

Understanding Adipocyte Differentiation

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Gregoire, Francine M., Cynthia M. Smas, and Hei Sook Sul. Understanding Adipocyte Differentiation. *Physiol. Rev.* 78: 783–809, 1998.—The adipocyte plays a critical role in energy balance. Adipose tissue growth involves an increase in adipocyte size and the formation of new adipocytes from precursor cells. For the last 20 years, the cellular and molecular mechanisms of adipocyte differentiation have been extensively studied using preadipocyte culture systems. Committed preadipocytes undergo growth arrest and subsequent terminal differentiation into adipocytes. This is accompanied by a dramatic increase in expression of adipocyte genes including adipocyte fatty acid binding protein and lipid-metabolizing enzymes. Characterization of regulatory regions of adipose-specific genes has led to the identification of the transcription factors peroxisome proliferator-activated receptor- γ (PPAR- γ) and CCAAT/enhancer binding protein (C/EBP), which play a key role in the complex transcriptional cascade during adipocyte differentiation. Growth and differentiation of preadipocytes is controlled by communication between individual cells or between cells and the extracellular environment. Various hormones and growth factors that affect adipocyte differentiation in a positive or negative manner have been identified. In addition, components involved in cell-cell or cell-matrix interactions such as preadipocyte factor-1 and extracellular matrix proteins are also pivotal in regulating the differentiation process. Identification of these molecules has yielded clues to the biochemical pathways that ultimately result in transcriptional activation via PPAR- γ and C/EBP. Studies on the regulation of these transcription factors and the mode of action of various agents that influence adipocyte differentiation will reveal the physiological and pathophysiological mechanisms underlying adipose tissue development.

I. INTRODUCTION

White adipose tissue (WAT) is the major energy reserve in higher eukaryotes, and storing triacylglycerol in periods of energy excess and its mobilization during energy deprivation are its primary purposes. Recently, there has been a dramatic increase in the incidence of obesity resulting from an excess of WAT. Obesity is a prevalent health hazard in industrialized countries (145, 298) and is closely associated with a number of pathological disor-

ders, including non-insulin-dependent diabetes, hypertension, cancer, gallbladder disease, and atherosclerosis. With regard to this wide range of health implications, the need to develop new and effective strategies in controlling obesity has become more acute. Recently, progress has been made in understanding the process of adipocyte differentiation and in the cloning of genes mutated in several monogenic mouse models of obesity. This has not only allowed us to begin to understand the cellular and molecular basis of adipose tissue growth in physiological and

pathophysiological states, but has also provided means to develop therapeutic strategies for the treatment and prevention of obesity. This review addresses regulation of WAT development at the cellular and molecular levels. We summarize information gained from studies in preadipocyte cell lines and attempt to incorporate results from often neglected primary preadipocyte studies.

II. ADIPOSE TISSUE AS A SECRETORY ORGAN

Mature adipocytes, the main cellular component of WAT, are uniquely equipped to function in energy storage and balance under tight hormonal control. However, with the recent realization that adipocytes secrete factors known to play a role in immunological responses, vascular diseases, and appetite regulation, a much more complex and dynamic role of WAT has progressively emerged. Leptin, the obese (*ob*) gene product, is a hormone that is primarily made and secreted by mature adipocytes and binds to its receptor in the hypothalamus. Studies indicate leptin may function in regulating body fat mass. Loss of fat stores decreases leptin levels and increases neuropeptide Y levels; this leads to increased food intake. Conversely, weight gain increases leptin levels leading to decreased food intake; melanocyte-stimulating hormone is necessary for this response (31, 201). Leptin levels are elevated in human obesity and in animal models of obesity. More recently, the leptin receptor has been detected in peripheral tissues. This suggests additional roles for leptin, including modulation of insulin action in liver (45), production of steroids in the ovary (310), and direct effects on adrenocortical steroidogenesis (23). Leptin also has a role in reproductive physiology (39, 178) and is involved in hematopoietic and immune system development (18, 173). This rapidly growing list, clearly not exhaustive, indicates that leptin has a much broader range of action than initially perceived.

Immune system-related proteins produced by adipocytes include adipsin, acylation stimulation protein (ASP), adipocyte complement-related protein (Acrp30/AdipoQ), tumor necrosis factor- α (TNF- α), and macrophage migration inhibitory factor (MIF). With the exception of TNF- α , their physiological function remains to be elucidated. Nonetheless, these adipocyte-derived factors might also be involved in either the control of energy homeostasis or insulin resistance. Acylation stimulation protein is generated by the interaction of complement factor D (identical to adipsin), factor B, and complement C3, components of the alternate complement pathway (41); ASP may be involved in regulating energy storage by stimulating triacylglycerol synthesis and glucose transport (44, 168, 252). Acrp30/AdipoQ displays sequence homology with C1q, the first component of the classical complement activation pathway. Like adipsin, Acrp30/AdipoQ is abundant in nor-

mal serum, and its secretion is enhanced by insulin. This suggests that it could function as a signaling molecule from adipocytes and might therefore regulate energy homeostasis (119, 229). As addressed in detail in section VI A, TNF- α not only inhibits adipocyte differentiation, but treatment of mature adipocytes with TNF- α reduces the expression of adipocyte genes (202, 259, 260, 273, 274). In addition, TNF- α may contribute to the insulin resistance that accompanies obesity and non-insulin-dependent diabetes mellitus; TNF- α levels are elevated in WAT of obese rodents and humans. This may contribute to insulin resistance by inhibiting insulin-stimulated tyrosine kinase activity of the insulin receptor (114–117, 135). Treatment of adipocytes with TNF- α was recently reported to increase secretion of MIF, a proinflammatory cytokine. Therefore, MIF may mediate the above-mentioned contribution of TNF- α to insulin resistance (112).

Vascular function-related proteins that are secreted by adipocytes include angiotensinogen and plasminogen activator inhibitor type 1 (PAI-1). White adipose tissue contains all the main components of the renin-angiotensin system such as angiotensinogen, angiotensin converting enzyme, angiotensin II, and angiotensin receptors (130). Angiotensinogen could play a role in regulating adipose tissue blood supply and fatty acid efflux from fat (73). Angiotensin II, the cleavage product of angiotensinogen, has been implicated in adipose tissue growth by stimulating production of prostacyclin by mature fat cells and thereby promoting adipocyte differentiation via a paracrine/autocrine mechanism (53). Because angiotensin II increases lipogenesis in both human and 3T3-L1 adipocytes, it may also be involved in the control of adiposity through regulation of lipid synthesis and storage in adipocytes (127). Plasminogen activator inhibitor type 1 is produced by adipose tissue, and treatment of 3T3-L1 adipocytes with transforming growth factor- β (TGF- β) significantly increases PAI-1 production. Higher PAI-1 levels have been reported in omental fat compared with subcutaneous fat. This may be correlated with increased PAI-1 levels noted in central obesity and may be involved in the development of vascular diseases associated with abdominal obesity (5, 164, 241).

Taken together, these studies clearly establish that the adipocyte behaves as an endocrine as well as a paracrine/autocrine cell. Along with its active role in regulating energy balance, WAT has the potential to play a dynamic role in a variety of other physiological processes, including the autoregulation of adipose tissue growth and development.

III. ORIGIN OF ADIPOSE CELLS AND ADIPOSE TISSUE

The origins of the adipose cell and adipose tissue are still poorly understood, and the molecular events leading

to the commitment of the embryonic stem cell precursor to the adipocyte lineage remain to be characterized. In most species, WAT formation begins before birth, as assessed by morphological studies performed on human, pig, mouse, and rat embryos (59, 204, 205, 245). The chronology of WAT appearance, however, is strictly dependent on the species as well as the adipose depot (59, 218, 245). White adipose tissue expansion takes place rapidly after birth as a result of increased fat cell size as well as an increase in fat cell number. Even at the adult stage, the potential to generate new fat cells persists. It has been demonstrated that fat cell number can increase when rats are fed a high-carbohydrate or high-fat diet (67, 68, 176). Increase in fat cell number is also observed in severe human obesity. However, the relative contribution of fat cell size and fat cell number to human adipose tissue growth on nutritional stimulation remains to be clarified. Regardless, early differentiation markers of adipocyte differentiation can be detected even in adipose tissue derived from very old mice (138). Moreover, fat cell precursors isolated from adult WAT of various species, including humans, can be differentiated *in vitro* into mature adipocytes (21, 58, 98, 104, 160, 213, 264). The potential to acquire new fat cells from fat cell precursors throughout the life span is now undisputed.

Although the developmental origin of fat cells is not known, several studies on multipotent clonal cell lines have suggested that the adipocyte lineage derives from an embryonic stem cell precursor with the capacity to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts, and myocytes. Treatment of the murine embryonic cell line C3H10T1/2 with a demethylating agent generates loci of muscle, cartilage, and fat cells (143, 265); 25% of colonies contained myofibers, 7% adipocytes, and 1% chondrocytes. The C3H10T1/2 cells may represent multipotential stem cells that are blocked at the mesodermal pathway. That a single gene could convert C3H10T1/2 cells into myoblasts has been demonstrated with the identification and characterization of the MyoD family of regulatory genes (54). The lower clonal frequency of conversion of C3H10T1/2 cells to adipocytes as compared with myocytes may indicate that more genes are required to activate adipocyte development. However, these different frequencies of conversion may also somewhat reflect culture conditions that could selectively promote the differentiation of one cell lineage over another. There is also evidence that a common bone marrow stromal cell type may give rise to adipogenic or osteogenic cells with several indications of a reciprocal relationship in the differentiation of these two cell types (84). For example, bone morphogenetic proteins of the TGF- β superfamily act as potent osteogenic agonists (83), whereas TGF- β inhibits adipocyte differentiation, as discussed in section VI A. Furthermore, the antidiabetic compounds thiazolidinediones, recently discovered to act through perox-

isome proliferator-activated receptor- γ (PPAR- γ), an adipogenic transcription factor described in section VI A, reduce bone marrow density and increase bone marrow adipocytes (84). Teratocarcinoma-derived C1 cells also behave as progenitor cells with osteoblast, chondroblast, or adipoblast cell fates (206). This suggests a close ontogenetic relationship between these connective tissue cell types. Osteogenic, chondrogenic, and adipogenic cells may arise from sclerotomal cells. The recent cloning of the basic-helix-loop-helix (bHLH) transcription factors twist and scleraxis has provided hints about the initial events that might precede and/or lead to the formation of the adipose lineage. In mouse embryo, these genes appear to play a significant role in the development of mesodermal tissues. Twist is expressed in early somites, and its expression is restricted to the sclerotome and excluded from myotome upon somite compartmentalization. Twist may be essential for the establishment of mesodermal cell fate and may be involved in the subdivision of the mesoderm lineage later in development (85). Significant levels of scleraxis can only be detected after somite compartmentalization, when it is expressed in sclerotome but not in myotome. Scleraxis could be a regulator of gene expression within the mesenchymal cell lineages that give rise to connective tissues (50). The precise role of these genes in adipocyte determination remains to be established.

IV. IN VITRO MODELS OF ADIPOCYTE DIFFERENTIATION

For the past 20 years, *in vitro* systems have been extensively used to study adipocyte differentiation. This has led to a dissection of the molecular and cellular events taking place during the transition from undifferentiated fibroblast-like preadipocytes into a mature round fat cells. Various preadipose cell lines and primary cultures of adipose-derived stromal vascular precursor cells have been used. Table 1 summarizes the characteristics of the most commonly employed preadipose cell models. Preadipose cell lines as well as primary preadipocytes are already committed solely to the adipocyte lineage, although they may represent different stages of adipocyte development. The most frequently employed cell lines are 3T3-F442A and 3T3-L1. These were clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19-day mouse embryos (89, 90, 92). 3T3-C2 cells derive from the same source but are not preadipocyte in nature. They do not differentiate into adipocytes and can be used to compare the responses of differentiation-defective with differentiation-competent cell types. The TA1 cell line was established by treating CH310T1/2 mouse embryo fibroblast cells with the demethylating agent 5-azacytidine (37, 143, 265). Ob17 cells and their derivatives were generated from

TABLE 1. *In vitro* models of adipocyte differentiation

Cell Lines	Origin/Stage of Development	Inducing Agents Used for Differentiation	Reference No.
ES cells	Mouse blastocyst*	Retinoic acid	52
CH3 10T1/2	Mouse embryo†	Demethylating agent 5'-azacytidine	143, 265
TA1	Derived from 5-azacytidine-treated 10 T1/2‡	10% FBS, insulin, and Dex	37
3T3-L1	17- to 19-day disaggregated mouse embryo‡	10% FBS, Dex and MIX, insulin (high concentration)	224
3T3-F442A	Same as above	10% FBS, insulin	179, 256
Ob17	Epididymal fat pads of adult <i>ob/ob</i> mouse‡	8% FBS, insulin, and T ₃	82
Primary Cultures [§]	Source/Age	Inducing Agents Used for Differentiation	Reference No.
Rat	Subcutaneous, epididymal, retroperitoneal/newborn (48 h), 4 wk old, or adult	Insulin (low concentration in 10% FBS, high concentration in serum free, accelerated)	57, 96, 97, 236, 284, 293
Mouse	Subcutaneous/8–12 day old	Serum free; insulin, HDL, Dex	160
Rabbit	Perirenal/4 wk old	Serum free; insulin, Dex	213
Pig	Perirenal, subcutaneous/fetal, newborn (1–7 day old)	Serum free; insulin with or without glucocorticoids	40, 108, 264
Human	Subcutaneous (abdominal)/variable age	Serum free; insulin (high concentration) and glucocorticoids	58, 63, 104

The most frequently used *in vitro* models of adipocyte differentiation are summarized. § General comments on primary cultures: committed to adipocyte lineage and most likely correspond to a later stage of adipocyte development than preadipose cell lines; extent of differentiation depends on age of donor and fat depot; and clonal expansion step appears to be not critical for human preadipocyte differentiation (63). FBS, fetal bovine serum; MIX, methylisobutylxanthine; Dex, dexamethasone; HDL, high-density lipoprotein; T₃, 3,3',5-triiodothyronine. * Totipotent cell line. † Multipotent. ‡ Already committed to adipocyte lineage.

adipose precursors present in the epididymal fat pads of genetically obese (*ob/ob*) adult mice (187). In addition to these models, embryonic stem (ES) cells have recently been shown to differentiate into mature adipocytes *in vitro* (52) and will likely provide a useful system for investigating the initial steps of adipogenesis. *In vitro*-differentiated adipocytes have many characteristics of adipose cells *in vivo*. Subcutaneous injection of preadipose cells in nude mice leads to development of mature fat pads that are histologically indistinguishable from WAT; this is convincing evidence that adipose cell acquisition occurs by a similar mechanism *in vivo* (91, 278).

Because the stage of differentiation and the lineage of preadipocyte cell lines have not been well established, primary cultures have been particularly useful for validating results obtained in preadipose cell lines. Primary preadipocytes have been successfully cultured from a number of species including humans and have several advantages over preadipose cell lines (96–98, 104, 106, 138, 160, 191, 236). Primary cells are diploid and may therefore reflect the *in vivo* context better than aneuploid cell lines. Second, they can be derived from adipose tissue obtained from various species at different postnatal stages of development and from various adipose depots (21, 57, 58). The latter is of particular interest, since molecular and biochemical differences in fat pads have been observed (96, 150, 151, 169, 212, 308). Such differences may have major physiological consequences, since excessive centralized fat accumulation (upper body obesity) is associated with increased morbidity in humans (139). On the other hand,

the potential cellular heterogeneity of the stromal vascular preadipose cell population is a drawback of primary cultures. This, however, may be a minor concern, since nearly 100% differentiation can be achieved when preadipocytes derived from young animals are adequately treated (57, 97, 236). For primary preadipocyte cultures, differentiation capacity is clearly donor dependent and decreases significantly with age (21, 58, 98, 138). The molecular basis for this reduced differentiation capacity is not known. It may, however, indicate that preadipose cells derived from aged donors represent a subpopulation of precursor cells arrested at distinct stage(s) of adipocyte development. These cells may therefore require a different subset of as yet to be characterized signals to undergo adipocyte differentiation (63, 98).

During the growth phase, cells of preadipocyte lines as well as primary preadipocytes are morphologically similar to fibroblasts. At confluence, induction of differentiation by appropriate treatment leads to drastic cell shape changes. The preadipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical characteristics of the mature white adipocyte. The nature of the induction is dependent on the specific cell culture model system employed. A variety of differentiation protocols have been developed for preadipose cell lines and for primary preadipocytes. Embryonic stem cells can be differentiated into adipocytes at high efficiency *in vitro* if early-developing ES cell-derived embryoid bodies are exposed to retinoic acid (RA) for a precise time, followed by treatment with standard adipo-

genic hormones (52). 3T3-L1 preadipocytes spontaneously differentiate over a period of several weeks into fat-cell clusters when maintained in culture with fetal calf serum. This can be accelerated by the inducing agents dexamethasone and methylisobutylxanthine (MIX), a phosphodiesterase inhibitor. High concentrations of insulin have been used in combination with these inducing agents. The identification of agents that accelerate differentiation, but that are not required to maintain the differentiated phenotype, provides insights to the biochemical pathways that may function during adipocyte differentiation.

Although preadipocytes from various sources are similar in many regards, their responsiveness to inducing agents varies considerably. This may represent differences in the stage of maturation at which the preadipocytes were originally harvested during cloning of cell lines or the age and/or source of tissue from which primary cells are isolated. The effects of the various inducing agents on adipocyte differentiation are discussed in detail in section VI. In most cases, primary preadipocytes derived from young animals require only either low concentrations (1–10 nM) of insulin in the presence of fetal calf serum, or high concentrations (1–10 μ M) in serum-free medium (57, 97, 104, 264). Depending on the species, the age of the donor, and/or the adipose depot source, agents such as glucocorticoids and MIX are either necessary to trigger the differentiation program or act only to accelerate it (57, 58, 96, 97, 104, 264, 293, 309). For established cell lines as well as for primary cells, the development of serum-free culture conditions has confirmed the positive role of these inducers and the involvement of insulin-like growth factor I (IGF-I), glucocorticoid, and cAMP signaling pathways. For preadipose cell lines, addition of insulin at supraphysiological concentrations does not affect the number of differentiated cells but does serve to accelerate lipid accumulation. In contrast, for most primary preadipocytes, which have a very low degree of spontaneous differentiation, insulin increases the number of differentiated cells; few lipid-containing cells are observed in the absence of insulin (95, 104, 264). Depending on the culture system, the insulin/IGF-I signaling pathway may either be critical for differentiation or necessary only to achieve the maximal rate of triacylglycerol accumulation that accompanies adipocyte differentiation.

V. PROCESS OF ADIPOCYTE DIFFERENTIATION

An overview of the stages of adipocyte differentiation is presented in Figure 1. The committed preadipocyte maintains the capacity for growth but has to withdraw from the cell cycle before adipose conversion. During adipocyte differentiation, acquisition of the adipocyte phenotype is characterized by chronological changes in the ex-

pression of numerous genes. This is reflected by the appearance of early, intermediate, and late mRNA/protein markers and triglyceride accumulation. These changes take place primarily at the transcriptional level, although posttranscriptional regulation occurs for some adipocyte genes (180, 295). In addition to the activation of genes, those genes that are inhibitory to adipogenesis or simply unnecessary for adipose cell function are repressed. Changes in gene expression during the early and late stages of adipocyte maturation have been characterized mainly through the use of preadipose cell lines. These have been extensively reviewed by others (47, 166, 249) and are summarized below. The limited information that is available on the details of the molecular events occurring during primary preadipocyte differentiation is also incorporated.

A. Growth Arrest

In preadipose cell lines as well as in primary preadipocytes, growth arrest and not cell confluence or cell-cell contact per se appears to be required for adipocyte differentiation. Confluent 3T3-F442A cells shifted to methylcellulose-stabilized suspension culture still undergo differentiation (196). This indicates that although confluence in these cells leads to growth arrest, cell-cell contact is not a prerequisite for adipocyte conversion. Primary rat preadipocytes plated at low density in serum-free medium can also differentiate in the absence of cell-cell contact (277). Two transcription factors, CCAAT/enhancer binding protein α (C/EBP- α) and PPAR- γ , have been shown to transactivate adipocyte specific genes, and are discussed in section VII. Both C/EBP- α and PPAR- γ also appear to be involved in the growth arrest that is required for adipocyte differentiation. McKnight and co-workers (276) have demonstrated the antimitotic activity of C/EBP- α through the use of a C/EBP- α -estrogen receptor fusion protein. Activation by estrogen treatment results in cessation of cell growth as assessed by cell number and DNA synthesis (276). Darlington and co-workers (268) have reported that C/EBP- α increases p21/SDI-1 mRNA and protein levels, but not other cell cycle components. Moreover, antisense p21/SDI-1 eliminates growth inhibition brought about by C/EBP- α (268). This indicates that C/EBP- α may function by increasing p21/SDI-1 levels. Spiegelman and colleagues (6) have showed that PPAR- γ is sufficient to induce growth arrest in fibroblasts and in adipogenic simian virus 40 large T antigen-transformed cells. In these PPAR- γ -expressing cells, cell cycle withdrawal is accompanied by a decrease in the DNA binding and transcriptional activity of the E2F/DP-1 complex because of phosphorylation of DP-1 and a decrease in the expression of the serine/threonine phosphatase PP2A catalytic subunit. Therefore, C/EBP- α and PPAR- γ may act

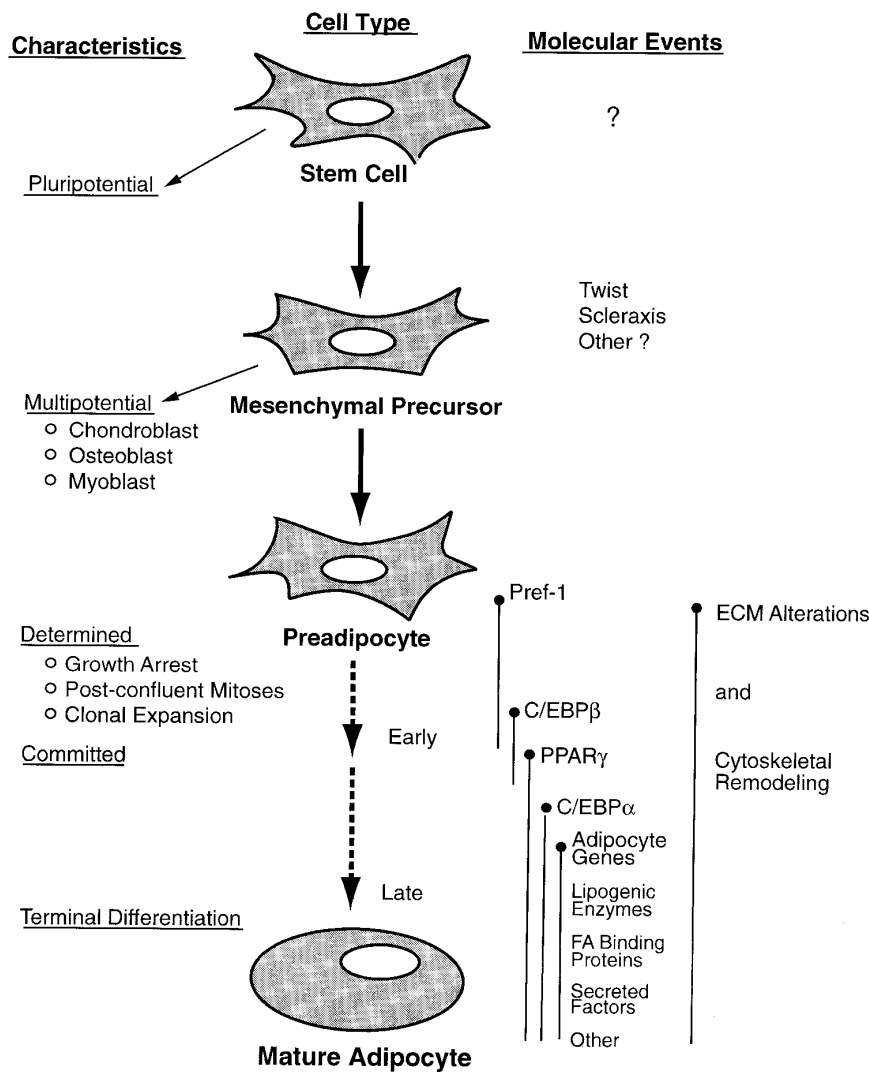


FIG. 1. Overview of stages in adipocyte differentiation. Our current understanding of adipocyte differentiation indicates that a pluripotent stem cell precursor gives rise to a mesenchymal precursor cell with the potential to differentiate along mesodermal lineages of myoblast, chondroblast, osteoblast, and adipocyte. As discussed in text, given appropriate environmental and gene expression cues, preadipocytes undergo clonal expansion and subsequent terminal differentiation. Selected molecular events accompanying this process are indicated to *right*, with their approximate duration reflected by the solid line. PPAR- γ , peroxisome proliferator-activated receptor- γ ; C/EBP, CCAAT/enhancer binding protein; pref-1, preadipocyte factor-1; ECM, extracellular matrix; FA, fatty acid.

cooperatively to bring about growth arrest (6). Although C/EBP- α and PPAR- γ expression increases dramatically during adipocyte differentiation, the low level of these factors expressed in preadipocytes may be sufficient to mediate growth arrest that precedes differentiation.

B. Clonal Expansion

After growth arrest at confluence, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through subsequent differentiation steps. Studies on preadipose cell lines have shown that growth-arrested cells undergo at least one round of DNA replication and cell doubling. This has been proposed to lead to the clonal amplification of committed cells (196). For 3T3-F442A and Ob17 cells, an increase in DNA synthesis precedes expression of late mRNA markers, and inhibition of DNA synthesis prevents the formation of fat cells (9, 147). However, primary preadipocytes

derived from human adipose tissue do not require cell division to enter the differentiation process (63). In these cells, inhibition of mitosis with cytosine arabinoside does not impair adipocyte development, indicating that clonal amplification of committed cells is not a critical step. These cells may have already undergone potential critical cell divisions in vivo and may therefore correspond to a later stage of adipocyte development.

Examination of growth-related proteins indicates potential differences between clonal expansion and pre-confluent cell growth in 3T3-L1 preadipocytes. Retinoblastoma proteins pRB, p107, and p130 bind the E2F/DP complex to inactivate growth-promoting transcriptional activities. A recent report indicates changes in the expression of retinoblastoma proteins during differentiation of 3T3-L1 cells, including a transient increase in p107. This appears specific to clonal expansion, since this change is not detected during serum-stimulated cell growth (217). The role of E2F and RB family members during adipogenesis is unknown and needs further examination. Similarly,

another group of growth arrest-specific (*gas*) genes shows a distinct expression pattern during clonal expansion. *Gas6* appears to be preferentially expressed during clonal expansion of postconfluent preadipocytes, whereas *gas1* and *gas3* are expressed in serum-starved preadipocytes (244). Combined, these observations suggest differential regulation of the cell cycle in preconfluent proliferation versus postconfluent hormonally stimulated clonal expansion. An intracellular receptor for the immunosuppressive drug FK506, FKBP51, also transiently accumulates during the clonal expansion phase. Interestingly, whereas FK506 itself does not have any effect on clonal expansion and adipocyte differentiation, at high concentrations it reverses the inhibitory effect of rapamycin, another immunosuppressant that shares intracellular receptors (FKBPs) with FK506 (305, 307). Regardless of its mechanism, inhibition of proliferation by rapamycin is accompanied by inhibition of mitogen-activated serine-threonine S6 kinase, indicating that clonal expansion is necessary for subsequent adipocyte differentiation of 3T3-L1 cells. This also suggests that a phosphorylation-dephosphorylation mechanism may be involved during the clonal expansion period. Interestingly, a transient increase in levels of the phosphatase inhibitor HA2 occurs during the clonal expansion phase. Constitutive expression of HA2 blocks differentiation only during the clonal expansion period but not during later stages of differentiation. Moreover, inhibition of adipocyte differentiation by HA2 can be overcome by treatment with the phosphatase inhibitor vanadate, suggesting that a critical tyrosine dephosphorylation is necessary during clonal expansion (157).

C. Early Changes in Gene Expression

Although it is helpful to schematize the stages of adipocyte differentiation into a hierarchy of molecular events, an accurate chronology of the earliest steps in adipocyte differentiation has not been elucidated. Growth arrest and clonal expansion are accompanied by complex changes in the pattern of gene expression that can differ with the cell culture models and the specific differentiation protocols employed. Expression of lipoprotein lipase (LPL) mRNA has often been cited as an early sign of adipocyte differentiation (3, 47, 98, 123, 166). Lipoprotein lipase is secreted by mature adipocytes and plays a central role in controlling lipid accumulation (48, 86). However, LPL expression occurs spontaneously at confluence and is independent of the addition of agents required for adipocyte differentiation (8, 9, 277). This suggests that LPL expression may reflect the growth-arrest stage rather than being an early differentiation step. It is also synthesized and secreted by other mesenchymal cell types including cardiac muscle cells and macrophages (49, 267). Because LPL expression is not adipocyte specific and it is indepen-

dent of the additional agents required for adipocyte differentiation, classification of LPL as an early marker of adipocyte differentiation remains somewhat questionable.

At least two families of transcription factors, C/EBP and PPAR, are induced early during adipocyte differentiation. The early expression of C/EBP and PPAR is logical given their subsequent involvement in terminal differentiation by transactivation of adipocyte-specific genes, described in section VII. Peroxisome proliferator-activated receptor- γ is largely adipocyte specific and is expressed at low but detectable levels in preadipocytes. Its expression rapidly increases after hormonal induction of differentiation. It is easily detectable during the second day of 3T3-L1 adipocyte differentiation, and maximal levels of expression are attained in mature adipocytes (27, 38). Induction of PPAR- δ appears to precede that of PPAR- γ . Expression of PPAR- δ , however, is rather widespread. It is detected in a variety of tissues as well as in several cultured cell lines, including the CH310T1/2, 3T3-C2, and NIH 3T3 (7). A transient increase in the expression of C/EBP- β and C/EBP- δ isoforms precedes the increase in PPAR- γ expression (27, 167, 299). The subsequent decrease of C/EBP- β and C/EBP- δ in early to mid stages of differentiation is concomitant with the induction of C/EBP- α mRNA. This increase in C/EBP- α expression occurs slightly before the expression of adipocyte-specific genes (27, 153, 167). Another transcription factor induced very early during adipocyte differentiation is sterol regulatory element binding protein-1c (SREBP-1c)/adipocyte determination and differentiation factor 1 (ADD1), a bHLH-leucine zipper protein that is involved in cholesterol metabolism (26) and may also participate in adipocyte gene expression (136, 137).

During adipocyte differentiation, cells convert from a fibroblastic to a spherical shape, and dramatic changes occur in cell morphology, cytoskeletal components, and the level and type of extracellular matrix (ECM) components. Many of the studies on the effect of cytoskeletal and ECM components in adipocyte differentiation predate the characterization of adipocyte transcription factors. It is likely that these changes could influence the expression and action of PPARs and/or C/EBPs during adipocyte differentiation. Decrease in actin and tubulin expression is an early event in adipocyte differentiation that precedes overt changes in morphology and the expression of adipocyte-specific genes (255). These changes in cell shape reflect a distinct process in differentiation and are not the result of accumulated lipid stores. 3T3 preadipocytes can undergo biochemical and morphological differentiation even in conditions when triglyceride accumulation is blocked by deprivation of biotin, a cofactor for fatty acid synthesis (148), or by addition of lipolytic agents (258). A switch in collagen gene expression is also an early event of adipocyte differentiation. The relative concentrations of fibroblast-expressed type I and type III procollagen mRNA decline by 80–90% during 3T3-L1 differentiation,

and secretion of type IV collagen and entactin/nidogen increases (13, 290). Expression of α_2 -collagen type VI mRNA is first detectable upon confluence in Ob1771 cells and rises sharply after confluence (51). It reaches maximal levels 4 days postconfluence and gradually decreases to 50% of maximal levels during differentiation (51). Increased production of soluble and cell-associated chondroitin sulfate proteoglycan-I (versican) has been reported during 3T3-L1 differentiation, and this may account for the observed increase in culture medium viscosity (29). The amount of pericellular fibronectin, as well as cellular synthesis of fibronectin, decreases by four- to fivefold during differentiation of 3T3-F442A cells (10). Preadipocyte factor-1 (pref-1), a recently described preadipocyte protein with epidermal growth factor (EGF)-like repeats, discussed in section VI B, has been hypothesized to be involved in maintaining the preadipose phenotype (246–248). A dramatic decrease in pref-1 expression accompanies adipocyte differentiation; it is abundant in preadipocytes and is not detectable in mature fat cells. It is the only known gene whose expression is completely downregulated during adipocyte differentiation.

D. Late Events and Terminal Differentiation

During the terminal phase of differentiation, adipocytes in culture markedly increase *de novo* lipogenesis and acquire sensitivity to insulin. The activity, protein, and mRNA levels for enzymes involved in triacylglycerol metabolism including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearyl-CoA desaturase (SCD1), glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase increase 10- to 100-fold (200, 256, 291). Glucose transporters (80), insulin receptor number, and insulin sensitivity increase. During adipocyte differentiation, there is a loss of β_1 -adrenergic receptors and an increase in the β_2 - and the β_3 -subtypes; this results in an increase in total adrenergic receptor number (69, 70, 101, 152). In addition to increases in mRNAs for proteins directly related to lipid metabolism, adipocytes also synthesize other adipose tissue-specific products. These include the following: aP2, an adipocyte-specific fatty acid binding protein also identified as 422 (19, 256); FAT/CD36, a putative fatty acid transporter (125, 239); and perilipin, a lipid droplet-associated protein (94). In addition, adipocytes produce a number of secreted products, discussed in section II. These include the following: monobutyrin, an angiogenic agent; adipsin, a homolog of the serine protease complement factor D; Acrp30/AdipoQ; PAI-1; and angiotensinogen II (5, 41, 61, 119, 127, 229). Leptin is also increased during *in vitro* terminal differentiation of adipocytes, although its level is much lower than that detected in adipose tissue (165). Peroxisome proliferator activated receptor- γ and/or C/EBP- α is implicated in the

coordinate activation of several of these genes, including aP2, GLUT4, SCD1, phosphoenolpyruvate carboxykinase (PEPCK), and leptin (113, 162, 175, 269, 270). The function of PPAR- γ and C/EBP- α in adipocyte differentiation is presented in section VII.

Experiments with BALB/c 3T3 mesenchymal stem cells indicate that cells that have progressed beyond a specific stage in the differentiation process are committed to subsequent terminal differentiation and can neither dedifferentiate nor reenter mitosis (287, 294). However, recent evidence indicates that the precise stage beyond which adipocytes can be considered terminally differentiated is not clearly defined. Partially differentiated human preadipocytes, evidenced by substantial cytoplasmic lipid accumulation, are still capable of cell division as assessed histologically and by flow cytometry (210). The progressive dedifferentiation of primary mature adipocytes followed by cell division has also been reported (262). Tumor necrosis factor- α treatment of mature TA1 or 3T3-L1 adipocytes or newly differentiated primary human adipocytes results in decreased expression of adipocyte markers and loss of lipid, with the development of morphological changes resulting in long, spindle-shaped cytoplasmic extensions (202, 273, 274). These cells therefore come to resemble preadipocytes. However, recent evidence indicates that although TNF- α -treated adipocytes and preadipocytes appear to share many similar morphological characteristics and gene expression patterns, they likely differ considerably. As described in section VI B, pref-1 expression is easily detected in preadipocytes and is absent from mature adipocytes; pref-1 levels are not restored by TNF- α of mature adipocytes. Pref-1 expression may reflect a fundamental difference between naive preadipocytes and those that result from TNF- α treatment (301). True reversion to a preadipose phenotype, if it takes place *in vivo*, could play a role regulating adipocyte number and consequently adipose tissue mass. The molecular and cellular events occurring during this process warrant more detailed study.

VI. FACTORS THAT MODULATE ADIPOCYTE DIFFERENTIATION

The growth and differentiation of animal cells are controlled by communication between individual cells or between cells and the extracellular environment. Adipocyte differentiation therefore requires the cell to process a variety of combinatorial inputs during the decision to undergo differentiation. Hormones and growth factors with a role in adipocyte differentiation act via specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events. Identification of agents or molecules that modulate the process in either a positive or negative manner provides insight into the signal transduction pathways involved. Extracellular matrix proteins may

TABLE 2. *Modulation of adipocyte differentiation by hormones, cytokines, and growth factors*

Agent	Preadipose Cell Lines (3T3-L1/3T3-F442A/Ob17/TA1)	Primary Cultures (Rat/Mouse/Rabbit/Pig/Human)	Reference No.
Insulin	+ (Accelerate lipid accumulation)	+ (Required)	37, 95, 104, 179, 224, 264
Glucocorticoids	+ For most, - (F442A only)	+ (Required or accelerate)	78, 96, 104, 179, 213, 224, 264
3,3',5-Triiodothyronine	+ (Ob17 only)	No effect	57, 103, 104, 213, 230, 236, 264, 293
Retinoic acid	-/+ (Concentration dependent)	-/+ (Concentration dependent)	60, 225, 226, 263
Growth hormone	+ (Required for most)	No effect or - (serum vs. serum free)	35, 81, 93, 102, 107, 283, 284
IGF-I	+ (Required for 3T3-L1)	+ (Most)	57, 190, 211, 251, 283
EGF, TGF- α	- For most, + (serum free)	- (Most)	105, 163, 230, 233, 282
TGF- β	- (Potent inhibitor)	- (Potent, effect irreversible)	160, 203, 237, 253, 282
FGF	-/No effect	No clear effect (rat)	105, 109, 185, 230, 236, 282
PDGF	-/+ Or no effect	No clear effect (human)	105, 109, 184, 230
IL-1, interferon- γ , TNF- α	-/No effect (low TNF- α)	- (Rat, mouse, human)	95, 134, 193, 199, 202
PGF $_{2\alpha}$	- (Most)	- (Rat)	122, 174, 188, 235, 282
PGI $_2$	+ (Ob17)	+ (Rat, human)	36, 186, 282
Forskolin, dibutyryl cAMP	-/+ (Concentration dependent)	+ (Rat)	243, 293, 296, 304
TPA	- (Most)	- (Rat)	185, 240, 242

IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; TGF, transforming growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; IL-1, interleukin-1; TNF- α , tumor necrosis factor- α ; PG, prostaglandin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

play an important role in modulating adipocyte differentiation by permitting the morphological changes and adipocyte-specific gene expression that accompany differentiation. The combination of hormones and growth/differentiation factors that trigger or potentiate adipocyte differentiation has been extensively examined and is summarized in Table 2. All preadipose cell lines exhibit some degree of spontaneous conversion when grown in the presence of fetal calf serum, indicating serum is integral to the differentiation process. However, serum components are neither well characterized nor easily controllable. The development of chemically defined serum-free media suitable for the differentiation of preadipose cell lines and primary preadipocytes has aided the assessment of the precise hormonal requirements for differentiation. Although the full complement of inducing agents required for differentiation varies with each cell culture model, IGF-I, cAMP, and glucocorticoids are generally considered necessary for the induction of differentiation either in serum-containing or in serum-free media. In addition to stimulatory factors, agents that suppress the differentiation of preadipocytes have also been identified. These may act either by their mitogenic properties, as for various growth factors, or by other independent mechanisms, as in the case of pref-1. Recent work has begun to clarify how these exogenous signals may influence transcription factors such as C/EBP and PPAR- γ that are critical for activation of adipocyte genes and adipocyte differentiation.

A. Hormones and Signal Transduction Pathways Regulating Adipocyte Differentiation

1. Growth hormone and IGF-I

Studies addressing the role of growth hormone (GH) and IGF-I in adipocyte differentiation illustrate potential

problems in comparing results obtained with different cell culture models under various culture conditions. A role for GH in adipocyte differentiation was first reported by Green et al. (93). Growth hormone has been shown to be necessary for differentiation of 3T3-F442A cells to adipocytes, and it has been suggested that GH promotes differentiation and sensitizes the cells to the mitogenic effects of IGF-I for clonal expansion (46, 93, 102). Sonenberg and co-workers (46, 102) suggested that GH may promote 3T3-F442A adipose conversion by inducing an antimitogenic state that is accompanied by decreased synthesis of the ECM proteins fibronectin and α -I collagen. The role of GH is also recognized in studies employing Ob1771 preadipocytes; in serum-free medium, GH increases the extent of adipocyte differentiation (35). Growth hormone stimulates IGF-I gene transcription and, as discussed below, IGF-I is necessary for adipocyte differentiation. It has been suggested that IGF-I secreted from Ob1771 cells could act in an autocrine/paracrine fashion to induce differentiation of Ob1771 cells (133). In contrast to observations in preadipose cell lines, no stimulatory effect of GH is observed in primary preadipocyte cultures. Initial reports on the effect of GH on rat and human preadipocytes indicate a lack of GH action (283). Because these studies were performed under serum-containing conditions, the effects of GH may not have been detectable. This concern was addressed in serum-free studies wherein GH was demonstrated to inhibit differentiation of rat, porcine, and human preadipocytes (81, 107, 283, 284). Although GH markedly stimulates IGF-I production in rat preadipocytes, which in turn promoted cell proliferation, the antiadipogenic action of GH is not related to growth promotion mediated by IGF-I. Addition of an anti-IGF-I monoclonal antibody prevents the stimulatory effect on cell proliferation but not the reduction of differentiation (284).

In addition to the known lipolytic activity of GH, inhibition of adipocyte differentiation may explain the effect of GH on adipose tissue mass *in vivo*. The conflicting results for the action of GH on the differentiation of preadipose cell lines and primary preadipocyte cultures highlight a substantial biological difference between these two models. Each may represent different stages of the adipocyte lineage; it has been proposed that the requirement for GH is obviated in primary cultures by their prior *in vivo* exposure to circulating GH. These cells may therefore be primed for subsequent sensitivity to other adipogenic agents (2, 283).

A requirement of IGF-I or pharmacological concentrations of insulin in adipocyte differentiation has been clearly demonstrated. Rubin and colleagues first reported that IGF-I is an essential factor for 3T3-L1 adipocyte differentiation, using fetal calf serum depleted of GH, insulin, and IGF-I by charcoal and ion-exchange resin treatment. Under both serum-containing and serum-free conditions, IGF-I has a dose-dependent action on 3T3-L1 preadipocyte differentiation (230, 251). Insulin-like growth factor-I also stimulates adipogenesis of primary rat, rabbit, and porcine preadipocytes (57, 190, 211), indicating that this growth factor may be an essential regulator of fat cell formation. In addition to IGF-I, clonal and primary preadipocytes also secrete insulin-like growth factor binding proteins (IGFBPs) in a differentiation-dependent manner, indicating that IGFBPs may be important in modulating IGF-I action in adipogenesis (22, 40, 190, 283). The mechanisms of action of IGF-I/IGFBPs are not well understood, but they are most likely acting in an autocrine/paracrine manner.

The adipogenic effects of IGF-I indicate the involvement of a phosphorylation-dephosphorylation mechanism, subsequent to IGF-I receptor tyrosine phosphorylation, in intracellular signaling during adipocyte differentiation. Transfection of either normal or transforming alleles of H-Ras apparently bypasses the need for IGF-I or high concentrations of insulin in the 3T3-L1 system (17, 207). The observation that transfected Raf-1 oncogenes also induce 3T3-L1 preadipocyte differentiation and that a dominant-negative Raf-1 blocks this process indicates that Raf proteins act downstream from Ras. However, transfection of Raf-1 induces only partial differentiation, indicating that Raf-independent pathways downstream of Ras may be involved in adipocyte differentiation. In this case, Raf-1-initiated signals did not activate the mitogen-activated protein kinase (MAPK) or RS kinase, suggesting a functional dissociation between Raf-1 and MAPK/RSK activation in Ras signaling pathways leading to 3T3-L1 differentiation (208). This agrees with the observation that MAPK activation is not required for the differentiation process (71). The role of Ras as an integral component of the differentiation-promoting IGF-I signaling pathway was also recently demonstrated for C3H10T1/2-derived

preadipocytes, where downregulation of *c*-Ras before confluence abolishes their differentiation (209). Recently, a serine/threonine kinase Akt (PKB) also has been demonstrated to be involved in adipocyte differentiation. Akt is activated by insulin and certain growth factors, and evidence indicates it functions as a downstream effector of phosphatidylinositol 3-kinase pathway. Expression of constitutively active Akt in 3T3-L1 cells results in their spontaneous differentiation into adipocytes in the absence of the normal inducing agents dexamethasone/MIX/insulin, suggesting the involvement of Akt-mediated signaling in adipocyte differentiation (142).

2. Other growth factors and cytokines

Unlike IGF-I, other growth factors and cytokines are generally considered as inhibitors of adipocyte differentiation. This is perhaps because of their mitogenic effects, since cell growth and differentiation are usually mutually exclusive. As discussed in section VA, growth arrest is requisite for differentiation. Several studies indicate a role for those growth factors that function through the EGF receptor, such as EGF and TGF- α , in adipose tissue development. Transforming growth factor- α inhibits differentiation of 3T3-F442A and rat preadipocytes, and transgenic mice overexpressing TGF- α have a 50% reduction of total body fat (163, 233). Epidermal growth factor inhibits differentiation of mouse, rat, and human preadipocytes (105, 233, 282), and subcutaneous administration of EGF to newborn rats results in a substantial decrease in fat pad weight, which suggests a delayed formation of adipocytes from preadipocytes (238). However, EGF is not always inhibitory. Differentiation of 3T3-L1 preadipocytes grown in serum-free medium has been reported to depend on EGF or platelet-derived growth factor (PDGF) (230). Chronic treatment of porcine preadipocyte cultures with EGF does not significantly alter their differentiation (Gregoire et al., unpublished data).

The role of basic fibroblast growth factor (bFGF) and PDGF in adipocyte differentiation is not clear. Basic fibroblast growth factor has been shown to have antiadipogenic effects in several preadipocyte cell lines under serum-containing conditions (109, 184, 185), whereas it has no effect in serum-free conditions (230). Moreover, in serum-free conditions, exposure of human preadipocytes to varying concentrations of bFGF has no effect on the number and morphology of differentiating cells (105) and either a modest or no stimulatory effect on rat preadipocytes (236, 282). Platelet-derived growth factor also has been reported to either inhibit (109, 184), have no effect (230), or promote (16) differentiation of preadipose cell lines. In addition, despite the obvious mitogenic activity seen in human preadipocyte cultures, PDGF did not substantially affect adipogenesis (105). Taken together, these reports suggest that the inhibitory effects of these growth

factors may depend on the origin, the state of development of the target preadipocytes, and culture conditions. In most cell culture models, TGF- β is a potent inhibitor of adipocyte differentiation (160, 203, 237, 253, 282). A possible mechanism for TGF- β inhibition of adipocyte differentiation may be via increasing synthesis of ECM components. The ECM influences adipocyte differentiation, as discussed in section viC, and TGF- β increases synthesis of ECM components (110). Inhibition of *in vitro* adipocyte differentiation, assessed by triglyceride accumulation and expression of various marker mRNAs, is also reported for a number of cytokines. Interleukin-11 has a dose-dependent inhibitory effect for both 3T3-L1 differentiation and for adipose conversion of bone marrow stroma-derived H-1/A cells. Inhibition is dominant over the effect of standard inducing agents (193, 194). Interferon- γ and interleukin-1 β inhibit the adipoconversion of 3T3-derived preadipocyte cell lines and primary rodent preadipocytes (95, 134, 199). Tumor necrosis factor- α decreases LPL synthesis and inhibits adipocyte differentiation. As mentioned in section vD, when applied to mature adipocytes at fairly high doses for a long period, TNF- α has been shown to cause loss of intracytoplasmic lipids, wherein the cells resemble a preadipocyte phenotype (273, 274). This TNF- α -mediated reversal of adipocyte differentiation has been shown to be associated with the downregulation of C/EBP- α and induction of *c-myc* expression (189, 259, 297). In addition, TNF- α treatment causes a rapid decrease in the levels of PPAR- γ mRNA and protein, as well as a parallel decrease in PPAR- γ DNA binding activity that precedes the decrease in C/EBP- α and p2. This suggests that the downregulation of PPAR- γ may be a mechanism whereby TNF- α exerts its effects in the mature adipocyte (301, 311). Furthermore, it has been shown that PPAR- γ is a phosphoprotein that undergoes EGF-stimulated MEK and MAPK-dependent phosphorylation. This phosphorylated form is less active in transactivation of adipocyte genes and in promoting adipogenesis (1, 30, 118). This therefore suggests that some of the growth factors inhibitory to adipocyte differentiation might act through the MAPK pathway to phosphorylate PPAR- γ . However, insulin treatment, which is known to increase lipid accumulation during adipocyte differentiation, also results in PPAR- γ phosphorylation (311). Further studies are needed to address this apparent discrepancy.

3. Nuclear hormone superfamily

Members of the nuclear hormone superfamily, including glucocorticoids, 3,3',5-triiodothyronine (T_3), and RA, influence adipocyte differentiation. Their action in adipocyte differentiation is not well characterized at the molecular level, but these hormones in general exert nuclear effects by binding to their respective intracellular hormone receptors. Although some general conclusions can be

made, the variability of serum composition must be taken into account, since serum itself may provide various factors. Dexamethasone, a synthetic glucocorticoid, is a component of the dexamethasone/MIX differentiation cocktail established for 3T3-L1 cells by Rubin et al. and dexamethasone is also routinely used for the differentiation of other preadipocyte cell lines (37, 78, 99, 224). It is also employed for the differentiation of primary preadipocytes derived from different fat depots from various species, including rodents, rabbits, pigs, and humans (96, 104, 160, 213, 264). Furthermore, dexamethasone is used for optimal adipocyte differentiation even in studies where transfection of PPAR- γ and/or C/EBP- α was employed to induce adipocyte differentiation of fibroblasts (272, 300). The role of PPAR- γ and C/EBP in adipocyte differentiation is discussed in detail in section vii. Depending on the origin of the cells and culture conditions, glucocorticoid treatment is either required for differentiation or acts to only accelerate this process. In serum-free medium, differentiation of porcine, rabbit, and human preadipocytes appears strictly dependent on the addition of glucocorticoids (104, 213, 264), whereas extensive differentiation of rat preadipocytes occurs in the absence of glucocorticoids (57, 97). It has been demonstrated in 3T3-L1 cells that glucocorticoids induce expression of C/EBP- δ . This increase may contribute to the formation of C/EBP- δ -C/EBP- β heterodimers, which in turn may lead to PPAR- γ expression (299). In studies with Ob1771 preadipocytes, glucocorticoid effects have been shown to be mediated through increased metabolism of arachidonic acid leading to an increase in production of prostacyclin, which in turn increases intracellular cAMP (4).

The ability of RA to affect various differentiation processes including the terminal events of the adipocyte differentiation program has been recognized for several years. When used at supraphysiological concentration, RA inhibits adipocyte differentiation of preadipocyte cell lines and primary porcine preadipocytes (60, 263). Retinoic acid addition either before or after treatment with inducing agents does not affect differentiation, indicating that RA acts at an early stage in differentiation. This finding is supported by the observation that RA treatment prevents induction of C/EBP- α and interferes with the mechanisms that induce as well as maintain PPAR- γ expression. These actions of RA seem to be predominantly mediated by liganded RA receptors (RARs) rather than retinoid X receptors (RXRs) (38, 302). Moreover, recent evidence indicates that the inhibitory effects of RA occur before PPAR- γ expression by blocking C/EBP- β induction (232). In contrast to the inhibitory effects observed for supraphysiological concentration of RA, concentrations close to the receptor dissociation constant act as potent adipogenic inducers for Ob17 cells and rat preadipocytes; this specifically involves the RAR- α subtype (225, 226). A critical role of RA in adipocyte differentiation has been recently highlighted by the finding that pretreatment of differentiating ES cell-derived em-

bryoid bodies with RA for a short period of time results in a high degree of adipogenesis. In this case, the role of RA in these very early events of the adipocyte differentiation program can be distinguished from that of RA on terminal differentiation described above, since neither adipogenic hormones nor potent activators of PPARs could substitute for RA (52).

3,3',5-Triiodothyronine also has been implicated in the terminal differentiation of Ob17 preadipocytes (82). The role of T_3 appears to be restricted to the Ob17 preadipocyte cell line, since no clear requirement for T_3 is observed in other preadipocyte culture models, including the 3T3-L1 and rat, porcine, or human primary preadipocyte cultures (57, 103, 104, 230, 236, 264, 293).

4. Prostaglandins

Several lines of evidence indicate that arachidonate metabolites may play an important physiological role in adipose tissue metabolism and development. Mature adipocytes and cultured preadipocytes produce significant amounts of prostaglandins (PGs), including $\text{PGF}_{2\alpha}$, PGE_2 , PGD_2 , and PGI_2 (122, 215). Prostaglandin E_2 is a strong antilipolytic compound, and $\text{PGF}_{2\alpha}$ and PGI_2 have been shown to modulate preadipocyte differentiation. Prostaglandin D_2 and its 15-deoxy- J_2 derivative may be endogenous ligands for PPAR- γ and therefore act as adipogenic signals (141). As presented in section VII, treatment with synthetic PPAR- γ agonists stimulates adipose conversion of 3T3-L1 preadipocytes (27).

Prostaglandin $F_{2\alpha}$ inhibits differentiation of various preadipose cell lines and primary rat preadipocytes (122, 174, 188, 216, 235, 282). Prostanoid FP receptor agonists have been recently reported to be potent inhibitors of differentiation for 3T3-L1 cells and primary rat preadipocytes, confirming the involvement of a FP prostanoid receptor in the inhibition of adipocyte differentiation (34, 234). In 3T3-L1 cells, FP receptor stimulation causes a transient increase in intracellular calcium, activation of a calcium/calmodulin-dependent protein kinase (CaM kinase), and an increase in DNA synthesis that is associated with the inhibition of differentiation. The addition to differentiating cells of KN-62, a CaM kinase inhibitor, in the presence of the FP receptor agonist reverses the inhibition of differentiation and suggests a critical role for a CaM kinase in adipocyte differentiation (174). However, the precise role of CaM kinases in this process remains to be clarified, since temporal activation of CaM kinase type II has been recently reported to be an obligatory step for adipogenesis. Blockage of CaM kinase type II activation with either KN-62 or KN-93 prevents the conversion of 3T3-L1 fibroblasts to adipocytes. This effect was dependent on the timing of inhibitor addition (285). A different mode of action for $\text{PGF}_{2\alpha}$ has been proposed for primary rat preadipocytes. In these cells, $\text{PGF}_{2\alpha}$ stimulates

mRNA expression and production of TGF- α , both in undifferentiated and differentiated cells. Both $\text{PGF}_{2\alpha}$ and TGF- α , which are inhibitors of adipocyte differentiation, are produced locally in adipose tissue. Therefore, stimulation of TGF- α expression by $\text{PGF}_{2\alpha}$ could represent an amplification mechanism to modulate preadipocyte differentiation and adipocyte function within adipose tissue (156). In contrast to the inhibitory role of $\text{PGF}_{2\alpha}$, a potent and specific adipogenic role has been attributed to prostacyclin (PGI_2). This prostanoid is one of the major metabolites of arachidonic acid both in preadipose and adipose cells, and it has been previously described as an autocrine/paracrine adipogenic effector for Ob1771 preadipose cells and primary rodent and human preadipocytes (35, 186, 282). Carbacyclin, a stable prostacyclin analog, has been shown to act by means of two intracellular signaling pathways known to synergize in inducing adipocyte differentiation, i.e., concomitant elevation of cAMP and free intracellular calcium (280, 281). Prostacyclin has also been reported to be an activator of the three known mammalian PPARs (α , δ , and γ) and to be the most effective activator for PPAR- δ described to date. This suggests that in addition to the biological effects of prostacyclin mediated by its cell-surface receptor, its ability to promote differentiation may also be mediated by PPARs (15, 28, 111). The paracrine adipogenic effect of PGI_2 has also been reported to be controlled by angiotensin II. This effect is mediated through the AT_2 subtype of the angiotensin II receptor. Ob1771 adipose cells challenged with this vasoactive peptide produce PGI_2 . Prostacyclin released by differentiated Ob1771 cells is able to induce preadipose cells to differentiate into adipose cells (53). In vivo, this paracrine mode of action may represent a crucial biological signal in the hyperplastic development of adipose tissue known to occur once adipose cells reach their maximal size. The role of PGE_2 and PGD_2 in adipocyte differentiation is less clear. Depending on the cell culture model systems, they have been reported to either inhibit or have no effect on preadipocyte differentiation (34, 235).

5. cAMP, G proteins, and protein kinase C

Methylisobutylxanthine accelerates the differentiation of preadipose cell lines and primary preadipocytes. As with glucocorticoids, MIX is routinely used for the differentiation of a variety of preadipocytes. Methylisobutylxanthine has been shown to increase expression of C/EBP- β , and this increase is required for subsequent PPAR- γ expression and adipocyte differentiation. The role of C/EBP- β in adipogenesis is presented in detail in section VII. The precise mode of action of MIX is not resolved. Methylisobutylxanthine is known to inhibit phosphodiesterases and block A_1 adenosine receptor in a competitive manner. It also stimulates adenylyl cyclase activity by blocking the inhibitory regulatory protein G_i

(198). This indicates that MIX may function through increasing cAMP accumulation. However, contradictory results have been obtained in studies that address whether the cAMP elevating agents dibutyryl cAMP or forskolin can replace the effects of MIX in stimulating adipocyte differentiation (243, 293, 296, 304). In studies on 3T3-F442A preadipocytes, modulation of adipogenesis by cAMP has been reported to depend on the increase in intracellular cAMP levels achieved by forskolin treatment; forskolin concentrations in the nanomolar range promote differentiation, whereas concentrations above the micromolar range inhibit the differentiation process (304).

The G proteins $G_{s\alpha}$ and $G_i\alpha$ have been shown to mediate adipocyte differentiation in 3T3-L1 cells in a manner apparently independent of adenylyl cyclase (87, 288). Antisense oligodeoxynucleotides to $G_{s\alpha}$ accelerate adipocyte differentiation, and agents that activate $G_{s\alpha}$ block differentiation. Likewise, expression of the inhibitory subunit $G_{\alpha_{1,2}}$ promotes lipid accumulation. These effects of $G_{s\alpha}$ and $G_{\alpha_{1,2}}$ are exerted at ambient or elevated intracellular cAMP levels, demonstrating that this critical role of G proteins in adipocyte differentiation is independent of adenylyl cyclase (288). Constitutive expression of $G_{\alpha_{1,2}}$ and $G_{s\alpha}$ chimeras has been used in 3T3-L1 cells to define the specific regions of these proteins responsible for repression of adipogenesis. The domain of $G_{s\alpha}$ requisite for regulation of adipogenesis maps to a region that includes switch domains I and II. These have been found to be spatially distinct from the domains that regulate adenylyl cyclase; this is consistent with the inability of cAMP to influence the adipocyte differentiation process (286). A potential role for the G protein $G_q\alpha$ in the control of adipose tissue physiology and/or preadipocyte differentiation has been recently reported. Ablation of $G_q\alpha$ by antisense RNA under the control of PEPCK promoter, which directs expression to WAT and liver in transgenic mice, causes increased body mass and hyperadiposity that persists through adult life (79). Absence of $G_q\alpha$ abolishes the A_1 adrenergic regulation of lipolysis, apparently predisposing the mice to fat accumulation. However, because the basis for hyperadiposity in these $G_q\alpha$ -deficient mice remains to be established, $G_q\alpha$ may also control adipogenic conversion in vivo (79). Rat primary subcutaneous preadipocytes in culture display a higher capacity to differentiate than epididymal preadipocytes (97). This site specificity of differentiation capacity was recently shown to be related at least in part to the differences in $G_q\alpha$ subunit expression (56). During adipogenesis, no major site differences are found in the amount of $G_{s\alpha}$ and $G_i\alpha$ subunits. However, $G_q\alpha$ dramatically decreases in subcutaneous-derived cells while it remains constant in epididymal-derived cells. The $G_q\alpha$ subunit mediates phospholipase C- β activation leading to diacylglycerol formation and protein kinase C (PKC) activation (65), an event known to downregulate adipogenesis of preadipocyte cell lines as well as rat

primary preadipocytes (184, 240, 242). Therefore, compared with epididymal preadipocytes, the higher capacity of subcutaneous preadipocytes to differentiate into adipocytes seems to correlate with a decrease in $G_q\alpha$ expression and a decrease in $G_q\alpha$ -mediated PKC activation (56).

Although the negative effect of PKC activation on adipocyte differentiation appears well established, the role played by the different PKC isoforms in this process remains unsettled. Protein kinase C is a family containing at least 11 isoforms divided into three major groups based on their structure and mode of activation (55). Several PKC isoforms are expressed in mature isolated rodent adipocytes as well as in 3T3-L1 preadipocytes and adipocytes. Differentiation-dependent changes in isoform expression occur during adipogenesis (66, 74, 149, 171). Although total PKC activity is reduced during 3T3-L1 differentiation, not all isoforms have lowered expression. Protein kinase C- ϵ expression occurs only with differentiation, and upregulation of this isoform does not occur in cells treated with the inhibitory agent TNF- α . During TNF- α -induced inhibition of adipocyte differentiation, downregulation of the PKC- α isoform was blocked; this suggests that downregulation of PKC- α is a discrete step in the 3T3-L1 differentiation program (171). Protein kinase C- α and PKC- ζ also decrease during rat preadipocyte differentiation, and the possible involvement of PKC- ζ in the postreceptor signaling pathway of insulin is suggested by studies in these primary cultures (149). Ectopic expression of PKC- η is also reported to alter the expression of cyclins and cdk inhibitors and induce adipogenesis in NIH 3T3 fibroblasts (161). These findings indicate that the PKC pathway of signal transduction is part of a highly complex system that likely exerts negative as well as positive effects on the adipocyte differentiation process.

B. Pref-1, an EGF Repeat-Containing Inhibitor of Adipocyte Differentiation

Comparison of the genes that are regulated by the adipogenic inducing agents dexamethasone/MIX in 3T3-L1 cells and the closely related but differentiation-defective 3T3-C2 cells has resulted in the identification of preadipocyte factor-1 (pref-1). 3T3-C2 cells, which do not undergo differentiation in response to the adipogenic inducing agents dexamethasone/MIX, express approximately threefold higher pref-1 levels than 3T3-L1 preadipocytes. Recent evidence has demonstrated that pref-1 is a EGF repeat-containing transmembrane protein that inhibits adipocyte differentiation and suggests that this molecule may link adipocyte differentiation signals from the extracellular environment to the cell interior (246–248, 250). Expression of pref-1 in 3T3-L1 preadipocytes decreases to undetectable levels during their differentiation to mature adipocytes. Fetal calf serum, an essential

component for *in vitro* adipocyte differentiation, dramatically downregulates *pref-1* levels. Taken together, these observations indicate that *pref-1* may not only be regulated during adipocyte differentiation, but suggest a regulatory role for *pref-1* in adipocyte differentiation. Preadipocyte factor-1 does not appear to have the mitogenic effects (C. Li, C. M. Smas, and H. S. Sul, unpublished data) normally associated with the inhibitory actions of growth factors and cytokines described in section *VIA2*. This suggests that *pref-1* may function via a unique mechanism to inhibit adipocyte differentiation.

1. Inhibitory action in adipogenesis

The inhibitory action of *pref-1* on adipocyte differentiation has been demonstrated by two approaches: 1) interfering with the normal downregulation of *pref-1* that occurs during adipocyte differentiation by constitutive expression of *pref-1* in 3T3-L1 preadipocytes, and 2) addition of a soluble form of the *pref-1* ectodomain to culture media during and after the dexamethasone/MIX differentiation treatment. To determine the effect of persistent *pref-1* expression on adipocyte differentiation, two independent pools of several hundred clones transfected with the *pref-1* expression construct have been examined for their ability to differentiate in response to the adipogenic agents dexamethasone/MIX. Although a high degree of differentiation is observed in controls of nontransfected 3T3-L1 preadipocytes or stable pools of 3T3-L1 harboring the reverse orientation of the *pref-1* cDNA, differentiation is drastically reduced in the *pref-1*-transfected cultures. These cultures maintain fibroblast morphology and contain few fat cells. Oil Red O staining reveals little to no lipid accumulation and markedly lower expression of *aP2* and *SCD1* mRNAs. Examination of the cultures by light microscopy reveals that the decrease in lipid staining in the *pref-1*-transfected cells is because of a decrease in the total number of cells that differentiate to adipocytes, not to a generally lower amount of lipid per cell (248).

The inhibitory effects of the *pref-1* ectodomain have been shown by addition of soluble *pref-1*-glutathione-*S*-transferase (GST) fusion protein to 3T3-L1 preadipocytes during the differentiation period. In the presence of soluble *pref-1*, only 10% of cells undergo adipocyte differentiation in response to the dexamethasone/MIX treatment, in comparison with the high degree of differentiation in GST-treated and untreated control cultures. The levels of mRNA for fatty acid synthase, *SCD1*, and *aP2* in the *pref-1*-treated cells are 20% that of GST-treated or untreated controls. Moreover, *C/EBP- α* and *PPAR- γ* mRNAs are similarly decreased. The inability of 3T3-L1 cells to express adipocyte transcription factors in the presence of soluble *pref-1* suggests that its inhibitory effects are exerted early in differentiation. The inhibitory effects of soluble *pref-1* are confirmed by experiments wherein 3T3-L1 preadipo-

cytes are subjected to differentiation in the presence of conditioned media from *pref-1*-transfected COS cells. Conditioned media obtained from mock-transfected COS cells did not affect adipocyte differentiation of 3T3-L1 cells as judged by the number of lipid-containing cells. However, conditioned media from *pref-1*-transfected COS cells drastically reduce adipocyte differentiation in response to dexamethasone/MIX treatment. These data, and the fact that *pref-1* antibody blocks the inhibitory action of soluble *pref-1*, suggest a specific inhibitory effect of *pref-1* in adipocyte differentiation and are consistent with the existence of a specific *pref-1* receptor. These findings indicate that, although synthesized as a transmembrane molecule, the *pref-1* ectodomain alone is sufficient for its inhibitory effects.

2. Generation of soluble *pref-1*

The inhibitory actions of the *pref-1* ectodomain indicate that the cytoplasmic tail is not required for the *pref-1* adipo-inhibitory function, although this region may have an as yet unidentified function. Processing of transmembrane precursors to generate active factors has been shown for a variety of molecules. For example, all soluble factors that function through the EGF receptor derive from cleavage of transmembrane precursors, as does the kit ligand. Transfection studies in COS cells reveal that processing of transmembrane *pref-1* occurs at two sites in the extracellular domain. In addition to full-length transmembrane *pref-1*, a cell-associated protein of 25 kDa has been identified through addition of a myc epitope tag to the extreme COOH terminus of *pref-1* (246). This 25-kDa protein is predicted to correspond to a residual membrane portion of full-length *pref-1* after a cleavage in the extracellular domain. Pulse-chase analysis of the media of *pref-1*-transfected COS cells indicates that the major soluble form of *pref-1* is 50 kDa and the existence of 31- and 24- to 25-kDa soluble *pref-1* proteins. The time course of disappearance of cell-associated and increase in soluble *pref-1* is consistent with a precursor-product relationship. Addition of a consensus phosphorylation site for A-kinase to the second EGF repeat of the *pref-1* extracellular domain, and subsequent phosphorylation, has determined that the 50-kDa and 24- to 25-kDa soluble forms contain the second EGF repeat and likely derive from the NH₂ terminus of the ectodomain. The 31-kDa soluble *pref-1* is not phosphorylated by these means, and its origins are unclear. Taken together, these predict two processing events: a membrane-proximal cleavage to generate the 50-kDa soluble *pref-1* and a membrane-distal cleavage that may generate the smaller soluble forms. These observations and the fact that the complete ectodomain acts to inhibit adipocyte differentiation indicate that generation of soluble *pref-1* from a transmembrane precursor extends the inhibitory range of action of the molecule.

3. Mode of action

Analysis of the soluble products that are generated from not only full-length pref-1, as above, but by three additional alternately spliced pref-1 forms indicate that the generation of the 50-kDa soluble pref-1 is dependent on the alternate pref-1 transcript expressed. Whereas each of the four alternately spliced forms of pref-1 resulted in production of the smaller soluble forms, only the two longest of the alternately spliced pref-1 cDNAs generate the largest soluble form. This indicates that the spliced-out sequence includes a processing site and localizes the cleavage to within a 22-amino acid juxtamembrane sequence. It therefore appears that the mode of pref-1 function, juxtacrine or paracrine, depends on the specific alternate pref-1 transcript expressed. However, the fact that the pref-1 ectodomain alone inhibits adipocyte differentiation indicates generation of the inhibitory signal does not require the pref-1 cytoplasmic region. This suggests that the pref-1 molecule, in either transmembrane or soluble form, probably functions as a ligand to initiate/maintain signals inhibitory to adipogenesis. It is highly unlikely pref-1 acts through the EGF receptor. The spacing and conservation of amino acids that are required for EGF-receptor interaction are absent in pref-1. Preadipocyte factor-1 does not affect DNA synthesis (Li et al., unpublished data) and therefore lacks the mitogenic activity that is normally associated with EGF receptor function.

The role of EGF repeats in other molecules leads to several hypotheses for pref-1 action in adipocyte differentiation. The EGF repeat unit is a 35- to 40-amino acid sequence characterized by highly conserved spacing of six cysteine residues that form three disulfide bonds. Members of the EGF-repeat family of proteins are membrane-bound or secreted proteins that act on cell growth and differentiation in an astonishing array of biological settings (12, 154, 192). A single EGF-like domain is the functional unit of EGF, TGF- α , and other growth factors that interact with the EGF receptor (33). The EGF-repeat family also includes proteins of the blood-clotting cascade, the LDL receptor, and several multidomain ECM proteins and cell adhesion molecules with a demonstrated role in cell guidance and development. Strong evidence for the role of EGF-repeat molecules in the regulation of differentiation and development is provided by the function of the transmembrane protein Notch and its ligands Delta and Serrate in *Drosophila*. Although the exact molecular mechanism remains to be elucidated, the ligand-Notch extracellular domain interaction brings about the association of the intracellular domain of Notch with Su(H), transcriptional activation of specific genes, and subsequent cell fate determination (14, 144). The identification and characterization of vertebrate notch homologs indicate signaling via EGF-repeat domains will likely be an important mechanism in mammalian cell lineage decision, as indicated by the effects of Notch in muscle differentiation and T-

lymphocyte development (192, 219, 220, 292). Although the mechanism of pref-1 inhibition of adipocyte differentiation remains to be determined, given the importance of cell shape modulation and the ECM environment in adipocyte differentiation, discussed in section viC, transmembrane pref-1 may possibly function by the interaction of its EGF-like domains with EGF-like or other protein domains present in ECM molecules, thereby maintaining the preadipose phenotype. These could include versican and laminin; both contain EGF-repeat domains and are modulated during adipocyte differentiation. Additionally, transmembrane and/or soluble pref-1 may act as a ligand for an as yet unidentified preadipocyte receptor molecule, to maintain signal transduction pathways inhibitory to adipogenesis. Identification of proteins that interact with pref-1 will lend valuable insights into the mechanisms of pref-1 action and will elucidate how the inhibitory signals of pref-1 are balanced against the differentiation-promoting effects of PPAR- γ and C/EBP.

C. Extracellular Matrix Components

Links between exogenous hormones and transcriptional regulators have begun to emerge, thereby allowing some integration of these two aspects in our understanding of the control of adipocyte differentiation. In contrast, knowledge of the molecular mechanisms regarding the effect of the ECM on adipocyte differentiation is lacking. These studies are particularly crucial if we are to fully understand the process in tissues, where differentiation occurs in close association with the stromal-vascular cell population. Cell adhesion molecules and ECM components modulate the interaction of cells with their environment in a manner that influences cell differentiation and migration. This may lead to cytoskeletal network rearrangement and an intracellular cascade of signal transduction that influences differentiation. Additionally, a physical connection between the ECM and nuclear matrix, via the cytoskeleton, has been proposed (129). The ECM, through altering cell spreading, may expose the cell surface to various growth factors present in the environment (181), and ECM components have been demonstrated to bind and sequester growth factors (303). The effect of ECM components on cell differentiation is demonstrated in general by the fact that a decrease in tissue-specific markers often occurs upon plating of primary cells onto tissue-culture dishes. Interaction between cell-matrix and nuclear events has been evidenced by numerous studies including β -casein induction by basement membrane substratum of mammary epithelial cells (261), basement membrane inhibition of keratinocyte differentiation (88), transactivation of the cytotactin/tenascin and neural cell adhesion molecule (N-CAM) promoters by specific homeodomain proteins (128), and modulation of N-CAM in dissociated versus aggregated p19 embryonal cells (170).

How the ECM influences adipocyte differentiation is yet to be addressed in appropriate molecular detail.

The ECM of adipose tissue interconnects adipocytes and gives rise to fat cell clusters *in vitro* and to fat lobules of adipose tissue *in vivo*. During adipocyte differentiation, drastic changes occur in cell morphology, cytoskeletal components, and the level and type of ECM components secreted as described in section *vC*. An early ultrastructural change seen in *in vivo* adipocyte differentiation is the deposition of collagen at the cell-ECM border and extracellular basement membrane biogenesis (183). Fibroblasts in developing fat tissue have loose interactions with the ECM that may be necessary for the morphological changes that accompany differentiation or to keep cells in close proximity and thereby increase their exposure to juxtacrine differentiation signals. Electron microscopy reveals that preadipose 3T3-F442A cells have limited granular ECM deposits. In contrast, 3T3-F442A adipocytes appear interconnected by abundant ECM rods and fibers and resemble the fat cell clusters seen in lobules of adipose tissue (146). These changes may be related to the loose interaction of the basement membrane with adipose cells that has been observed in fat tissue. Many of the ECM components, for example, laminin and entactin/nidogen, are known to interact with each other and the cell surface. Modulation of ECM components could permit release of cell-cell adhesion and remodeling of cell components. These changes might be necessary for cellular reorganization and could provide a permissive or instructive environment for the expression of adipocyte genes.

Subjecting preadipocytes to differentiation conditions in the presence of ECM components demonstrates their influence on adipocyte differentiation. Type I and III collagen, fibronectin, and poly-L-lysine, as well as β -integrins are negatively correlated with the differentiation of preadipocyte cell lines (11, 24, 221, 257, 290), whereas increases in type IV collagen, entactin, and an unorthodox laminin complex accompany the adipocyte differentiation process (13, 195). Culture of 3T3-F442A preadipocytes on fibronectin matrices decreases gene expression of lipogenic enzymes and leads to decreased triglyceride accumulation (257), possibly by interfering with the cytoskeletal and morphological changes necessary for adipocyte differentiation. The inhibitory effect of fibronectin is not observed by its addition to culture media, and this inhibition is overcome by keeping cells in a rounded configuration. These observations indicate that the inhibitory effect of fibronectin requires cell spreading. Cytochalasin D, an agent that disrupts actin filaments, overcomes the inhibitory effects of fibronectin (257), further indicating that cytoskeletal rearrangement is a prerequisite for terminal differentiation. In a separate study, differentiation of 3T3-F442A cells has been shown to be accelerated by cytochalasin D treatment, perhaps by allowing earlier cytoskeletal remodeling. Furthermore, with long-term cytochalasin D treatment, a small por-

tion of differentiation-defective 3T3-C2 cells undergo morphological and biochemical conversion to adipocytes (197). Addition of soluble fibronectin and growth on fibronectin matrices markedly decreases differentiation of ST-13 preadipocytes. This is reversed by an antibody against the $\alpha_5\beta_1$ -integrin and by a short peptide corresponding to the cell attachment domain of fibronectin. Interestingly, differentiation of these cells, on the other hand, is stimulated by the addition of a thermolysin digest of fibronectin (76). The adipocyte-stimulating activity is in the NH₂-terminal fibrin binding domain and can be activated by use of specific matrix metalloproteinases (76, 77). These experiments suggest that fibronectin has the ability to both inhibit and stimulate adipocyte differentiation, depending on the state of the molecule.

As described in section *vC*, changes in expression of specific types of collagen accompany adipocyte differentiation. Use of ethyl-3,4-dihydroxybenzoate (EDHB), a specific inhibitor of active collagen synthesis, highlights the role of collagen in modulating adipocyte differentiation. Exposure of TA1 cells to EDHB preceding confluence and during the initial stage of differentiation prevents cells from entering the terminal differentiation program; this demonstrates that differentiation of preadipose cells into adipocytes requires active synthesis of collagen (124). A role for proteoglycans in adipocyte differentiation is suggested by the observation that proteoglycan metabolism is altered during 3T3-L1 preadipocyte differentiation (29, 182). Levels of versican/CSGP-I, a chondroitin 4-sulfate proteoglycan, are increased early during 3T3-L1 cell differentiation. This increase appears to correlate with the increased viscosity of the culture medium that occurs during differentiation. The role of ECM in the differentiation process is confirmed in primary preadipocyte studies (108, 214, 215, 279). The source or type of ECM dictates the influence of ECM substrata on primary porcine preadipocyte differentiation (108). Laminin, in particular, may play a critical role in morphological changes of porcine preadipocyte development. All of the above studies clearly indicate that ECM components modulate adipocyte differentiation, perhaps by release of cell-cell adhesion, thereby permitting changes in cell morphology or volume (29). It remains to be determined whether ECM components could be directly involved in initiating or transducing signals for adipose differentiation or merely allow cells differential access to the growth and/or inhibitory factors present in the surrounding environment.

VII. TRANSCRIPTION FACTORS CRITICAL FOR ADIPOCYTE DIFFERENTIATION

Adipocyte differentiation involves communication of extracellular signals and those of the ECM environment to the nucleus. This leads to a coordinate regulation of

adipocyte-specific gene expression resulting in the mature adipocyte that is highly specialized for energy storage and homeostasis. As presented above, many classes of molecules transduce inductive and inhibitory signals from the environment. Although the full complement of proteins involved in this process remains to be determined, ultimately the PPAR and C/EBP family of transcription factors must function cooperatively to transactivate adipocyte genes and thereby bring about adipocyte differentiation. The role of transcription factors of the PPAR and C/EBP families in adipocyte differentiation has been extensively reviewed recently (27, 153, 167), and we present an overview only.

A. PPAR Family

The PPARs belong to type II nuclear hormone receptor family and form heterodimers with the RXR (131, 231). The PPARs are activated by a variety of structurally dissimilar compounds, including the thiazolidinedione class of antidiabetic drugs, Wy-14, 463, and clofibrate. The PPARs regulate transcription through binding of PPAR-RXR heterodimers to a response element consisting of a direct repeat of the nuclear receptor hexameric DNA recognition motif (PuGGTCA) spaced by one nucleotide (DR-1) (271). Peroxisome proliferator-activated receptor- γ is the most adipose specific of the PPARs, and it is induced before transcriptional activation of most adipocyte genes. Low but detectable expression of PPAR- γ also occurs in liver and hematopoietic cells (25, 62). The expression of PPAR- γ has been shown to be sufficient to induce growth arrest as well as to initiate adipogenesis in exponentially growing fibroblast cell lines, demonstrating its critical role in the regulation of adipocyte differentiation (6, 120, 272). Moreover, retroviral-mediated ectopic expression of PPAR- γ in the presence of PPAR activators decreases myoblast-specific gene expression and directs these cells into the adipocyte lineage (120). Both synthetic and natural ligands of PPAR- γ have recently been identified. Thiazolidinediones, a new class of drugs which increase insulin sensitivity, can directly bind and activate PPAR- γ and have been shown to stimulate adipose conversion (140, 155, 227). Thiazolidinediones are strongly adipogenic for fibroblasts and myoblasts, especially for those expressing PPAR- γ , with 15-deoxy-D-PGJ₂, the most potent activator (72, 141). Whether 15-deoxy-D-PGJ₂ is the major endogenous ligand for PPAR- γ is not yet known (27). The observations reporting that PPAR- γ is highly expressed in adipocytes and that its ectopic expression is capable of triggering the entire program of adipogenesis in fibroblast and muscle cells has led to the hypothesis that PPAR- γ can regulate development of the adipocyte lineage (272). Although PPAR- γ plays a critical role in the induction and maintenance of the fully differentiated adipocyte phenotype, its participation in the adipocyte lineage determination

process remains unclear. Retinoic acid but not potent PPAR activators can commit ES cells into the adipocyte lineage, suggesting that RAR may be involved in the initial steps of adipose cell development (52).

The role of other PPAR isoforms in adipogenesis remains to be resolved. Peroxisome proliferator-activated receptor- α was also reported to be able to induce significant adipocyte differentiation in response to strong PPAR activators. Because this isoform is only weakly expressed in adipocytes and has been reported to be less adipogenic than PPAR- γ , its precise role in triggering and/or maintaining the adipocyte phenotype remains unclear (28). Although PPAR- δ is not adipocyte specific, this PPAR isoform is highly expressed in adipose tissue. It is upregulated very early during adipocyte differentiation and has been suggested to play an important role in adipogenesis (7). Involvement of PPAR- δ in the *trans*-differentiation of myoblasts to adipoblasts triggered by either fatty acids or thiazolidinediones has been recently postulated by Grimaldi et al. (100). However, a role for PPAR- δ in triggering *trans*-differentiation seems very unlikely given that the level of PPAR- δ is high in myoblasts and is only marginally increased with thiazolidinedione treatment (266). Moreover, transcriptional activation of PPAR isoforms by various PPAR activators demonstrates that thiazolidinediones are potent activators of PPAR- γ but not PPAR- δ (28). Transfection of 3T3-C2 cells with a PPAR- δ expression construct has been reported to confer fatty acid inducibility on the aP2 gene and the FAT gene (7). However, retroviral expression and activation by bromopalmitate of PPAR- δ in NIH 3T3 cells does not stimulate adipogenesis (28). Although a partial role of PPAR- δ in mediating the transcriptional effects of fatty acids on preadipocyte differentiation cannot be ruled out, its role in adipose tissue development still remains to be demonstrated.

B. C/EBP Family

Members of the C/EBP family were the first transcription factors demonstrated to play a major role in adipocyte differentiation. These transcription factors have a basic transcriptional activation domain and an adjoining leucine zipper motif, which provides the ability for homo- and heterodimerization. Isoforms of C/EBP are expressed in tissues, such as liver, that metabolize lipid and cholesterol-related compounds at high rates (43). Studies of adipocyte differentiation, primarily in 3T3-L1 cells, provide extensive evidence for C/EBP- α function in adipocyte differentiation (27, 153, 166, 167). Although not strictly adipocyte specific, C/EBP- α is expressed just before the transcription of most adipocyte-specific genes is initiated. CCAAT/enhancer binding protein- α binds and transactivates the promoters of several adipocyte genes, including aP2, SCD1, GLUT-4, PEPCK, leptin, and the insulin recep-

tor. Mutation of the C/EPB- α site in these genes abolishes transactivation (121, 132, 172, 223). In some instances, constitutive expression of C/EPB- α is sufficient to induce differentiation of 3T3-L1 cells in the absence of hormonal agents, and expression of antisense C/EPB- α mRNA in 3T3-L1 preadipocytes prevents differentiation (158, 159). Moreover, C/EPB- α can efficiently promote adipogenesis in a variety of mouse fibroblastic cells, including those that have little or no spontaneous capacity to develop into adipocytes (75). In view of its known antimitotic activity, C/EPB- α has also been indicated to function in the termination of the mitotic clonal expansion that occurs early in the differentiation program and seems to be involved in the maintenance of the adipocyte phenotype through autoactivation of the C/EBP- α gene (42). Together, these findings provide evidence that C/EPB- α is both required and sufficient to induce adipocyte differentiation. Generation of C/EPB- α knockout mice supports the physiological role of C/EPB- α in adipose tissue development, since C/EBP- α null mice fail to develop WAT (289). However, this study probably addresses the role of C/EPB- α in adipocyte maturation rather than its potential function in the initiation and/or commitment of mesenchymal precursors. Although mature white adipocytes are absent in C/EPB- α ($-/-$) mice, the presence of committed preadipocytes already expressing early markers of differentiation has not been assessed, and their existence cannot be excluded. Fine-tuning of the control of adipocyte gene expression by C/EBP proteins involves homo- and heterodimerization between the C/EBP- α , C/EBP- β , and C/EBP- δ isoforms. Each isoform has a distinct temporal and spatial expression pattern during adipocyte differentiation (32). Whereas C/EBP- α expression occurs relatively late in differentiation, the β - and δ -isoforms of C/EBP are present in preadipocytes, and their levels increase transiently early in differentiation. By late differentiation, C/EBP- β decreases to 50% of its initial level, and C/EBP- δ is nearly undetectable (32). Neither C/EBP- α , β , nor δ is adipocyte specific (20, 32), although C/EBP- α is expressed at high levels in the mature adipocyte and adipose tissue (32, 306). CCAAT/enhancer binding protein- β and possibly C/EBP- δ seem to function early as transcriptional activators in the sequence of events leading to adipocyte differentiation. Conditions that cause expression of either the β - or δ -isoforms accelerate adipogenesis in 3T3-L1 preadipocytes (167). Ectopic expression of C/EBP- β , particularly the liver-enriched transcriptional activator protein spliced form, activates the expression of PPAR- γ in NIH 3T3 fibroblasts exposed to dexamethasone, insulin, and fetal bovine serum and stimulates their conversion into committed preadipocytes (300). Expression of PPAR- γ is induced by coexpression of C/EBP- β and C/EBP- δ . Although this suggests that these two C/EBP isoforms may heterodimerize to directly regulate PPAR- γ (299), the increased PPAR- γ expression may also be the indirect effect

of onset of adipocyte differentiation resulting from C/EBP- β action. Taken together, current data suggest that an increase in C/EBP- β above a threshold level induces expression of PPAR- γ . Upon ligand activation, PPAR- γ , in concert with C/EBP- α , leads to the full adipocyte differentiation program.

The observation that in 3T3-L1 preadipocytes C/EBP- β and C/EBP- δ levels are increased by MIX and dexamethasone, respectively (32, 306), has led to the suggestion that C/EBP- β and C/EBP- δ might relay the adipogenic effects of these agents early in adipocyte differentiation. Expression of ectopic C/EBP- β in NIH 3T3 cells obviates the MIX requirement for their differentiation, indicating that C/EBP- β may be a primary effector of MIX action in adipocyte differentiation. The observed induction of C/EBP- δ by dexamethasone could lead to the formation of C/EBP- δ -C/EBP- β heterodimers, and these were postulated to be more transcriptionally active than C/EBP- β homodimers. However, examination during the initial stages of differentiation of dexamethasone-treated NIH 3T3 fibroblasts that ectopically express C/EBP- β indicates that C/EBP- δ only minimally contributes to functional C/EBP complexes (299). Moreover, dexamethasone remains an absolute requirement for adipocyte differentiation of NIH 3T3 fibroblasts ectopically expressing high levels of both C/EBP- δ and C/EBP- β , indicating that C/EBP- δ cannot substitute for the role of dexamethasone in the differentiation process (299).

C/EBP homologous protein-10 (CHOP-10) is another member of the C/EBP family. It displays overall sequence similarity to C/EBP proteins in the DNA binding and dimerization domain. However, the basic region of CHOP-10 deviates considerably in sequence from that of other C/EBP proteins, and CHOP-10-C/EBP heterodimers are unable to bind to a common class of C/EBP sites. With respect to such sites, CHOP-10 appears to function as a negative modulator of the activity of C/EBP transcription factors (275). CHOP-10 is absent under normal conditions and is induced by cellular stresses; its function under normal circumstances is still unclear.

C. bHLH Family

A potential role of bHLH in activating the adipocyte differentiation program has recently been suggested by the observation that overexpression of Id3 prevents adipocyte differentiation (177). Ids are a group of ubiquitous nuclear proteins that possess the helix-loop-helix domain but are missing the basic DNA binding domain. Ids are believed to block muscle differentiation by competing with myogenic bHLH proteins for dimerization with their bHLH binding partner, E12/E47, which binds to an E-box motif to transactivate muscle-specific genes (126). In 3T3-F442A cells as well as in primary preadipocyte cultures,

downregulation of Id2 and Id3 has been shown to precede adipocyte differentiation. A fall in Id3 seems to be a specific event of the adipocyte differentiation program; its mRNA declines very early after confluence and is not stimulated when fresh serum is added to confluent 3T3-F442A cells. On the other hand, Id2 and Id3 mRNA levels are not reduced in the differentiation-defective 3T3-C2 cells (177). These results indicate that Id3 may be a negative regulator of fat cell formation by preventing an as yet unidentified bHLH protein from activating the adipocyte differentiation program. To date, there is no candidate for such a protein. SREBP-1c/ADD1 is a bHLH protein that is expressed abundantly in adipose tissue (136) but is considered not capable of heterodimerization with Ids (177); SREBP-1c/ADD1 displays a dual DNA specificity, binding to both an E-box motif and non-E-box motif (SRE-1) (137). A role in adipocyte differentiation is suggested by the early increase of its mRNA levels during adipocyte differentiation and by its transactivation of several key lipogenic genes (64, 136). SREBP-1c/ADD1 has been shown to increase fatty acid and fat synthesis, and this has been attributed in part to its proposed influence on PPAR- γ activity; it has been hypothesized to be involved in the generation of endogenous ligands for PPAR- γ (27). Expression of SREBP-1c/ADD1 is also increased during osteoblast differentiation, where it is correlated with expression of an osteoblastic phenotype-related gene, indicating that increased expression of this protein is not specific to adipocyte differentiation. However, this observation appears to further illustrate the close relationship between the osteoblast and adipoblast lineages (228).

Another bHLH protein, twist, discussed in section III, is expressed at high levels in WAT and brown adipose tissue, primarily in stromal cells. A decrease in twist expression is observed upon differentiation of 3T3-L1 cells and primary preadipocytes. Like Ids, twist is not adipocyte specific; a similar pattern of expression occurs in osteogenic cell lines (85). Twist, when ectopically expressed, inhibits myoblast differentiation by preventing transactivation of muscle target genes by MEF2. In addition, twist, like Ids, dimerizes with E proteins, thereby blocking binding of myogenic bHLH transcription factor to DNA (222, 254). Although modulation of twist expression during adipocyte differentiation strengthens the potential role of this bHLH transcription factor in adipose tissue development, its precise role in this process remains difficult to predict at this point.

Intensive efforts dedicated to the isolation of regulatory genes for adipocyte differentiation have resulted in the identification of the C/EBP and PPAR family of transcription factors, which play a central role in the terminal differentiation of adipocytes. In addition, the expression pattern of the above-described helix-loop-helix transcription factors in the adipocyte lineage is consistent with the concept that other transcription factors may function in,

or modulate, different stages in adipocyte development. Use of new cell culture models such as ES cells, recently shown to differentiate at high efficiency into adipocytes in certain culture conditions (52), may provide a system for the identification of new adipogenic regulatory genes or those that function at earlier stages.

VIII. CONCLUSIONS

The elucidation of the cooperative function of PPAR- γ and C/EBP- α as the key transcription factors for adipocyte gene activation and differentiation has been pivotal to our understanding of adipogenesis at the molecular level. A variety of agents influence adipocyte differentiation, although their effects are somewhat dependent on the preadipose cell culture models and culture conditions employed. With this variability in mind, a general picture emerges, suggesting that the multitude of diverse regulatory signals likely converges at a few classic signal transduction pathways. We can just now begin to connect the dots from hormonal and growth factor signals to specific nuclear events. It is likely a detailed picture will emerge over the next several years. The inhibitory actions of pef-1, a member of the EGF-repeat family, suggest that new classes of important regulatory molecules remain to be discovered. Less is known about the signaling processes subsequent to cell-cell or cell-matrix interactions that modulate transcriptional activity and adipocyte differentiation, and this area warrants future detailed examination. Understanding how the various molecules regulate adiposity and energy balance in physiological and pathophysiological situations remains an important area for future research and may lead to the development of novel therapeutic approaches to human obesity.

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