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TRANSCRIPTIONAL CONTROL OF ADIPOGENESIS

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■ **Abstract** The major transcriptional factors involved in the adipogenic process include proteins belonging to the CCAAT/enhancer binding protein family, peroxisome proliferator-activated receptor γ, and adipocyte determination and differentiation dependent factor 1, also known as sterol regulatory element-binding protein 1. This process has been characterized with the aid of cell lines that represent various stages in the path of adipocyte commitment, ranging from pluripotent mesodermal fibroblasts to preadipocytes. Molecular analyses have led to a cascade model for adipogenesis based on timed expression of CCAAT/enhancer-binding proteins and peroxisome proliferator-activated receptor γ. Gene targeting and transgenic-mouse technologies, which allow the manipulation of endogenous genes for these transcription factors, have also contributed to the understanding of adipogenesis. This review aims to integrate this information to gain an understanding of the transcriptional regulation of fat cell formation.

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INTRODUCTION

A major function of adipose tissue is to store excess energy as triglycerides under conditions of nutritional excess (36). These stores are mobilized to provide the organism with sustenance during starvation. Recognition of the importance of adipose tissue in normal physiology as well as pathophysiology has increased with the

discovery that fat cells secrete important hormones, including leptin (43). Excess adipose tissue is a hallmark of obesity, the prevalence of which has skyrocketed to epidemic proportions in the past few decades (143). Guidelines recently released by the National Institutes of Health suggest that ∼55% of the people in the United States are obese (132). The cosmetic and health ramifications of obesity have led to a multi-billion-dollar diet industry. The failure of dietary therapy for maintenance of weight loss, together with the high morbidity and mortality of obesity-associated conditions including type II diabetes, hypertension, and coronary heart disease (143), has added a sense of urgency to the basic research on mechanisms underlying adipose tissue development. This review focuses on transcription factors that are involved in the development of adipose tissue.

MODELS AVAILABLE FOR STUDYING ADIPOCYTE DEVELOPMENT

During embryonic development, mesenchymal stem cells give rise to tissues of mesodermal origin, which include adipose, cartilage, and bone tissues (25). Development of adipose tissue occurs at different stages in various species (51). In mice, white adipose tissue (WAT) is almost completely absent at birth and develops post-natally (119). Brown adipose tissue (BAT) develops earlier than WAT. The functions of WAT and BAT are slightly divergent. The former is involved in the storage of excess nutrients in times of surplus and releases these stores when food is scarce, under the influence of appropriate signals (122). The role of BAT is mainly in adaptive non-shivering thermogenesis (78). This tissue expresses the family of uncoupling proteins (UCP), which short-circuits the mitochondrial β-oxidation pathway, resulting in the loss of energy as heat (18). Ablation of BAT in mice results in obesity caused by the increased metabolic efficiency of these animals (79). BAT stores are not present in humans as in rodents, although the expression of UCP-1 in WAT implies the presence of some BAT cells in WAT deposits (44). The development of BAT has not been as extensively studied as that of WAT, although the major transcription factors that are expressed in WAT are also expressed in BAT (110).

Much of our current understanding of the transcriptional control of adipogenesis comes from in vitro cell culture systems. One genre of model system may be related to the mesenchymal stem cells described above. This system includes the 3T3 and the C3H10T1/2 cell lines, which are pluripotent and can be differentiated into adipocytes, osteocytes, or chondrocytes when exposed to an inhibitor of DNA methylation such as 5-azacytidine (133). Other model cell lines, such as the 3T3-L1, 3T3-F442A (47, 48, 50), and Ob1771 cells (89), are already committed to the adipogenic pathway. In fact, injection of 3T3-F442A preadipocytes into mice gives rise to normal fat pads (49). These cells are therefore considered to be preadipocytes. Under appropriate hormonal stimuli, often a cocktail of insulin, dexamethasone, and the phosphodiesterase inhibitor isobutylmethylxanthine,

preadipocyte cell lines can be differentiated into cells that morphologically and ultrastructurally resemble mature adipocytes. Subsequent biochemical and molecular analyses of these cells have proven that they possess the functional characteristics of fat cells. Thus, these cell lines have been vital for the discovery of the transcriptional regulators of adipogenesis. Recently, ES cells have been differentiated into adipose lineages by using appropriate stimuli (27, 101). These cells will no doubt provide a valuable resource for the identification of early genes involved in commitment to the adipocytic lineage.

Advances in transgenic technologies have permitted manipulation of adipocyte development in the context of intact animals, especially mice. Creation of null (knockout) mutations and overexpression of mutant genes in adipose tissue have allowed investigators to test hypotheses about the roles of specific transcription factors in adipogenesis as well as to create new animal models.

TRANSCRIPTION FACTORS INVOLVED IN ADIPOGENESIS

This review focuses on the three transcription factor families that have emerged as critical adipogenic regulators during studies of model systems. They are peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBP), and adipocyte determination and differentiation dependent factor 1 (ADD-1), also known as sterol regulatory element-binding protein 1 (SREBP-1).

Peroxisome Proliferator-Activated Receptor γ

PPARγ is a member of the nuclear hormone receptor superfamily (Figure 1). It exists as two isoforms, PPARγ1 and PPARγ2, which are transcribed from the same gene by the use of alternate promoters (160). PPARγ2 is 30 amino acids longer than PPARγ1 and is the adipocyte-specific isoform of this receptor. Northern analysis shows that PPARγ1 is also predominantly expressed in adipose tissue (21). However, expression of PPARγ1 is more widespread in tissues, including (but not restricted to) the colon (31a, 71, 104, 105), macrophages (88, 98, 139), and cells of the vasculature (57). Recently, a third human PPARγ mRNA species that gives rise to a protein identical to PPARγ1 has been identified (34). This mRNA, termed PPARγ3, is transcribed from a novel promoter in the PPARγ gene, and its expression is restricted to adipose tissue and the small intestine. PPARγ is conserved in all mammalian species, and, interestingly, a PPARγ homolog in *Xenopus laevis* is primarily expressed in the fat body of that organism (31).

Role of Peroxisome Proliferator-Activated Receptor γ in Adipocyte Differentiation The role of PPAR in adipocytic differentiation was discovered independently in two ways. Our laboratory found that PPAR ligands could substitute for the adipogenic hormones during the differentiation of preadipocytes to

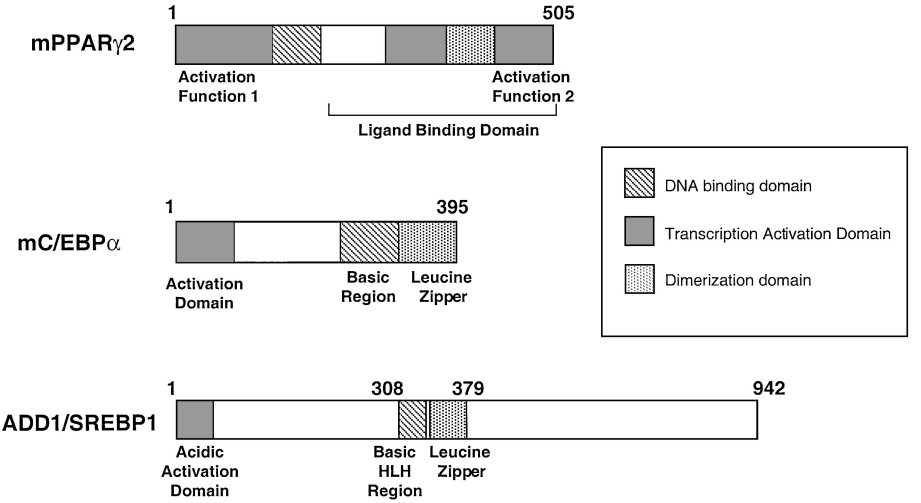

Figure 1 Schematic representations of structures of the three classes of transcription factors involved in adipocyte differentiation.

adipocytes (20). Subsequently, we found that PPARγ levels are dramatically induced during the differentiation of preadipocytes to adipocytes (21). Spiegelman and colleagues cloned PPARγ2 as a component of adipose regulatory factor-6, which confers adipocyte-specific expression to the murine adipocyte protein 2 (aP2) gene (also known as adipocyte-specific fatty acid-binding protein, or 422) through adipose regulatory elements 6 and 7, which are *cis*-acting elements in the aP2 enhancer (136). It was found that these adipose regulatory elements are hormone response elements for PPAR and that the PPARγ/RXRα heterodimer functions as a transactivator of the adipose regulatory factor-6 complex. Subsequently, PPAR response elements were found in many adipocyte genes, including those encoding lipoprotein lipase (106), phosphoenolpyruvate carboxykinase (135), and acyl-CoA synthetase (107). Ectopic expression of PPARγ2 in NIH 3T3 fibroblasts results in the adipogenic conversion of these cells in the presence of PPARγ ligands and hormonal stimuli of dexamethasone, isobutylmethylxanthine, and insulin (137). The component of the differentiation cocktail responsible for PPARγ induction is dexamethasone (145). Ectopically expressed PPARγ is also able to *trans*-differentiate myoblast lines into adipocytes lines (56).

Ligand Regulation of Peroxisome Proliferator-Activated Receptor γ Activity

PPARγ is related structurally to nuclear receptors for hormones and other small lipophilic ligands. It possesses the modular structure described for nuclear hormone receptors, including a ligand-independent N-terminal transactivation domain (1), a central DNA-binding domain, and a carboxy-terminal ligand-binding domain. Initially, it was found that fatty acids and their derivatives could activate

PPARγ in transactivation assays (31,46, 62). Prostaglandins such as PGJ_2 and PGD_2 were able to activate the receptor in transactivation and adipogenesis assays (155). The determination of the involvement of PPARs in the process of adipogenesis led to a high level of interest in identifying ligands for the receptor. Two groups identified 15-deoxy-$\Delta^{12,14}$ PGJ_2 as a potent activator of PPARγ (41, 65). In a ligand-binding assay, it was demonstrated that this compound could bind PPARγ. However, to our knowledge, this prostaglandin has not been demonstrated to occur naturally in adipocyte cell lines or in adipose tissue. Other naturally occurring substances that are ligands for PPARγ include oxidized fatty acids (139). It is possible that PPARγ functions as an integrator of multiple low-lipophilicity ligands. Ligands for RXRα, the heterodimeric DNA-binding partner for PPAR (67), for example, LG100268, are able to induce the differentiation of 3T3-L1 cells into adipocytes (108). In the presence of both PPARγ and RXRα ligands, a synergistic effect is observed (108). This effect is also observed in vivo in *ob/ob* mice, in which RXR ligands such as LG100268 act as insulin-sensitizing agents. Addition of thiazolidinediones (TZDs) to the treatment regimen enhances the glucose-lowering ability of LG100268 (87).

The most potent ligands for PPARγ are the TZDs, a novel class of antidiabetic drugs (53). Three of these drugs, troglitazone [Resulin (22)], rosiglitazone [Avandia (5)], and pioglitazone [Actos (84)], have been approved by the Food and Drug Administration for the treatment of type II, non insulin-dependent diabetes mellitus in the United States. The ability of these drugs to activate PPARγ correlates well with their ability to induce adipocyte differentiation, as well as their hypoglycemic activities in rodents and humans (9, 144), although there are a few exceptions (10, 96). The correlation between PPARγ activation and antidiabetic potency also extends to non-TZD PPARγ activators (10, 154). The mechanistic link between the activation of PPARγ and the hypoglycemic effects produced by these drugs in vivo has not been clearly established. One possible source of the insulin sensitizing effects of these drugs could be through their ability to block TNF-α-induced inhibition of insulin signaling (92a). Leptin gene expression in adipocytes is also inhibited by TZDs (54, 61). Although the level of PPARγ expression is highest in adipocytes, TZDs are effective hypoglycemic agents in a murine model of lipoatrophic diabetes (14). Whether this is caused by activation of PPARγ in residual fat cells or in other tissues, such as liver and skeletal muscle, remains to be determined.

Other Peroxisome Proliferator-Activated Receptors Other members of the PPAR family include PPARα and PPARδ. The term "peroxisome proliferator-activated receptor" derives from the observation that peroxisome proliferators, including the fibrate class of hypolipidemic drugs, are ligands for PPARα, the first member of this subfamily to be discovered (58). Thus, the term is actually a misnomer for PPARγ and PPARδ, which are not activated by these drugs. The ligand-binding domain is poorly conserved within the PPAR family, in contrast with the similarities seen in members of the thyroid hormone receptor and retinoic

acid receptor families. The DNA-binding domain shows a higher degree of conservation than the ligand-binding domain, which explains the ability of the PPAR isoforms to activate similar target genes (29).

PPARα is expressed predominantly in the liver (58). Although the function of PPARα in the liver is the regulation of fatty acid catabolism (29), several investigators (13, 20, 155) have found that expression and/or activation of PPARα results in the terminal differentiation of fibroblasts into adipocytes. A naturally occurring ligand for PPARα is 8(*S*)-hydroxyeicosatetraenoic acid (40, 66, 155), and this compound is weakly adipogenic in 3T3-L1 cells. PPARδ is expressed in preadipocytes (21, 137) and may be induced to higher levels early in the differentiation program in some adipogenic model systems (3). Experimental studies in which PPARδ is expressed ectopically in fibroblasts demonstrated that this isoform is not adipogenic in the presence of PPARδ ligands and that it may even interfere with the adipogenic program by affecting the regulation of downstream factors in that program (13). Recently, when PPARδ was ectopically expressed in fibroblastic 3T3C2 cells, concomitant exposure to ligands for both PPARγ and PPARδ was found to achieve lipid accumulation (8).

Regulation of Peroxisome Proliferator-Activated Receptor γ Activity by Phosphorylation Post-transcriptional regulation of PPARγ by mitogen-activated protein (MAP) kinase was discovered by several groups (1, 15, 55, 158). PPARγ2 is phosphorylated at the serine 112 position (amino acid 84 of PPARγ1, serine 114 in the human PPARγ2) by MAP kinase. A mutated form of the receptor (serine 112 to alanine) that could not be phosphorylated was found to have increased adipogenic activity compared with the wild-type receptor in virus-infected NIH 3T3 cells (55). The non-phosphorylatable PPARγ is also better able to activate transcription from reporter genes containing PPAR-binding sites (1, 55). Reciprocally, phosphorylation reduces the adipogenesis and transactivation caused by PPAR (15, 113). Activation of the MAP kinase pathway by either 12-0-tetradecanoyl phorbol-13-acetate or mitogens such as epidermal growth factor and platelet-derived growth factor blocks the adipocyte differentiation program, in part by phosphorylation of PPARγ (15, 55). A preadipocyte-derived prostaglandin, PGF2α, also inhibits adipogenesis through cell surface receptors activating the MAP kinase pathway to increase the phosphorylation of PPARγ (97). The role of insulin in the activation of PPARγ is not clear. Under some circumstances, insulin can potentiate the activation of PPARγ by its ligand (158), which is in contrast to the ability of insulin to activate the MAP kinase pathway. In any case, it is likely that the activation of MAP kinase by insulin does not have an important role in adipogenesis (38).

The decreased activity of phosphorylated PPARγ is not caused by an impaired ability to bind DNA (15), an inability to bind RXR (113), or defective nuclear localization (55). Rather, the phosphorylated form has a lower affinity for activating ligand than the nonphosphorylated form owing to a phosphorylation-induced conformational change in the receptor (113). The role of PPARγ phosphorylation

in vivo is unclear; the levels of the phosphorylated and unphosphorylated receptor are unchanged during the course of adipogenesis in 3T3-L1 cells (97).

A non-phosphorylatable mutant (Pro115Gly) of PPARγ is found in a small subset of obese humans (99). Like the S112A mutant, this mutant also has increased adipogenicity in fibroblasts. The obese subjects bearing this mutation appear to have less severe insulin resistance than control obese subjects.

Peroxisome Proliferator-Activated Receptor γ Coactivators Advances in the field of transcriptional regulation have identified several proteins that function as coactivators for nuclear hormone receptors (45, 83). They include the p160 class SRC-1/NcoA-1/ERAP160, GRIP-1/TIF-2/SRC-2, pCIP/ACTR/RAC-3/AIB-1/TRAM-1/SRC-3, and CBP/p300. Both groups of coactivators interact with PPARγ, although CBP may function more specifically with PPARγ (108). Another putative coactivator is PPAR-binding protein, which was initially identified in a screen for PPAR coactivators (159) and has subsequently been identified in complexes with thyroid hormone receptor (156), vitamin D receptor (95), and the orphan receptor RORα (4). All of these molecules have been shown to interact with PPARγ in a ligand-dependent manner and to potentiate transcriptional activation by PPARγ. These coactivators are ubiquitously expressed and do not exhibit receptor specificity.

Two additional molecules have been identified as coactivators of PPARγ. PPAR gamma coactivator 1 (PGC-1) was identified as a PPARγ-interacting protein in BAT (94). PGC-1 expression is induced in response exposure to cold and is associated with mitochondriogenesis (146). PGC-1 also interacts with other nuclear receptors, including estrogen receptor and thyroid hormone receptor, and is not a classical coactivator in that its association with PPARγ is ligand-independent. Interestingly, PGC-1 was recently shown to coordinate the recruitment of SRC-1 and CBP/p300 to PPARγ (93). The N-terminus of PPARγ was used to identify PGC-2, which interacts with PPARγ in a ligand-independent manner and increases transactivation by PPARγ as well as other nuclear receptors, but not PPARα (19).

Gene Targeting of Peroxisome Proliferator-Activated Receptor γ Ablation of the PPARγ gene in mice results in a placental-lethal phenotype (7, 68). PPAR$\gamma^{-/-}$ embryos do not survive past E10 because of myocardial thinning in the mutant embryos. Barak, Evans and colleagues obtained a live PPAR$\gamma^{-/-}$ pup by aggregation of two-celled PPARγ embryos with wild-type tetraploid embryos, a situation in which the wild-type embryos contribute exclusively to the development of extraembryonic tissues (7). The PPARγ-null pup survived to term but succumbed to a variety of metabolic disorders by post-natal day 5. This animal lacked adipose tissue, proving that PPARγ is required for fat cell development in vivo. As in other cases of lipodystrophy, this mouse exhibited a fatty liver (85, 117). It is interesting that the PPAR$\gamma^{+/-}$ mice were less prone to develop insulin resistance when fed a high-fat diet than were their wild-type littermates (68), possibly owing to the reduced adipocyte size and decreased production of TNF-α and fatty acids

in the heterozygotes. PPARγ-null ES cells have been used to derive chimeric mice (101). In these animals, PPARγ-null cells do not contribute to the formation of fat cells. PPARγ-null ES cells are also incapable of adipocytic differentiation in vitro. Thus, in addition to the sufficiency of PPARγ for adipogenesis that was determined from cell culture models, gene targeting studies prove that PPARγ is required for fat cell genesis in vivo as well as in vitro.

CCAAT/Enhancer-Binding Protein

C/EBPs belong to the basic leucine zipper (bZIP) family of transcription factors (Figure 1). The carboxy terminus of the protein contains the basic DNA-binding region and the leucine zipper, which is responsible for the dimerization potential of these proteins (28). Of the six members of the C/EBP family that have been identified, adipose tissue expresses C/EBPα, C/EBPβ, C/EBPδ, and C/EBPζ.

Role of CCAAT/Enhancer-Binding Proteins in Adipocyte Differentiation The expression of the various C/EBPs during adipogenesis follows a distinct temporal pattern. C/EBPβ and C/EBPδ are expressed early in response to hormonal stimulation, whereas C/EBPα is expressed late in the differentiation program and is responsible for the expression of many adipocyte marker genes (28, 81). Several adipocyte-specific genes, such as those encoding stearoyl-CoA desaturase, GLUT-4, 422/aP2, phosphoenol-pyruvate carboxykinase, and UCP, have C/EBP-binding sites in their promoters (24, 60, 90, 91). The delay in expression of C/EBPα is explained by the presence of a transcriptional repressor, C/EBPα undifferentiated protein or AP-2α, in preadipocytic 3T3-L1 cells (59). Also, the widely expressed transcriptional factor Sp1 is found to inhibit the binding of C/EBPs to the adjacent C/EBP promoter (130) in preadipocytes, thereby preventing the expression of C/EBPα protein. Data from several laboratories indicate that expression of C/EBPα is critical to the adipogenic program. The conditional expression of C/EBPα in fibroblasts is sufficient for the conversion of these cells into adipocytes without any additional hormonal stimulation (42, 75). Moreover, when production of C/EBPα protein was inhibited by expression of antisense C/EBP mRNA, lipid accumulation in 3T3-L1 preadipocytes and expression of fat-specific marker genes were blocked (74), indicating that this factor is necessary for adipogenic conversion. There exist multiple translation products for C/EBPα, caused by alternate start sites, whose levels change over the course of differentiation, which may be a mechanism of regulation of this process (76). Of the proteins translated, p42 possesses the most significant adipogenic and antimitotic properties (76).

Initiation of the adipogenic protocol at the molecular level in the preadipocyte begins with the expression of C/EBPβ and C/EBPδ. Overexpression of C/EBPβ in 3T3-L1 preadipocytes led to the adipose conversion of these cells in the absence of dexamethasone, insulin, and isobutylmethylxanthine (152). When expressed in

an inducible manner in pluripotent NIH-3T3 cells, C/EBPβ induces PPARγ gene expression in a variety of cell culture systems (109, 148). By contrast, induction of C/EBPα during C/EBPβ-induced adipogenesis is cell-type dependent (145, 152). This indicates that the adipogenic function of C/EBPα can be replaced by C/EBPβ. The induction of GLUT-4, the insulin-responsive glucose transporter, was shown to be C/EBPα-independent in the presence of PPARγ ligand in cell lines ectopically expressing C/EBPβ and C/EBPδ (149).

C/EBPζ, also termed CHOP-10 or gadd153, binds other C/EBP proteins (100). These heterodimers cannot bind DNA, and therefore CHOP-10 acts as a dominant-negative inhibitor of the other C/EBP proteins. However, although it was originally described as being induced during adipogenesis, the CHOP-10 expression is not part of the adipogenic program but rather is dependent on glucose deprivation of cells (17). This is consistent with the emerging physiological role of CHOP-10 as a mediator of the cellular stress response (33, 39).

Phosphorylation of CCAAT/Enhancer-Binding Proteins C/EBPs are regulated by phosphorylation, and this is important in the regulation of their activity in adipogenesis. C/EBPα is phosphorylated by glycogen synthase kinase 3, whose activity is antagonized by insulin via the phosphatidylinositol 3-kinase pathway (102). The phosphorylation of C/EBPβ may play an important role in the clonal expansion stage of adipocyte differentiation (see below). This factor is also serine-phosphorylated by cAMP, thereby increasing its ability to transactivate the acetyl-CoA carboxylase gene (128).

Gene Targeting of CCAAT/Enhancer-Binding Proteins Mouse models lacking C/EBPα, C/EBPβ, and/or C/EBPδ illustrate the importance of C/EBPs in adipogenesis. C/EBPα-null mice die because of hypoglycemia within 8 h of birth (37, 141). This phenotype is likely caused by the fact that C/EBPα is abundant in the liver, where it regulates genes involved in energy metabolism. Rescue of a small percentage of the mice by glucose injection is possible (141). When compared to their wild-type littermates at 32 h post-partum, these C/EBP α-null mice did not exhibit any subcutaneous inguinal WAT. Although interscapular BAT is present, there is aberrant lipid accumulation in this tissue. Also, the expression of UCP in C/EBP α-null mice is reduced despite normal expression of fatty acid synthase, GLUT-4, and 422/aP2 (141). It is possible that other C/EBP isoforms are able to substitute for transactivation of these genes but not for transactivation of UCP-1. Consistent with other models discussed above, in vitro differentiation experiments utilizing C/EBP α-null fibroblasts demonstrated that ectopic expression of PPARγ induces adipogenesis in the presence of PPAR ligand (147).

Whereas the C/EBP$\delta^{-/-}$ mice are born at the expected Mendelian frequency and normally survive post-natally, some of the C/EBP β-null mice die within the first few weeks after birth (129). In comparison, the compound homozygotes,

C/EBP$\beta^{-/-}$ C/EBP$\delta^{-/-}$, have a much lower survival rate (15%) (129). The survivors, in each case, did not suffer any gross defects and were able to survive independent of any support. All three types of mice showed impaired lipid accumulation in their BAT as well as WAT tissue. In the double knockouts, the size of the epididymal WAT pads was reduced dramatically in comparison to that of their wild-type littermates. Intriguingly, in these mice, examination of the levels of message of the fat-specific markers, lipoprotein lipase, 422/aP2, and phosphoenolpyruvate carboxykinase was not altered in either WAT or BAT. The only gene whose level was affected by the lack of C/EBPβ and C/EBPδ was UCP-1 in BAT. Unexpectedly, the levels of C/EBPα and PPARγ were not altered in either WAT or BAT. This is surprising, especially since embryonic fibroblasts isolated from C/EBP$\beta^{-/-}$ C/EBP$\delta^{-/-}$ mice do not undergo adipogenic conversion and fail to express PPARγ and C/EBPα. This implies that, in vivo, there might exist alternate pathways that could substitute for the lack of the upstream activators of the adipogenic program.

Studies of CCAAT/Enhancer-Binding Protein in Transgenic Mice Transgenic mice expressing a dominant-negative bZIP protein under the control of the adipocyte-specific aP2 promoter exhibit a lipodystrophy phenotype (85) that is very similar to that observed with the expression of diphtheria toxin (103) or constitutively active SREBP-1c (117) from the same promoter. In this case, the mutant bZIP protein forms stable heterodimers with C/EBPs and fos/jun and specifically abolishes their DNA-binding ability (85). These mice do not develop WAT, and BAT levels are dramatically reduced, consistent with the requirement of C/EBP proteins for normal adipogenesis. The mutant mice display organomegaly, fatty livers, and hyperlipidemia and are severely insulin resistant. This loss-of-function animal model emphasizes the importance of the C/EBP family of proteins in adipogenesis in vivo. Leptin administration apparently does not alleviate the insulin resistance of these mice (44a).

ADD1/SREBP

ADD1, a 98-kDa basic helix-loop-helix leucine zipper transcription factor (Figure 1), was identified as an adipocyte factor that bound to an E-box motif (CANNTG) in the promoter of the fatty acid synthase gene (138). ADD1 is the rat homolog of the family of membrane-bound proteins known as the SREBPs that regulate cholesterol metabolism (12). SREBP-1, which is the human homolog of ADD1, was independently cloned from human HeLa cells on the basis of its ability to bind the sterol response element (153). This protein exists in the endoplasmic reticulum in the membrane-bound state; in the absence of cholesterol it is proteolytically cleaved and released into the cytosol, and it enters the nucleus to increase the transcription of genes (142). There exist two forms of SREBP-1, namely SREBP-1a and SREBP-1c (ADD1), which arise owing to alternate

transcriptional start sites of the same gene (118). Almost all of the SREBP found in 3T3-L1 cells is isoform SREBP-1a, whereas in mouse adipose tissue the level of SREBP-1c exceeds that of SREBP-1a by threefold (118). In rats, ADD1 is expressed in other tissues besides WAT, such as the liver, kidney, and gut, but the highest level of expression is seen in brown fat (138).

ADD1/SREBP-1 regulates the expression of enzymes in the cholesterol biosynthetic pathway, as well as enzymes involved in fatty acid synthesis and uptake (64, 77, 82, 114, 115, 142). Recent data demonstrate that the effects of ADD1/SREBP on fatty acid synthase are through the E-box sequence and not through the sterol response element (63). The increased levels of ADD1 message in 3T3-L1 cells in response to insulin treatment suggest that this protein is an insulin response factor (63).

Role of ADD1/SREBP-1 in Adipocyte Differentiation The expression of ADD1 in 3T3-L1 preadipocytes is first detected 24 h after adipogenic hormones are added (64). Ectopic expression of ADD1 is not sufficient to convert NIH-3T3 cells into adipocytes; however, the presence of a PPARγ activator causes a 15%–20% increase in the extent of differentiation (64). The ectopic expression of a dominant-negative form of this transcription factor that bears a point mutation in the DNA-binding domain results in suppression of the adipocyte phenotype and expression of adipocyte markers (64). Recent data suggest that the retroviral expression of ADD1 in 3T3-L1 preadipocytes increases the expression of PPARγ (35). Simultaneous ADD1 expression increases the transcriptional activity of PPARγ/RXRα on the aP2/422 promoter, possibly by increasing the formation of the endogenous ligand for PPARγ (64a).

Gene Targeting of ADD1/SREBP Targeted disruption of the SREBP-1 gene results in death of the 50%–80% of the homozygous null progeny (114a). The surviving mice were normal, and did not show alterations in their adipose mass and levels of lipogenic and other adipose genes. It is possible that the presence of low levels of SREBP-2 in fat compensated for the lack of SREBP-1. Targeted disruption of the SREBP-2 gene results in intrauterine death of the homozygous pups (114a). An adipose-specific knock-out of both SREBP-1 and -2 may be helpful in resolving the role of this factor in adipogenesis.

Studies of ADD1/SREBP in Transgenic Mice Overexpression of ADD1/SREBP in fat tissue in vivo results in mice with severely compromised levels of fat (117). Thus, in vivo, increased expression of ADD1/SREBP-1 seems to interfere with development of fat. To compensate for reduced adipose mass, these animals have fatty livers. Also, in accordance with the symptoms of patients with congenital lipodystrophy, these animals are severely diabetic. These animals have elevated levels of preadipocyte factor-1 and TNF-α, although it is not known whether this is causal. Interestingly, administration of leptin reverses the diabetic

condition of these animals by a mechanism independent of its ability to decrease food intake (116).

Other Transcription Factors Implicated in Adipocyte Differentiation

There is evidence for expression and roles in adipogenesis of DNA-binding proteins, besides the transcriptional regulators discussed above. STATs (signal transducers and activators of transcription) 1, 3, 5A, 5B, and 6 are expressed in adipocytes, suggesting that the STAT family of transcription factors may play a role in adipogenesis (6, 26). The expression of STAT proteins was shown to be dependent on PPARγ ligands (124). Three members of the homeobox transcription factor (Hox) family, are also expressed and differentially regulated during adipocyte differentiation (26). In addition, adipocyte enhancer-binding protein 1 acts as a transcriptional repressor because of a novel carboxypeptidase activity and is down-regulated during adipocyte differentiation (52). Recently, it was also shown that this protein can bind the γ_5 subunit of the heterotrimeric G protein, although the relative importance of this phenomenon is not known (92). The levels of the proto-oncogenes products c-*fos* and *jun*-B peak a few hours after induction of adipogenesis, whereas levels of c-*jun* remain constant throughout the adipogenic program (123) c-*fos* is involved in negative regulation of certain adipocyte genes through the AP-1 site (30).

In addition to PPAR, other members of the nuclear hormone receptor superfamily that are involved in adipocyte development are the glucocorticoid receptor and the retinoic acid receptor (RAR). The role of glucocorticoid receptor during adipocyte differentiation has not been dissected at the molecular level. Dexamethasone, a ligand for this receptor, is an important component of the hormonal stimulus used to achieve adipocyte differentiation in vitro (51). Recent evidence indicates that stimulation of adipogenesis by dexamethasone may be mediated by its ability to decrease the levels of preadipocyte factor-1, an anti-adipogenic epidermal growth factor repeat-containing protein that is expressed in preadipocytes (120, 121). RARs inhibit adipocyte differentiation in the presence of their ligands such as all-*trans* retinoic acid (ATRA). This effect is likely mediated by inhibition of the transactivating function of C/EBPs, particularly C/EBPβ (109).

The role of transcriptional repressors during adipogenesis has not been extensively studied. The transcription factor Sp1 was found to negatively regulate the gene encoding C/EBPα through an Sp1 site, which overlaps with the C/EBP-binding site in the C/EBPα promoter (130). The levels of Sp1 decrease during the course of adipocyte differentiation in 3T3-L1 cells. A decrease in Sp1 levels during adipocyte differentiation was promoted by isobutylmethylxanthine, a component of the standard differentiation cocktail (130). Repression of the C/EBPα gene is brought about independently through a transcription factor of the AP-2α family called C/EBPα undifferentiated protein. The levels of this transcriptional repressor are decreased during the course of adipocyte differentiation of 3T3-L1 cells (59).

EVENTS INVOLVED IN ADIPOCYTE DIFFERENTIATION

The adipogenic program in preadipocyte cell lines consists of several sequential steps that have been well characterized, as represented in Figure 2. Detailed descriptions of these events can be found in past reviews (81). Briefly, the sequence of events that precedes terminal differentiation includes the following:

1. Determination of a preadipocytic fate (the nature of the signals or events involved in this process remains to be elucidated).
2. Growth arrest at confluence (when confluence is reached, cells arrest in the G_0/G_1 stage of the cell cycle. This step is required for the initiation of differentiation events).
3. Clonal expansion (this process involves synchronous entry of all cells into S phase of the cell cycle, leading to one or two rounds of mitosis; it occurs when appropriate adipogenic stimuli are presented to the cells and is characterized by the expression of C/EBPβ and C/EBPδ).
4. Terminal differentiation (this process is accompanied by an exit from the cell cycle; PPARγ, C/EBPα, and many of the adipocyte markers are expressed at this stage, and the cells assume their characteristic rounded morphology and visibly accumulate lipid droplets).

The transcription factor that is the master regulator for adipocytic commitment of mesodermal stem cells still remains elusive. Two basic helix-loop-helix

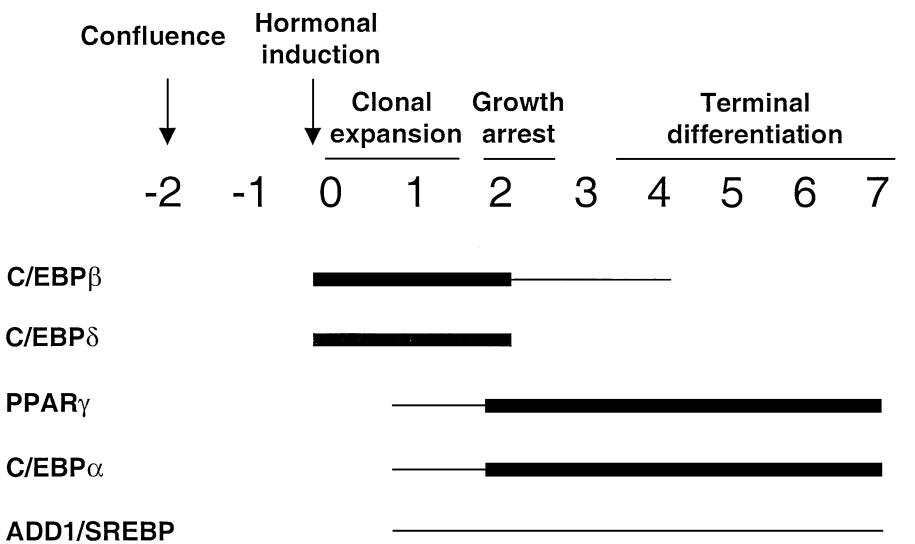

Figure 2 Temporal expression of the major transcriptional factors involved in differentiation of preadipocytic cell lines. *Thick line*, higher expression level; *thin line*, lower expression level.

transcription factors, twist (70) and scleraxis (11), that are important for the formation of tissues of mesodermal lineage have been identified. It is not known whether these factors have a role in fat development. One requirement of a master regulatory factor is that its expression is adipose-specific, thereby excluding the C/EBP class of proteins, which are extensively expressed in non-adipose tissues (72). PPARγ2 is adipose-specific; however, its expression does not occur until approximately 24 to 48 h after hormonal stimulation of preadipocytic cell lines has been initiated (21). Recently, protocols involving the high-frequency conversion of pluripotent ES cells into adipocytes have been described (27, 101). This system could potentially provide a resource for identifying the genetic switch that commits a pluripotent stem cell to a preadipocytic lineage.

ATRA blocks adipogenesis when added 24 to 48 h after exposure of 3T3-L1 cells to differentiation medium containing adipogenic hormones (20, 69, 127). By using ligands that can discriminate between the two subclasses of retinoic acid receptors (RAR and RXR), and by examining the pattern of expression of RAR and RXR in the differentiating adipocyte, we showed that the inhibition of adipogenesis was an RAR-mediated event (151). Ectopic expression of RAR in these cells extends the window of ATRA sensitivity of these cells. The effects of RAR ligand at the stage of PPARγ induction are mediated at least in part through the interference by RAR with C/EBPβ-mediated transcription (109). Interestingly, when C/EBPα is ectopically expressed in 3T3-L1 cells, ATRA blocks the adipogenic conversion of these cells, even though normally C/EBPα is expressed at a stage much later in the adipogenic program, after cells have ceased to be sensitive to inhibition by ATRA (109). This phenomenon can be explained by the possibility that C/EBPα can substitute for C/EBPβ in the transcriptional process. Further, our work demonstrated that ATRA could block adipogenesis in cells that express both PPARγ and C/EBPα even in the presence of PPARγ ligand, provided that the retinoid was added at the time of gene transduction into the cells (112). This implies that the expression of PPARγ and C/EBPα is not sufficient to cause ATRA insensitivity of the inhibition of adipogenesis. The exit of cells from the cell cycle, characterized by the hypophosphorylation of the retinoblastoma protein, is important to the status of ATRA sensitivity of the cells. Conversely, ligands for the RXR family of receptors promote the conversion of preadipocytes to adipocytes (108). This property may be specific to a subset of RXR ligand-specific since LG100268, but not SR11237, has this property (108, 151).

It has been demonstrated that ligand-activated PPARγ can cause cell cycle arrest in fibroblasts and transformed HIB-1B cells by inhibiting the DNA-binding and transcriptional activity of the E2F-DP complex (2). This is caused by the increased phosphorylation of these proteins brought about by the decreased expression of protein phosphatase 2A. These data suggest a role for PPARγ in the withdrawal from the cell cycle that precedes the onset of cellular differentiation. Recently, PPARγ was demonstrated to regulate the expression of p18 and p21 cyclin-dependent kinase inhibitors in 3T3-L1 preadipocytes, which may provide another mechanism by which this protein causes exiting of cells from the cell cycle (86).

The cytokine (TNF-α) inhibits adipocyte differentiation through activation of TNF receptor 1 (111, 150). Treatment with TNF-α down-regulates many genes in adipocytes, such as those encoding PPARγ (157), C/EBPα, and GLUT-4 (125, 126). This inhibition might be caused by the ability of TNF-α to block the clonal expansion that is required for the stimulation of adipogenesis (80). An alternate pathway by which TNF-α might block adipogenesis is the activation of the Erk-1/2 MAP kinase pathway, perhaps via phosphorylation of PPARγ. Indeed, the presence of PD98059, an agent that specifically blocks the Erk MAP kinase pathway, abolishes the inhibitory effects of low concentrations of TNF-α on adipocyte differentiation (38). It is interesting that, although prevention of Erk activation has little or no effect on adipogenesis (32, 38, 97), inhibition of p38 MAP kinase completely blocks fat cell differentiation (32). The transcriptional target of p38 MAP kinase is not known for certain, although the C/EBPs are potential candidates.

The mechanisms involved in the regulation of the levels of C/EBP proteins during adipocyte differentiation have been well elucidated. C/EBPβ and C/EBPδ are abundantly expressed in the initial stages of differentiation (16, 152). These factors turn on the expression of C/EBPα via a C/EBP-binding site on the promoter of the gene encoding C/EBPα (23). Levels of C/EBPα in terminally differentiated cells are maintained by auto-regulation of the gene through this C/EBP-binding site (23). C/EBPα is known to be antimitotic (76, 140) via the activation of the p21 protein, a cyclin-dependent kinase inhibitor (134), and the expression of this transcription factor is characterized by exiting of cells from the clonal expansion stage. A tyrosine phosphorylation event is known to occur during the termination of clonal expansion and the increase of adipocyte gene expression (73). In a recent series of experiments using immunofluorescence techniques, Tang & Lane (131) demonstrated that the maintenance of the mitotic phase is accompanied by a centromeric localization of C/EBPβ and C/EBPδ, which presumably prevents these factors from binding their response element on the gene encoding C/EBPα. This would, in turn, prevent premature expression of C/EBPα, resulting in exiting the cell cycle. The mechanism by which C/EBPβ gradually acquires DNA-binding capacity has been suggested to involve post-translational modifications of the protein, primarily phosphorylation (131), which would disrupt the compact secondary conformation of the protein and expose its DNA-binding sites. Further examination of the possibility of the existence of such a mechanism through identification of the protein kinases that may be involved in such a process is required.

SUMMARY AND CONCLUSIONS

Our understanding of the regulation of adipocyte development has grown exponentially during the past few years. The availability of new techniques will no doubt help to sustain this rapidly growing field of research over the next few years. Further, adipose tissue-specific ablation of transcription factors such as PPARγ

and C/EBPα is important because of the diverse roles of these proteins in physiology. Integrating information obtained via cell culture with new data gathered from genetic animal models will be critical in establishing the hierarchy of events involved in the process of formation of the fat cell. This may some day lead to the development of novel therapies to combat the rapid rise of obesity among the populations of developed nations.

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