Turnover of acyl-CoA-binding protein in four different cell lines measured by using two-dimensional polyacrylamide-gel electrophoresis

Carsten L. BUUS,* Karsten KRISTIANSEN† and Jens KNUDSEN*

*Institute of Biochemistry and †Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

Acyl-CoA-binding protein (ACBP), also named diazepam-binding inhibitor or endozepine, is a 10 kDa protein for which a surprisingly large number of biological activities has been suggested. Some of these would seem to require a rapid intracellular turnover of the protein. In this paper we report on the turnover of ACBP in cell lines derived from mouse, rat and man. ACBP was identified in two-dimensional gels by using specific antibodies. Cells were labelled with [³⁵S]methionine and chased for

various periods of time. Total protein was extracted, subjected to two-dimensional PAGE, and radioactivity in the spot containing ACBP was determined by liquid-scintillation counting. ACBP half-life was determined, and varied from 25 to 53 h depending on the cell line and the growth conditions. In all cases, radioactivity in ACBP was lost slightly faster than radioactivity in total protein. These results are discussed in relation to the possible function suggested for ACBP.

INTRODUCTION

Acyl-CoA-binding protein (ACBP) is an 86-amino acid cytosolic protein which was purified from bovine liver on the basis of its ability to induce synthesis of medium-chain-length fatty acids by goat mammary-gland fatty acid synthetase (Mogensen et al., 1987). The sequence of ACBP is highly conserved in eukaryotic species from yeast to man (Knudsen et al., 1989; J. Knudsen, unpublished work). The secondary structure of ACBP, recently determined by n.m.r. spectroscopy, contains four α -helices comprising residues 1–15, 22–35, 52–60 and 68–85 (Andersen et al., 1991). The ACBP/ligand-binding specificities have been characterized, and ACBP has been shown to bind with high affinity acyl-CoA esters with chain length in the range C₁₄–C₂₂ (Rosendal et al., 1993), whereas no binding of non-esterified fatty acids, CoA or 3'-dephosphoacyl-CoA esters was observed (Rasmussen et al., 1990; Rosendal et al., 1993). The site of hydrophobic interaction has been shown by photoaffinity labelling to reside within amino acid residues 23–38 (Hach et al., 1990). The lack of binding of 3'-dephosphoacyl-CoA esters indicates that electrostatic interaction between the negatively charged phosphate group in acyl-CoA and positively charged groups in the binding site is necessary for binding (Rosendal et al., 1993). The fact that overexpression of recombinant bovine ACBP in yeast induces a large increase in acyl-CoA ester pool size shows that *in vivo* ACBP can bind long-chain acyl-CoA esters and acts as an intracellular acyl-CoA supplier (Mandrup et al., 1993). Such a housekeeping function is in accordance with the fact that ACBP has been found in all tissues tested (Mikkelsen and Knudsen, 1987; Knudsen et al., 1989) and that the rat ACBP gene has all the characteristics of a typical housekeeping gene (Mandrup et al., 1992).

Diazepam-binding inhibitor (DBI), initially purified from rat brain (Guidotti et al., 1983), is identical with ACBP (Knudsen et al., 1989). It was suggested that DBI or its processing products octadecaneuropeptide [DBI-(33–50)] and triakontatetrapeptide [DBI-(17–50)] perform a neuromodulation activity by either interaction with the type-A receptor for γ -aminobutyric acids (Guidotti et al., 1983; Ferrero et al., 1986) or modulation of neurosteroidogenesis by interaction with the mitochondrial

benzodiazepine receptor located in the outer mitochondrial membrane in glial cells (Papadopoulos et al., 1991).

Steroid synthesis in adrenal cell responds rapidly to stimulus by adrenocorticotropin (ACTH), and this response is inhibited by protein-synthesis inhibitors and rapidly restored on removal of these inhibitors (Garren et al., 1965; Crivello and Jefcoate, 1978). An 8.2 kDa protein which stimulated pregnenolone synthesis in purified bovine adrenal mitochondria was isolated from bovine adrenal gland. This protein was claimed to be identical with ACBP except that it was missing the two C-terminal amino acid residues (Besman et al., 1989). Synthesis of ACBP in the adrenal gland was shown to be stimulated by ACTH, and the half-life of ACBP was estimated to be less than 2 min (Yanagibashi et al., 1988; Hall et al., 1988; Hall, 1991). Recent results obtained by labelling of adrenocortical and Leydig cell lines *in vivo* did not confirm this observation. Although ACTH stimulated steroid synthesis in these cell lines, the synthesis and degradation of ACBP were not affected significantly and the half-life of ACBP was estimated to be more than 3 h (Brown et al., 1992).

Both the involvement in steroid-hormone synthesis and the neuromodulator activity would seem to require a high turnover rate of the protein, whereas a housekeeping function in acyl-CoA metabolism would be compatible with a slow turnover. A more detailed study of the turnover rate of the protein would therefore add to our understanding of the possible functions of this protein.

In the present study we have determined the rate of turnover of ACBP in four different cell lines. In all four cell lines, ACBP was found to be stable. The half-lives determined varied between 25 and 53 h depending on cell line and growth conditions, thus supporting our notion that ACBP should be considered a housekeeping protein.

MATERIALS AND METHODS

Cell culture

3T3-L1 and 3T3-C2 mouse embryo fibroblasts, H-4-II-E rat hepatoma cells and HepG2 human hepatoblastoma cells were obtained from the American Type Culture Collection (Rockville,

MD, U.S.A.). 3T3-L1 and 3T3-C2 cells were maintained at 37 °C in 80 cm² flasks (Nunc, Roskilde, Denmark) in a humidified atmosphere containing 7% CO₂ in air in 60% Dulbecco's modified Eagle's medium (DMEM), 30% Medium 199 (Gibco, Paisley, Scotland, U.K.) and 10% calf serum (Imperial Laboratories) and supplemented with antibiotics (penicillin, 100 µg/ml; streptomycin, 100 µg/ml; amphotericin, 2.5 µg/ml). HepG2 and H-4-II-E cells were maintained as described above but in Eagle's minimum essential medium (Sigma, St. Louis, MO, U.S.A.); HepG2 cells were supplemented with 10% calf serum and H-4-II-E cells were supplemented with 10% fetal calf serum plus 10% calf serum.

For experiments, cells were inoculated at a density of 6×10^3 /cm² in Nuclon microwell plates (Nunc) and cultured in a one-to-one mixture of DMEM and Ham's F12 medium (DMEM/F12, Sigma) containing serum, 15 mM Hepes and antibiotics. 3T3-C2 cells were cultured to confluence with medium containing 10% calf serum. Differentiation of confluent 3T3-L1 cells was induced by replacing calf serum with fetal calf serum and exposing the cells to dexamethasone (1 µM) for 48 h and then to insulin (10 µg/ml). HepG2 and H-4-II-E cells were cultured in DMEM/F12 with the serum composition as described for cell maintenance. H-4-II-E cells were cultured to confluence. As HepG2 cells do not show clear cell-contact growth inhibition, experiments were performed at the time when slightly overgrown islands appeared.

[³⁵S]Methionine labelling

The cell layer was washed once in low-methionine labelling medium (DMEM/F12) and then labelled for 24 h in 100 µl portions of low-methionine labelling medium containing 500 µCi/ml [³⁵S]methionine (specific radioactivity > 1000 Ci/mmol; NEN; NEG-009T) for 3T3 cells and 250 µCi/ml for H-4-II-E and HepG2 cells. The concentration of unlabelled methionine was 0.0067 mM. Cells were then chased with high-methionine chase medium (DMEM/F12 containing 2.5 mM methionine) and two wells were harvested after a chase of 0, 12, 24, 48 and 96 h. Lysis of cells was performed as follows: the cell layer was carefully washed three times with prewarmed (37 °C) PBS (7.45 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 2.68 mM KCl and 137 mM NaCl, pH 7.4), and then 25 µl of 2D-lysis buffer [9.8 M urea, 2% (v/v) ampholyte pH 3.5–10, 2% (v/v) Nonidet P40 and 100 mM dithiothreitol] was added and the microwell was sealed and agitated for 20 min. The extract from each well was collected and stored at –80 °C.

Protein determination and lipid analysis

All assays were performed using wells on the same culture plate as was used for pulse-chase experiments.

Total protein was determined in four individual wells. The cell layer was washed once with PBS (37 °C), then 200 µl of buffer (25 mM glycylglycine, 150 mM KCl, 5 mM MgSO₄, 5 mM disodium EDTA and 1 mM dithiothreitol, pH 7.4) was added and cells were disrupted by sonication for 2 s at 25 W with a Branson sonicator. Homogenates were collected and stored at –80 °C until assayed for total protein by the modified Lowry procedure as described by Schacterle and Pollack (1973). BSA was used as standard.

Lipid accumulation was determined in three individual wells. Cells were labelled for 24 h in 200 µl of medium containing 0.18 µCi of [1-¹⁴C]acetate (specific radioactivity 53 mCi/mmol) before extraction. The cell layer was washed once with prewarmed PBS and the cells were disrupted by adding 200 µl of methanol

followed by sonication for 2 s using a Branson sonicator at 25 W. Methanol was collected and sonication in methanol was repeated four times. Lipids were extracted as described by Bligh and Dyer (1959). The lower lipid-containing phase was recovered with a drawn-out Pasteur pipette, and the solvent evaporated under a stream of nitrogen. Lipids were redissolved in 100 µl of chloroform and counted by liquid-scintillation counting using OptiPhase HiSafe 3 scintillation fluid.

ACBP content was determined in four individual wells using the sandwich e.l.i.s.a. procedure described by Hansen et al. (1991). The cell layer in the microwell was washed once with PBS (37 °C) and sonicated for 2 s in 200 µl of Tris-buffered saline (8.0 mM Tris/HCl, 2.7 mM KCl, 137 mM NaCl and 0.02% NaN₃, pH 9.0) and stored at –80 °C until assayed.

Determination of [³⁵S]methionine incorporation into total protein

Two portions (0.6 µl) were taken from the extract of each pulse-chase well and plated on small pieces of 3 MM filter paper (approx. 5 mm²). The filters were carefully floated on a trichloroacetic acid solution (10%, w/v; 0 °C) with carrier methionine (1 mg/ml) and were allowed to stand for 30 min. The temperature was raised to 85 °C, and the filters were allowed to stand for a further 30 min and then washed twice for 15 min in trichloroacetic acid (5%, w/v; 0 °C) with methionine and once for 15 min in ethanol (96%, v/v). The filters were dried and counted by liquid-scintillation counting using OptiPhase HiSafe 3 scintillation fluid. Double determinations deviated less than 6% from the mean using this method.

Preparation of 100 000 g supernatant used for identification of ACBP in two-dimensional polyacrylamide gels

Cells from five to eight culture flasks (80 cm²) were scraped off with a rubber 'policeman' and harvested by centrifugation at 400 g for 10 min. The supernatant was aspirated and the pellet was dispersed in 3.0 ml of PBS (pH 7.4, 0 °C) with protease inhibitors [0.22 mM phenylmethanesulphonyl fluoride (PMSF), 0.2 µg/ml leupeptin, 0.4 µg/ml antipain, 0.08 µg/ml Tos-Phe-CH₂Cl, 0.04 µg/ml Tos-Lys-CH₂Cl]. The cells were disrupted by Potter-Elvehjem homogenization and centrifuged for 30 min at 100 000 g. The supernatant was made 0.5 mM with dithiothreitol and concentrated using a Centricon-3 micro concentrator (Amicon, Danvers, MA, U.S.A.). Then proteins were freeze-dried and redissolved in 2D-lysis buffer and stored at –80 °C.

Immunoprecipitation

H-4-II-E cells were cultured to confluence in 35-mm diam. culture wells (Nunc) and labelled for 24 h with 100 µCi of [³⁵S]cysteine/[³⁵S]methionine (specific radioactivity 1165 Ci/mmol; NEN-Du Pont) in 1 ml of medium as described above. At the end of the labelling period, the cell monolayers were washed twice with PBS (37 °C), and then 1 ml of detergent buffer (50 mM Tris/HCl, 150 mM NaCl, 1.0% Nonidet P-40, 0.5 mM dithiothreitol and 0.02% NaN₃, pH 7.5) with protease inhibitors as above was added to each culture well. The cells were lysed by agitation for 60 min at room temperature, and the lysates were collected and centrifuged for 5 min at 5000 g. The supernatants were transferred to tubes containing 25 µl of Protein A-Sepharose coated with either anti-(rat ACBP) IgG or preimmune IgG (control). The tubes were agitated end-over-end at 4 °C for 10 h. The Sepharose beads were pelleted by centrifugation, and the supernatant was concentrated and made 0.5 mM with dithiothreitol using a Centricon-3 microconcentrator. The concentrated

supernatant was freeze-dried and redissolved in 2D-lysis buffer. The Sepharose was washed twice with detergent buffer, drained and immunoprecipitated proteins were extracted by mixing with 2D-lysis buffer.

Two-dimensional PAGE

Non-equilibrium pH-gradient electrophoresis (NEPHGE) was performed with 5% (v/v) Ampholine carrier ampholyte pH 3.5–10 (LKB, Bromma, Sweden) as previously described by O'Farrell et al. (1977). First-dimension rod gels (130 mm × 1.5 mm diameter) with 4% acrylamide. Samples were applied and run at constant current (0.1 mA/gel) until the voltage had reached 300 V and then at constant voltage (400 V) for 4.5 h. First-dimension gels were frozen in buffer [60 mM Tris/HCl, 100 mM dithiothreitol, 2% (w/v) SDS and 10% (v/v) glycerol, pH 6.8] immediately after electrophoresis and stored at -80°C . Electrophoresis in the second-dimension was essentially as described by Schagger and von Jagow (1987): gels were 1 mm thick and 140 mm wide, and the height of the stacking gels were 20 mm and that of separation gels was 130 mm. Stacking gels were 4% acrylamide and the separation gels were 15% acrylamide; both stacking and separation gels contained 2.6% cross-linker of total acrylamide. First-dimension gels were quick-thawed and, without equilibration, mounted on the top of the second-dimension gel by using buffer, pH 6.8, containing 1% (w/v) agarose, 60 mM Tris/HCl, 2% (w/v) SDS and 100 mM dithiothreitol; electrophoresis was performed at 10 mA/gel for 2 h and then at 25 mA/gel overnight. Gels were fixed in 50% (v/v) methanol/20% (v/v) acetic acid for 1 h and in 25% (v/v) ethanol/5% (v/v) acetic acid for 10 min and finally prepared for fluorography by soaking in Amplify (Amersham) for 20 min. Gels were dried and exposed to X-ray film (AGFA Curix RP2) at -70°C . The gels were matched with the developed X-ray film and the gel plugs were carefully excised and counted. Molecular-mass standard proteins ranging from 2.5 to 17.0 kDa were from Sigma.

Western blotting and identification of ACBP

Extracts (20–60 μg of protein) from [^{35}S]methionine-labelled cells were mixed with 100 000 *g* fraction proteins (150 μg) from unlabelled cells and subjected to two-dimensional gel electrophoresis as described above. Proteins were blotted on to Hybond-ECL 0.45 μm nitrocellulose membrane (Amersham) by using a semidry blotter (Kem-En-Tec, Copenhagen, Denmark). Transfer conditions were 0.8 mA/cm² for 1 h at room temperature, and cathode and anode buffers consisted of 0.1 M Tris, 0.1 M Tricine and 15% (v/v) methanol, pH 8.25, and 0.2 M Tris/HCl and 15% (v/v) methanol, pH 10.4, respectively. After transfer the membrane was dried and exposed to X-ray film (Hyperfilm-TM; Amersham). After exposure, the membrane was blocked for 1 h in diaminopropane buffer (20 mM diaminopropane, 150 mM NaCl, 0.05% Tween-20 and 0.02% NaN₃, pH 10.0) containing 3% BSA, washed for 5 min in diaminopropane buffer without BSA and then incubated overnight at room temperature in diaminopropane buffer containing 3% BSA and 0.28 $\mu\text{g}/\text{ml}$ rabbit anti-ACBP antibody. The membrane was washed twice for 5 min in diaminopropane buffer without BSA and once for 5 min in TBST (50 mM Tris, 150 mM NaCl and 0.05% Tween-20, pH 7.9) followed by incubation for 3 h in TBST containing 3% BSA (pH 7.9) and peroxidase-conjugated pig anti-(rabbit IgG) antibody (DAKO, P217 diluted 1:1000; Dakopat, Copenhagen, Denmark). Finally, the membrane was washed five times for 10 min each in TBST (pH 7.9), and immunoreactive spots were visualized by the enhanced chemi-

luminescence detection system (Amersham) as recommended by the manufacturer.

Calculation of the half-life

Radioactivity in ACBP or total protein, corrected for radioactive decay, was plotted in a semilogarithmic plot as a function of chase time. The slope of the curve was fitted by linear-regression analysis assuming that the loss of radioactivity followed first-order kinetics, and the rate of turnover was calculated in terms of half-life ($t_{1/2}$).

RESULTS AND DISCUSSION

Our approach to the determination of ACBP turnover involved pulse-chase experiments combined with determination of radioactivity in ACBP separated from other proteins by two-dimensional gel electrophoresis.

Preliminary isoelectric-focusing experiments using the Phast-System (Pharmacia) showed that native mouse and rat ACBP have pI values of about 9, whereas native human ACBP has a pI of 5.9 (results not shown). To identify ACBP in two-dimensional gels, cultures of mouse 3T3-L1 and 3T3-C2 cells, rat H-4-II-E cells and human HepG2 cells were labelled with [^{35}S]methionine. Total protein was extracted, mixed with an unlabelled 100 000 *g* protein fraction, and subjected to two-dimensional gel electrophoresis. Proteins were blotted on to nitrocellulose membranes and the distribution of [^{35}S]methionine-labelled proteins was visualized by autoradiography. The location of ACBP on the same membrane was then determined by using specific antibodies and enhanced chemiluminescence detection (Figures 1–3). The two-dimensional gel electrophoresis protocol used gave an even distribution of low-molecular-mass proteins, and, by superimposing the autoradiographs, unambiguous identification of ACBP in extracts from all types of cells was achieved. In accordance with the results obtained by isoelectric focusing under non-denaturing conditions, ACBP from mouse 3T3-L1

Figure 1 Identification of rat ACBP by two-dimensional NEPHGE

(a) Labelled proteins (approx. 200 μg) extracted from rat H-4-II-E cells were separated by two-dimensional gel electrophoresis and blotted on to a nitrocellulose membrane. The blot was exposed to hyperfilm-TM for 5 days. (b) The immunoreactive spot on the same blot was identified by using the enhanced chemiluminescence technique. The blot was exposed for 2 min. Only one immunoreactive spot could be detected and the entire blot therefore is only part of the gel shown. The left side of the gel is the basic part. For experimental details, see the Materials and methods section.

Figure 2 Western blot and fluorograms of [^{35}S]methionine-labelled HepG2 cells

Labelled proteins (approx. 200 μg) extracted from HepG2 cells were separated by two-dimensional gel electrophoresis and blotted on to a nitrocellulose membrane. The membrane was exposed to hyperfilm-TM for 5 days and then immunostained by the enhanced chemiluminescence technique using monospecific rabbit anti-(rat ACBP) antibody. (a) Immunodetection of human ACBP (pI 5.9). (b, c) Fluorograms of cells labelled for 24 h and chased with unlabelled methionine for 12 h (b) and 48 h (c). The position of ACBP is indicated with an arrow. Only one immunoreactive spot could be detected on the entire blot and therefore is only a part of the gel shown. The left side of the gel is the basic part.

and 3T3-C2 cells and rat H-4-II-E cells was found in the basic half of the gels, whereas ACBP from human HepG2 cells was found just to the acidic side of the gels.

Initially a double-labelling strategy using ^3H -labelled ACBP as internal standard was attempted. However, purification and freeze-drying of the ^3H -labelled ACBP introduced microheterogeneity which resulted in the appearance of a number of radioactive spots in two-dimensional gels.

In contrast, no microheterogeneity was observed when ACBP in crude cell extracts was analysed. Therefore the double-labelling strategy was abandoned and the turnover of ACBP was determined from the recovery of ^{35}S -labelled ACBP from the gels. To examine the reproducibility of this procedure, equal amounts of labelled cell extracts were loaded on to four gels. From each gel, 12 low-molecular-mass proteins were excised and the amount of radioactivity was determined. The mean $\pm$ S.D. was calculated for each protein. In the most unfavourable case, the S.D. was 17.3 % of the mean. This accuracy was judged to be sufficient for the turnover studies.

Contamination of the ACBP spot with co-migrating protein could result in serious errors in the determination of the ACBP turnover rate. We therefore investigated this problem using immunoprecipitation. An H-4-II-E cell lysate was treated with Protein A-Sepharose coated with either affinity-purified rabbit anti-ACBP antibodies or preimmune IgG, and the treated lysates were subjected to two-dimensional gel electrophoresis. Figure 4 shows that the spot identified as ACBP was completely absent

Figure 3 Western blot and fluorograms of proteins from 3T3-L1 and 3T3-C2 cells labelled with [^{35}S]methionine

(a) Immunodetection of murine ACBP in differentiated 3T3-L1 cells was carried out as described for HepG2 and H-4-II-E cells (Figures 1 and 2). Immunostaining of the entire Western blot showed only one immunoreactive spot, therefore only part of the gel is shown. The position of ACBP (about pI 9.0) is indicated with an arrow. (b-e) Fluorograms of proteins from cells labelled for 24 h and chased for 0 h (b), 12 h (c), 24 h (d) and 96 h (e). These four gels were exposed simultaneously to the same film. The two spots marked with asterisks in b to e represent tentatively identified strongly labelled 3T3-L1 adipocyte marker protein. (f) Fluorogram of confluent 3T3-C2 cells labelled for 24 h and chased for 96 h. The left side of the gel is the basic part. The ACBP spot is indicated with an arrow. For experimental details, see the Materials and methods section.

from the lysate treated with the anti-ACBP antibodies, but clearly present in the lysate treated with preimmune IgG. Two-dimensional gel electrophoresis of the immunoprecipitates

Figure 4 Immunoprecipitation of ACBP in lysates of H-4-II-E cells

An ^{35}S -labelled H-4-II-E cell lysate was treated with Protein A-Sepharose coated with either preimmune IgG (a) or affinity-purified rabbit antibodies (b) and subsequently analysed by two-dimensional NEPHGE followed by autoradiography. In (a) ACBP is indicated by an arrow and the corresponding location in (b) is indicated. For experimental details, see the Materials and methods section.

Figure 5 Semilogarithmic plot showing the kinetics of loss of radioactivity in ACBP (a) and trichloroacetic acid-precipitable proteins (b) from H-4-II-E cells labelled with [^{35}S]methionine and chased

The curves were fitted by using linear-regression analysis assuming first-order degradation kinetics. For experimental details, see the Materials and methods section.

demonstrated that ACBP was present in the precipitates with the anti-ACBP antibodies, but absent from precipitates with pre-immune IgG (results not shown). Thus no H-4-II-E proteins comigrate with rat ACBP. Hypothetically, proteins co-migrating with ACBP could be present in the mouse cell lines 3T3-L1 and 3T3-C2 or the human HepG2 cell line. However, the presence of clearly resolved and symmetrical ACBP spots in lysates from these cell lines and the similarity of the turnover rate of ACBP in all cell lines (see below) argue that this is not the case.

ACBP is abundant in liver (Mikkelsen and Knudsen, 1987).

Table 1 Loss of radioactivity in ACBP and total trichloroacetic acid-precipitable protein

The rate of loss is given as the half-life. The correlation coefficient and the number of measuring points used for the calculations are indicated in parentheses. For experimental details see the Materials and methods section.

Cell line	Half-life for ACBP (h)	Half-life for total trichloroacetate-precipitable protein (h)
3T3-C2, confluent	25 ($r^2 = 0.95$, $n = 9$)	33 ($r^2 = 0.93$, $n = 10$)
3T3-L1, adipocyte	40 ($r^2 = 0.99$, $n = 7$)	47 ($r^2 = 0.97$, $n = 8$)
H-4-II-E, confluent	35 ($r^2 = 0.96$, $n = 10$)	43 ($r^2 = 0.94$, $n = 10$)
HepG2, growing	53 ($r^2 = 0.85$, $n = 10$)	63 ($r^2 = 0.98$, $n = 10$)

Hence, ACBP turnover was analysed in two liver-derived cell lines, the rat H-4-II-E cell line and the human HepG2 cell line. Figure 5 illustrates the loss of radioactivity in ACBP and total protein in a pulse-chase experiment using rat H-4-II-E cells. In these cells, ACBP was degraded with a half-life of 35 h. Radioactivity in total protein was lost at a slightly lower rate corresponding to a half-life of 43 h. In HepG2 cells, ACBP appeared to be even more stable. In these cells the half-life of ACBP was determined to be 53 h. Similarly to rat H-4-II-E cells, radioactivity in total protein was lost at a slightly lower rate corresponding to a half-life of 63 h (Table 1). The two cell lines HepG2 and H-4-II-E behaved slightly differently in culture. The H-4-II-E cells showed cell growth contact inhibition at confluence and therefore had a constant cell number during the whole chase period. The HepG2 cells, in contrast, did not show cell growth contact inhibition and therefore continued to grow during the chase period with the result that total protein increased from 53 to 77 μg per well.

The mouse 3T3-L1 cell line is a subline of the fibroblast line 3T3 which, on reaching confluence, is able to differentiate into adipocytes (Green and Kehinde, 1974). We have shown that this differentiation results in a marked increase in the level of ACBP and its cognate mRNA (Hansen et al., 1991). The related subline 3T3-C2 is refractory to differentiation and remains fibroblastic (Mackall et al., 1976). We have employed these cell lines to examine whether differentiation from the preadipocyte state to the adipocyte state was associated with marked changes in the stability of ACBP. The extent of differentiation was followed by measuring protein content, ACBP content and total lipid synthesis (Figure 6). The result shows that the 3T3-L1 cells were almost fully differentiated on day 5 and the pulse-chase experiment was therefore started at this time point. The results also showed that the lipogenic capacity was high and constant throughout the chase period. The differentiated 3T3-L1 cells incorporated 40–50 % of the added [^{14}C]acetate into extractable lipids, whereas 3T3-C2 only incorporated 1–2 %. The ACBP concentration in 3T3-L1 cells was increased to 600 ng/mg of protein on differentiation, a value similar to those reported by Hansen et al. (1991). The induction of ACBP was delayed compared with induction of lipid synthesis as previously observed by Hansen et al. (1991).

The half-lives of ACBP in 3T3-C2 fibroblasts cultured without insulin and 3T3-L1 adipocytes cultured with insulin were 25 and 40 h respectively (Table 1). This difference in half-life is most likely caused by insulin. The half-life of glutamine synthetase, a protein that is also induced during the differentiation of 3T3-L1 cells, changed from 24 h in 3T3-L1 adipocytes cultured without insulin to 32 h in 3T3-L1 adipocytes stimulated with supraphysio-

Figure 6 Differentiation of 3T3-L1 cells into adipocytes

Cells were grown to confluence in micro wells and stimulated with standard medium containing dexamethasone (Dex; 1 μ M) or insulin (10 μ g/ml) as shown by horizontal bars. Labelling with [³⁵S]methionine (L) and the chase period are also indicated by horizontal bars. □, Content of total protein per well; ●, lipid synthesis (d.p.m. in extractable lipids after labelling for 24 h with 0.18 μ Ci of [1-¹⁴C]acetate per well); ▲, ACBP content per well determined by e.l.i.s.a. Every time point represents the mean value of three to four measurements from parallel wells; vertical bars are $\pm$ S.D. ($n=1$).

logical concentrations of insulin (Miller et al., 1983). Insulin has also been reported to decrease the rate of protein degradation in fibroblasts (Hershko et al., 1971) and adipocytes (Minemura et al., 1970). The observed change in ACBP half-life may not affect the ACBP level relative to total protein as the half-life of total trichloroacetic acid-precipitable protein is affected to the same extent (Table 1).

Two low-molecular-mass proteins (labelled with asterisks in Figures 3b–3e) were observed to be strongly labelled in 3T3-L1 adipocytes when compared with confluent 3T3-C2 cells. The most abundant of these proteins was positioned just to the acidic side of mouse ACBP (pI about 9.0). This protein is likely to be the 15 kDa cytosolic fatty-acid-binding protein 422 (aP2), a marker protein for 3T3-L1 adipocytes for which a pI of 8.5 has been reported (Hresko et al., 1990). The half-life of the tentatively identified protein 422 (aP2) was determined to be 39 h ($r^2 = 0.95$), a half-life almost identical with that determined for ACBP (40 h; Table 1).

As in H-4-II-E and HepG2 cells, radioactivity in ACBP in 3T3-L1 and 3T3-C2 cells was also lost at a slightly higher rate than radioactivity in total protein (Table 1).

In conclusion our results show that ACBP is a rather stable protein in the cell lines analysed. There is a good correlation between content of ACBP mRNA and ACBP-like immuno-reactivity in different peripheral organs and in different regions in the brain (Costa and Guidotti, 1991), indicating that the translational efficiency and breakdown of ACBP is approximately the same in all tissues. However, although it cannot be excluded that the turnover might be higher in, for example, steroidogenic tissue, the present findings are in agreement with previously

published results which showed that the half-life for ACBP in MA-10 mouse Leydig cells and Y-1 mouse adrenal cells exceeded 3 h (Brown et al., 1992).

The long half-life observed in the present study is in keeping with our view that ACBP is a housekeeping protein, a notion that is further substantiated by the finding that the rat ACBP gene has all the characteristics of a typical housekeeping gene (Mandrup et al., 1992). The fact that ACBP specifically binds long-chain acyl-CoA esters with high affinity (Mikkelsen and Knudsen, 1987; Knudsen et al., 1989; Rasmussen et al., 1990; Rosendal et al., 1993) and that, when overexpressed in yeast, it induces an increased long-chain acyl-CoA ester pool (Mandrup et al., 1993) strongly indicates that a basic function of ACBP is related to acyl-CoA metabolism.

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