ATP citrate-lyase and glycogen synthase kinase-3β in 3T3-L1 cells during differentiation into adipocytes

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ATP citrate-lyase (CL), acetyl-CoA carboxylase (ACC) and glycogen synthase kinase-3β (GSK-3β) levels were measured in cytosol from 3T3-L1 cells during differentiation from fibroblasts into fat-cells. Protein levels were estimated from immunoblots using specific antisera. Cytosol from confluent cells contain significant amounts of GSK-3β, which fell during differentiation of these cells into adipocytes. CL from confluent cells was found to be mostly in the form of a single protein band of apparent mass 110 kDa. Levels of CL and ACC increased during cell differentiation into adipocytes. During the first 3 days of differentiation, CL migration changed, and it was expressed as a complex of protein bands of apparent mass 110 kDa, 113 kDa and 115 kDa. At later stages of differentiation, when these cells had assumed the phenotype of fat-cells, they expressed CL mainly as protein bands of 110 and 113 kDa. When samples containing these bands were treated with alkaline phosphatase, the 113 kDa protein band collapsed into the 110 kDa species. This suggests that the slower-migrating species of CL is a higher-order phosphorylation state of the same protein. Furthermore, when purified CL, mostly expressed as the 110 kDa species, was phosphorylated with cyclic AMP-dependent protein kinase alone or together with GSK-3 and resolved by SDS/PAGE, the phosphorylated CL now migrated more slowly as the 113 kDa and 115 kDa forms. CL phosphorylation was hormone-regulated, since, in samples from fat-cells that had the complex two-band pattern, when cultured in medium without serum or hormones, CL migration reverted to a single band of 110 kDa, similar to confluent cells. Treatment of these 'down-regulated' cells with insulin rapidly induced substantial amounts of the 113 kDa species, with a concomitant decrease in the 110 kDa species.

INTRODUCTION

Glycogen synthase kinase-3 (GSK-3) was identified as a protein kinase that phosphorylates glycogen synthase and causes its inactivation [1–4]. Insulin causes dephosphorylation of these GSK-3 target sites, resulting in enzyme activation. How insulin causes this dephosphorylation is unknown, but it had appeared unlikely that inhibition of GSK-3 activity accounted for this effect [4].

At about the same time as identification of GSK-3, a protein kinase that specifically phosphorylates ATP citrate-lyase (CL) was described [5,6], named ATP citrate-lyase kinase and subsequently renamed multi-functional protein kinase [7] to emphasize its spectrum of substrates and its F_A activity. *In vitro*, this kinase also phosphorylated glycogen synthase and acetyl-CoA carboxylase (ACC), decreasing their activities [7,8], and phosphorylated inhibitor-2 [7]. GSK-3 was also found to phosphorylate inhibitor-2 and the protein-synthesis initiation factor eIF-2B [9].

As studies on the insulin effects on CL phosphorylations [10,11] suggested that sites specifically phosphorylated by CL kinase were decreased, and as direct assay of minimally treated supernatants from insulin-treated adipocytes [12] demonstrated a decrease in the activity of this kinase when assayed with glycogen synthase or CL as substrates, we proposed that insulin decreased the activity of this kinase. Furthermore, we suggested that this was an important mechanism in the control of enzymes of metabolism by insulin [7,13].

CL kinase is now known to be identical with GSK-3, and, by analogy with the GSK-3/glycogen synthase relationship, it was proposed that insulin decreased the activity of GSK-3 as one mechanism to account for the known insulin control of glycogen synthase activity [14]. Recently, using eIF-2B as substrate, Welsh and Proud [9] found that insulin treatment of CHO cells also markedly decreased GSK-3 activity.

Molecular cloning revealed the existence in mammals of two highly related proteins, termed GSK-3α (the first one purified) and GSK-3β [15]. These proteins, of 51 kDa and 46 kDa respectively, are similar in their catalytic properties. GSK-3 is highly phosphorylated on tyrosine *in vivo*, and its activity varies as a function of this phosphorylation [16]. In addition, GSK-3β activity (not GSK-3α) is specifically inhibited by phosphorylation by various forms of protein kinase C [17].

The mechanism by which insulin affects both protein phosphorylation and dephosphorylation [18–23] is not understood. Three key proteins involved in metabolism, glycogen synthase (EC 2.4.1.11), ACC (EC 6.4.1.2) and CL (EC 4.1.3.8) [24–26], are affected by insulin in this complex manner. Studies on CL have shed some light on this phenomenon [11,27]. CL has four phosphorylation sites, three structural and one catalytic histidyl site. The structural sites are spaced four residues apart (-SGSTST$_{446}$PAPS$_{450}$RTAS$_{454}$FSESR-) [11]. Serine-454 is phosphorylated by cyclic-AMP-dependent protein kinase (A-kinase) [28,29], by an insulin-stimulated kinase [30] and by S6 kinase [31]. Threonine-446 and serine-450 are phosphorylated only by GSK-3 [11]. The lyase phosphorylation sequence strongly

Abbreviations used: DMEM, high-glucose Dulbecco's minimal Eagle's medium; GSK-3, glycogen synthase kinase-3; A-kinase, cyclic-AMP-dependent protein; CL, ATP citrate-lyase; ACC, acetyl-CoA carboxylase; IM+, Induction medium plus; IM−, Induction medium minus; LBM, low basal medium; TBS, Tris-buffered saline; ECL, enhanced chemiluminescence.

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resembles the configuration about glycogen synthase site-3. This similarity is of special interest, as it is known that insulin decreases the phosphorylation of sites-3 a–d, affecting its enzyme activity, albeit in skeletal muscle [4,32].

CL, a tetramer (molecular mass 440 kDa) of four identical subunits, is a principal enzyme of both fatty acid and cholesterol biosyntheses [33]. It catalyses the conversion, in the cytosol, of citrate and CoA into oxaloacetate and acetyl-CoA. CL activity varies as a function of hormone and metabolic state [34–36], and these activity changes in part are due to changes in the amount of enzyme under control of transcription [37]. It has not been possible to show that CL activity is regulated by phosphorylation.

As insulin effects on the phosphorylation of epididymal fat-cell CL were substantial [18,38,39], an investigation of both CL and one of its specific kinases (GSK-3) was undertaken in the murine 3T3-L1 cell line [40–42]. We found that, for CL, its level and phosphorylation state change in a complex manner in response to insulin treatment and differentiation, and for GSK-3, its level changes with differentiation, consistent with the observed concomitant changes in CL phosphorylation.

EXPERIMENTAL

Materials

CL and ACC were purified from rat liver [7,43]. Rabbit polyclonal antibodies against purified rat CL and ACC holoenzymes were generated by standard techniques and are the same antibodies as used in our prior immunoprecipitation studies [7,44]. Anti-trypE/GSK-3β and anti-trypE/GSK-3α antibodies that have been fully characterized [15] were used to blot for GSK-3 isozymes in 3T3-L1 cell fractions. The catalytic subunit of A-kinase was purified from rabbit skeletal muscle [45]. GSK-3α was purified from calf brain and rat liver [14]. P-81 chromatography paper was purchased from Whatman. Tween-20, insulin, dexamethasone and protein kinase inhibitor peptide were from Sigma. Intestine alkaline phosphatase (molecular-biology grade, 3 units/μg of protein) was from Boehringer. Acrylamide, as a 40% solution, was from BDH, and bisacrylamide, as a 2% solution, was from National Diagnostics Corp. Reagents for Western blotting were from Bio-Rad Corp. Enhanced chemiluminescence (ECL) reagents were from Amersham. Ecolume was purchased from ICN Radiochemicals. [γ-^{32}P]ATP (sp. radioactivity 6000 Ci/mmol) was from New England Nuclear. other chemicals were of the highest grade available. Protein was measured by the Bradford Coomassie R250 dye technique (Bio-Rad).

Cells and cell culture conditions

The differentiation of 3T3-L1 cells closely follows the schema of Rubin et al. [42]. 3T3-L1 cells, purchased from A.T.C.C., are grown to confluency in 10 cm plastic plates in DMEM (high-glucose Dulbecco's minimal Eagle's medium) containing 10% calf serum, with glutamine and non-essential amino acids added to final concentrations of 2 mM and 0.1 mM respectively (maintenance medium). Cells are maintained in an atmosphere of air/CO_2 (19:1) at 37 °C. At 1 or 2 days post-confluency, the medium is changed to one containing 10% fetal-calf serum, 5 μg/ml insulin, 0.5 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine in DMEM with added glutamine and non-essential amino acids (Induction medium plus; IM+). After 72 h, the medium is changed to one containing 10% fetal-calf serum and 2.5 μg/ml insulin in DMEM with added glutamine and non-essential amino acids (Induction medium minus; IM−). The cells swell and synthesize triacylglycerols, and by 2–3 days of culture in IM− they are filled with fat droplets and the plate is covered by an opaque film of fat. To 'down-regulate' these cells, adipocytes are cultured in a medium consisting of DMEM with added glutamine and non-essential amino acids and 0.15% albumin (low basal medium; LBM).

FTO-2b hepatoma [46,47] cells are grown in medium consisting of DMEM/F12 (1:1, v/v), with 10% fetal-calf serum. At the start of the [^{32}P]P_i experiment, cells are cultured for 24 h in media without fetal-calf serum. At the conclusion of all experiments, plates are rapidly rinsed twice with warm PBS. Cells are frozen in liquid N_2 by floating the plate in the cold liquid and stored at −70 °C. Cells are harvested by addition of 300 μl of buffer A to each frozen plate. Buffer A is 40 mM β-glycerol phosphate, 0.2% Nonidet P40, 5 mM $MgCl_2$, 40 mM NaF, 20 mM *p*-nitrophenyl phosphate, 1 mM EDTA, 1 mM EGTA, 100 μM sodium vanadate, 0.2 mM phenylmethanesulphonyl fluoride, 500 μg/l leupeptin and aprotinin and 2 mM dithiothreitol, final pH 7.0. The cell slurry is harvested and after 10 min the mixture is homogenized in a Dounce homogenizer (20 strokes) and centrifuged at 6000 ***g*** for 6 min. The supernatant is centrifuged at 30000 ***g*** for 20 min to obtain the mitochondrial pellet, and the supernatant is again centrifuged at 104000 ***g*** for 60 min to obtain the microsomal-glycogen pellet and the cytosol fraction.

Western blotting of CL, ACC and GSK-3β

Samples are treated with 4× sample buffer (60 mM Tris/HCl, pH 6.8, 14.4 mM 2-mercaptoethanol, 25% glycerol, 2% SDS and 0.1% Bromophenol Blue), heated in a boiling-water bath, and samples containing 20 μg of protein are resolved by SDS/PAGE (7% acrylamide), gel dimensions being 13.5 cm × 14.7 cm × 0.12 cm for samples containing CL and ACC. Electrophoresis is at a constant voltage of 90 V for 16 h with 25 mM Tris/HCl, 192 mM glycine and 0.15% SDS, pH 8.7, as the buffer. To detect GSK-3β, samples of the supernatant (20 μg of protein) are resolved on a 10%-acrylamide gel with dimensions of 9.8 cm × 14.7 cm × 0.12 cm. Electrophoresis is at a constant voltage of 180 V for 4 h.

Electrotransfer of proteins to nitrocellulose ('Genie Transfer' apparatus, Idea Scientific Co.) is at 24 V, with the transfer buffer consisting of 25 mM Tris/HCl, 192 mM glycine and 20% methanol. The buffer contained 0.02% SDS for the transfer of CL and ACC, and 0.05% SDS for the transfer of GSK-3β. After blocking with a non-fat dry milk solution, the nitrocellulose is washed with Tris-buffered saline (TBS: 10 mM Tris, 150 mM, NaCl, pH 7.5) and then incubated with the primary antibody in TBS with 1% albumin and 0.05% Tween-20 (dilution being 1:1000, 1:1000 and 1:450 for CL, ACC and GSK-3β respectively). Blots are washed with TBS containing 0.2% Tween-20, and then incubated with horseradish peroxidase conjugate (dilution 1:20000) for 45 min in TBS with 1% albumin and 0.05% Tween-20. Blots were washed with TBS/Tween and revealed by using the ECL detection system. Based on appearance, exposures are varied from 3 to 180 s to obtain a satisfactory image. CL and ACC band intensities are quantified by a Joyce–Loebel optical densitometer [38].

Alkaline phosphatase treatment

Cytosolic proteins in buffer A were diluted with an alkaline buffer (final concns. 50 mM glycine, pH 10, 2 mM $MgCl_2$, 0.1 mM phenylmethanesulphonyl fluoride and one-quarter the buffer A concentrations). Alkaline phosphatase (7 units;

1 unit/μg), was added to a final volume of 25 μl containing 0.5 μg/μl protein, and the sample was incubated overnight.

RESULTS

Electrophoretic mobility of CL from 3T3-L1 cells treated with insulin

To determine CL electrophoretic-mobility patterns from 3T3-L1 fat-cells and the effect of insulin on them, cells were grown to confluency and differentiated into adipocytes. They were washed with PBS and cultured for an additional 12 h in LBM to remove them from hormone and serum stimulation. Insulin was added to some plates (Figure 1), and cytosol was prepared.

CL, from either confluent cells or differentiated cells cultured in medium without hormones (LBM) (lanes 1, 3 and 5), migrated as a major band of 110 kDa, with a diffuse and faint component migrating more slowly. CL from fat-cells (lane 2) migrated as two bands of apparent mass 113 and 110 kDa. Insulin addition to fat-cells cultured for 12 h in LBM (lanes 4 and 6) rapidly caused a significant portion of its CL to migrate as a band of 113 kDa. We also observed that insulin addition (1 nM) to LBM media during the 12 h of culture in medium without hormones completely prevented the 'down-regulation' of these two bands to one.

Effect of alkaline phosphatase treatment on the migration of CL

Cytosol from adipocytes was treated with highly purified alkaline phosphatase, incubated overnight, and after electrophoresis and protein transfer to nitrocellulose, CL was detected by Western blotting. CL from control fat cells (Figure 2, lanes 1 and 3) migrated as two bands. Alkaline phosphatase treatment (lanes 2 and 4), caused CL to migrate as a single band of 110 kDa, consistent with CL blots from confluent 3T3-L1 cells and LBM-cultured fat-cells. These results demonstrate that heterogeneity of CL bands on gel electrophoresis is due to differences in phosphorylation *in vivo* of the CL subunit.

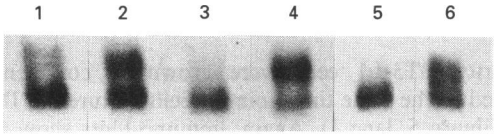

Figure 1 Electrophoretic mobility of CL from 3T3-L1 cells treated with insulin

3T3-L1 cells were differentiated into fat-cells as described in the text. Cells were harvested and cytosol proteins were resolved by SDS/PAGE and transferred to nitrocellulose for Western blot analysis, revealed by the ECL technique. Lanes: 1, confluent cells; 2, fat-cells cultured in IM− for 2.5 days; 3, fat-cells cultured in LBM for 12 h; 4, fat-cells cultured in LBM for 12 h, followed by addition of insulin (1 nM) for 20 min. Lanes 5 and 6 are from a different experiment: 5, fat-cells cultured in LBM for 25 h; 6, fat-cells cultured in LBM for 12 h, followed by additional culture for 13 h with insulin (1 nM). The load in each well was 20 μg of protein.

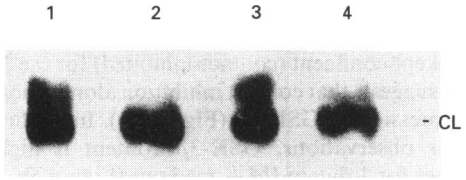

Figure 2 Effect of alkaline phosphatase treatment on the electrophoretic mobility of CL

Cytosol protein from 3T3-L1 fat-cells (grown in IM− for 3 days) was treated with alkaline phosphatase, resolved by gel electrophoresis, and CL was detected as described in the text. Lanes: 1, fat-cells incubated at 37 °C overnight; 2, fat-cells incubated with alkaline phosphatase overnight at 37 °C; 3, fat-cells incubated at 25 °F; 4, fat-cells incubated with alkaline phosphatase at 25 °C. The load in each lane was 10 μg of protein.

Incorporation of [^{32}P]P_i into FTO-2b-cell CL

Insulin increases the incorporation of [^{32}P]P_i into CL in rat epididymal fat-cells [16,39,48]. To determine whether, *in vivo*, newly phosphorylated CL from insulin-treated cells migrates more slowly than does CL from controls, [^{32}P]P_i incorporation into CL from control (serum-starved) and insulin-treated FTO-2b cells was studied.

Cells grown without sera for 24 h were incubated in phosphate-free DMEM containing 100 μCi/ml [^{32}P]P_i for 1 h. As shown by Western blot analysis (Figure 3, lane 2), insulin treatment caused a substantial amount of CL to migrate as the 113 kDa species. The insulin-induced increase in CL radioactivity is associated with the more slowly migrating band. In the control, the radioactive CL band, though less intense, also migrates a little less rapidly than the substrate CL species (110 kDa), suggesting that all newly phosphorylated CL migrates less rapidly. The small amount of radioactivity associated with the control 110 kDa region of the gel may represent non-specifically adsorbed [^{32}P]P_i associated with CL or another radiolabelled protein. In addition, the results in Figure 4 show that CL from another insulin-sensitive cell line when treated with insulin behaves similarly on Western-blot analysis to CL from 3T3-L1 cells.

Effect of 3T3-L1 cell differentiation on the amounts of cytosolic CL, ACC and GSK-3β

Cytosol from 3T3-L1 cells at various time points during differentiation were immunoblotted for the three enzymes of interest (Figure 4). This Figure is a composite of findings from three sequential and independent differentiation series, each of which luminesced at slightly different intensities. An additional seven series were carried out that confirmed each observation at least three times. At pre-confluency and confluency (Figure 4b, lanes 1 and 2), one major CL band of apparent mass 110 kDa was seen. During differentiation of these cells in IM+, which

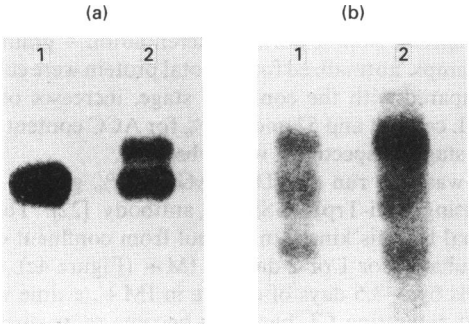

Figure 3 Effect of insulin on the incorporation of ^{32}P[P_i] into CL and on its electrophoretic mobility in FTO-2b cells

Cells were cultured for 24 h in serum-free media. Cells were washed in phosphate-free DMEM, and this medium containing 100 μCi/ml [^{32}P]P_i was added and incubations were continued for 1 h. Cells were harvested and CL in the cytosol was detected. (**a**) Lane 1, control; lane 2, insulin (2 nM) for 10 min. (**b**) Autoradiogram of the nitrocellulose immunoblot: lanes 1 and 2 correspond to the samples in (**a**). The load in each well was 20 μg of protein.

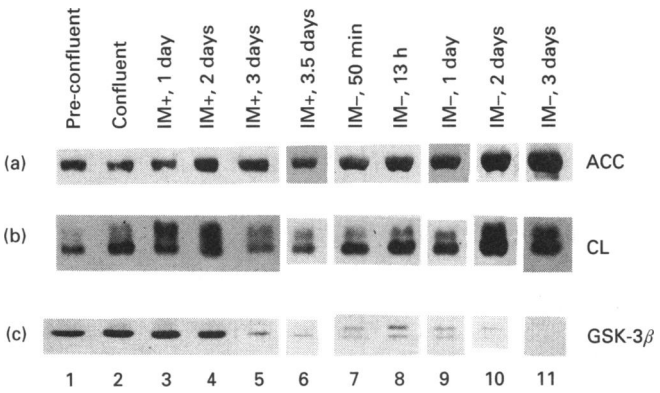

Figure 4 Effect of 3T3-L1 cell differentiation on the amounts of CL, ACC and GSK-3β in the cytosol

Confluent 3T3-L1 cells were differentiated into adipocytes. Cells were harvested at different time points and enzymes of interest were detected by ECL: (**a**) ACC; (**b**) CL; (**c**) GSK-3β, run on a 10% gel. Lanes are described in the Figure. This is a selected composite of three separate experiments. For quantification, all values were normalized to the confluent.

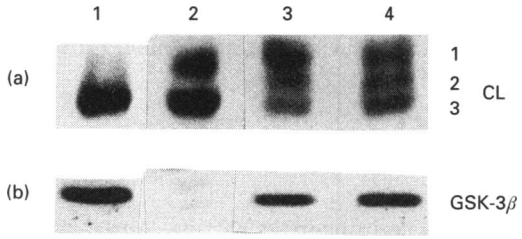

Figure 5 CL immunoblot patterns from cells during differentiation

Confluent 3T3-L1 cells were differentiated into adipocytes. Protein (20 μg) was loaded in each lane of a 7.5%-acrylamide gel, and electrophoresis and immunoblot detection by ECL were performed as described in the text. (**a**) Western blots of CL; (**b**) Western blots of GSK-3β. Lanes: 1, post-confluent cells, 3 days; 2, cells differentiated into fat-cells (IM− for 15 hours); 3, cells cultured in IM+ for 1 day; 4, cells cultured in IM+ for 1 day and in IM− for 30 min. The load in each well was 20 μg of protein.

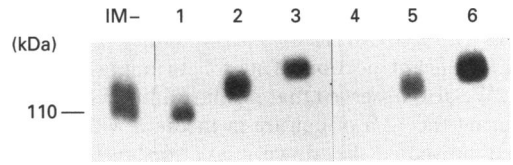

Figure 6 CL immunoblot patterns of purified enzyme phosphorylated *in vitro* by A-kinase and GSK-3

Rat liver CL (7.5 μg) purified without addition of phosphatase inhibitors [7] was phosphorylated with A-kinase or with A-kinase plus GSK-3 in buffer (total volume 100 μl), consisting of 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 μM ATP containing 60 μCi/nmol [γ-^{32}P]ATP. A 150 ng portion of total protein was electrophoresed as described. Lanes: 1 and 4, control CL: 2 and 5, A-kinase-phosphorylated CL: 3 and 6, A-kinase + GSK-3-phosphorylated CL. Lanes 1–3, immunoblots, 4–6, autoradiogram. 'IM−' indicates position of 20 μg of cytosolic protein from fat-cells cultured in IM− that was electrophoresed adjacent to the experimental samples as marker.

contains insulin, dexamethasone and isobutylmethylxanthine, CL gives more diffuse blots, with a substantial portion migrating with an apparent mass of 115 kDa (lanes 3–6). Interestingly, the CL blot pattern changed after only 12 h of culture in IM+. However, a reproducible and substantial increase in CL content was not found until culture in IM−, after at least 3 days of culture in IM+ (lanes 7–11), a period in which there is increased lipogenesis and gross accumulation of lipid. CL from cells cultured in IM− shows a substantial portion of its mass migrating more slowly than CL from confluent cells. However, this upper band migrates a little faster and as a more discrete band (apparent mass 113 kDa) than that found in samples from IM+ treated cells. This phenomenon was noted after only 50 min of culture of these cells in IM−.

With respect to ACC (Figure 4a), there is an obvious increase in enzyme content during the differentiation of these cells into adipocytes. This increase starts during culture in IM+ and parallels the increase in CL during culture in IM−. Our experience in measuring CL and ACC contents is as follows: samples from 10 independent series of cells undergoing differentiation at three time points (cells that are confluent, cells incubated for 2–3 days in IM+, and cells incubated for 0.5–3 days in IM−) were blotted for CL and ACC contents by ECL technique. Total grains measured by densitometry for CL or ACC for a specific sample during differentiation ÷ grains for the confluent sample normalized for the total protein were calculated. When compared with the confluent stage, increases of 14 and 48% for CL content and 52 and 232% for ACC content in IM+ and IM− stages respectively were observed.

Cytosol was also run on SDS/PAGE (10% gels) to blot for GSK-3β using anti-TrpE/GSK-3β antibody [22]. There is a strong signal for this kinase in cytosol from confluent cells and in cells incubated for 1 or 2 days in IM+ (Figure 4c). GSK-3β content falls by 3–3.5 days of culture in IM+, a time when the most slowly migrating CL band has become more discrete and migrates a little more rapidly, at 113 kDa. This is a period in which there is some morphological differentiation of these cells into adipocytes. GSK-3 content remains low during final differentiation of these cells into adipocytes. The decrease in GSK-3β with differentiation has been observed in 10 independent 3T3-L1 cell-differentiation experiments.

Because these new results suggest that the CL subunit migrates as a minimum of three forms, efforts were made to identify them with clarity. 3T3-L1 cells were grown to confluency and maintained to the same time point as cells cultured in IM+ for 3 days (Figure 5, lane 1). Again, immuno-blots show that CL from confluent cells migrates as a single band with an apparent molecular mass of 110 kDa. After 3 days of growth in IM+, cells were cultured for an additional 15 h in IM− (lane 2). CL now migrates as two bands with apparent molecular masses of 113 and 110 kDa respectively. Other cells, after 1 day of growth in IM+ (lane 3), were refed with IM− for 30 min (lane 4). Cytosol from cells cultured for 1 day in IM+ (lane 3), show a substantial portion of CL as higher-molecular-mass species, migrating with apparent molecular masses of 115, 113 and 110 kDa. Culture of these cells for just 30 min in IM− demonstrates a shift of the bands to the lower-molecular-mass forms (Figure 5a, lanes 3 and 4).

Figure 5 demonstrates two additional points. GSK-3β remains high in cells kept confluent (contact-inhibited) for the 3-day time period. This suggests that contact inhibition alone is not sufficient to induce repression of GSK-3β (Figure 5b). In confirmation of our previous observations, GSK-3β content is high in cells cultured either for 1 day in IM+ medium (Figure 5b, lane 3) or for 2 days (results not shown).

To prove that the CL subunit can exist in three phosphorylated forms, purified enzyme was phosphorylated with A-kinase alone or together with rat liver purified GSK-3 [7]. After SDS/PAGE, various CL species were detected by Western blotting. [γ-^{32}P]ATP was added to monitor newly phosphorylated species, and autoradiograms of these blots were developed. As shown in Figure 6 (lane 1), rat liver CL purified without the addition of

phosphatase inhibitors migrates predominantly as a band of 110 kDa. Phosphorylation with A-kinase alone (lanes 2 and 5), which phosphorylates serine-454, slows its migration, so that CL now migrates as the 113 kDa species. Phosphorylation with both kinases (lanes 3 and 6), which phosphorylates three sites, slows migration even more, so that CL now migrates as 115 kDa species. The autoradiogram of this blot (lanes 4–6) confirms these findings and also demonstrates that both kinases added together phosphorylate CL synergistically [27,49].

DISCUSSION

3T3-L1 fibroblasts cultured with proper factors differentiate into adipocytes [41,42,50] and express co-ordinately multiple gene products leading to regulated triacylglycerol synthesis and degradation providing for controlled energy storage and release [42,51,52]. In this paper, immunoblots were used to determine the relative amounts of CL and ACC, two enzymes important in fat metabolism, and GSK-3, a kinase that is now thought to play an important regulatory role in metabolism.

CL, in 3T3-L1 cells, consists of distinct phosphorylated species in significant abundance. These phospho-forms are affected in a predictable manner by both insulin and differentiation. In 3T3-L1 fat-cells that had been 'down-regulated' by culture in medium without serum or hormones, insulin treatment caused CL to migrate as two bands rather than one, the more slowly migrating species at 113 kDa (Figure 1). Furthermore a more complex pattern of CL bands was found when confluent cells were treated with agents that stimulate insulin-controlled protein kinases, other mitogen-stimulatable kinases, and A-kinase. These CL species were consistent with there being a minimum of three forms of CL that reflect various phosphorylation states (Figure 5). As changes *in vivo* were rapid and were seen on immunoblots, they were deemed substantial. CL in FTO-2b cells also consists of distinct phospho-species that are in significant abundance and respond to insulin in an analogous fashion to those described by 3T3-L1 adipocytes.

Evidence that CL multiple band patterns represent higher-order phosphorylation states of the same protein rests on three observations. Alkaline phosphatase treatment of CL from fat-cells collapsed the two distinct protein bands to one. [^{32}P]P_i incorporation experiments showed that newly phosphorylated protein, whether insulin-enhanced phosphorylated protein or newly phosphorylated control protein, migrated as the 113 kDa species and not the more rapidly migrating form, indicating that the phosphorylated protein migrates precisely with the upper band. In addition, by using purified lyase as substrate for phosphorylation by A-kinase and GSK-3, it was shown that these three phosphorylation states can be duplicated by appropriate kinase-directed phosphorylations and can be resolved by our immunoblot procedure.

Others [23], studying CL that had been purified from insulin- and glucagon-stimulated ^{32}P-labelled hepatocytes, concluded that both hormones only stimulate phosphorylation of the same serine and that no other residues are affected. Reports in the literature [53], our previous observations [7,10,11,18,27,44,49] and the data in the present paper suggest that the above conclusion is not correct. With respect to the literature, studies on phosphorylation *in vitro* by GSK-3, of either glycogen synthase prepared from diabetic or insulin-treated diabetic animals [39,50] or CL prepared from control and insulin-treated fat-pads [7], show that *in vivo* physiological treatments affect the phosphorylation state of these purified substrates, as the ability of these purified substrates to be phosphorylated *in vitro* by GSK-3 was affected in a predictable manner.

Why the findings reported in the present paper were not noted by others is now clear. CL is phosphorylated on three sites in adipose and liver tissues [11]. Threonine-446 and serine-450 are sites that are phosphorylated by GSK-3, and serine-454 is the site phosphorylated by both A-kinase [11,28,29] and an insulin-stimulated kinase [30,31]. Our data suggest that CL is in the dephosphorylated form (null) in confluent 3T3-L1 cells, in mature fat-cells cultured in LBM, in FTO-2b cells when these hepatoma cells are incubated in medium without serum, and in purified CL. If CL in previous reports had been in the null form, the enzyme would have been a poor substrate for phosphorylation by GSK-3 [12]. In addition, these sites would have been missed, as in the h.p.l.c. analysis of trypsin-treated ^{32}P-labelled CL the digest was first clarified by $HClO_4$ precipitation [28]. This is now known to precipitate the large hydrophobic peptide B, which contains threonine-446 and serine-450 [11].

CL from pre-confluent, confluent and post-confluent cells is mostly in the dephospho state (null form). During the first stage of differentiation, when cells are cultured in IM+, blots of cytosolic protein show CL as a prominent band of 110 kDa and diffuse bands of higher-molecular-mass proteins up to 115 kDa. During this period, insulin, DX, other 'factors' and cyclic AMP are all elevated. When medium is changed to one containing 'factors' plus insulin, bands of 113 kDa and 110 kDa become prominent, and the presumed most highly phosphorylated form disappears.

It was predicted, on the basis of h.p.l.c. analysis and peptide sequencing of CL from hormone-treated rat epididymal fat-pads [11,27], that CL from 3T3-L1 fat-cells treated with insulin should contain highly phosphorylated serine-454 and minimally phosphorylated threonine-446 and serine-450, whereas adipocytes treated to increase cyclic AMP levels without a concomitant decrease in GSK-3 activity should contain CL with all three residues phosphorylated. These two CL forms distinguish two different physiological states. Thus, at least two forms of CL should be generated during differentiation of 3T3-L1 cells into adipocytes. Three CL forms were found, as there exists the null form (110 kDa) and the 113 kDa and 115 kDa species.

CL and ACC levels increase in 3T3-L1 cells with differentiation [35,36,54]. However, a direct association of specific phosphorylation changes with changes in CL specific activity has not been shown, and we have been unsuccessful in demonstrating changes in specific activity that correlate with large and rapid insulin-induced changes in CL phosphorylations. As hormone- and differentiation-induced changes in the phosphorylation state of CL have been shown, detailed studies of CL activity as a function of the amount of each phospho-species, the effect of allosteric effectors on activity and the cellular distribution of CL should be undertaken. Furthermore, as the currently used CL assay system using malate dehydrogenase [38] is not satisfactory when using concentrated homogenates from fat-cells, efforts to develop a more sensitive and specific assay system for CL activity are underway.

GSK-3 content was studied, as this kinase uniquely phosphorylates two phosphorylation sites on lyase. As noted in Figures 4 and 5, cytosolic GSK-3β content is high in pre-confluent and confluent cells. GSK-3 content is unchanged for the first 2 days of culture in IM+, but fall rapidly on day 3 of culture, a period in which cells become committed to differentiate into fat-cells [55]. The finding that GK-3β content in 3T3-L1 cells decreases during the middle and latter stages of cellular differentiation into adipocytes is consistent with the hypothesis [7,11,12,14,27] that GSK-3 activity is low during anabolic states and increased fat biosynthesis. We [7,8] and others [9] proposed

that regulation of GSK-3 plays a minute-to-minute role in the control of glycogen and fat metabolism. We suggested [12] that a likely mechanism for an insulin-induced rapid decrease in GSK-3 activity is by insulin's effects on other kinases that target GSK-3, as leaving out phosphatase inhibitors from extraction buffers kept GSK-3 activity high [12]. Recently, the time during which the actions of insulin and dexamethasone result in commitment to adipocyte differentiation has been determined [55], and is similar to the time period during which GSK-3 content falls.

An unanswered question in the regulation of metabolism by insulin is that, whereas the tyrosine kinase activity of the insulin receptor is increased by insulin action, stimulation of glycogen synthase and ACC activities is mediated by insulin-dependent dephosphorylations [56]. This paradox can be resolved if, in addition to an insulin-induced increase in protein phosphatase-1 activity due to the stimulation of an insulin-stimulated kinase [57,58], GSK-3 activity is decreased by some kinase on the insulin-stimulated pathway. This kinase is not likely to be mitogen-activated protein (MAP) kinase [59], but could be MAP kinase-activated protein kinase-1 or p70 S6 kinase, as suggested by Sutherland et al. [60]. A decrease in GSK-3 content and its presumed activity, and in the short term, a decrease in GSK-3 activity by its phosphorylation by either protein kinase C [17] or an insulin-enhanced kinase such as that described by Sutherland et al. [60], or dephosphorylation of its regulatory phosphotyrosine [16], would result in an increase in glycogen synthase activity.

The present paper describes *in vivo* the regulation of GSK-3 content during a normal physiological process. Its regulation could be at the gene level as well as at several other steps, including mRNA and protein stability, and translation. Studies on the short-term regulation of GSK-3 activity and the control of GSK-3 content will define an important signalling pathway.

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