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REGULATION OF AORTIC CARBOXYPEPTIDASE-LIKE PROTEIN (ACLP) DURING 3T3-L1 ADIPOGENESIS.

by

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ABSTRACT

The 175 kD aortic carboxypeptidase-like protein (ACLP), suggested to be involved in smooth vascular muscle cell differentiation, has been shown to be expressed in 3T3-L1 preadipocytes. In this study we demonstrate that ACLP protein expression is transiently but significantly down-regulated by day 2 of an 8-day 3T3-L1 differentiation. ACLP protein down-regulation correlates with increases in cell number, suggesting a potential link between ACLP and clonal expansion. The transient modulation of ACLP is shown to be partly due to transcriptional regulation. Analysis of the individual components of differentiation cocktail indicate that all components are necessary to induce maximal ACLP downregulation and, as such, this event is differentiation-dependent. Although ACLP overexpression had no apparent effect on adipogenesis, its pattern of expression, unique to post-mitotic proliferation, indicates a potential role for ACLP in adipose tissue development and warrants further investigation to elucidate its function during preadipocyte differentiation.

DEDICATION

To my family, friends, and teachers.

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"The devil has put a penalty on all things we enjoy in life. Either we suffer in health or we suffer in soul or we get fat."

- Albert Einstein

I. INTRODUCTION

A. Obesity

There has been a progressive rise in the prevalence of obesity over the past two decades. With over 30% of adults in North America now considered to be obese (1,2), obesity has become an epidemic. Current knowledge of human obesity has progressed beyond the simple generalizations of the past. Formerly, it was the mainstream view that obesity was caused by the single adverse behavior of inappropriate eating in the setting of attractive foods. However, in light of many great discoveries in the field of obesity research, we now know that there are, in fact, many underlying biological alterations that can predispose an individual to this disease. The study of animal models of obesity, biochemical alterations in humans and experimental animals, and the complex interactions of psychosocial and cultural factors that create susceptibility to obesity indicate that this disease in humans is quite complex and deeply rooted in biologic systems. Thus, it is almost certain that obesity is a multifactorial disease and that, as such, it has numerous subtypes.

White adipose tissue (WAT) is a normal constituent of the body that serves the important function of storing energy as triacylglycerol for mobilization in response to metabolic demands. Obesity is defined as the excess accumulation of body fat frequently resulting in a significant impairment of health. According to the guidelines set by the World Health Organization (WHO), obesity is clinically characterized by a body mass index (BMI) of 30 kg/m^2 (body weight in kg/[height in meters]^2) or greater and those with a BMI of 25 to 30 kg/m^2 are classified as overweight. Some of the disorders

associated with obesity include type 2 diabetes mellitus, hypertension, dyslipidemia, and ischaemic heart disease (3,4,5,6,7). Most of the secondary conditions that arise from obesity have one common cause – insulin resistance (8). Insulin resistance in obese individuals may be due to an inhibitory action of the cytokine TNFα (tumour necrosis factor alpha) on the insulin signal transduction pathway (9). Elevated levels of plasma TNFα, as a result of its increased secretion from adipose tissue, have been reported in obese individuals (10). Obesity-related hypertension may reflect the stimulatory effects of both insulin and the cytokine leptin (product of the *ob/ob* gene secreted by adipocytes) on the sympathetic nervous system (11).

Excess fat accumulation is initially associated with an increase in adipocyte size (hypertrophy), followed by an increase in adipocyte number (hyperplasia) (12). Although the etiologic mechanisms underlying obesity require much clarification, it is an imbalance between energy intake and expenditure that ultimately leads to obesity. In a state of positive energy balance, adipocytes grow in size and ultimately reach a finite volume. It is believed that at this point, an as yet unidentified paracrine signal(s) triggers the formation of new adipocytes from the precursor cells (preadipocytes) in a process termed adipogenesis (13,14,15). Other studies have reported that there can also be direct nutritional effects on preadipocyte differentiation. Experiments using preadipocyte cultures suggest that increases in insulin levels upon feeding can result in adipogenesis (16). Additionally, experiments using mice have shown that the levels of the transcription factor adipocyte determination dependent factor (ADD) 1, responsible for the expression of lipogenic genes, are elevated in the fed state (17).

Both genetic and environmental factors are likely to be involved in the pathogenesis of obesity. While excessive caloric intake and/or insufficient physical activity may trigger obesity, metabolic and endocrine abnormalities may prime an individual to becoming obese. The discovery that various mutations in the peroxisome-proliferator-activated receptor gamma2 (PPARγ2), a key regulator of adipogenesis, are associated with obesity has certainly strengthened this view. In one study, screening of obese subjects revealed that a common Pro115Gln mutation in PPARγ2 could accelerate adipogenesis when expressed in murine fibroblasts (18). A proline to alanine residue-mutated polymorphic variant of PPARγ2 in humans, known as Pro12Ala PPARγ2, can also result in an enhancement of preadipocyte differentiation by up-regulating the expression of adipogenic genes (19). Moreover, a recent study has shown a strong correlation between a saturated fatty acid-rich diet, the Pro12Ala polymorphism, and BMI (20).

It is important to understand that, although excess fat can be detrimental to health, so can its complete absence. The study of two different models of genetically fat-less mice has revealed that they suffer from type 2 diabetes, fatty liver, and other metabolic alterations (21,22), demonstrating that total loss of fat can lead to similar metabolic disruptions as does obesity. As discussed earlier, the metabolic disorders associated with obesity can be ultimately linked to insulin resistance. In these individuals, the excess accumulation of triacylglycerol leads to a diminished capacity for further lipid storage, giving rise to lipid accumulation in the liver and elevated levels of circulating lipids which, in turn, can lead to the development of artherosclerotic plaques and other conditions associated with high lipid levels. In lipoatrophic patients or the fat-less mice,

similar conditions as obesity arise due to a lack of a site for storage of lipids, leading to an elevated blood lipid profile.

There are a number of causes that can lead to a loss of adipose tissue (lipoatrophy) in humans including immune responses (23), infectious/drug-induced lipoatrophy (24), and genetic causes such as the Dunnigan-type familial partial lipodystrophy (25,26). In nearly all cases, lipoatrophy is associated with type 2 diabetes mellitus. All the metabolic disorders, including insulin resistance, appear to be directly due to a lack of adipose tissue, as these are reversed to normal in lipoatrophic mice upon adipose tissue transplantation (27). Furthermore, a new class of drugs, known as thiazolidinediones (TZD), that act as PPARγ ligands and result in increased insulin sensitivity and in adipogenesis, have been shown to reduce insulin resistance and thereby hyperglycemia and hypertriglyceridemia (28,29). The use of this class of drugs may prove effective in combating the metabolic disorders associated with lipodystrophy (30).

B. Adipose Tissue Development

1. General Overview

a. Origin of Adipose Tissue

The developmental origin of adipocytes remains unclear but studies have suggested that they derive from multipotent mesodermal cells that originate from an embryonic stem cell precursor (31,32,33). In addition to differentiating into the adipocyte lineage, these precursor cells can also commit to the chrondrocyte, osteoblast, and myocyte lineages (34). It is known that WAT starts to develop before birth in mice, rats, and humans (35,36,37). Soon after birth, there is an expansion of WAT due to a growth in adipocyte size and number. It is now recognized that, throughout the lifetime of both animals and humans, there exists a population of preadipocytes, within the stromal vascular compartment of adipose tissue, capable of replication and differentiation into mature adipocytes. Thus, having a solid underlying knowledge of the replication and differentiation of preadipocytes is important for our understanding of adipose tissue growth in health and obesity.

b. Role of Adipose Tissue

In addition to serving as a site for storage of excess energy in the form of triacylglycerol, recently it has been realized that WAT is also responsible for the secretion of various factors known to play a role in regulation of appetite, vascular diseases, and immunological responses. Adipocytes secrete tumor necrosis factor alpha (TNFα) which may act in an autocrine or paracrine fashion to decrease insulin sensitivity (9,38,39). Other secreted factors include adipsin (40), acylation stimulation protein (ASP) (41), adipocyte complement-related protein (Acrp30/AdipoQ) (42,43), and macrophage migration inhibitory factor (MIF) (44). Secreted proteins involved in vascular functions include angiotensinogen and plasminogen activator inhibitor type 1 (PAI-1) (45). Adipocytes also secrete leptin, a cytokine product of the obese (*ob*) gene that plays a role in regulating body fat by acting as a satiety factor upon binding to its receptor in the hypothalamus (46,47). However, as there have been very few reported cases of human obesity due to a lack of leptin or leptin action, it appears that the major role of leptin is beyond that of its control over food intake and energy expenditure. It has been suggested that leptin plays a greater role in protecting and preserving the body during periods of negative energy balance (starvation) than it does in obesity (48).

Recently, a new factor has been found to be secreted by adipocytes – resistin. Resistin contains a unique cysteine-rich repeat motif and has been shown to induce insulin resistance (49). In cell culture studies, the same protein was independently cloned as adipose tissue specific secretory factor (ADSF) and was shown to inhibit adipogenesis (50).

c. Adipose Tissue Remodeling

The cellularity of adipose tissue is influenced by the integration of at least three undergoing processes: preadipocyte proliferation, preadipocyte differentiation, and apoptosis (51,52). A fine balance between the three processes is crucial for the normal homeostasis of adipose tissue. Inherent genetic differences between adipose depots may explain why in some disease states we observe depot-specific effects, as in Cushing's syndrome and in highly active anti-retroviral therapy (HAART)-induced lipodystrophy syndrome (53). In both of these syndromes, there is peripheral lipoatrophy and truncal obesity, ie: adipose tissue redistribution.

Adipose tissue redistribution is also seen in diabetic patients on TZD treatment. TZDs lead to an increase in subcutaneous adipose tissue while decreasing or having no effect on visceral fat depots (54,30), a redistribution that is opposite to that observed in HAART-induced lipodystrophy.

2. Preadipocyte Differentiation

a. Cell Line Models

The establishment of immortalized preadipose cell lines nearly 30 years ago has made it possible to extensively study and dissect the molecular and cellular events involved in preadipocyte differentiation. Numerous preadipose cell lines, representing different stages of differentiation, have been used to elucidate the underlying signaling pathways of preadipocyte differentiation. Of these, the 3T3-L1 preadipocytes have been the most extensively studied.

3T3-L1 cell line. The 3T3-L1 cells were isolated from Swiss 3T3 cells derived from disaggregated 17- to 19-day mouse embryos and are committed to the adipocyte lineage (55). The 3T3-L1 preadipocytes are well characterized with respect to their genetic program of differentiation which is very similar to that of human preadipocytes (34,32) and, as such, they are the most frequently employed cell line in the study of preadipocyte differentiation. Further evidence that adipose cell formation occurs by a similar mechanism *in vivo* is given by the development of mature fat pads that are histologically indistinguishable from WAT following subcutaneous injection of related preadipocytes (3T3-F442A) into athymic mice (56,57,58).

3T3-C2 cell line. The 3T3-C2 cells are derived from the same source as the 3T3-L1 cells. However, this cell line is differentiation-incompetent (55) so that it can be used as a control cell line to determine whether a response is differentiation-dependent. It has been shown that 3T3-C2 cells do enter the early stage of preadipocyte differentiation (termed the mitotic clonal expansion phase; see below) but that they are unable to completely differentiate and attain the full mature adipocyte phenotype (59).

Primary Cultures. Since preadipose cell lines are murine in origin, aneuploid, and may represent different stages of differentiation, the use of primary cultures, although very difficult and limited (60,61), has been useful for verifying results obtained from preadipose cell lines. There are several advantages in using primary cell cultures. Primary cells can be obtained from different adipose depots and, as such, they closely represent the *in vivo* condition and they can be more useful in studying depot-specific responses. One major disadvantage of primary cultures, however, is the cellular heterogeneity of the stromal vascular fraction which requires laborious treatments in order to isolate the preadipocyte population. These treatments include collagenase digestion of fibrous tissues, differential centrifugation, and size-exclusion filtrations (60). Additionally, the extent of differentiation of primary preadipocytes is donor-dependent, decreasing with age (62,63,60) and increasing with body fat (64). Other drawbacks include availability and accessibility limitations to acquiring human preadipocytes which are normally obtained from patients undergoing elective surgery, and their reduced differentiation upon subsequent passages. Recently, O'Rahilly's group has reported that addition of a thiazolidinedione to the differentiation medium may allow limited

subpassages of human subcutaneous preadipocytes in primary culture while maintaining their capacity to differentiate (65).

b. Cellular and Molecular Events of Preadipocyte Differentiation.

The differentiation of 3T3-L1 preadipocytes occurs during an eight day period that is categorized by the chronological expression of various genes needed for conversion from preadipocyte into mature adipocyte. Gene markers of early differentiation include lipoprotein lipase (LPL), the α_2 chain of type VI collagen, the transcription factors CAAT/enhancer binding protein (C/EBP) β, C/EBPδ, and the adipocyte determination and differentiation factor 1 (ADD1; also known as sterol regulatory element binding protein 1 or SREBP1). The appearance of peroxisome proliferator-activated receptor gamma (PPARγ) and C/EBPα mark the intermediate stages of differentiation while glucose transporter type 4 (GLUT4), adipsin, fatty acid synthase, and glycerol phosphate dehydrogenase are indicative of late stage or terminal differentiation. The expression of these genes by typical Western analyses can be used to determine the stage of preadipocyte differentiation. The morphological changes that accompany the differentiation of preadipocytes include a change from a flat fibroblastic look to a more spherical phenotype with accumulation of lipid globules in the cytoplasm. Preadipocyte differentiation can be divided into three major stages: growth arrest, clonal expansion, and terminal differentiation.

Growth Arrest. Preadipocytes proliferate, reach confluence, and form cell-cell contacts, at which point they undergo growth arrest – the first step towards differentiation. At this growth-arrested stage, known as the G_0 stage of cell cycle, the cells are ready to differentiate and early markers of differentiation can be detected (66).

Although growth arrest is necessary for differentiation, some studies have suggested that in certain cell lines, such as the 3T3-F442A cells (67), cell confluence and cell-cell contact need not be present. However, in most other *in vitro* cell line models, including the 3T3-L1 preadipocytes, cell-cell contact is a pre-requisite for successful differentiation. The exact molecular mechanism underlying growth arrest due to contact inhibition is unknown. Garces and colleagues have demonstrated that cell-cell contact and activation of Notch-1, a transmembrane receptor member of the epidermal growth factor-like family of proteins, are implicated in the control of PPARγ gene expression through mechanisms independent of the hormonal induction of preadipocyte differentiation (68).

Induction of Differentiation. Upon undergoing growth arrest, the preadipocytes become primed for differentiation which can be induced by a hormonal cocktail consisting of high doses of insulin, the glucocorticoid dexamethasone, and the cAMP elevating agent 3-methyl-isobutylxanthine (IBMX) (60,69,70). No single component of the differentiation cocktail can alone induce full differentiation. It is thought that dexamethasone and IBMX potentiate differentiation once it has been initiated by insulin or IGF-1 (71). A brief discussion of the mechanisms of action of each of the components of the differentiation stimulus follows.

Hormones such as insulin and IGF-1 are responsible for the activation of an elaborate signaling cascade that ultimately culminates in the expression of key transcription factors required for the expression of adipogenic genes. Some of the intermediate enzymes involved in this process include phosphoinositide 3-kinase (PI 3-kinase), mitogen activated protein kinase (MAPK), protein kinase B (PKB), and the serine/threonine kinase p70 S6 kinase (72). 3T3-L1 preadipocytes have nearly twice as many IGF-1 receptors as insulin receptors which can also bind insulin albeit with a much lower affinity (69); for this reason, a much higher concentration of insulin than IGF-1 is required to induce differentiation (73). Upon adipocyte differentiation, the number of insulin receptors and insulin sensitivity increase (73).

Other studies suggest that differentiation can be induced by insulin acting on its own receptor. Inhibition of the insulin receptor gene expression impairs the ability of 3T3-L1 cells to undergo differentiation, suggesting a more direct role for the insulin signalling pathway through the insulin receptor (74). In a series of very elegant experiments in which the cytoplasmic portion of the insulin receptor was fused to the extracellular ligand binding domain of the human colony stimulating factor-1 (CSF-1), Chaika and colleagues demonstrated that 3T3-L1 cells expressing this chimeric receptor were able to undergo differentiation when induced with CSF-1, supporting the notion that the stimulation of the insulin receptor is sufficient to initiate differentiation (75). Also, Gagnon and Sorisky demonstrated that insulin was capable of inducing differentiation at nanomolar concentrations when 3T3-L1 preadipocytes were incubated at lower glucose concentrations (76).

Glucocorticoids such as dexamethasone have been shown to induce the expression of the transcription factor C/EBPδ that leads to the induction of the adipogenic transcription factor PPARγ (77). Another study has suggested that the effect of glucocorticoids may be mediated by an increase in arachidonic acid metabolism leading to elevations in intracellular cAMP concentration (66). Additionally, glucocorticoids have been implicated in the repression of preadipocyte factor-1 (pref-1), an epidermal growth factor repeat domain-containing protein with an anti-adipogenic function, by a mechanism that is not completely clear but, as discussed later, might involve stabilization of matrix-cytoskeleton interactions (78,79). As will be discussed later, one study has shown that the glucocorticoid dexamethasone is also involved in the growth-arrest of cells prior to terminal differentiation (80).

The exact mechanism of IBMX action is unknown. However, similar to glucocorticoid action, IBMX has been shown to induce PPARγ through activation of another transcription factor, C/EBPβ. A possible mechanism may involve cAMP since it has been demonstrated that IBMX inhibits phosphodiesterase and stimulates adenylyl cyclase by blocking the inhibitory regulatory protein G_i leading to elevated intracellular cAMP (81). In turn, cAMP may mediate its effects through activating cAMP-dependent transcription factors such as cAMP response-element binding protein (CREB), cAMP response-element modulator protein (CREM), and activating transcription factor 2 (ATF2) (82,83). Reusch and colleagues have demonstrated CREB is responsible for activating several adipocyte-specific gene promoters such as phosphoenolpyruvate carboxykinase (PEPCK), fatty acid binding protein (FABP [aP2/422]), and fatty acid synthetase (FAS) (84).

Mitotic Clonal Expansion. Upon the onset of differentiation, the growth-arrested preadipocytes exit the G_0 stage and re-enter the cell-cycle at the S stage for 1-2 rounds of mitotic replication, termed the clonal expansion phase (32,85). The purpose of the clonal expansion appears to be two-fold: first, replication of DNA results in the de-repression of genes required for differentiation and, second, it gives rise to the clonal amplification of committed cells. The molecular processes implicated during this phase are still under investigation.

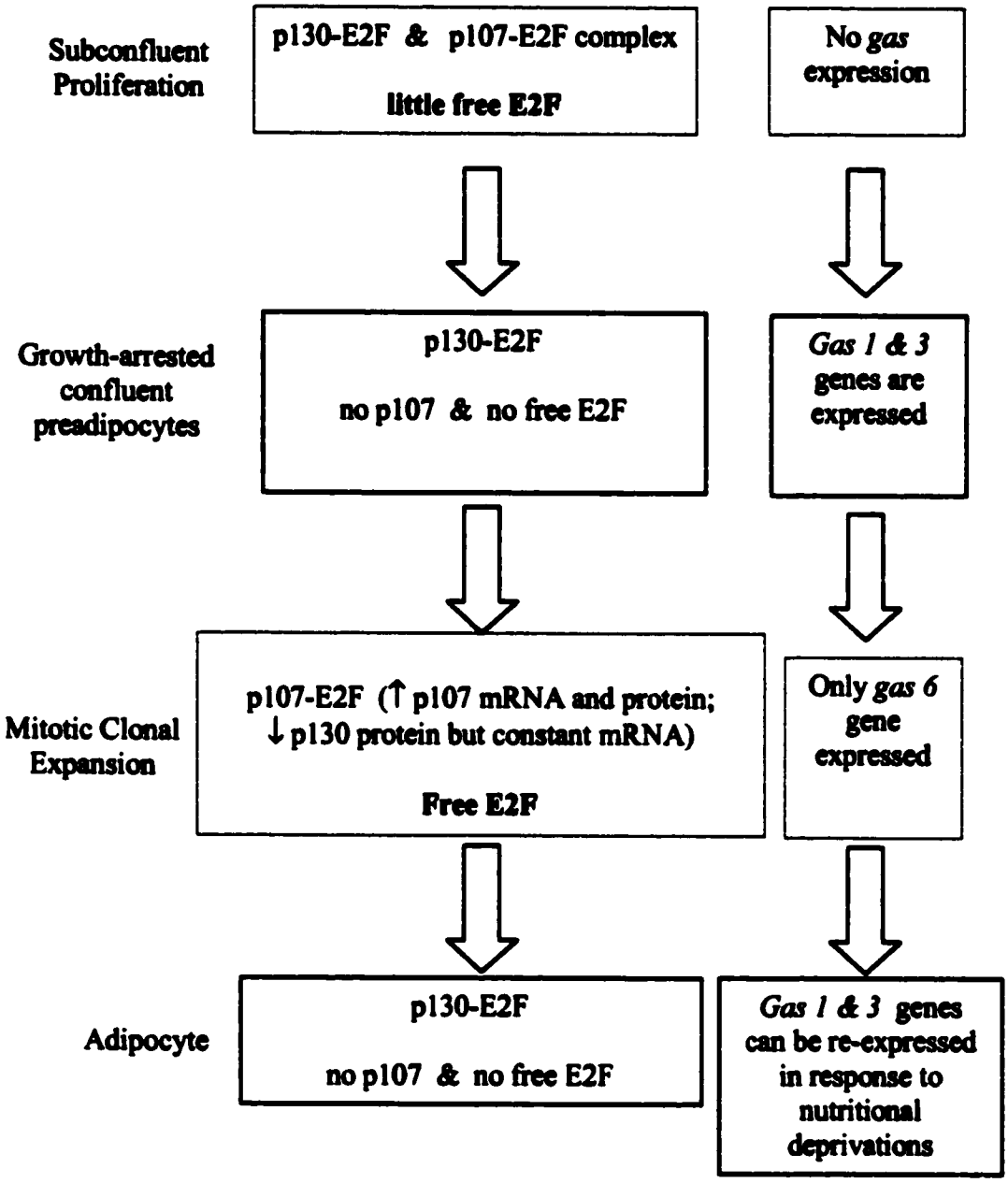
The E2F transcription factors are DNA-binding proteins that are important in regulating the transcription of genes associated with regulation of cell-cycle and differentiation (86,87). It has been shown that at growth-arrest, prior to the onset of the clonal expansion phase, the family of E2F transcription factors (E2F1-5), involved in the expression of adipogenic genes required for the G_0-S cell cycle transition and entry into the clonal expansion phase, is sequestered and rendered inactive by hypophosphorylated retinoblastoma proteins pRB and p130 (59,88,89,90). Cyclin-dependent kinases (CDKs) are known to be responsible for inducing entry into the cell cycle (91). CyclinCDK complexes consist of a catalytic serine/threonine kinase subunit and a regulatory cyclin subunit. CyclinCDK complexes function to hyperphosphorylate retinoblastoma proteins, resulting in their inactivation and the release of E2F transcription factors that will, in turn, regulate the genes required for S phase (92). The activity of cyclinCDK complexes is regulated by a number of factors such as the controlled synthesis and degradation of cyclins, the phosphorylation state of the kinase subunit, and the binding of inhibitory factors. For example, it has recently been shown that increases in intracellular calcium

levels lead to the degradation of p27, a member of the cyclin-dependent kinase inhibitor family (CDKI), resulting in the release and activation of the cyclinCDK complex (93).

It has been shown that during subconfluent preadipocyte proliferation, the transcription factors E2F are bound to the retinoblastoma proteins p130 as well as p107 with little free E2F detected. However, upon reaching confluence and undergoing growth-arrest, all E2F becomes bound to p130 only (59). During the mitotic clonal expansion phase, E2F again switches binding partners and becomes bound to p107 with the additional presence of free E2F (Figure 1). It has been observed that during this p130:p107 switch, both p107 mRNA and protein are up-regulated but p130 mRNA remains constant while its protein levels are down-regulated, suggesting a transcriptional and a translational regulation, respectively (59). Upon exiting the clonal expansion phase and undergoing the second growth-arrest, the E2F/p130/p107 expression pattern reverts back to that of the contact-inhibited, growth-arrested, day 0 preadipocytes. It has been shown that, in addition to inhibiting the normal insulin signal transduction, another inhibitory action of TNFα on preadipocyte differentiation includes the disruption of the normal pattern of p130 and p107 expression, resulting in a complete inhibition of differentiation (94).

Another unique characteristic of the clonal expansion phase is in the expression pattern of a group of growth arrest-specific (*gas*) genes. *Gas6* is only expressed during the clonal expansion of post-confluent preadipocytes whereas *gas1* and *gas3* are expressed in serum-starved-quiescent and confluent-growth-arrested preadipocytes, and in response to nutritional deprivation in mature adipocytes (95) (Figure 1).

Figure 1 The p130:p107 switch and *gas* gene expression characteristic of mitotic clonal expansion.


Subconfluent Proliferation
p130-E2F & p107-E2F complex
little free E2F
No gas expression
Growth-arrested confluent preadipocytes
p130-E2F
no p107 & no free E2F
Gas 1 & 3 genes are expressed
Mitotic Clonal Expansion
p107-E2F (↑ p107 mRNA and protein; ↓ p130 protein but constant mRNA)
Free E2F
Only gas 6 gene expressed
Adipocyte
p130-E2F
no p107 & no free E2F
Gas 1 & 3 genes can be re-expressed in response to nutritional deprivations

Taken together, the p130:p107 switch and the expression of gas 6 during clonal expansion suggest a different underlying molecular mechanism for post-confluent mitosis versus subconfluent proliferation.

As mentioned earlier, the differentiation of the 3T3-C2 fibroblasts appears to be blocked at a stage after the mitotic clonal expansion, since these cells have been shown to undergo clonal expansion including a p130:p107 switch that is similar to that observed in the 3T3-L1 cells (59). However, in the 3T3-C2 cells, the decrease in p130 protein during clonal expansion is not as dramatic and a highly phosphorylated form of p130 is detected (59) which has been shown to correlate with a loss in its ability to bind E2F (96). These results, along with the observation that growth-arrested, confluent 3T3-C2 cells are able to undergo proliferation upon hormonal stimulation, provide further evidence that 3T3-C2 fibroblasts are differentiation-resistant due to a deficit that lies downstream of mitotic clonal expansion. This deficit may be due to a lack of the transcription factor PPARδ, as one study has demonstrated that the ectopic expression of PPARδ in these cells can promote the induction of PPARγ and differentiation (97).

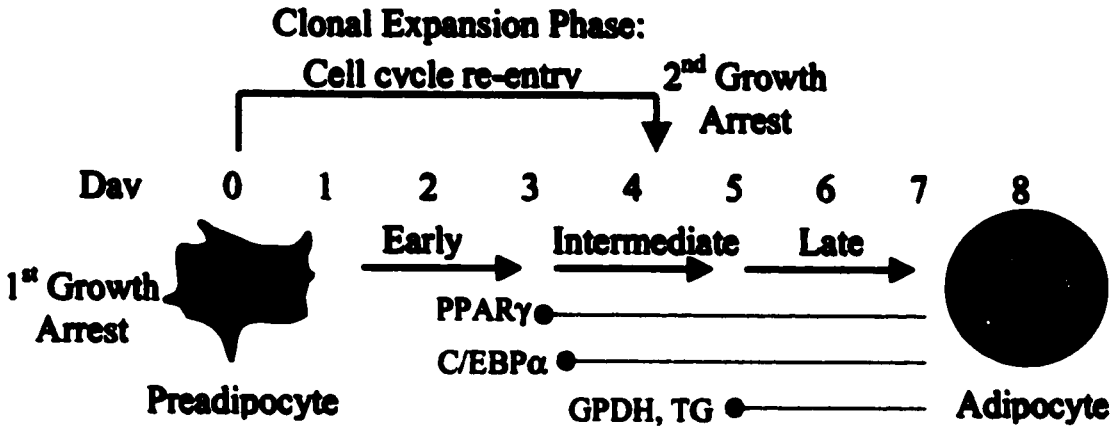
Terminal Differentiation. After the clonal expansion phase, the cells undergo a second growth-arrest that is thought to be mediated by the marked appearance of C/EBPα and PPARγ. It has been shown that C/EBPα has a potent anti-mitotic activity (98) which may involve activation of p21/SDI-1 (99). Additionally, it has been demonstrated that ligand activation of PPARγ is sufficient to induce growth arrest in NIH-3T3 fibroblasts (retrovirally infected to express PPARγ) and 3T3-F442A preadipocytes by a mechanism that may involve a decrease in the DNA binding and transcriptional activity of the

E2F/DP-1 complex (100). Recently, yet another group of transcription factors known as the GATA family, specifically GATA-2 and GATA-3, have been implicated in the preadipocyte-adipocyte transition. It has been shown that these transcription factors mediate their effect, in part, by inhibiting the expression of PPARγ so that their down-regulation is required for the normal progression of preadipocyte differentiation (101).

Another study has suggested that the glucocorticoid dexamethasone is necessary for this growth-arrest by a mechanism that involves the down-regulation of the growth arrest-associated gene 2 (gas2) and that without dexamethasone the cells undergo a growth-arrest that is similar to the postconfluent growth-arrest (95). At this point, termed the G_d stage, the cells are ready to acquire the mature adipocyte morphology and to synthesize and accumulate fat. This phase is characterized by the induction of a vast number of proteins and enzymes involved in triacylglycerol metabolism, including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase, glucose transporter 4 (GLUT4), insulin receptor, leptin, glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase (GPDH), and fatty acid synthase (34,102,103,104).

The general overview of the events occurring during preadipocyte differentiation is illustrated in Figure 2.

Figure 2 General overview of 3T3-L1 preadipocyte differentiation. Preadipocytes follow an 8-day differentiation protocol that is classified into early, intermediate, and late or terminal differentiation. Confluent, growth-arrested cells re-enter the cell cycle (day 0) upon stimulus to differentiate, undergo 1-2 rounds of mitosis (clonal expansion phase) and undergo a second growth-arrest around day 4, after which, they are primed to enter terminal differentiation and acquire the full adipocyte phenotype.


Clonal Expansion Phase:
Cell cycle re-entry
2nd Growth Arrest
Day 0 1 2 3 4 5 6 7 8
1st Growth Arrest
Early
Intermediate
Late
PPARγ
C/EBPα
GPDH, TG
Preadipocyte
Adipocyte

Extracellular Matrix Rearrangement. The extracellular matrix (ECM) is a dynamic network that, in addition to providing a structural support, plays an active role in differentiation and cellular migration. During preadipocyte differentiation, cells undergo significant morphological changes reflecting alterations in their cytoskeleton and components of their ECM. A decrease in actin and tubulin expression is one of the earliest events of differentiation (105). There is also a decline of type I and type III collagen while secretion of type IV collagen and entactin increases (106,107).

Integrins are another class of ECM cell-surface receptors that function as adhesion molecules by linking the ECM to the cytoskeleton. During differentiation, preadipocytes need to detach from the ECM to allow for morphological changes. To achieve this, the cells down-regulate the levels of integrins and the synthesis of ECM (108). Exposure of preadipocytes to transforming growth factor β (TGFβ) suppresses differentiation by resulting in an increased expression of integrin receptors and stabilization of collagen and fibronectin components of the ECM (109,110). Furthermore, the treatment of 3T3-L1 preadipocytes with TGFβ also appears to impair insulin signal transduction in these cells (111). Similarly, the preadipocyte factor 1 (pref-1) may also be involved in inhibition of differentiation by stabilizing the matrix-cytoskeleton interaction through its EGF-like repeats, similar to that of many ECM proteins and cell adhesion molecules.

A large family of enzymes known as matrix metalloproteinases (MMPs) which include collagenases, gelatinases, stromelysins, and membrane-type MMPs (112) have been implicated in matrix turnover, especially in renal cell physiology (113). In a variety of cell types, integrins have been shown to be involved in matrix turnover by binding to

proteases (114,115). It has been shown that integrins can directly bind to MMP-2, localizing ECM degradation to the cell surface (116). Focal changes in cell-matrix contacts, as a results of this pericellular proteolysis, results in a variable and transient alteration in cellular attachment/detachment. Interestingly, MMP-1, involved in cleavage of native fibrillar collagen, has been found to be up-regulated upon prolonged collagen activation of a member of a novel class of tyrosine kinase receptors known as discoidin domain receptor 2 (DDR2) (117,118) which will be introduced later. It has also been shown that in adipocytes, the secretion of TNFα is dependent on the cleavage of the membrane-bound pro-TNFα by an MMP (119).

Aortic carboxypeptidase-like protein (ACLP), a newly identified protein in vascular smooth muscle and 3T3-L1 preadipocytes, has been found to contain features which suggest its potential interaction with the ECM.

C. Aortic Carboxypeptidase-like Protein

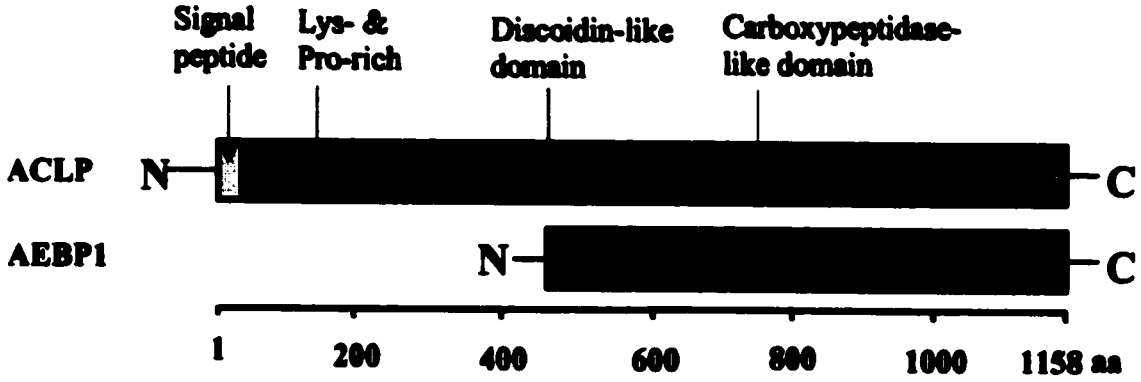
1. Characteristics

As its name suggests, aortic carboxypeptidase-like protein (ACLP) was initially identified during the screening of human aorta proteins that interact with transcription factors E12 and E47, the products of the E2A gene (120). ACLP is a 175 kD protein that contains a signal peptide sequence, a proline- and lysine-rich repeating motif, a discoidin I-like domain, and a C-terminal domain that is 39% identical to carboxypeptidase E (Figure 3). The potential functions of these domains are discussed below.

Since ACLP contains a putative signal peptide sequence, it is possible that it is secreted in the ECM. Signal peptide sequences also play a role in the trafficking of proteins, directing them into pathways that lead to their insertion into the plasma membrane. However, since ACLP lacks any hydrophobic membrane-spanning domain, its signal peptide potentially serves in its secretion. Moreover, a possible role for ACLP in the ECM is suggested by its discoidin-like domain, as will be discussed shortly.

Its proline- and lysine-rich motif are characteristic of the activating domains of many transcription factors. However, due to the fact that ACLP has only been found to be localized in the cytosol and in close association with the plasma membrane (120) and because it lacks a DNA binding domain, it is unlikely that it functions as a transcription factor.

Figure 3 Comparison of human ACLP and AEBP1. Starting from the N-terminus ACLP contains: Signal sequence peptide, lysine- and proline rich repeating motif, discoidin I-like domain (DLD), carboxypeptidase-like domain (CLD). AEBP1 is missing the signal peptide sequence, the lysine- and proline-rich motif, and part of the DLD.



	ACLP	AEBP-1
Protein Size on Western	176 kD	79 kD
mRNA size on Northern	3.9 kb	3.9 kb
Localization	Non-nuclear	Nuclear
Carboxypeptidase activity	Absent	Present
Protein Expression during 3T3-L1 adipogenesis	Transient downregulation	Downregulated

The discoidin-like domain of ACLP has significant identity with Discoidin I. Discoidin I is a lectin produced by the cellular slime mold *Dictyostelium discoideum* (121). Discoidin I is concentrated intracellularly early in development and it is subsequently externalized into the glycoconjugate rich slime coat surrounding the maturing aggregate where it binds to polysaccharides rich in galactose and N-acetylgalactosamine (122). It is thought that discoidin plays a role in facilitating cellular migration and aggregation (123) and, as such, the discoidin-like domain may play a similar role in ACLP. The discoidin-like domain of ACLP may also be involved in anchoring the protein to the plasma membrane by binding to phospholipids since, although ACLP lacks a transmembrane-spanning domain, it has been observed to be membrane-associated (120).

A group of novel non-integrin matrix receptors have recently been identified, designated the discoidin domain receptors (DDRs) based on their homology to the *Dictyostelium discoideium* protein discoidin I (117,124). DDRs are receptor tyrosine kinases that are activated by specific types of collagen so that they may serve as sensors for the changes in integrity of the matrix surrounding the cells. DDRs have been found to be expressed in a vast number of tissues including brain, keratinocytes, colon, kidney, lungs, thyroid, pancreas, heart, skeletal muscle, and connective tissue (125,126,127,128,129). Unfortunately, literature on the expression of this class of receptors in adipose cell lines is lacking, but given the expression of these receptors in such a wide variety of tissues, and their presence in cell lines that come from the same precursor cell as adipocytes, it would not be surprising if they were also expressed in adipose tissue.

At its C-terminus, ACLP contains a carboxypeptidase-like domain that is 39% identical to carboxypeptidase E (CPE). CPE is an enzyme involved in the processing of a multitude of inactive prohormone precursors such as pro-opiomelanocortin, pro-enkephalin, and pro-insulin (130). CPE is also involved in the activation of the precursor forms of prohormone convertases PC 1/3 and PC2 (131). In addition to its carboxypeptidase activity, CPE serves as a sorting receptor in the regulated secretory pathway of the various prohormones (130,132). While its soluble form has a carboxypeptidase function, its membrane-bound form has low enzymatic activity when associated with the membranes of secretory granules and, instead, functions as a prohormone receptor (133). The proteolytic cleavage of its tail yields the enzymatically more active soluble form of CPE (133,134). Despite significant similarity between CPE and the carboxypeptidase-like domain of ACLP, *in vitro* studies have failed to demonstrate a carboxypeptidase activity for ACLP (120). This may be due to a divergence of residues in ACLP from those of the carboxypeptidase family (135). Some of these differences include an asparagine residue (amino acid 763) in human ACLP instead of a histidine that is involved in zinc binding in carboxypeptidases; an asparagine (amino acid 852) and a tyrosine (amino acid 874) residues in human ACLP instead of a tyrosine and a glutamic acid residues, respectively, that are catalytically important in carboxypeptidases; and a negatively charged glutamic acid residue (amino acid 700) in human ACLP replaces a positively charged arginine residue present in the substrate recognition pocket of carboxypeptidases that is involved in stabilizing the C-terminal carboxyl group of the substrate (120).

Layne and colleagues have shown that ACLP expression is drastically up-regulated at the level of both protein and mRNA during differentiation of the multipotent mouse neural crest cells, Monc-1, into smooth muscle cells (120). Additionally, they have observed an increase in ACLP mRNA and protein expression in cultured mouse and rat aortic smooth muscle cells during quiescent periods induced by serum withdrawal (120).

Although ACLP mRNA has been found in a variety of tissues including the brain, colon, kidney, and to a lesser extent in the stomach, spleen, and thymus, ACLP protein has so far only been found in aortic smooth muscle cells and in adipose tissue (120), suggesting tissue-specific translational regulation.

2. ACLP in Adipose Tissue

So far, there have been no studies performed to determine the role of ACLP in adipose tissue. All that is simply known is that its levels of expression in 3T3-L1 preadipocytes are much lower compared to that of vascular smooth muscle cells and differentiated Monc-1 cells (120). As will be presented here for the first time, our experiments show that ACLP expression is transiently, but significantly, modulated during preadipocyte differentiation.

3. ACLP vs. AEBP1

Adipocyte Enhancer Binding Protein 1 (AEBP1) was cloned in 1995 and identified as a transcriptional repressor of the adipose *P2* gene (136), also known as *aP2* or *422*, whose product is the adipocyte lipid-binding protein ALBP (103,137,138). AEBP1 is a 79 kD protein that has been reported to demonstrate a carboxypeptidase activity that is believed to be involved in its transcriptional repression function by proteolytically cleaving and inactivating transcription factors (136). It has been demonstrated that the level of AEBP1 expression is reduced during adipogenesis to allow transcription of genes required for normal progression of differentiation (136). Additionally, it has been shown that AEBP-1 activity can be modulated by its interaction with a Gγ subunit of a heterotrimeric G protein (139). Besides its direct transcriptional repression via its carboxypeptidase activity, AEBP-1 may also inhibit adipogenesis by maintaining MAPK in its active phosphorylated form (140).

Like ACLP, AEBP1 also contains an N-terminus domain that is 35% identical to discoidin I. In fact, GenBank™ analysis of their cDNA reveals that the 3' end of human ACLP cDNA (corresponding to the C-terminal of the protein) is highly identical to that of mouse AEBP1 (136). This has resulted in much controversy over the nature of these two proteins (Figure 3). It has been suggested that ACLP is the authentic protein and that the AEBP1 cDNA is incomplete and simply a truncated ACLP clone that is missing the ACLP signal peptide sequence, the proline- and lysine-rich motif, and part of the discoidin domain (120).

II. OBJECTIVE

Based on results demonstrating modulation of ACLP expression during vascular smooth muscle cell differentiation and suggesting a potential role for ACLP in the development of the VSMC lineage (120), the purpose of this study was to (a) characterize the expression of ACLP at the level of both transcription and translation during 3T3-L1 preadipocyte differentiation by performing Northern and Western analyses, (b) investigate ACLP protein expression in primary preadipocyte cultures, and (c) examine the role of ACLP by performing over-expression experiments and determine its effects on preadipocyte differentiation.

III. MATERIALS AND METHODS

A. Preadipocyte Cultures and Preadipocyte Differentiation.

3T3-L1 and 3T3-C2 preadipose cell lines were obtained from American Type Culture Collection and grown to confluence in standard growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C in a 10% CO_2 atmosphere. Differentiation was induced on day 0 (2-day post-confluent cells) by addition of 100 nM insulin, 0.5 mM isomethylbutylxanthine (IBMX), and 0.25 μM dexamethasone. After 48 hours (day 2), the medium was replaced with DMEM supplemented with 10% calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 100 nM insulin and changed every 2 days up to day 8.

3T3-F442A preadipocytes (generous gift from Dr. Howard Green, Harvard University) were grown to confluence in DMEM/10% CS/1% PS. Two days post-confluence (day 0) cells were induced to differentiate in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin, and 100 μM insulin. Media was changed every two days for an 8-day differentiation period. Mouse white adipocyte homogenate was a kind gift from Drs. A. Melnyk and J. Himms-Hagen (University of Ottawa, Canada).

For experiments involving exposure of preadipocytes to individual components of the differentiation medium, growth-arrested day 0 (2 days post-confluence) cells were exposed to growth media (DMEM supplemented with 10% calf serum and 1% pen-strep)

containing either dexamethasone (D), IBMX (M), insulin (I), or DMI (normal differentiation) at above-mentioned concentrations. After 48 hours, cells exposed to growth medium plus D or M were switched into growth media only, while those exposed to growth medium plus insulin were kept in this medium (growth medium plus insulin) for the entire 8 days. Medium was changed every 2 days. For analysis of ACLP protein expression during subconfluent 3T3-L1 preadipocyte proliferation, cells were grown in normal growth media and harvested at 60% confluence.

B. Cell Counts

In order to analyze the mitotic clonal expansion stage of preadipocyte differentiation, 3T3-L1 and 3T3-C2 cells were trypsinized with 0.2 ml of trypsin followed by the addition of 0.8 ml of the growth media (to stop the trypsin action) on days 0 to 8 of an 8-day differentiation protocol and subjected to visual cell counting using a brightline haemocytometer (VWR Scientific) and a Nikon TMS microscope.

C. Western blot analysis.

At specific time points along the 8-day differentiation protocol, cultured cells were washed two times with PBS and scraped in 1X Laemmli lysis buffer (141) containing 60mM Tris pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.002% bromophenol blue, 710 mM β-mercaptoethanol, and 1 mM of the tyrosine phosphatase inhibitor sodium orthovanadate. Samples were passed five times through a 26.5 gauge

needle in order to shear any DNA that would otherwise interfere with protein migration through the gel, and then boiled for 5 minutes to denature the protein. The Lowry procedure (142) was used to quantify the protein content, using a Sigma protein assay kit. In this assay, an alkaline cupric tartrate reagent complexes with the peptide bonds within the protein, forming a purple-colour upon addition of the phenol reagent. Absorbance is read at 750 nm and the protein concentration is determined using bovine serum albumin as a standard. Equal amounts of protein from the various samples were run on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The amount of protein loaded varied from 40 μg to 150 μg between different experiments, however, it was constant within a given experiment. The proteins were transferred electrophoretically to nitrocellulose membranes in 16.8 mM Tris, pH 8.6, 192 mM glycine, 3.5 mM SDS, and 20% methanol transfer buffer for a period of 1.5 hours at 70 V. Next, the blots were rinsed twice with PBS containing 0.1% polyoxyethylenesorbitan monolaurate (Tween 20) followed by one hour incubation in the same solution containing 5% nonfat dry milk to block non-specific antibody binding. Blots were incubated with anti-ACLP serum (a generous gift from Dr. Matthew Layne, Harvard University) diluted 1:1000 (in PBS, 3% BSA, 0.02% sodium azide) and then with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit serum diluted 1:5000. Polyclonal anti-ACLP antibody was generated by immunizing New Zealand White rabbits with 100 μg of the C-terminal half of mouse ACLP encoding amino acids 615-1128. This ACLP antibody does not recognize AEBP1 (120). Membranes were then processed with an enhanced chemiluminescence reagent (NEN Life Science Products) to detect the horseradish peroxidase activity by its reaction with luminol (oxidation), which results in emission of light that can be detected with a

photosensitive Kodak™ film. To perform additional probings on the same membranes, the blots were first stripped of antibodies by washing them three times with a stripping solution (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) for 10 minutes at 60°C followed by blocking and incubation with the appropriate antibodies.

D. ***Northern blot analysis.***

At specific time points, cells were scraped in 600 μL lysis buffer consisting of 4M guanidine thiocyanate, 25 mM sodium citrate, 0.5% N-lauryl sacosine, and 0.1 M β-mercaptoethanol. Cell lysates were passed five times through a 23.5 gauge needle followed by the addition of 60 μL 2 M, sodium acetate pH 4.0, 600 μL water-saturated phenol, and 120 μL chloroform. The mixture was then centrifuged at 10,000 x g for 30 minutes at 4°C. The RNA-containing upper aqueous layer was transferred to a new tube, 600 μL of isopropanol was added, and the RNA allowed to precipitate overnight at -20°C. Next, RNA was spun down by centrifuging at 18,000 x g for 30 minutes at 4°C. The RNA pellet was resuspended in 100 μL lysis buffer followed by the addition of 300 μL of ethanol. RNA was once again allowed to precipitate overnight at -20°C followed by centrifugation at 18,000 x g for 30 minutes at 4°C. The pellet was then washed with 300 μL 70% ethanol and centrifuged once again as before. The RNA pellet was dissolved in 20-50 μL diethyl pyrocarbonate (DEPC) water and spectrophotometrically quantified at 260 and 280 nm using a Pharmacia Biotech Ultrospec. Two to four micrograms of RNA was prepared in a sample loading buffer consisting of 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mM EDTA, 6% formaldehyde, 50%

deionized formamide, 2 μl of 1:10 diluted ethidium bromide, and 1 μl RNA loading dye. The samples were heated at 55°C for 15 minutes, and then loaded onto a gel consisting of 1% agarose, 3% formaldehyde, 20 mM MOPS, and 1mM EDTA. The running buffer consisted of 20 mM MOPS, 1 mM EDTA, and 3% formaldehyde. The gel was initially run at 90 V until the entrance of the dye into the gel followed by 50 V for about one hour. The gel was viewed under UV light through a camera connected to a computer using the image Quant software to determine the presence of the 18S and 28S ribosomal RNA bands in order to confirm the integrity of the samples and confirm equal loading. The gel was washed for 5 minutes at room temperature in 0.9 M NaCl, 90 mM tri-sodium citrate.$2H_2O$, pH 7. RNA was transferred at room temperature through capillary action onto a H-bond nitrocellulose membrane in 3 M NaCl, 0.3 M tri-sodium citrate.$2H_2O$, pH 7. Next, the membrane was baked at 80°C for 4 hours followed by prehybridization in 0.6 M NaCl, 60 mM tri-sodium citrate.$2H_2O$, 0.002% Ficoll, 0.002% polyvinyl pyrrolidone, 0.002% BSA, 1mg/ml salmon sperm DNA, and 0.5% SDS, pH 7 for 4 hours at 60°C.

The probe was generated from the 0.7 kb cDNA fragment obtained by a Sac I restriction digest of the full size ACLP cDNA (a generous gift from Dr. Matthew Lane, Harvard University). The probe was radiolabeled using $^{32}\gamma$P-dCTP and Amersham Multiprime Probe Labeling System. RNA on the membrane was allowed to hybridize overnight at 60°C with the ACLP probe. The membrane was washed at room temperature with 0.3 M NaCl, 30 mM tri-sodium citrate.$2H_2O$, 1% SDS, pH 7 for 15 minutes, then another wash with the same solution at 42°C for 15 minutes, another wash with 0.15 M NaCl, 15 mM tri-sodium citrate $2H_2O$, 1% SDS, pH 7 for 15 minutes

followed by a wash with the same solution at 60°C for 15 minutes and a final wash with 15 mM NaCl, 1.5 mM tri-sodium citrate $2H_2O$, 1% SDS, pH 7 at 60°C for 8 minutes. To detect the ACLP mRNA bands, the membrane was exposed to a Kodak X-AR film and/or to a phosphor screen followed by scanning of the screen by a Typhoon 8600 Variable Mode Imager (Amersham Pharmacia Biotech).

E. DNA Preparation for Transfections

The plasmid constructs pLXSN-ACLP and pLXSN were a generous gift from Dr. Lane, Harvard University. In order to generate more of these plasmids, the constructs were first introduced into DH5α competent E. Coli (Life Technologies) by heat-shock transformation and allowed to grow followed by plasmid isolation using the QIAGEN Plasmid Maxi Kit: 0.7 μg of DNA was added to 50 μL of E. Coli and mixed by pipetting up and down. The cells were incubated on ice for 30 minutes and mixed every 15 minutes. Next, the cells were heat-shocked for 2 minutes at 42°C and placed on ice. 500 μL of LB media was added and the cells were incubated in a shaker at 37°C for 1 hour. The cells were then plated on a 100 mm agar dish containing 0.1 mg/mL ampicillin and incubated overnight at 37°C. Colonies were picked the next morning and grown in 5 mL LB media containing 0.1 mg/mL ampicillin for 6 hours at 37°C with vigorous shaking. Next, 200 uL of the starter culture was transferred into 100 mL of LB media containing 0.1 mg/mL ampicillin and incubated at 37°C for 10 to 12 hours with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C and then processed with the Qiagen Maxi Kit in order to isolate the plasmid constructs of

interest. DNA yield was determined by measuring the absorbances at 260 nm and 280 nm.

F. The Retroviral System of Infection

Thre retroviral system of gene transduction, first established in the 1980s, is a very reliable and efficient method of introducing a novel gene into a host genome by taking advantage of the integration machinery of naturally occuring retroviruses.

Retroviral gene delivery is quickly becoming the method of choice for gene expression in cells that render themselves difficult to other methods because it is generally faster, more efficient and more reliable, and has broader utility than alternative gene transfer protocols such as plasmid transfections.

Many modifications are made to wild-type retroviruses in order to create safe and effective gene expression systems. The most significant modification is the removal of genes involved in viral replication (gag, pol, and env genes) from the retroviral genome. The remaining necessary retroviral elements are used to form the retroviral vector. The vector retains the ψ^+ packaging signal so the RNA can be recognized and packaged into viral particles. The vector also contains long terminal repeats (LTRs) for insertion into the host cell genome and for their promoter and processing functions. Lastly, a selectable marker, such as antibiotic resistance, and the gene of interest are included in the vector.

To generate infectious virus particles that carry the gene of interest, specialized packaging cell lines have been constructed that contain chromosomally integrated genes

for viral gag, pol, and env proteins, all of which are required in *trans* to make virus. The *gag* gene encodes internal structural proteins; the *pol* gene encodes reverse transcriptase (RT) and integrase; and the *env* gene encodes the viral envelope protein, which resides on the viral surface and facilitates infection of the target cell by direct interaction with cell type-specific receptors. Thus, the host range of the virus is dictated not by the DNA vector but by the choice of the *env* gene used to construct the packaging cell. Ecotropic viruses can only infect rat and mouse cells, whereas amphotropic and polytropic viruses have a much broader host range.

The packaging cell line is transfected with the vector DNA and, at this point, either stable viral producer cell lines may be selected (providing the vector has an appropriate selectable marker), or virus is produced transiently as mRNAs that are transiently transcribed from the vector are encapsidated, and bud off into the cell supernatant. These supernatants are collected and used to infect target cells. Upon infection of the target cell, the viral RNA molecule is reverse transcribed by RT (present in the virion particle), and the cDNA of the gene of interest, flanked by the LTRs, is integrated into the host DNA. Because the vector itself does not express viral proteins, once a target cell is infected, the LTR expression cassette is incapable of proceeding through another round of virus production.

G. Stable Transfections of GP+E86 retroviral packaging cells.

Gp+E86 cells are derivatives of mouse NIH-3T3 fibroblasts that can be used to produce retrovirus upon generation of stably transfected colonies. GP+E86 cells obtained from ATCC were plated at a density of 1 x 10^5 cells per 60 mm dish in DMEM/1% pen-strep/2.5% FBS/7.5% calf serum. 15 μg of pLXSN-ACLP and pLXSN were precipitated overnight at -20°C with 0.1 volume of 3M sodium acetate, pH 5.2 and 2 volumes of 95% ethanol. The next day, the cells were changed into fresh growth media. DNA was isolated by centrifugation at 18,000 x g at 4°C for 15 minutes and resuspension of dry DNA pellet in 450 μL 0.1X TE buffer, pH 8.0 (1 mM Tris, pH 9.0, 0.1 mM EDTA). 50 μL of 2.5 M $CaCl_2$ was added to the DNA, the mixture was transferred into a 15 mL conical tube containing 500 μL of 2X Hepes Buffer Saline (HBS), the mixture was vigorously mixed by generating air bubbles with a pipette gun, and then incubated at room temperature for 30 minutes. The DNA mixture was then added to the cells in a dropwise circular fashion. 24 hours later, the cells were washed 5 times with the growth media in order to remove the $CaCl_2$ precipitate. Also, a control sham-transfected dish was manipulated in an identical fashion. The following day, each dish was split into ten 100 mm dishes. 24 hours later, the cells were changed into growth media containing the antibiotic G418 (450 μg/ml) for selection. In about seven days, colonies were picked and each colony was plated in a 60 mm dish and grown under selection. Upon confluence, each colony was split into one 35 mm dish and three T25 flasks. Upon confluence, the 35 mm dish was scraped in 1X Laemmli lysis buffer for determination of ACLP protein expression by Western analysis, the three T75 dishes were used to collect the viral

supernatant which was stored at -85°C. The cells were frozen at -85°C overnight and then stored in liquid nitrogen.

H. Transient Transfections of 293T/17 Phoenix-Eco retroviral packaging cells

Phoenix-Eco cells, derivatives of 293 human renal epithelial cells, were generously provided by the Nolan laboratory at Stanford University. These cells have numerous advantages over GP+E86 cells including a faster protocol, due to the generation of transiently transfected clones, and the generation of higher viral titres than those obtained with GP+E86 cells. 15 μg of DNA (pLXSN-ACLP and pLXSN) was sterilized by ethanol precipitation overnight as described earlier. Phoenix cells were plated at a density of 0.5 x 10^5 cells per 60 mm dish in 4 mL DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Next day, one hour before transfection, cells were changed into fresh growth media. DNA was isolated by centrifugation at 18,000 x g for 15 minutes and resuspension of dry DNA pellet in 450 μL sterile water. 50 μL of 2.5 M $CaCl_2$ was added to the DNA and the mixture was transferred into a 15 mL conical tube containing 500 μL of 2X HBS and bubbled vigorously. The DNA mixture was then added to the cells in a dropwise circular fashion. 24 hours later, the cells were changed into fresh growth media. The next day (48 hours post-transfection) the media was replaced again and the viral supernatant was collected 24 hours later (from 48 to 72 hours post-transfection). The Phoenix cells were then scraped in 200 μL 1X Laemmli lysis buffer for determination of ACLP protein expression by Western analysis.

I. Infection of 3T3-L1 preadipocytes.

The GP+E86 viral supernatants derived from the three T75 flasks were combined (15 mL total) in a 15 mL conical tube and stored at -85°C until the 3T3-L1 cells were ready for infection. The Phoenix-Eco-derived viral supernatants, on the other hand, were not stored but were collected and used directly on 3T3-L1 cells that were set up for infection. 3T3-L1 preadipocytes were plated at a density of 6.3x 10^3 cells/cm^2 and infected 24 hours later (cells were plated 48 hours after Phoenix cell transfection). The viral supernatants were filtered through 0.45 μm syringe filters and polybrene was added to give a final concentration of 4 μg/mL. The media of 3T3-L1 cells was then replaced with the filtered viral supernatant. The cells were infected for 24 hours and then grown to cell confluence with normal growth media. One set of cells was scraped in 1X Laemmli lysis buffer to determine ACLP protein expression by Western analysis while another set was induced to differentiate.

J. Triacylglycerol Assay

At the end of the 8-day differentiation period, the cells were processed to determine their triacylglycerol (TG) content (143). First, they were washed twice with cold 1X PBS. TG was extracted by the addition of 1 mL of a 2:3 (v/v) mixture of isopropanol to heptane. After 30 minutes, the solution was removed and another 0.5 mL of the same mixture was added. After 15 minutes, the solution was removed, both fractions were combined, and samples were dried in a Savant SpeedVac SC200. The TG

pellet was redissolved in 300 μL of isopropanol, saponified with 150 μL 1.8 M potassium hydroxide, incubated at room temperature for 10 minutes, reacted with 300 μL of 3mM sodium metaperiodate and 300 μL of 1:250 acetyl acetone solution to yield a coloured product upon incubation at 60°C for 15 minutes that was quantified spectrophotometrically at a wavelength of 410 nm. Aliquots of Triolene (7.5, 15, 45, 60, and 75 μg) were used and processed in an identical fashion to obtain a standard curve. The TG-extracted cells were scraped in 200 μL of 1X Laemmli lysis buffer and their protein content determined by the Lowry method as outlined under Western analysis. In order to account for variation in cell number, the TG mass (μg) for each well was normalized by dividing it by its protein content (mg) which reflects the number of cells.

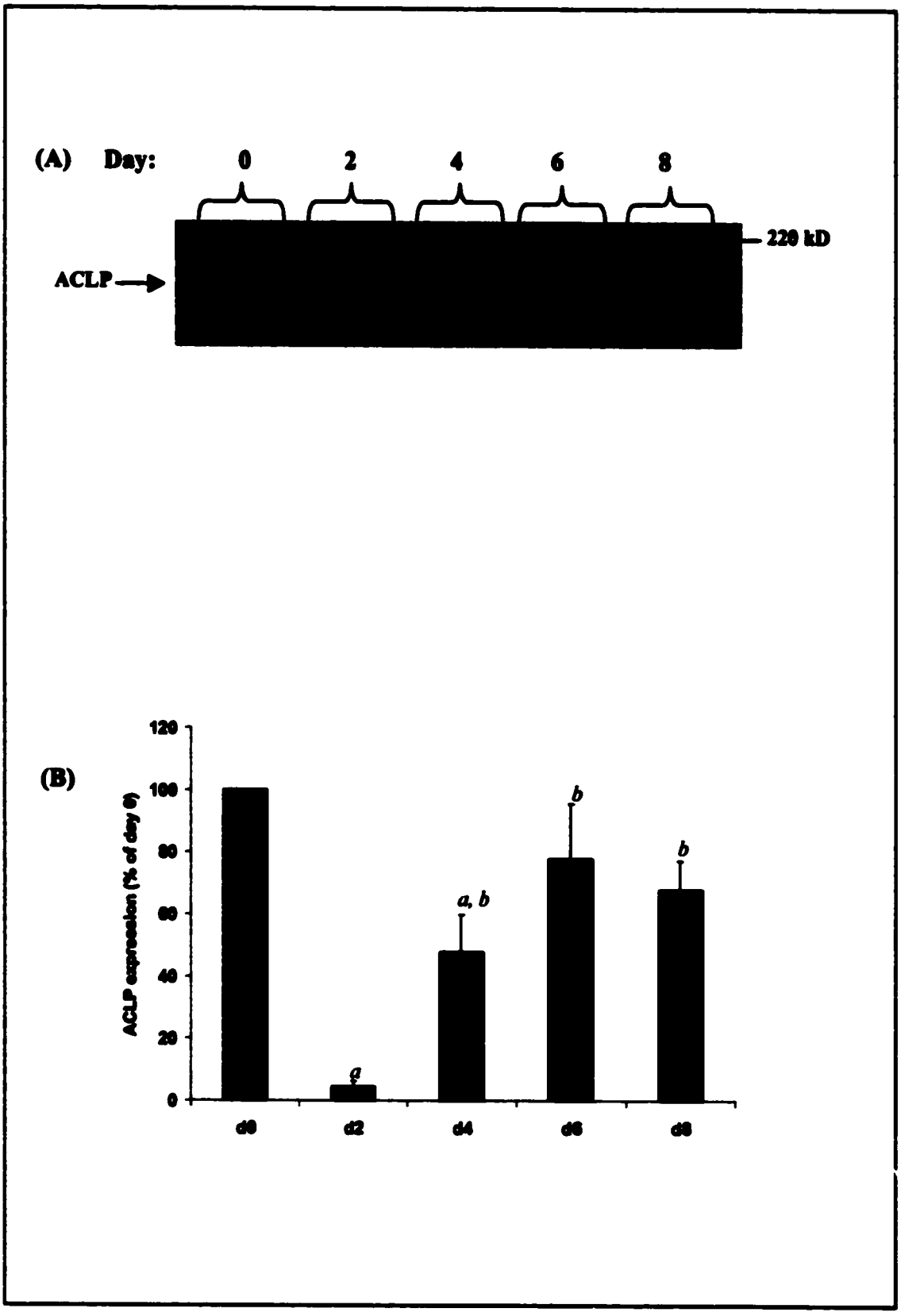
K. Statistical Analysis

Statistical analysis of data included a one-way analysis of variance (ANOVA) with Student-Newman-Keuls' post-test analysis. These analyses were performed with the aid of GraphPad InStat version 3.00 for Windows 95, GraphPad Software (San Diego California USA, www.graphpad.com).

IV. RESULTS

Our first approach towards characterizing the expression of ACLP during preadipocyte differentiation was to analyze its protein expression by Western blotting. Day 0 preadipocytes (2 days post-confluence) were induced to differentiate and total cell-lysates were obtained every two days up to day 8. With each sample in duplicate (two separate dishes), equal amounts of protein were loaded in all lanes (150 μg). Figure 4 A is a representative blot of 5 (days 0,8) and 3 (days 2,4,6) independent experiments while the combined densitometric and statistical analyses are illustrated in Figure 4 B. The level of ACLP protein expression during 3T3-L1 preadipocyte differentiation was observed to transiently but significantly decline on day 2 by about 10-fold and to revert back to near-normal levels by day 4. However, the levels on day 8 appear to still be somewhat less than that of day 0 (68 ± 9%).

Figure 4 Western-analysis of ACLP protein expression during differentiation of 3T3-L1 preadipocytes. (A) Western blot demonstrating the modulation of ACLP protein expression during an 8-day differentiation. Samples are in duplicate. 150 ug of protein was loaded across all lanes. (B) Densitometric analysis of Western blot bands expressed as percent of day 0 levels. Values represent mean ± S.E.M. (n=5 for day 0,8; n=3 for day 2,4,6). *a*, $p<0.001$ for day 2 versus day 0 and $p<0.01$ for day 4 versus day 0. *b*, $p<0.01$ for day 2 versus day 4 and $p<0.001$ for days 6 and 8 versus day 2 (ANOVA with Student-Newman-Keuls post-test analysis).


(A)
Day:
0
2
4
6
8
220 kD
ACLP
(B)
ACLP expression (% of day 0)
120
100
80
60
40
20
0
a
a, b
b
b
d0
d2
d4
d6
d8

In order to more precisely determine the timing of this modulation of ACLP, we decided to look closely at ACLP protein expression between days 0 and 4. Figure 5A shows the time-course Western analysis of ACLP expression. ACLP protein levels gradually decline to a minimum value of 9.6 ± 2.4% (mean ± S.E.M.; n=5) of day 0 levels and revert back to 72 ± 7% (mean ± S.E.M.; n=5) of day 0 levels by day 4 as shown by the densitometric analysis of the intensity of the bands in Figure 5C. To verify that the cells were undergoing differentiation, the blot was reprobed with PPARγ antibody. Indeed, we see a clear and significant upsurge in PPARγ expression (Figure 5B), indicating preadipocyte differentiation.

We next analyzed ACLP protein expression in the differentiation-resistant 3T3-C2 preadipocytes upon exposure to the same hormonal cocktail that induces differentiation of 3T3-L1 preadipocytes, in order to determine whether the ACLP down-regulation that is observed in the 3T3-L1 cells is differentiation-specific. Interestingly, we observed a pattern of ACLP protein expression that was similar to that of 3T3-L1 cells with a decrease to a minimum of 33 ± 9% (mean ± S.E.M.; n=3) of day 0 levels by day 2 and a reversal to day 0 levels bay day 4 (Figure 6 A and C), indicating that this event is, at least, independent of terminal differentiation. Moreover, although ACLP down-regulation may be necessary for differentiation, it is definitely not sufficient to induce this process, as indicated by a lack of PPARγ expression in 3T3-C2 cells (Figure 6B) that is consistent with absent morphological changes that would normally accompany differentiation.

Figure 5 Western blot analysis of ACLP and PPARγ expression between days 0 and 4 of the differentiation protocol of 3T3-L1 preadipocytes. (A) Western blot illustrating the gradual and transient ACLP down-regulation. Samples are in duplicate and 60 ug of protein was loaded across all lanes. (B) Western blot demonstrating the increase in PPARγ expression that accompanies differentiation. (C) Densitometric analysis of ACLP protein expression expressed as percentage of day 0 levels. Values represent averages of independent experiments (n=5 for day 0,2,4; n=3 for day 1,3) ± S.E.M. $p<0.001$ for all pairs except day 1 versus day 3 (ANOVA with Student-Newman-Keuls post-test analysis).

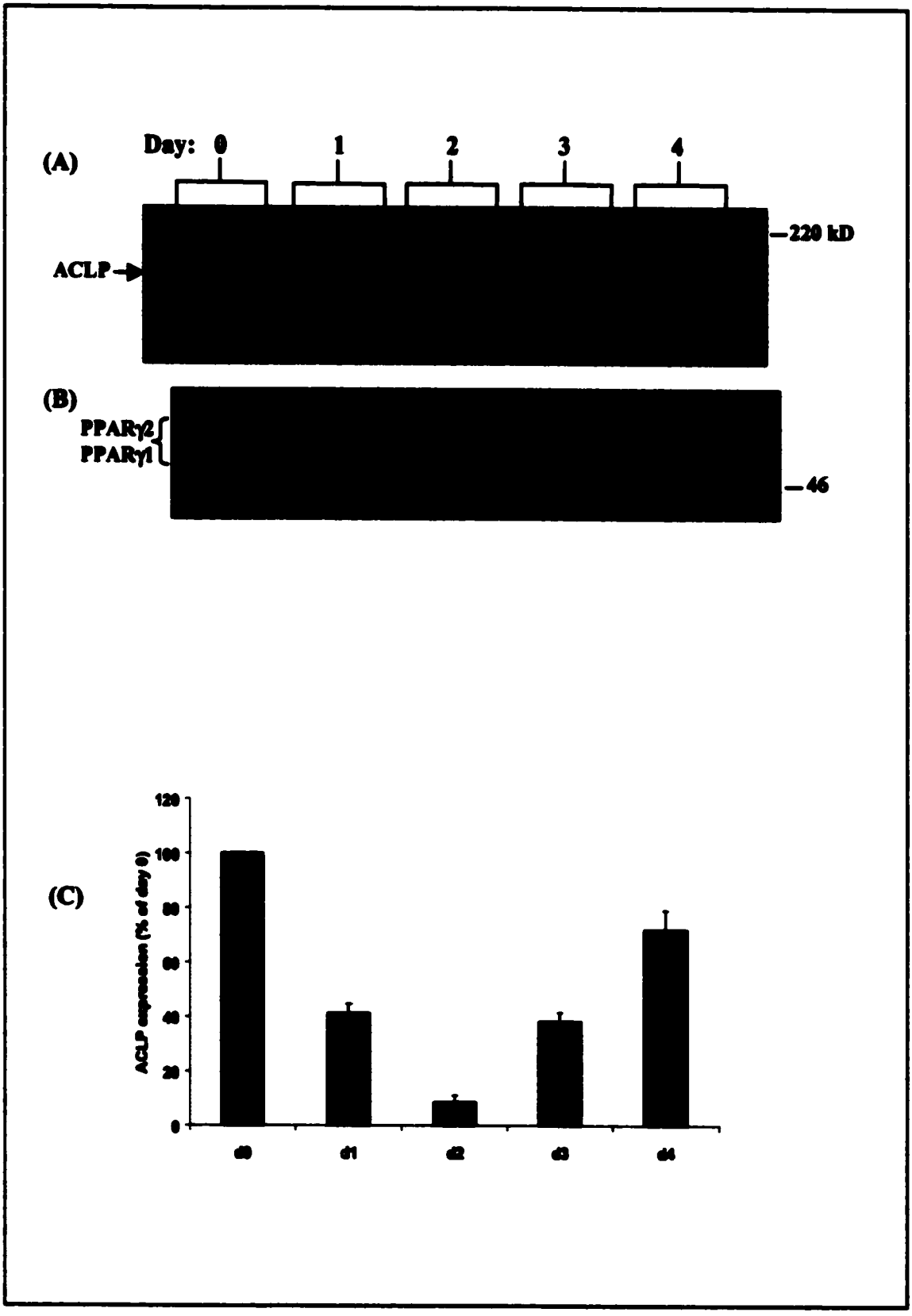
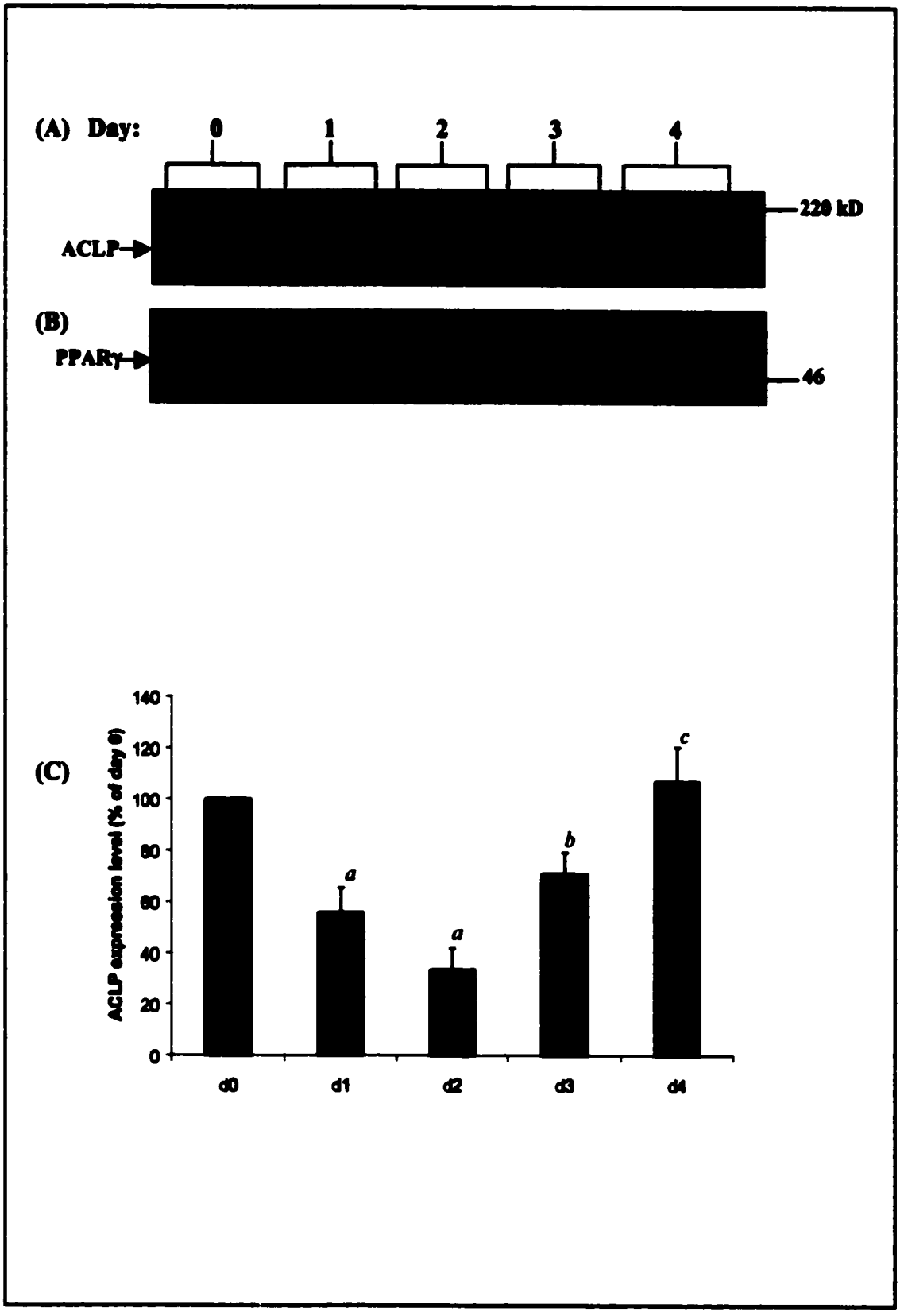

(A)
Day: 0
1
2
3
4
ACLP
220 kD
(B)
PPARγ2
PPARγ1
46
(C)
ACLP expression (% of day 0)
120
100
80
60
40
20
0
d0
d1
d2
d3
d4

Figure 6 Western blot analysis of ACLP expression in the 3T3-C2 cell line. (A) ACLP protein expression between days 0 and 4 upon exposure to the same hormonal cocktail that induces differentiation of 3T3-L1 preadipocytes. Samples are in duplicate and 70 ug of protein was loaded across all lanes. (B) Analysis of PPARγ expression. (C) Densitometric analysis of ACLP protein expression expressed as percent of day 0 levels. Values represent averages of three independent experiments (n=3) ± S.E.M. *a*, $p<0.05$ for days 1 versus day 0 and $p<0.01$ for day 2 versus day 0. *b*, $p<0.05$ for day 3 versus day 2. *c*, $p<0.05$ for day 4 versus day 3 (ANOVA with Student-Newman-Keuls post-test analysis).


(A) Day:
0
1
2
3
4
220 kD
ACLP
(B)
PPARγ
46
(C)
ACLP expression level (% of day 0)
140
120
100
80
60
40
20
0
a
a
b
c
d0
d1
d2
d3
d4

To gain further insight into the mechanism underlying the observed down-regulation of ACLP protein expression, Northern analysis was performed on RNA isolated from the cells at various times throughout the 8-day differentiation protocol as outlined under the methods section. Figure 7 reveals that the decrease in ACLP protein expression is paralleled by a decrease in ACLP mRNA levels. By day 2, ACLP mRNA decreases to a minimum value of 13 ± 8% (mean ± S.E.M.; n=4) of day 0 levels and revert back to only 31 ± 10% (mean ± S.E.M.; n=4) by day 4. In order to determine whether these levels would completely revert back to day 0 levels with more time, the Northern analysis was extended to day 8 (Figure 7B). Again, there is a marked decline in ACLP mRNA on day 2 and a gradual increase by day 4 and 8. On day 8, ACLP mRNA is still less than day 0 levels with a value of 60 ± 7% (mean ± SEM; n=4). Northern analysis of the 3T3-C2 cells reveals that in this cell line ACLP mRNA is also altered. We observe a decrease to 4.6 ± 1.7% (mean ± range; n=2) of day 0 levels by day 2 and a gradual reversion back to 71 ± 5% (mean ± range; n=2) of day 0 levels by day 4 (Figure 8).

Therefore, the general pattern of mRNA expression of ACLP during adipogenesis is consistent with that seen for ACLP protein expression. However, the extent of ACLP protein reduction was more pronounced for 3T3-L1 versus that of 3T3-C2 cells, whereas the decreases in ACLP mRNA were comparable in both cell lines.

Figure 7 Northern blot analysis of ACLP mRNA expression during differentiation of 3T3-L1 preadipocytes. (A) ACLP mRNA expression between days 0 and 4 of differentiation. Day 2 control undifferentiated cells (lanes labeled 2C) were kept in normal growth media for 2 days. Samples are in duplicate and 4ug of RNA were loaded across all lanes. (B) ACLP mRNA expression between days 0 and 8. Samples are in duplicate and 4 ug of RNA was loaded across all lanes. (C) Densitometric analysis of Northern blots expressed as percentage of day 0 levels. Values represent mean ± range for days 1, 2C, 3 (n=2) and mean ± S.E.M. for days 2, 4, 8 (n=4) (ANOVA with Student-Newman-Keuls post-test analysis).

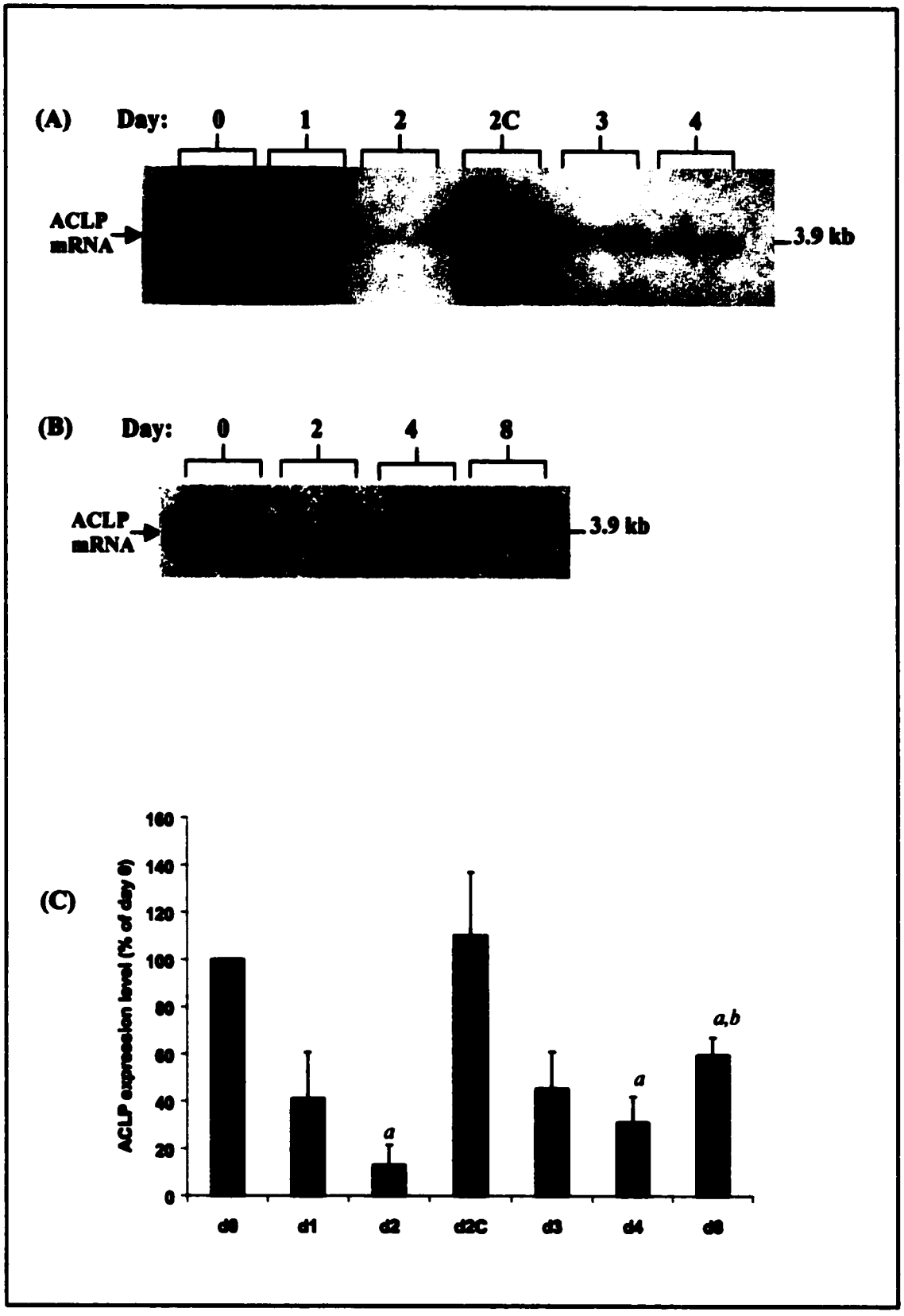
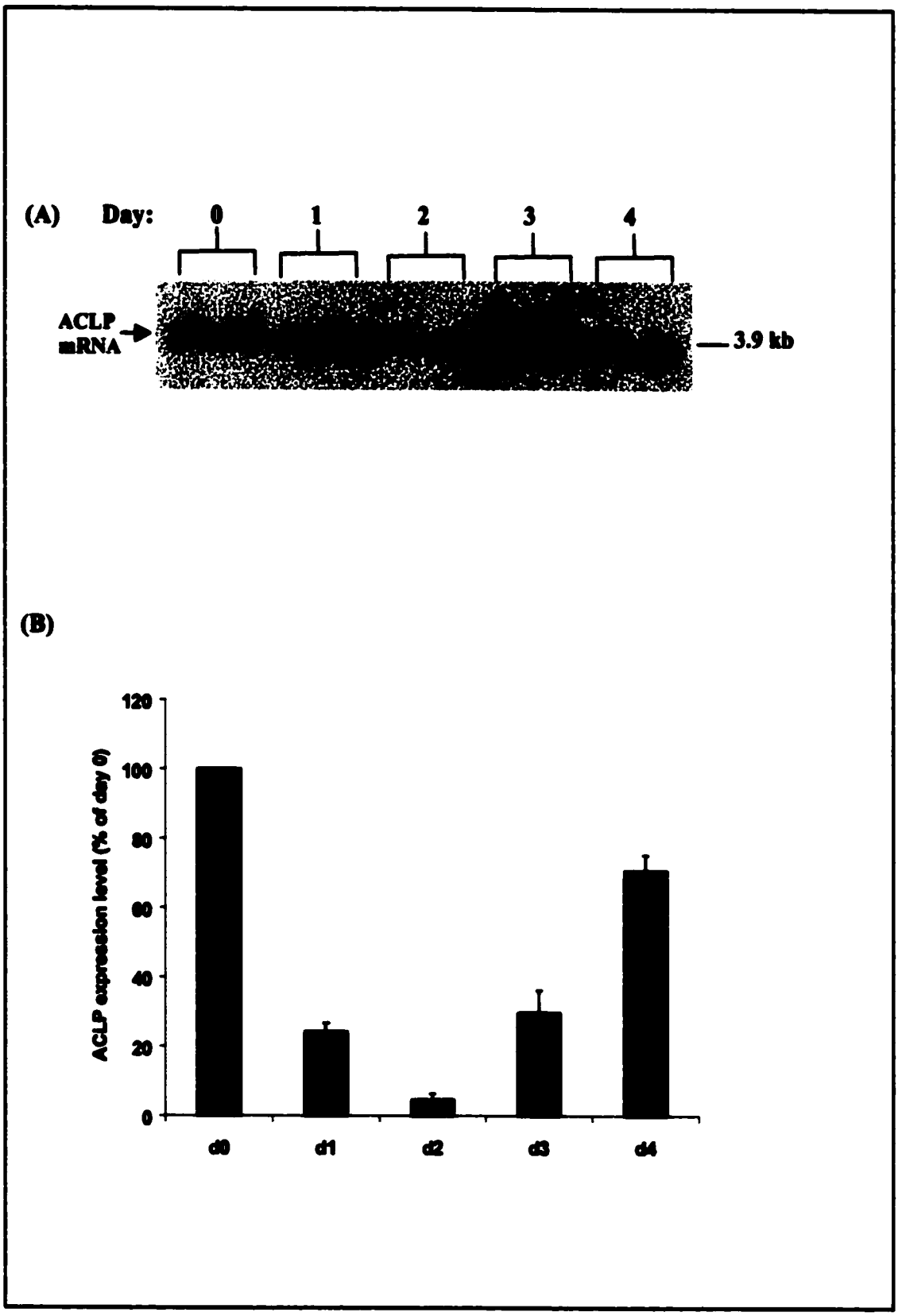

(A)
Day:
0
1
2
2C
3
4
ACLP mRNA
3.9 kb
(B)
Day:
0
2
4
8
ACLP mRNA
3.9 kb
(C)
ACLP expression level (% of day 0)
160
140
120
100
80
60
40
20
0
a
a
a,b
d0
d1
d2
d2C
d3
d4
d8

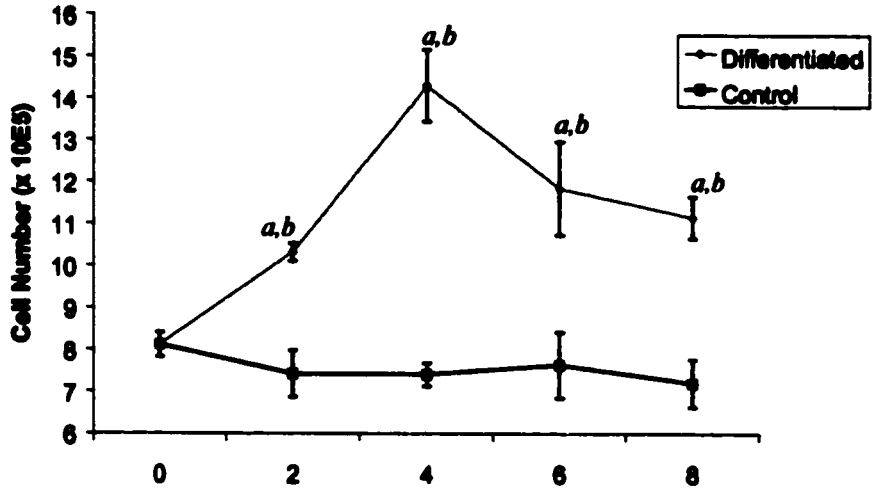
Figure 8 Northern blot analysis of ACLP mRNA expression in 3T3-C2 cells. (A) ACLP mRNA expression between days 0 and 4 upon exposure to hormonal cocktail that induces differentiation in 3T3-L1 cells. Samples are in duplicate and 4 ug of RNA was loaded across all lanes. Day 2 control undifferentiated cells (lane labeled 2C) were kept in normal growth media. (B) Densitometric analysis of the Northern blots expressed as percent of day 0 levels. Values represent mean of two independent experiments ± range.


(A)
Day:
0
1
2
3
4
ACLP mRNA
3.9 kb
(B)
ACLP expression level (% of day 0)
120
100
80
60
40
20
0
d0
d1
d2
d3
d4

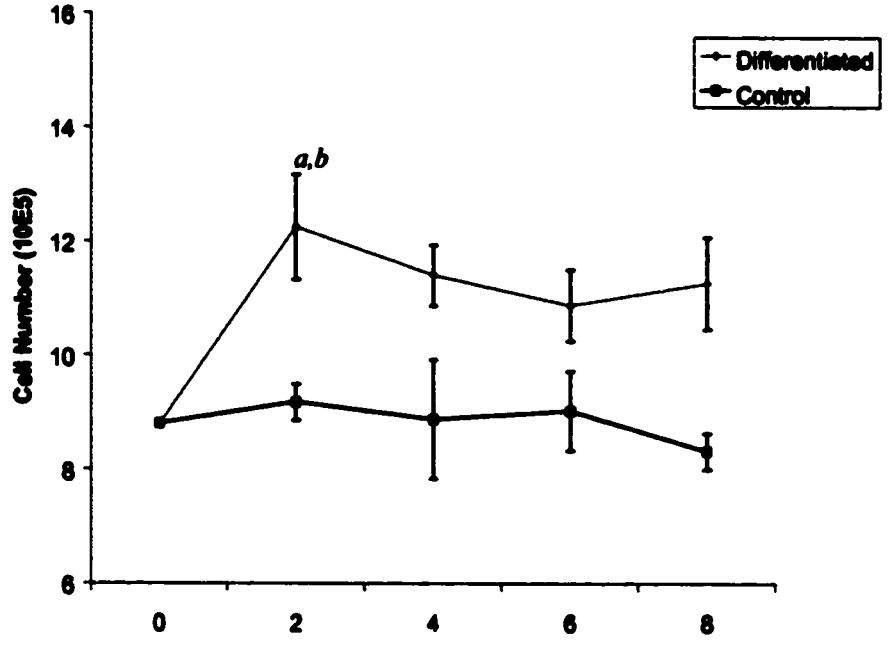
The timing of the decrease in ACLP mRNA and protein expression we have observed coincides with the early clonal expansion phase of adipogenesis. We investigated whether the extent of clonal expansion correlated with the decline in ACLP protein levels observed in the 3T3-L1 and 3T3-C2 cell lines. Cell count experiments were performed to compare the clonal expansion of these two cell lines. As expected, the 3T3-L1 cells roughly double in number by day 4 (76 ± 11% increase in cell number; mean ± S.E.M.; n=4) and maintain a constant cell population thereafter (Figure 9A). As reported in other studies (Richon, 1997), the 3T3-C2 cells also appeared to undergo a somewhat limited proliferation, increasing 39 ± 10% (mean ± S.E.M.; n=3) by day 2 (Figure 9B). It would appear that the steeper decline in ACLP protein expression occurring in 3T3-L1 cells is associated with a more prominent clonal expansion.

Figure 9 Characterization of clonal expansion in 3T3-L1 and 3T3-C2 cell lines. (A) Cell number counts between days 0 and 8 of 3T3-L1 differentiation. Values represent the means of four independent experiments (performed in duplicate) ± S.E.M. *a*, $p<0.05$, $p<0.01$ for day 2 differentiated versus day 0, day 4 differentiated versus day 0, and days 6, 8 differentiated versus day 0, respectively. *b*, $p<0.05$ for day 2, $p<0.001$ for day 4, $p<0.05$ for days 6 and 8 differentiated versus their corresponding control. (B) Cell number counts between days 0 and 8 of 3T3-C2 stimulation with hormonal cocktail. Values represent the means of three independent experiments (performed in duplicate) ± S.E.M. *a*, $p<0.05$ for day 2 differentiated versus day 0; *b*, $p<0.05$ for day 2 differentiated versus day 2 control. (ANOVA with Student-Newman-Keuls post-test analysis)

(A)


Cell Number (x 10E5)
16
15
14
13
12
11
10
9
8
7
6
0
2
4
6
8
a,b
a,b
a,b
a,b
Differentiated
Control

(B)


Cell Number (10E5)
16
14
12
10
8
6
0
2
4
6
8
a,b
Differentiated
Control

Optimal 3T3-L1 preadipocyte differentiation requires converging signaling pathways that are activated by dexamethasone, IBMX, and insulin. To determine whether ACLP down-regulation is a result of the combined effects or due to only one of these signaling pathways, 3T3-L1 cells were analysed with respect to ACLP protein expression upon exposure to the individual components of the differentiation stimulus.

Three independent experiments were performed and representative blots are shown for ACLP expression and PPARγ expression in Figure 10 A and B, respectively. Combined results and statistical analysis for ACLP expression are shown in Figure 10 C.

As shown in previous experiments, normal differentiation lead to a significant decline in ACLP protein levels by day 2 (5 ± 1%; mean ± S.E.M.; n=3) with a reversal to 64 ± 16% (mean ± S.E.M.; n=3) by day 4 (Figure 10A, lanes 2 and 6; Figure 10C).

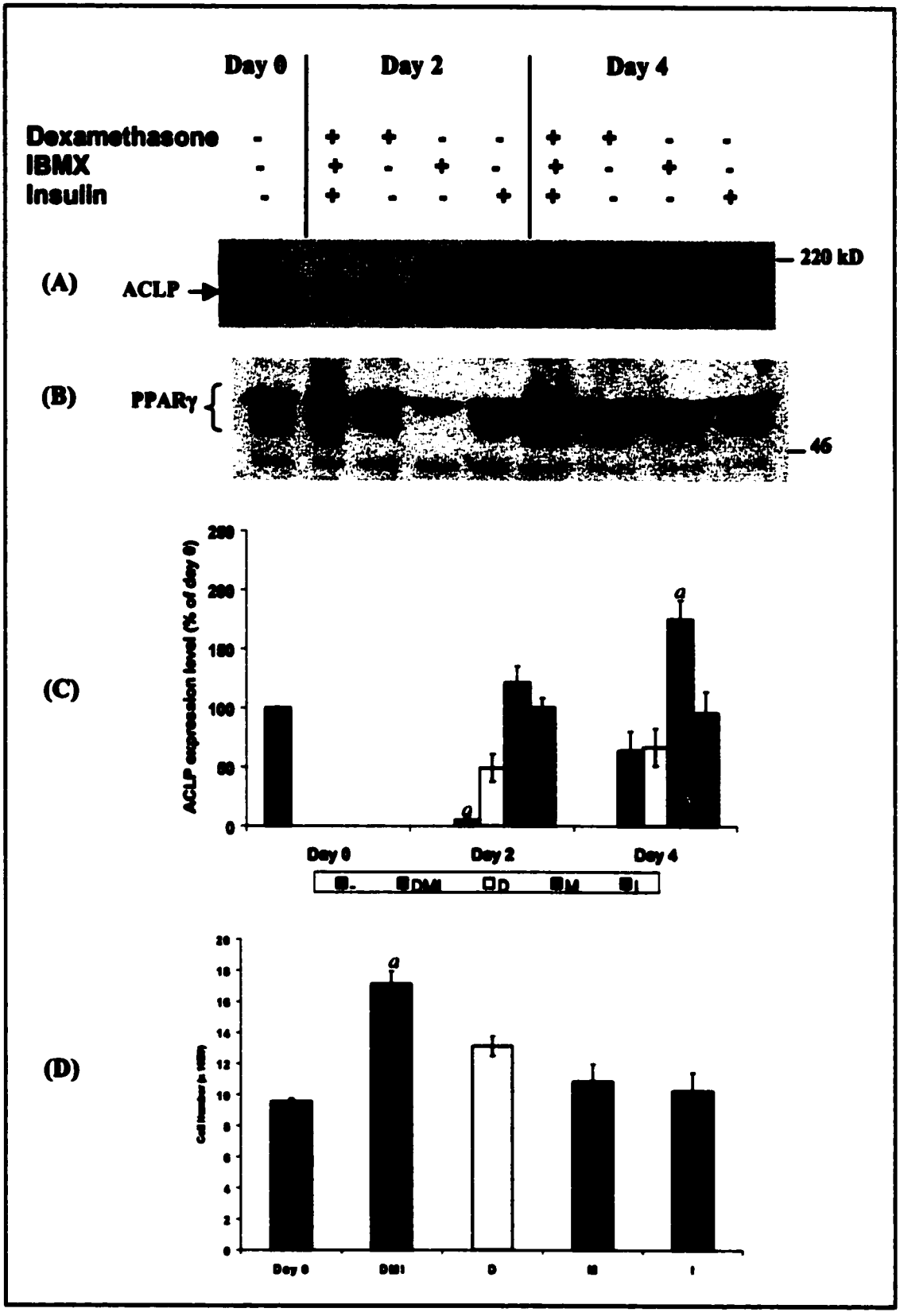
Insulin or IBMX alone were not capable of decreasing ACLP expression; however, it appeared that dexamethasone alone resulted in a slight down-regulation of ACLP on day 2 (49 ± 12%; mean ± S.E.M.; n=3) (Figure 10A, lane 3; Figure 10C). However, this decline in ACLP is not statistically significant ($p>0.05$, ANOVA with Student-Newman-Keuls post-test analysis). Interestingly, it appears that IBMX alone results in an increase in ACLP expression by day 4 (175 ± 16%; mean ± S.E.M.; n=3) (Figure 10A, lane 8; Figure 10C).

Additionally, the ability of these components to induce differentiation was analysed by reprobing the membrane with PPARγ antibody to detect PPARγ expression. As expected there was an induction of PPARγ in normally differentiated cells (Figure 10B, lanes 2 and 6). PPARγ levels remained comparable to day 0 levels in cells induced

with insulin or IBMX alone, but there was a slight increase on days 2 and 4 with dexamethasone alone (Figure 10B, lanes 3 and 7 respectively).

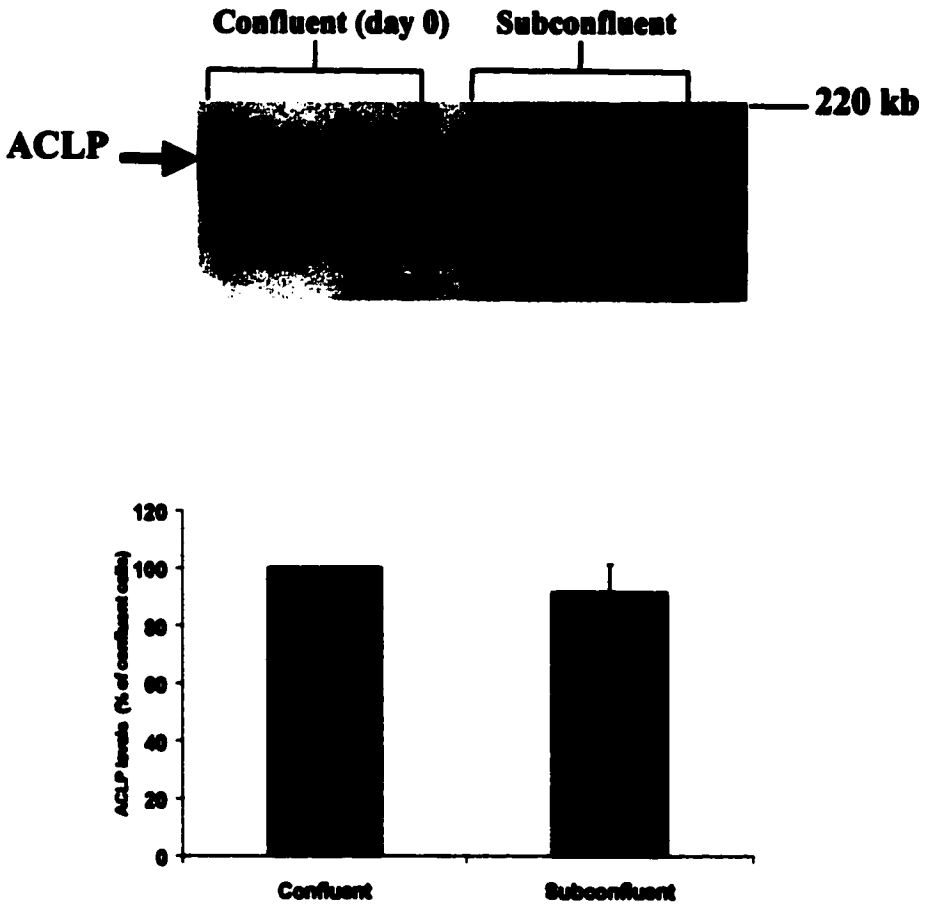
We decided to also test the effect of the each individual component on inducing clonal expansion. As shown in Figure 10D, normal differentiation (DMI) resulted in an increase in cell numbers similar to that observed previously in Figure 9 A. IBMX or insulin alone did not induce proliferation while it appears that dexamethasone alone resulted in a slight increase in cell number. However, this increase is not statistically significant ($p>0.05$, ANOVA with Student-Neuman-Keuls post-test analysis).

Figure 10 The effect of the individual components of the differentiation media on ACLP protein expression and cell numbers. (A) Western blot analysis of ACLP protein expression upon exposure to each component of the differentiation cocktail. 40 ug of protein was loaded across all lanes. (B) Analysis of PPAR expression. Blots shown are representative of three independent experiments. (C) Densitometric analysis of ACLP Western blot bands expressed percentage of day 0 levels (mean ± S.E.M.; n=3). *a*, $p<0.001$ for day 0 versus day 2 DMI and $p<0.01$ for day 0 versus day 4 IBMX. (D) Changes in cell number by day 4 in response to exposure to insulin, dexamethasone, or IBMX expressed as mean ± S.E.M. (n=3). *a*, $p<0.001$ for DMI versus day 0. (ANOVA with Student-Newman-Keuls post-test analysis).


Day 0
Day 2
Day 4
Dexamethasone
IBMX
Insulin
(A)
ACLP
220 kD
(B)
PPARγ
46
(C)
Day 0
Day 2
Day 4
(D)

We next examined whether the observed decline in ACLP level was specific only to the proliferation that occurs during the mitotic clonal expansion phase. To address this, we looked at the levels of ACLP expression in proliferating, subconfluent 3T3-L1 preadipocytes and compared it to those in growth-arrested, confluent (day 0) cells. If the observed decline in ACLP expression that occurs during adipogenesis is simply a general response that occurs in dividing preadipocytes, then we would expect to observe lower ACLP levels in pre-confluent cells undergoing normal cell-cyle mitosis versus growth-arrested confluent preadipocytes. However, as shown in Figure 11, the level of ACLP protein expression is unchanged in subconfluent proliferation, indicating that the decrease in ACLP observed during post-confluent clonal expansion is a specific feature of this phase of differentiation.

Figure 11 ACLP protein expression in subconfluent proliferation of 3T3-L1 preadipocytes versus confluent growth-arrested preadipocytes. Densitometric analysis of Western blots expressed as percentage of ACLP expression in confluent cells. Values represent mean ± range (n=2).


Confluent (day 0)
Subconfluent
220 kb
ACLP
120
100
80
60
40
20
0
Confluent
Subconfluent

The observations that ACLP expression is markedly down-regulated on day 2 of differentiation led us to hypothesize that the transient decrease in an as yet unknown function of ACLP during the mitotic clonal expansion phase may be necessary for the progression of differentiation. In order to test this hypothesis, retroviral systems for infection were employed to constitutively overexpress ACLP in 3T3-L1 preadipocytes and thus prevent the transient down-regulation of ACLP.

The first set of experiments was carried using the GP+E 86 packaging cells to generate the retrovirus. This approach is fairly time-consuming since it involves generation of stable GP+E 86 transfected colonies in order to obtain high titres of recombinant retrovirus. For this reason, the subsequent overexpression experiment was carried out using the more efficient Phoenix-Eco packaging cell line, a derivative of the 293 cells.

Results of three independent retroviral infection experimetns using the GP+E86 packaging cell line are shown in Figure 12. Figure 12A is a Western blot analysis demonstrating ACLP protein expression in 3T3-L1 cells infected with the ACLP-containing vector, with the empty vector only (pLXSN) or uninfected, on day 8 of preadipocyte differentiation. Figure 12B is the combined results of the three experiments showing triacylglycerol accumulation (normalized by protein content) on day 8 of preadipocyte differentiation. There are no significant differences in triacylglycerol content, and hence in the extent of differentiation ($p>0.05$; ANOVA with Student-Newman-Keuls post-test analysis).

Figure 12 Effect of ACLP overexpression on 3T3-L1 adipogenesis (GP+E 86 packaging cell line used to generate retrovirus). (A) ACLP protein expression on day 8 of differentiation of uninfected cells and cells infected either with retrovirus containing the pLXSN-ACLP or the empty vector (pLXSN). (B) Combined triacylglycerol accumulation normalized by protein content of cells shown in (A). Values represent mean ± S.E.M. (n=3 for ACLP and pLXSN, n=1 for Uninfected). Differences are not statistically significant ($p>0.05$) (ANOVA with Student-Newman-Keuls post-test analysis).

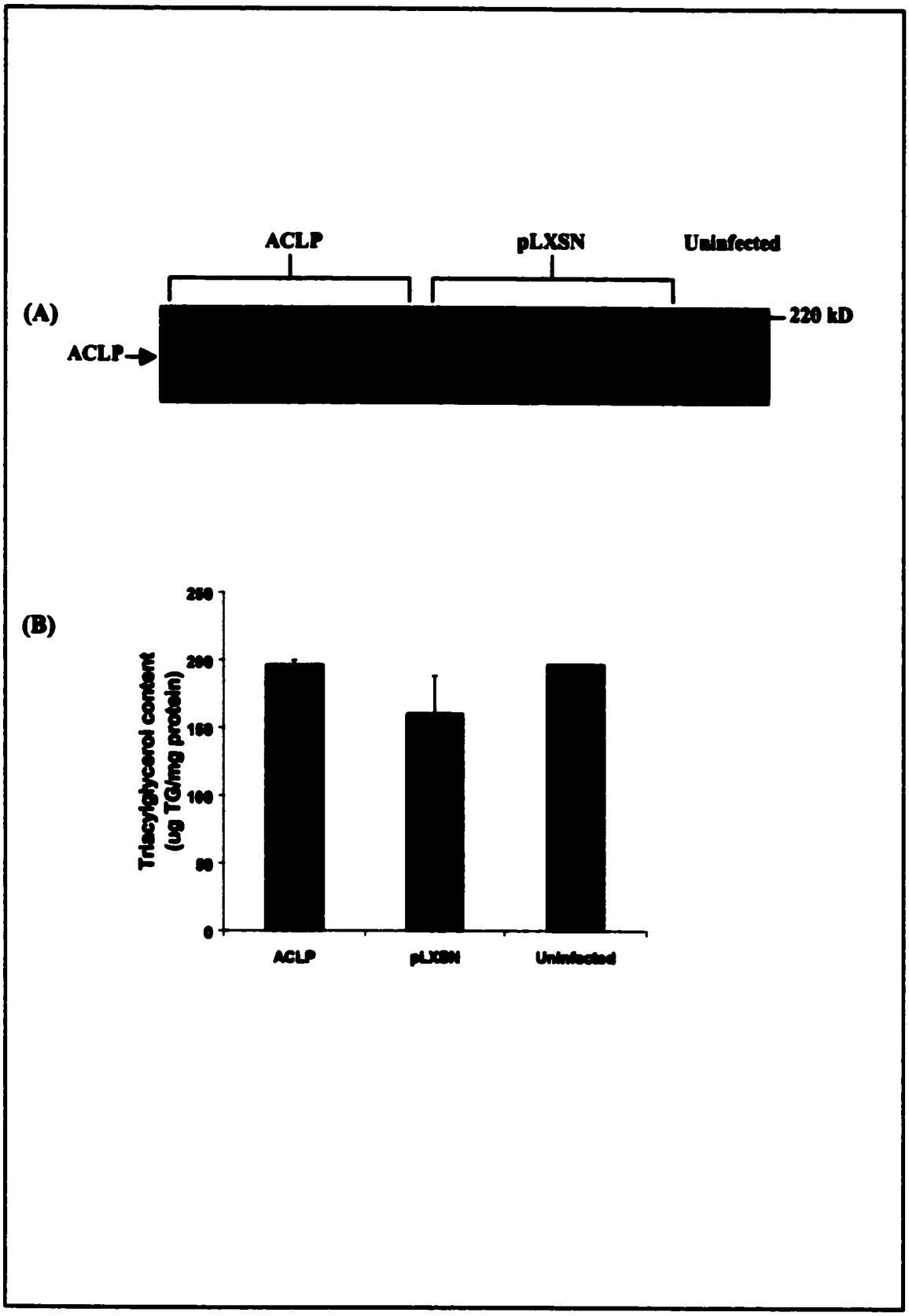
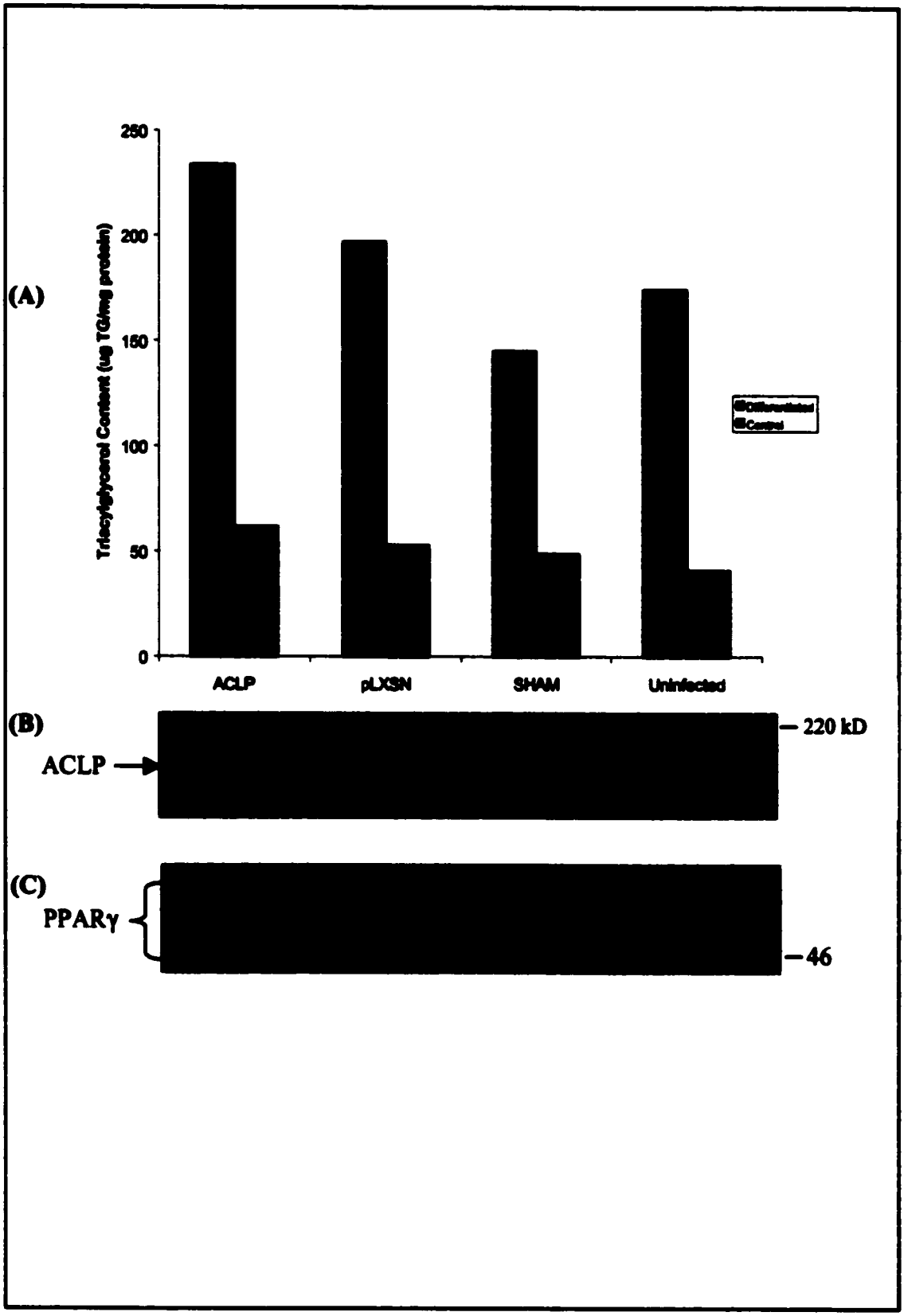

ACLP
pLXSN
Uninfected
(A)
220 kD
ACLP
(B)
Triacylglycerol content
(ug TG/mg protein)
200
150
100
50
0
ACLP
pLXSN
Uninfected

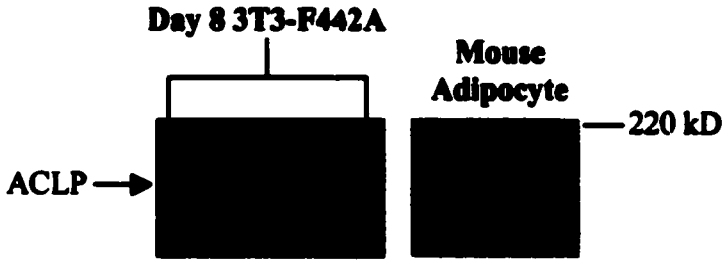
Figure 13 illustrates the extent of differentiation and ACLP protein expression in 3T3-L1 cells overexpressing ACLP (infected by retrovirus generated by Phoenix-Eco packaging cells) and control cells. Figures 13 demonstrates A) triacylglycerol accumulation (normalized by protein content), B) ACLP protein expression on day 8 of differentiation, and C) PPARγ expression. Again, as seen in the previous figure, ACLP overexpression, achieved by employing a different packaging cell line, does not have an effect on preadipocyte differentiation as measured by accumulation of triacylglycerol and PPARγ expression.

Figure 13 Effect of ACLP overexpression on adipogenesis of 3T3-L1 cells (Phoenix-Eco packaging cell line used to generate retrovirus). (A) Triacylglycerol accumulation (normalized by protein content) in four cell populations either infected to overexpress ACLP, infected with the empty vector (pLXSN), SHAM infected (with no DNA), or uninfected. (B) ACLP expression on day 8 of differentiation in cells of (A). (C) PPARγ expression in corresponding cells of (B).


(A)
Triacylglycerol Content (ug TG/mg protein)
250
200
150
100
50
0
ACLP
pLXSN
SHAM
Uninfected
(B)
ACLP
220 kD
(C)
PPARγ
46

ACLP protein expression was examined in two additional adipocyte cell culture models, the 3T3-F442A preadipocytes and primary mouse adipocytes. Western blot results are shown in Figure 14. The expression of ACLP protein is verified by the presence of 175 kD bands in both the 3T3-F442A adipocyte and in primary mouse adipocytes.

Figure 14 ACLP protein expression in 3T3-F442A cells and primary mouse adipocytes. Equal protein (25 μg) was loaded for all lanes (in duplicate for 3T3-F442A adipocytes).


Day 8 3T3-F442A
Mouse
Adipocyte
220 kD
ACLP

V. DISCUSSION

Obesity develops as a result of a positive energy balance which leads to an excess accumulation of triacylglycerol in existing adipocytes which can, in turn, stimulate the formation of new adipocytes from precursor preadipocytes.

3T3-L1 preadipocyte differentiation follows a specific program, in which the cells start a short finite phase of proliferation upon induction with the appropriate hormones and then enter terminal differentiation during which they attain the mature adipocyte morphology. During the course of differentiation, the expression of a myriad of genes is modulated, some transiently, to allow the normal progression of differentiation. By acquiring knowledge of these genes and their products, we may ultimately be able to design new therapeutic approaches to target their expression to interfere with and block the formation of new adipocytes. In this study we have examined the regulation of expression of the novel protein, ACLP, suggested to be involved in vascular smooth muscle cell differentiation (120), during 3T3-L1 adipogenesis.

There is a transient but significant down-regulation of ACLP protein expression down to a minimum level by day 2 of an 8-day differentiation protocol in both the 3T3-L1 and the 3T3-C2 cell lines which reverses to near-original (day 0) levels by day 4. However, the extent of ACLP protein down-regulation is more pronounced in the 3T3-L1 cells than in the differentiation-resistant 3T3-C2 cells.

The observed decrease in ACLP protein levels could arise from a decrease in the transcription of the *ACLP* gene. Northern blot analysis of 3T3-L1 preadipocyte differentiation demonstrates a decrease in ACLP mRNA on days 1 and 2 that closely resembles that of its protein, suggesting that transcriptional regulation of the *ACLP* gene

is, in part, responsible for the observed decline in the levels of ACLP protein. Another possible mechanism that cannot be entirely ruled out to account for the decrease in levels of ACLP mRNA is an increase in the rate of its mRNA degradation. In order to test for changes in mRNA stability, one could perform nuclear run-off experiments. However, since it appears that there is an equivalent drop in ACLP mRNA in the 3T3-C2 cells, perhaps post-translational control of the ACLP protein, such as regulation of its degradation, also plays a role to account for the more profound drop in ACLP protein in 3T3-L1 cells. This dual mechanism would ensure a strict and quick down-regulation of ACLP protein, even though the ACLP mRNA is down-regulated to the same extent in both cell lines. In fact, translational regulation is suggested to be the mechanism of control in many tissues such as the kidney and the colon where there is abundant expression of the ACLP mRNA but not of the protein (120). Further evidence for an active translational regulation in the 3T3-L1 cells comes from the observation that although ACLP protein expression returns to near-original levels by day 4, the levels of its mRNA, despite being higher than day 2 levels, are still much lower than day 0 levels. It is possible that in the 3T3-L1 cells, the observed reversal in protein expression from day 2 levels (seen by day 4) is due to the combination of enhanced rates of both transcription and translation, and, perhaps, decreased degradation of the ACLP protein.

Although the 3T3-C2 cells are described as differentiation-resistant, they are only so with respect to terminal differentiation since they are able to enter the early clonal expansion phase and undergo a limited proliferative phase as compared to their 3T3-L1 counterparts (59). Our cell count experiments carried out in order to characterize the increases in cell number that occurs during the clonal expansion phase of preadipocyte

differentiation are in agreement with those reported by Richon and colleagues (59). The 3T3-L1 cells rapidly increase in number upon the onset of differentiation reaching a maximum by day 4 (76 ± 11%) and maintaining a constant cell number thereafter. In contrast, the 3T3-C2 cells undergo a lesser degree of clonal expansion, reaching a maximum by day 2 (39 ± 10%) and maintaining constant cell numbers thereafter. It is very interesting to note that the extent of ACLP protein down-regulation correlates with the degree of clonal expansion, suggesting a potential link between ACLP and mitotic clonal expansion.

The 3T3-C2 cells do not express PPARδ and PPARγ which may account for their resistance to terminal differentiation. Indeed, the ectopic expression of PPARδ has been shown to induce PPARγ expression, rendering the 3T3-C2 cells differentiation-competent (97). Since the decrease in ACLP is intact in the clonal expansion phase of 3T3-C2 cells, the requirement for PPARδ, followed by PPARγ, must follow the ACLP modulation. However, since ACLP protein down-regulation is not as pronounced in this cell line, it is possible that the presence of signaling pathways downstream of PPARγ, involved in full differentiation, may potentiate the drop in ACLP by up-regulating the rate of ACLP protein degradation (as discussed above). Furthermore, these results suggest that although down-regulation of ACLP may be necessary, it is not a sufficient event for the induction of terminal differentiation in 3T3-C2 cells.

The presence of ACLP protein in two additional adipocyte cell culture models, the 3T3-F442A and primary mouse adipocytes, further strengthens the view that ACLP plays a role in the physiology of adipose tissue.

We studied the effect of the individual components of the differentiation medium on ACLP protein expression. Our results indicate that none of the three pathways, induced either by insulin, dexamethasone, or IBMX, on its own, is responsible for the full-fledged down-regulation of ACLP expression. Rather, this is a process that is dependent on the combined action of the three components of the differentiation cocktail and, as such, ACLP down-regulation is a differentiation-dependent event. Interestingly, we did observe a partial decline in ACLP levels in response to dexamethasone alone. However, we cannot determine whether this is due to a direct, differentiation-independent action of dexamethasone or due to partial induction of differentiation as observed by a slight increase in PPARγ expression. This observed effect of dexamethasone on PPARγ is consistent with the findings of Hamm and coworkers (144). Another interesting and unexpected observation was an increase in ACLP expression by day 4 upon exposure to IBMX alone for the first two days, suggesting that perhaps it is the presence of IBMX for the first two days that is responsible for the reversal in ACLP protein expression seen with the normal differentiation protocol (DMI).

Additionally, cell count experiments revealed that none of the three components of the differentiation medium alone could induce clonal expansion. However, there was a trend suggesting a slight increase in cell number with dexamethasone alone that, nonetheless, may reflect a partial induction of clonal expansion in response to dexamethasone that correlates with the observed partial increase in PPARγ and the partial decrease in ACLP expression.

Prospective work includes analysis of ACLP expression during 3T3-L1 differentiation induced by an alternate protocol that does not require dexamethasone, as exposure of cells to dexamethasone alone resulted in a partial drop in ACLP levels with a slight increase in PPARγ. This approach would involve the use of PPARγ agonists known as thiazolidinediones (TZDs). Alternatively, analysis of the expression of earlier molecular markers such as SREBP-1, C/EBPδ and C/EBPβ would be useful in determining whether the observed effects of dexamethasone alone were due to a partial induction of differentiation or due to a non-specific effect of dexamethasone, as the levels of these markers would be expected to be elevated in the former case. To further strengthen the evidence that ACLP down-regulation is a differentiation-dependent process, confluent 3T3-L1 preadipocytes could be subjected to continuous proliferation without the induction of differentiation. This can be achieved by exposing the cells to the non-adipogenic hormone platelet-derived growth factor (PDGF) and verifying that ACLP expression does not decline.

Examination of preconfluent proliferation revealed that ACLP protein expression levels are no different than in growth-arrested confluent cells, suggesting that the observed down-regulation of ACLP is specific to the proliferation that occurs during the mitotic clonal expansion phase. In contrast, Layne and colleagues have demonstrated that ACLP expression, both at the levels of protein and mRNA, is down-regulated in actively proliferating vascular smooth muscle cells as compared to quiescent cells (120). There would appear to be intrinsic differences between 3T3-L1 preadipocytes, which are non-differentiated, and VSMCs, which are already differentiated, with respect to ACLP regulation.

As discussed earlier in the introduction, another inherent difference between the mitotic clonal expansion and subconfluent proliferation has been shown to lie in the differential expression of the retinoblastoma proteins p130 and p107 (59). Thus, the differential expression of ACLP in subconfluent preadipocytes versus differentiating preadipocytes provides further support for the notion that, in 3T3-L1 cells, there exist two different cell cycles: one associated with hormonally stimulated mitotic clonal expansion and another associated with normal subconfluent proliferation.

In order to shed some light on the possible role of ACLP in adipogenesis, we overexpressed it in 3T3-L1 preadipocytes using a retroviral system of infection and analyzed its effects on differentiation, as measured by triacylglycerol accumulation and PPARγ expression. Based on our data revealing that ACLP levels drop severely in early differentiation, we hypothesized that ACLP may be a negative regulator. We anticipated that by overexpressing ACLP and, thus, preventing its transient down-regulation, we would have been able to inhibit adipogenesis. We performed four experiments, three with the NIH-3T3 derived GP+E86 and one with the 293T derived Phoenix-Eco packaging cell lines, in which the overexpression is clearly evident on day 8 of differentiation. The results of these experiments show that ACLP overexpression did not have an inhibitory effect on adipogenesis. Analysis of the infected cells demonstrates that ACLP is significantly overexpressed in day 8 adipocytes. It is expected that the overexpression levels of a constitutively expressed gene would not alter with time. However, given the characteristic down-regulation of endogenous levels of ACLP which may also be partly due to increased degradation, it is plausible that levels of ACLP protein may still be sufficiently down-regulated on day 2 of differentiation of ACLP-

overexpressing cells as to elicit normal differentiation. Nonetheless, these results could also mean that the down-regulation of ACLP is not necessary for adipogenesis. The precise levels of ACLP overexpression during a time-course of adipogenesis should be addressed in future experiments.

Interestingly, we observe that the level of PPARγ expression is higher in the infected cells (pLXSN-ACLP and pLXSN) compared to the uninfected controls (SHAM infected and uninfected). This was an unexpected result, although it agrees with what is normally seen visually with microscope analysis: infected cells usually accumulate more lipid droplets. Perhaps the actual insertion of DNA enhances adipogenesis in these cells.

The effect of ACLP overexpression on clonal expansion also needs to be precisely determined. Based on our measured cellular protein concentrations used for Western blot analysis, we can conclude that there was no major effect of ACLP overexpression on clonal expansion, as the increase in protein concentrations, reflecting the increase in cell number, was comparable to that of control cells undergoing differentiation. Additionally, further experiments are necessary to determine the effect of ACLP overexpression on earlier molecular events such as the expression of the transcription factors ADD1/SREBP-1, C/EBPβ, and C/EBPδ. The notion that a certain stimulus may have distinct effects on different molecular events of differentiation is not without precedence. For example, the protease inhibitor ritonavir has been shown to enhance adipogenesis as determined by late markers of differentiation including GPDH activity and triacylglycerol accumulation by enhancing ADD1/SREBP1 expression while having an inhibitory effect on intermediate markers such as PPARγ and C/EBPα (145). In another study, PPARγ expressing NIH-3T3 fibroblasts have been shown to convert to mature adipocytes as

determined by late makers of differentiation, including lipid accumulation and aP2 expression, but fail to synthesize C/EBPα and do not acquire insulin sensitivity (146).

Studies have shown that stabilization of ECM results in an inhibition of differentiation (108,109,110,111). A recent study by Layne's group demonstrates that ACLP is secreted into the ECM of collagen-rich tissues including the vasculature, dermis, and the developing skeleton where it is involved in embryonic development and in dermal wound healing (147). Thus, assuming that ACLP plays a role in stabilizing the ECM through its discoidin-like domain, it is expected that its transient decrease during the mitotic clonal expansion phase would be required to "free" the preadipocytes from the matrix to allow for proliferation and to enable for the matrix rearrangement that occurs during preadipocyte differentiation. However, as we have failed to observe an inhibition of differentiation with ACLP overexpression, then perhaps the overexpression levels achieved were not sufficient and were easily overcome by the ECM de-stabilizing molecular events, such as the downregulation of molecules with which ACLP may interact (eg: downregulation of collagen I, collagen III, and integrins) and up-regulation of MMP enzymes (112).

The nature of the relationship between ACLP and AEBP-1 still remains unclear and the truth about these two proteins lies beyond the scope of this study. Ro and colleagues argue that AEBP-1 is the authentic protein which exists in adipose tissue while Layne and colleagues believe that AEBP-1 is simply a truncated clone of ACLP. Nonetheless, both groups agree that AEBP1 and ACLP may have the same mRNA that hybridizes at a 4 kb band in Northern analysis (120,136). Ro has reported that the

expression of the 79 kD transcriptional repressor AEBP1 protein declines throughout preadipocyte differentiation as does its mRNA (136). However, we have been unable to detect AEBP-1. Instead, consistent with that of Layne's group, we only detect the 176 kD ACLP protein whose levels, as we show, are transiently but significantly down-regulated during 3T3-L1 adipogenesis. Like ACLP protein, the level of ACLP mRNA is also transiently down-regulated. Although the levels of ACLP mRNA increase beyond day 2 of differentiation, they still remain lower than day 0 levels by day 8. Thus, our Northern results may be in agreement with that of Ro and colleagues, although, Ro does not specify the extent of the decrease in AEBP1 mRNA.

Although demonstrated to be catalytically inactive by *in vitro* assays, ACLP contains a C-terminal domain that is 39% identical to carboxypeptidase E (CPE). Interestingly, CPE is found in two forms, one that is membrane-bound and inactive and another that is soluble and catalytically active. It has been reported that the catalytically inactive membrane-bound form CPE functions as a receptor on the Golgi membrane that serves to direct certain prohormones such as proinsulin through the regulated secretory pathway (130). Additionally, the cleavage of the membrane-bound CPE generates the soluble and catalytically active form that catalyzes the cleavage of proinsulin to insulin (130,133,134). However, most of the CPE remains in the membrane bound form with only about 10% being converted into the soluble form (130). Thus, like CPE, the carboxypeptidase-like domain of ACLP may have a non-catalytic function by serving a role in secretory pathways.

In summary, our results show, for the first time, that ACLP expression is transiently modulated during 3T3-L1 adipogenesis and, to a lesser extent, during hormonal stimulation of 3T3-C2 cells. Our results demonstrate that this event involves transcriptional regulation but we cannot disregard other possible regulations such as post-translational regulation (ie: degradation). The extent of ACLP down-regulation correlates with increases in cell number, suggesting a possible link between ACLP and mitotic clonal expansion. Moreover, ACLP modulation is an event that is specific to the proliferation of the clonal expansion phase. Overexpression of ACLP had no effect on adipogenesis, as determined by triacylglycerol accumulation and PPARγ expression.

The transient expression pattern of the *ACLP* gene, unique to post-mitotic proliferation of the clonal expansion phase, suggests a potential role for ACLP during adipogenesis. Our results warrant further investigation to precisely determine the role of ACLP and its mechanism of action. Combined, these results may bring us closer to elucidating the seemingly complex signaling cascade that is involved in preadipocyte differentiation with the ultimate goal of revealing the pathophysiological mechanisms that underly the development of obesity.

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VII. APPENDIX A

List of Abbreviations

aa	Amino acid
ACLP	Aortic carboxypeptidase-like protein
Acrp	Adipocyte complement-related protein
ADD-1	Adipocyte determination dependent factor 1
ADSF	Adipose tissue specific secrotory factor
AEBP1	Adipocyte enhancer binding protein 1
ALBP	Adipocyte lipid-binding protein
aP2	Adipocyte P2
ASP	Acylation stimulation protein
ATCC	American Type Culture Collection
ATF2	Transcription factor 2
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CAAT/enhancer binding protein
cAMP	Cyclic adenosine 5'-monophosphate
CDK	Cyclin-dependent kinase
CLD	Carboxypeptidase-like domain
CPE	Carboxypeptidase E
CREB	cAMP response-element binding protein
CREM	cAMP response-element modulator protein
CS	Calf serum
CSF-1	Colony stimulating factor 1
CTP	Cytidine 5'-triphosphate
DDR	Discoidin domain receptor
DEPC	Diethyl pyrocarbonate
DLD	Discoidin-like domain
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FABP	Fatty acid binding protein
FAS	Fatty acid synthetase
FBS	Fetal bovine serum
Gas	Growth arrest-specific
GLUT4	Glucose transporter type 4
GPDH	Glycerol-3-phosphate dehydrogenase
HAART	Highly active anti-retroviral therapy
HDL	High-density lipoprotein
HRP	Horseradish peroxidase

IBMX	Isobutylmethylxanthine (3-mehtyl-isobutylxanthine)
IGF-1	Insulin-like growth factor 1
LPL	Lipoprotein lipase
MAPK	Mitogen activated protein kinase
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
NIH	National Institute of Health
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PC	Prohormone convertase
PDGF	Platelet-derived growth factor
PEPCK	Phophoenolpyruvate carboxykinase
PI3-K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PPAR	Peroxisome-proliferator-activated receptor
Pref-1	Preadipocyte factor 1
Rb	Retinoblastoma
RER	Rough endoplasmic reticulum
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SREBP-1	Sterol regulatory element binding protein 1
TG	Triacylglycerol
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TZD	Thiazolidinedione
VSMC	Vascular smooth muscle cell
WAT	White adipose tissue
WHO	World Health Organization

VIII. APPENDIX B

Curriculum Vitae

Name: Kayvan Jasper Abaiian

Date of Birth: March 14, 1976

Place of Birth: Ottawa, Canada

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Education:

Bachelor of Science (Honours Biochemistry)
University of Ottawa
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Awards:

Ontario Graduate Student Scholarship
University of Ottawa
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Graduate School Excellence Scholarship
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Graduate School Admission Scholarship
University of Ottawa
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Excellence Scholarship
1996 to 1999

Dean's Honour List
1995 to 1999

Undergraduate Admission Scholarship
1995

Abstracts:

Abaiian KJ, Crapper T, Gagnon AM, Layne MD, Sorisky A. Regulation of Aortic Carboxypeptidase-like Protein (ACLP) during 3T3-L1 adipogenesis. In: Abstracts of the American Diabetes Association's 62nd Annual Meeting, Philadelphia, PA. June 22-26, 2001: 1715-PO.

IX. APPENDIX C

Contributions of Collaborators

I thank Sanjay Bhartia and Ann Nguyen for obtaining the preliminary data on ACLP protein expression, and Thet Crapper for performing cell count experiments and analyzing ACLP protein expression in 3T3-C2, 3T3-F442A and primary mouse cells.