR-mediated *trans*-activation. It is likely that at least part of this effect is due to sequestration and/or increased metabolism of PPAR ligands in the cytoplasm. Lipid-binding proteins have generally been regarded as cytoplasmic transport proteins. However, this view is almost exclusively based on results obtained by cellular fractionation, and it is well known that nuclear proteins may leak from the nucleus during fractionation. Preliminary results from our laboratory suggested that several lipid-binding proteins also locate to the nucleus. Consequently, we decided to determine the intracellular localization of ACBP, ALBP, and KLBP in CV-1 cells, the cell type that was used in the transfections described above. The lipid-binding proteins were transiently expressed in CV-1 cells and the localization was determined by immunofluorescence microscopy. CV-1 cells have an overall low expression of endogenous lipid-binding proteins, and do not express detectable amounts of ALBP and KLBP. In addition, the low expression of monkey ACBP is not detectable with the antimouse ACBP antibody. **Figure 6** shows that a significant fraction of the transiently expressed ACBP, ALBP, and KLBP localized to the nucleus. We never observed cells exhibiting an exclusively cytoplasmic localization. No immunostaining could be detected in cells transfected with the empty expression vector, and the addition of TTA did not result in an altered distribution of any of these lipid-binding proteins in the transfected CV-1 cells (results not shown). Thus, ACBP, ALBP, and KLBP are able to localize to the nucleus in transiently transfected CV-1 cells, and may occasionally predominantly localize to the nucleus.

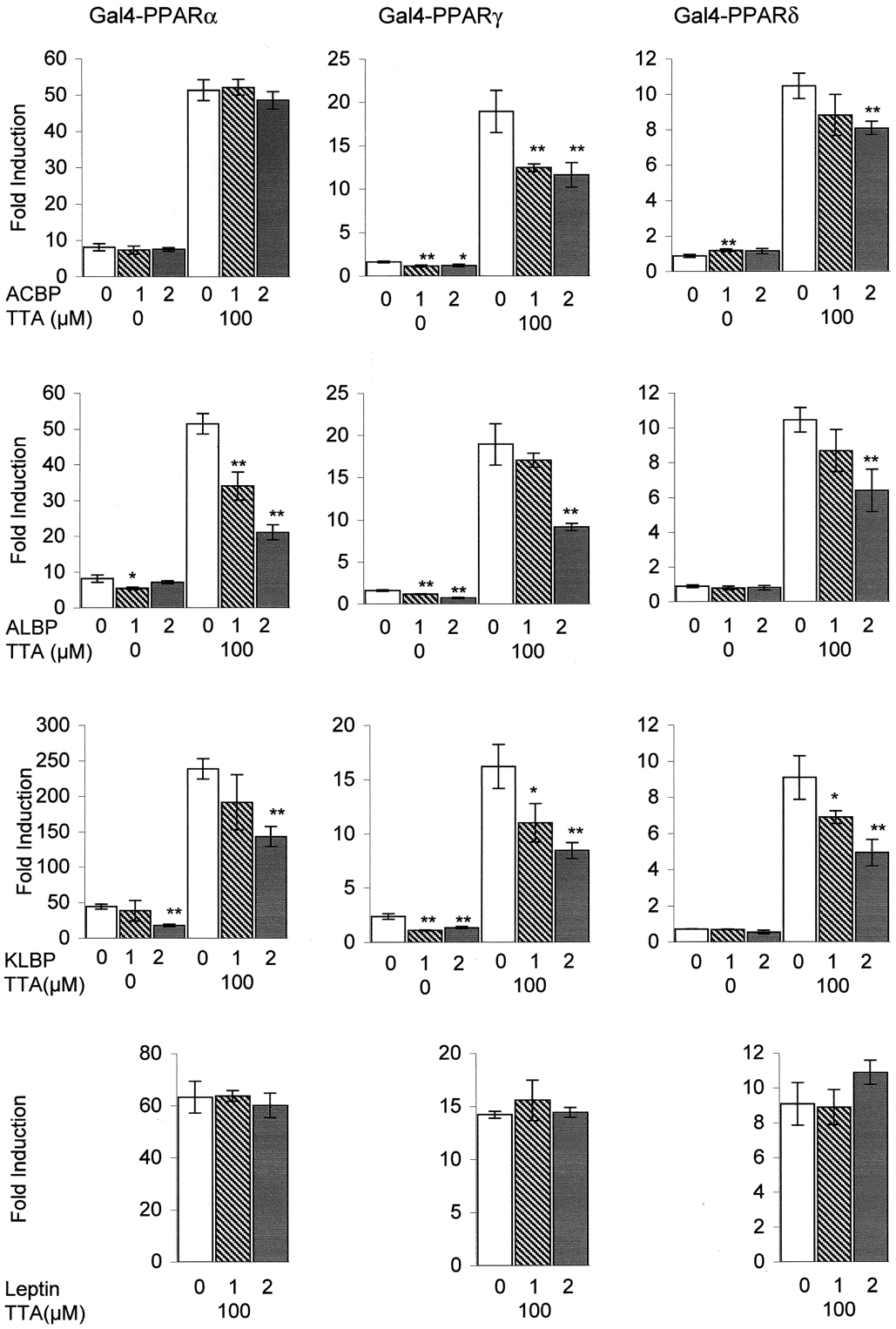

Fig. 3. The effect of lipid-binding proteins on GAL4-PPAR(LBD)-mediated *trans*-activation. CV-1 cells were transiently transfected with $pUAS_{Gal}$-LUC (1 μg), pSV-β-galactosidase-control (0.5 μg), and either pcDNA1-GAL4-PPARα(LBD) (0.25 μg), pcDNA1-GAL4-PPARδ(LBD) (0.25 μg), or pcDNA1-GAL4-PPARγ(LBD) (0.25 μg). Cells were treated or not with 100 μM TTA and cotransfected or not with pcDNA1-ACBP, pcDNA3-ALBP, pcDNA3-KLBP, or pOb1 as indicated. Equal DNA/promoter load was obtained by the addition of the empty pcDNA 3.1 vector. Luciferase activities were normalized to β-galactosidase activities. The normalized luciferase activities are shown as fold induction relative to that of the transfection without PPAR activator and without vectors expressing lipid-binding proteins. Each column and error bar represents the average and the standard deviation, respectively, of three independent transfections. Results were tested by single classification ANOVA. * $P < 0.05$; ** $P < 0.01$. The results are representative of three or more experiments.

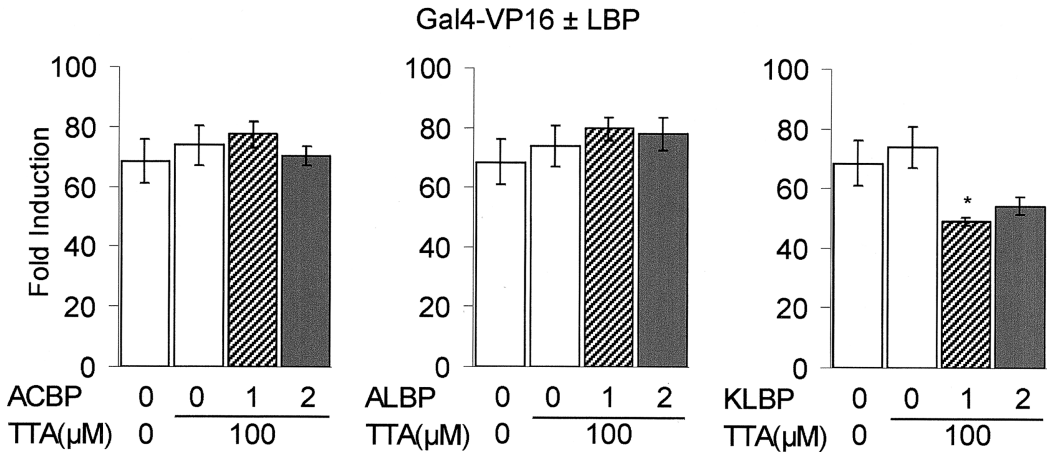

Fig. 4. Coexpression of ACBP and ALBP has no effect on GAL4-VP16-mediated *trans*-activation. CV-1 cells were transfected as indicated in Fig. 4 except that the GAL4-PPAR(LBD) expression vectors were replaced by the GAL4-VP16 (0.25 μg) expression vector. Each column and error bar represents the average and the standard deviation, respectively, of three independent transfections. Results were tested by single classification ANOVA. No significant differences were found. The results are representative of three experiments. * $P < 0.05$.

Expression and intracellular localization of endogenous ACBP, ALBP, and KLBP during adipocyte differentiation of 3T3-L1 cells

Induction of ACBP and ALBP expression during adipocyte differentiation has been well documented at the RNA and protein level (42, 43, 45). In addition, Northern blotting has shown that adipocyte differentiation is accompanied by increased levels of KLBP mRNA (44). Thus, to investigate the intracellular localization of lipid-binding proteins in a system where they are naturally expressed, we decided to determine the localization of endogenous ACBP, ALBP, and KLBP during adipocyte differentiation of 3T3-L1 cells. **Figure 7** shows that KLBP is induced in parallel with ACBP and ALBP during adipocyte differentiation of 3T3-L1 cells and, furthermore, demonstrates that the affinity-purified antibodies directed against ACBP, ALBP, and KLBP only recognize the cognate protein on Western blots. **Figure 8** illustrates the intracellular localization of ACBP, ALBP, and KLBP during adipocyte differentiation of 3T3-L1 cells. It has been reported that immunolocalization analyses of adipocytes often cause difficulties due to strong background fluorescence of the fat-laden cells. Therefore, immunostaining of the nuclearly localized C/EBPα was included as a positive control. Immunostaining of C/EBPα is clearly visible on day 2, and the intensity of this nuclear staining increased steadily in accordance with the increased expression visualized by Western blotting. Weak immunostaining of ACBP, ALBP, and KLBP was visible already on day 0. A clear induction could be observed on day 2 followed by a significant increase in the level of all three lipid-binding proteins up to day 10, in agreement with the results obtained by Western blotting. On day 0 and day 2, ACBP and ALBP as well as KLBP localized predominantly to the nucleus. After the increased expression of the three lipid-binding proteins during adipocyte differentiation, the cytoplasmic staining became more prominent, and the lipid-binding proteins became almost evenly distributed between the nucleus and the cytoplasm in the fully differentiated adipocytes.

DISCUSSION

By using conventional immunofluorescence microscopy and confocal laser scanning microscopy we showed that ACBP, ALBP, and KLBP localized to the nucleus as well as the cytoplasm of CV-1 and 3T3-L1 cells. In CV-1 cells transiently expressed ACBP, ALBP, and KLBP exhibited prominent nuclear localization. In no case was the intracellular localization of these three lipid-binding proteins confined exclusively to the cytoplasm. Addition of TTA did not affect the distribution of ACBP, ALBP, and KLBP in CV-1 cells.

A significant fraction of the endogenously expressed ACBP, ALBP, and KLBP localized to the nuclei of 3T3-L1 preadipocytes and adipocytes. The proteins appear to localize predominantly to the nuclei, especially in the early stages of the differentiation. However, firm conclusions on the relative intracellular distributions cannot be drawn from the present data because the small lipid-binding proteins during fixation and immunostaining may be differentially retained in the cytoplasmic and the nuclear compartments, respectively. The results obtained for ACBP are supported by a report demonstrating that ACBP also exhibits prominent nuclear localization in rat liver hepatocytes and the rat hepatoma cell line H4-IIE-C3 (56).

The size of ACBP, ALBP, and KLBP is sufficiently small to allow diffusion through the nuclear pores. A cluster of basic amino acid residues resembling a nuclear localization signal is present in the C-terminal region of ALBP and KLBP, and in the mammalian ACBPs a cluster of lysine residue is present in the extreme C-terminal region. However, this cluster is not conserved outside mammals, and is clearly absent in yeast ACBP, which nevertheless exhibit a

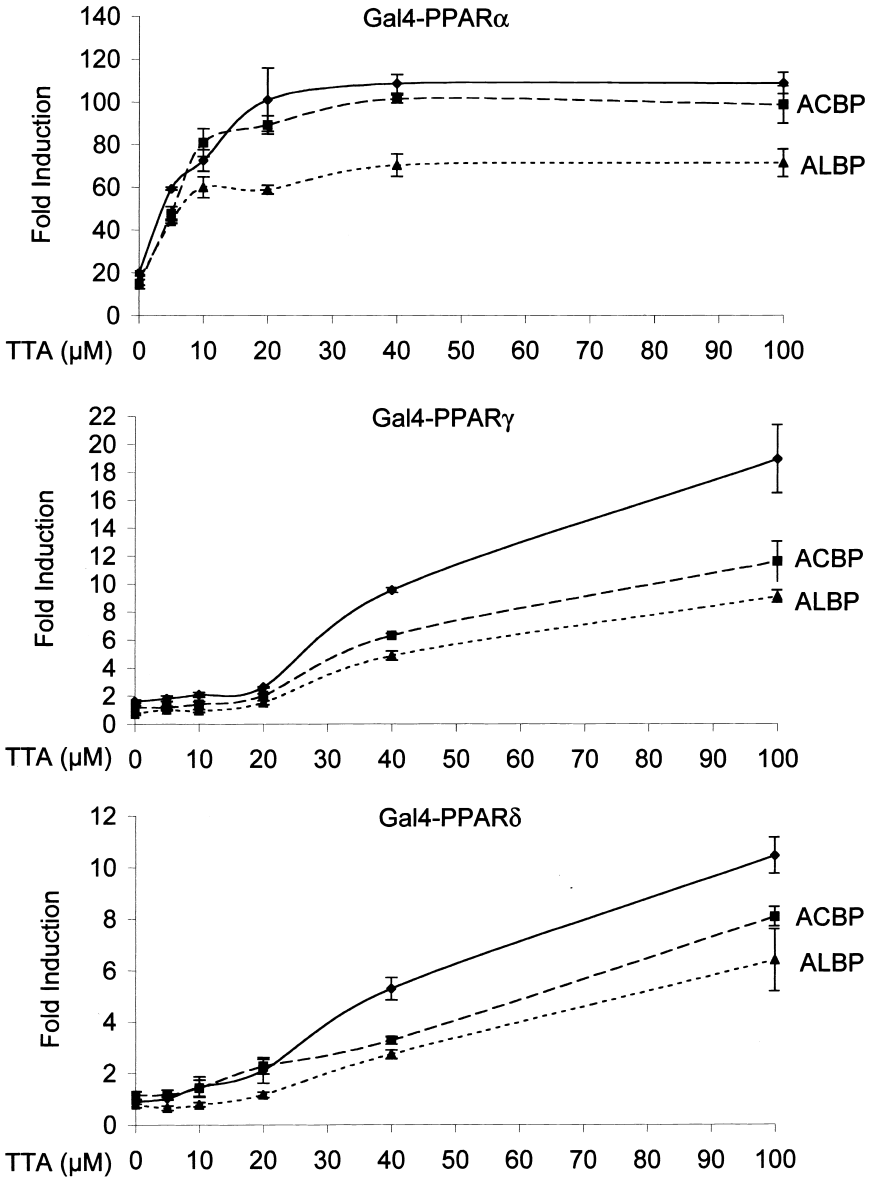

Fig. 5. Lipid-binding proteins shift the dose-response curve of PPAR activation by TTA to the right. CV-1 cells were transiently transfected with $pUAS_{Gal}$-LUC (1 μg), pSV-β-galactosidase-control (0.5 μg), and either pcDNA1-GAL4-PPARα(LBD) (0.25 μg), pcDNA1-GAL4-PPARδ(LBD) (0.25 μg), or pcDNA1-GAL4-PPARγ(LBD) (0.25 μg). Cells were treated or not with 5, 10, 20, 40, or 100 μM TTA and cotransfected or not (solid diamonds) with 2 μg of pcDNA1-ACBP (solid squares) or 2 μg pcDNA3-ALBP (solid triangles) as indicated. Equal DNA/promoter load was obtained by the addition of the empty pcDNA 3.1 vector. Luciferase activities were normalized to β-galactosidase activities. The normalized luciferase activities are shown as fold induction relative to that of the transfection without PPAR activator and without vectors expressing lipid-binding proteins. Each column and error bar represents the average and the standard deviation, respectively, of three independent transfections. The results are representative of three or more experiments.

prominent nuclear localization (C. Børsting, R. Hummel, A. J. Kal, A. Stoop, M. van den Berg, H. F. Tabak, J. Knudsen, and K. Kristiansen, unpublished observations). Further studies are needed to examine whether nuclear translocation of ALBP, KLBP, and ACBP depends on targeting signals. In this context it should be noted that controlled nuclear exclusion has been reported for the cellular retinoic acid-binding protein type I (57), which is comparable in size to ALBP and KLBP and devoid of a canonical nuclear localization signal.

The results from the transient transfections with the full-length PPARs as well as those with PPAR LBDs fused to the GAL4 DNA-binding domain indicate that ACBP and ALBP are able to attenuate the TTA-stimulated *trans*-activation by all PPAR subtypes and shift the TTA dose-response curve to the right. The attenuating effect is in general more pronounced for ALBP than for ACBP. In particular, GAL4-PPARα(LBD)-mediated *trans*-activation is only minimally affected by coexpression of ACBP and only when the concentration of TTA is low. This suggests that a competition exists

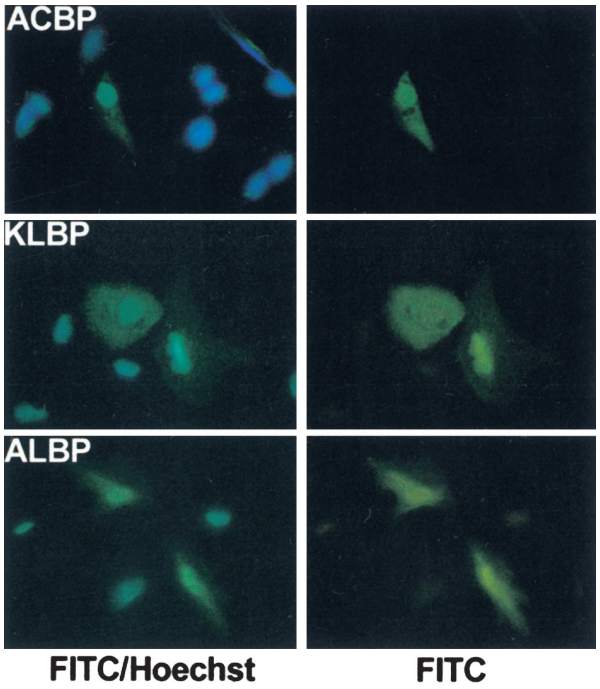

Fig. 6. ACBP, ALBP, and KLBP exhibit a nuclear as well as a cytoplasmic localization in transiently transfected CV-1 cells. CV-1 cells were transiently transfected with the appropriate expression vectors. The cells were fixed after 24 h, and the intracellular localization of the lipid-binding proteins was determined by immunofluorescence microscopy. Nuclei were counterstained with Hoechst 33258.

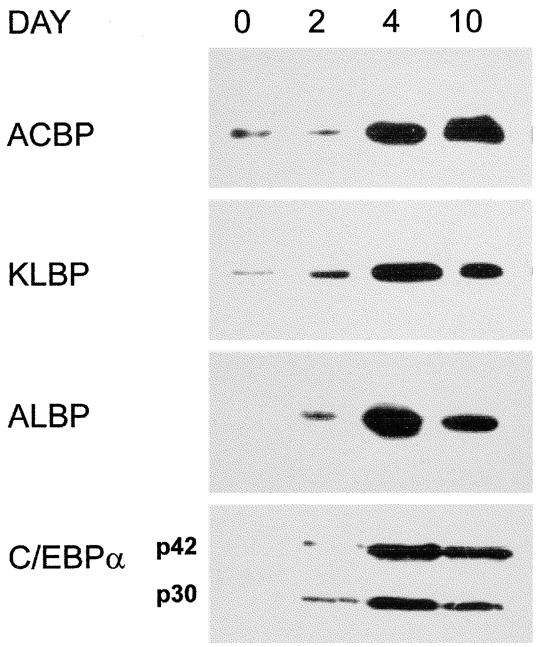

Fig. 7. Expression of lipid-binding proteins and C/EBPα during adipocyte differentiation of 3T3-L1 cells. Protein extracts were prepared from 3T3-L1 cells at different time points during adipocyte differentiation. The expression of ACBP, ALBP, KLBP, and C/EBPα was examined by Western blotting. The strength of the enhanced chemiluminescence signals can be compared only horizontally and does not allow comparison of the abundance of the individual proteins.

between binding of TTA to PPARα, which has a much higher affinity for TTA than the other PPARs, and processes furthered by high levels of ACBP (see below).

The effect of the ACBP and ALBP on *trans*-activation is specific for the PPARs because VP16-mediated *trans*-activation is not affected. KLBP, on the other hand, represses not only PPAR-mediated *trans*-activation but also VP16-mediated *trans*-activation, which make it impossible to draw firm conclusions about the effect of KLBP. However, it is worth noting that the magnitude of the KLBP-dependent repression of VP16-mediated *trans*-activation is less than that of PPAR-mediated *trans*-activation, suggesting that KLBP apart from a more general effect also exerts a specific attenuating effect on PPAR-mediated *trans*-activation. In an earlier study we found that coexpression of ALBP enhanced PPAR-mediated *trans*-activation modestly in the absence of exogenous ligands (58). However, we have since discovered that this was caused by differences in promoter load, that is, the transfections were performed without securing equal promoter load in the transfected cells.

The 3-thia-substituted TTA cannot be β-oxidized, but is activated to TTA-CoA. Thus, TTA treatment of cells presumably results in an accumulation of TTA-CoA in the cells (59). The attenuation of the TTA-induced PPAR *trans*-activation may have a metabolic as well as a sequestration component. In ALBP knockout mice the absence of ALBP expression in adipose tissue is only partially compensated for by an upregulation of the expression of KLBP (60). It was found that the total pool of fatty acids in adipose tissue was inversely correlated with the amount of FABP, suggesting that FABPs facilitate the metabolism of the fatty acids, thereby decreasing their availability as PPAR ligands (32). In addition, an analysis of fatty acid uptake in diabetic rats suggested that ALBP would mainly be involved in the efflux and not the influx of fatty acids (61). Overexpression of ALBP and other FABPs in L6 myoblasts has been shown to increase esterification of fatty acids (12). Finally, ACBP has been shown to be expressed at high levels in cells with a high fatty acid metabolism (18) and to relieve product inhibition of the long-chain acyl-CoA synthetase, thereby accelerating metabolism of fatty acids (62). Thus, it is possible that the attenuating effect of ALBP and ACBP on TTA-induced PPAR-mediated *trans*-activation is due to the ability of these lipid-binding proteins to increase esterification of TTA. In addition, ALBP may directly sequester TTA taken up by the cells, and it is possible that the ability of ALBP to both sequester and increase the metabolism of TTA accounts for the more pronounced effect of ALBP compared with ACBP on the attenuation of PPAR *trans*-activation, in particular PPARα-mediated *trans*-activation. Our finding that a significant amount of ALBP localizes to the nucleus, suggests that ALBP might sequester TTA in the nucleus as well as the cytoplasm.

It is noteworthy and fully in keeping with the preceding hypotheses that ACBP and ALBP do not suppress activation of PPARγ by BRL49653. Binding of BRL49653 by ACBP has not been reported, and it is unlikely that

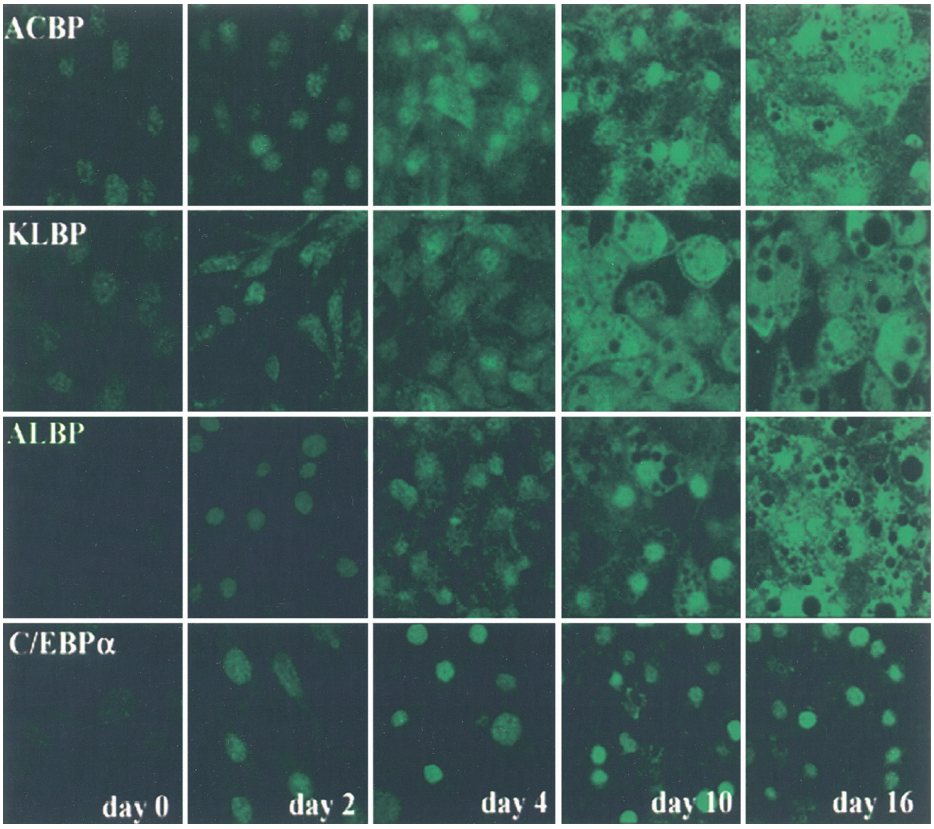

Fig. 8. Intracellular localization of lipid-binding proteins and C/EBPα during adipocyte differentiation of 3T3-L1 cells. 3T3-L1 cells were induced to differentiate, and cells were fixed on the indicated days during the differentiation process. After permeabilization, the cells were incubated with primary antibodies against ACBP, ALBP, KLBP, or C/EBPα. FITC-conjugated swine antirabbit-IgG was used as secondary antibody. The intracellular localization of ACBP, ALBP, KLBP, and C/EBPα was visualized by confocal laser scanning microscopy.

BRL49653 or a metabolite thereof would bind to ACBP. ALBP does bind thiazolidinediones, but the K_d of binding is in the micromolar range, and hence orders of magnitude above the K_d of binding of BRL49653 to PPARγ (63). Accordingly, the presence of ACBP or ALBP would not be expected to affect the metabolism of BRL49653 or the binding per se of BRL49653 to PPARγ.

The effects on PPAR-mediated *trans*-activation by overexpression of the lipid-binding proteins contrast the reported enhancement of retinoid-dependent *trans*-activation by expression of the cellular retinoic acid-binding protein II (64, 65). Similar to the lipid-binding proteins, the cellular retinoic acid-binding protein II seems to shuttle between the cytoplasm and the nucleus, but the collision-dependent transfer of ligand by the cellular retinoic acid-binding protein II clearly enhances *trans*-activation.

In conclusion, the prominent adipocyte lipid-binding protein ALBP does not appear to be positively involved in transducing the PPAR activating signal of exogenous ligands. Rather, it appears that ALBP as well as ACBP when expressed at high levels may act as negative regulators, possibly by sequestering and increasing the metabolism of fatty acids and other PPAR ligands. Several of the lipid-binding proteins including ALBP and ACBP have functional PPAR response elements in their promoter regions and are known to be induced by PPAR ligands including fatty acids (66, 67) (T. Helledie and S. Mandrup, unpublished results). Thus, increased levels of fatty acids will induce the expression of these lipid-binding proteins, which may sequester and/or enhance the metabolism of these fatty acids, thereby effectively reducing the availability of PPAR ligands. This might represent another example of negative feedback regulation ultimately involved in the maintenance of cellular homeostasis.

A report from our laboratory suggested that ACBP might be positively involved in the synthesis of endogenous ligands for PPARγ during adipocyte differentiation (53). However, the results presented in this article indicate that ACBP can also negatively affect activation of PPARs by exogenous ligands, possibly by binding acyl-CoA derivatives and thereby increasing the turnover of the ligands. In addition, overexpression of ACBP may also increase the size of the pool of acyl-CoA esters, which may function as PPAR antagonists (M. Elholm, I. Madsen, C. Jørgensen, A. Krogsdam, I. Kratchmarova, D. Holst, M. Göttlicher, J-Å. Gustafsson, T. Flatmark, R. Berge, J. Knudsen, S. Mandrup, and K. Kristiansen, unpublished observations). The effect of KLBP on transcription is intriguing, and certainly warrants further investigation. Pronounced differences distinguish the ligand-binding pockets of KLBP

and ALBP. Furthermore, the electrostatic surfaces of KLBP and ALBP differ significantly (68), suggesting that they may exhibit differences in their ability to interact with cellular proteins and membranes. How these differences affect the biological functions of ALBP and KLBP remains to be elucidated.

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