Role of PPARγ in Regulating a Cascade Expression of Cyclin-dependent Kinase Inhibitors, p18(INK4c) and p21(Waf1/Cip1), during Adipogenesis* (Received for publication, December 14, 1998, and in revised form, March 16, 1999)

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Molecular mechanisms coupling growth arrest and cell differentiation were examined during adipogenesis. Data are presented that document a cascade expression of members of two independent families of cyclin-dependent kinase inhibitors that define distinct states of growth arrest during 3T3-L1 preadipocyte differentiation. Exit from the cell cycle into a pre-differentiation state of post-mitotic growth arrest was characterized by significant increases in p21 and p27. During onset of irreversible growth arrest associated with terminal differentiation, the level of p21 declined with a concomitant, dramatic increase in p18 and a sustained level of p27. The expression of p18 and p21, regulated at the level of protein and mRNA accumulation, was directly coupled to differentiation. Stable cell lines were engineered to express adipogenic transcription factors to examine the active role of trans-acting elements in regulating these cell cycle inhibitors. Ectopic expression of peroxisome proliferator-activated receptor (PPAR) γ in non-precursor fibroblastic cell lines resulted in conversion to adipocytes and a coordinated increase in p18 and p21 mRNA and protein expression in a PPARγ ligand-associated manner. These data demonstrate a role for PPARγ in mediating the differentiation-dependent cascade expression of cyclin-dependent kinase inhibitors, thereby providing a molecular mechanism coupling growth arrest and adipocyte differentiation.

Adipocytes of white adipose tissue, as well as myocytes from heart and skeletal muscle, represent examples of terminal differentiation whereby the expression of a specialized phenotype is marked by cessation of cell proliferation and the accumulation of cells in the G1 phase of the cell cycle. In mammalian cells, phase transition is regulated by the phosphorylated states of various substrates including the retinoblastoma family proteins which mediate S phase progression (1). These substrates are phosphorylated by a dimer complex comprising a regulatory “cyclin” subunit and a catalytic cyclin-dependent kinase (cdk). Phosphorylating activity of cyclin/cdk complexes is further modulated by cyclin-dependent kinase inhibitors (CKIs), which are grouped into two distinct families based on sequence homology and targets of inhibition (2). To date, seven CKIs have been identified, including p15INK4a, p16INK4a, p18INK4c, and p19INK4d defining the INK4 family, and p21Cip1, p27Kip1, and p57Kip2, representing the CIP/KIP family. Recent reports have demonstrated that CKI expression is up-regulated during cell differentiation in vitro and in vivo (3, 4), suggesting that these cyclin cycle inhibitors may play a universal role in exit from the cell cycle and/or maintenance of the irreversible growth arrest which defines terminal differentiation.

Adipocyte differentiation is largely controlled by two families of transcription factors: the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) (5–7). Members of the C/EBP family (C/EBPα, C/EBPβ, and C/EBPδ) form heterodimers and homodimers via a leucine zipper motif with dimers binding to regulatory elements within target genes via basic DNA binding domains. Ectopic expression of various C/EBPs has been shown to convert non-precursor fibroblastic cell lines into fully differentiated adipocytes (8–10), whereas genetic knockouts in vitro and in vivo block adipocyte differentiation (11–13). The PPARs (PPARα, PPARδ, and PPARγ) define a family of ligand-activated nuclear hormone receptors that heterodimerize with the retinoid X receptor and bind to specific peroxisome proliferator-responsive elements located within the promoters of target genes. Through utilization of different start sites and alternate splicing, the PPARγ gene gives rise to two isoforms, γ1 and γ2. Tissue distribution of PPARγ2 is highly enriched in adipose tissue and ectopic expression in various non-precursor cell lines also gives rise to adipocyte differentiation (14). Although the natural ligand for PPARγ is still under investigation, a synthetic class of specific ligands, called thiazolidinediones (TZDs), greatly enhance transcriptional activity (15).

Current models of the molecular process of adipogenesis involve a cascade expression of C/EBPδ and C/EBPβ, followed by the expression of C/EBPs and PPARγ, which precede and regulate the expression of many genes representative of the mature adipocyte. Much of our understanding of the interplay between these and other trans-acting elements that regulate adipocyte differentiation has been made possible with the establishment of preadipocyte cell lines (e.g. 3T3-L1 and 3T3-F442A) that differentiate from proliferative, fibroblastic-like cells into mature adipocytes exhibiting nearly identical morphological and biochemical properties of white adipose tissue (16). After reaching a state of density-induced growth arrest, preadipocyte cell lines can be induced to differentiate with exposure to a combination of mitogen and hormonal agents.

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The abbreviations used are: cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MDI, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin; kb, kilobase pair(s); TNFα, tumor necrosis factor α; PPA, proliferating preadipocyte.

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Immediately after exposure to these agents, the cells reenter the cell cycle for a limited period of cell proliferation, commonly referred to as clonal expansion. This is followed by the establishment of a unique state of post-mitotic growth arrest, referred to as $G_{0}$, which has been reported to be permissive for subsequent differentiation (17). With the onset of differentiation, the cells enter a third state of growth arrest that is irreversible, where they are then considered to be terminally differentiated. Although numerous reports have recently emerged concerning the transcriptional regulation of adipocyte specific gene expression, little is known concerning the molecular events involved in the progression of clonal expansion and the establishment of distinct states of growth arrest that mark the progression toward terminal differentiation.

In this investigation, we demonstrate that induction of differentiation of 3T3-L1 preadipocytes results in gene expression representing classic cell cycle progression that switches to adipogenic gene expression concomitant with exit from the cell cycle. In addition, the data presented here document a cascade expression of members of two independent families of CKIs that define distinct states of growth arrest associated with adipogenesis. Moreover, the expression of p18 and p21 is shown to be regulated during the conversion of non-precursor fibroblasts into adipocytes by ectopic expression of the adipogenic transcription factor, PPARγ, providing a molecular mechanism coupling growth arrest and adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Stable Cell Lines**—The NIH-3T3 cell line ectopically expressing C/EBPβ and C/EBPδ under control of a tetracycline operator was created and described previously (18). Stable cell lines expressing PPARγ were derived by retroviral infection as described previously (19). Briefly, packaging cells (BOSC23) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). At approximately 80% confluence, the cells were transiently transfected with pBabe-derived PPARγ expression vector by calcium phosphate coprecipitation with chloroquine as described (20). Viral supernatants were collected 48 h after transfection, filtered, and applied with 4 μg/ml hexadimethrine bromide to proliferating Swiss and Balb/c fibroblasts for 20 min. Medium was then changed to DMEM containing 10% calf serum. After 48 h, cells were passaged if necessary and exposed to 2 μg/ml puromycin for selection. Resistant cells were propagated in puromycin until experimentation.

**Cell Culture and Induction of Differentiation**—Murine 3T3-L1 preadipocytes and fibroblast cell lines ectopically expressing adipogenic transcription factors were induced to differentiate into adipocytes as described previously (21). Briefly, cells were propagated in DMEM containing 10% calf serum (growth medium). At 2 days postconfluence, the medium was changed to DMEM containing 10% FBS supplemented with 0.5 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI; differentiation medium). After 48 h, cells were maintained in DMEM containing 10% FBS and 0.4 μM insulin throughout the remaining time course of experimentation. Maintenance medium was changed every 48 h until the cells were utilized for experimentation. Throughout the study, “time 0” refers to postconfluent cells immediately before chemical induction of differentiation with the addition of MDI to the culture medium. The term “post-MDI” refers to the time elapsed since the addition of MDI to the culture medium.

**RNA Analysis**—Total RNA was extracted from fibroblast cell lines with Trizol (Life Technologies, Inc.) according to manufacturer’s instructions with modifications. Briefly, cultured cells were washed in ice-cold phosphate-buffered saline, lysed with Trizol reagent, passed through a 21-gauge needle, and gently mixed (5:1) with chloroform. Following centrifugation, the aqueous phase was mixed with an equal volume of isopropyl alcohol and centrifuged. The resulting pellet was dissolved in RNAase-free water, mixed with an equal volume of chloroform/butanol (4:1), vortexed vigorously for 15 s, and centrifuged. The aqueous phase was collected and RNA precipitated with sodium acetate/ethanol. Following quantitation, 20 μg of total RNA was denatured in formamide and electrophoresed through formaldehyde/agarose gels. The RNA was blotted to Hybond-N nylon (Amersham Pharmacia Bio-tech), cross-linked, hybridized, and washed. Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (New England Biolabs Inc., Beverly, MA) and [α-32P]dCTP (NEN Life Science Products). Hybridization to the ribosomal 18 S subunit was used to quantitate equal loading.

**Protein Analysis**—Preparation and fractionation of isolated adipocytes from rat fat pads was performed as described previously (22). Cultured cells were washed with phosphate-buffered saline, lysed in Tris/SDS buffer containing Nonidet P-40 and protease inhibitors, vortexed, and centrifuged. Protein content of the supernatant was determined using a BCA kit (Pierce) according to manufacturer’s instructions. Following quantitation, proteins were separated by electrophoresis through SDS-polyacrylamide gels and transferred to polyvinyldene difluoride membrane (Bio-Rad). Following transfer, membranes were blocked with milk (5%) with the following antibodies: rat anti-adenyl cyclase (Cell Signaling Technology); p53 and cyclin D1 (Transduction Laboratories); p18, C/EBPα, and PPARγ (Santa Cruz); and Glut4 (23). Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce) according to manufacturer’s instructions.

**RESULTS**

Molecular Events Demonstrating a Switch between Adipocyte Growth and Differentiation—Cultured 3T3-L1 preadipocytes induced to differentiate are documented to undergo an early phase of clonal expansion, which precedes the acquisition of a fat-laden phenotype. Heretofore, few investigations have explored potential molecular mechanisms that may play a role in exit from clonal expansion and/or maintenance of growth arrest associated with terminal differentiation. To assess the switch between growth and differentiation, we initially characterized changes in gene expression during cell cycle progression that follows the induction of differentiation. Cells were cultured to 2 days post-confluence and induced to differentiate as described under “Experimental Procedures.” Total RNA was collected every 2 h for 30 h following a change from growth to differentiation medium and subjected to Northern analysis. As shown in Fig. 1A, 2-day post-confluent cells not exposed to chemical inducers (0 h) had entered a state of density-induced growth arrest, as indicated by the comparison of histone and cyclin gene expression to subconfluent, proliferating preadipocytes (PPA). Switching to differentiation medium consisting of DMEM supplemented with 10% FBS and MDI resulted in cell cycle progression with sequential activation of ornithine decarboxylase (early G1), cyclin D1 (mid G1), cyclin E (late G1), cyclin A (late G2/S), histone H2B (S phase), and cyclin B (G2/M) gene expression. The rapidity of early gene activation and the peak of histone expression, estimated at 18–20 h, suggests that reentry of these density-arrested preadipocytes into the cell cycle occurred immediately following the change to differentiation medium. The kinetics of cyclin gene expression presented here are consistent with reported changes in Rb phosphorylation (24) and E2F-binding complexes (25) determined for differentiating 3T3-L1 preadipocytes. Based on the additional observation of immediate early (c-Myc), delayed early (ornithine decarboxylase), and S-phase (histone H2B) gene activation reported here and elsewhere (26, 27), it appears that chemical induction of differentiation of these preadipocytes resulted in synchronous activation of cell cycle gene expression that begins in the very early phases of G1, possibly G0, and continues through to cell division.

Fig. 1A also compares the kinetics of clonal expansion with adipogenic transcription factor gene expression. Chemical induction of differentiation resulted in immediate activation of C/EBPβ and C/EBPδ, a process that has been shown to occur independent of protein synthesis and afforded by isobutylmethylxanthine and dexamethasone, respectively (28). Although it is still uncertain what role, if any, that C/EBPβ and C/EBPδ may play in the activation of post-confluent cell cycle progression, it is clear that cell proliferation kinetically preceded the expression of C/EBPα and PPARγ. The virtual absence of cyclin
and histone gene expression during late stages of differentiation (6 day; Fig. 1A) indicates that preadipocytes ceased proliferating at some point during the process of adipogenesis. To assess when the switch between growth and differentiation occurred, RNA was collected every 24 h for 9 days following the change to differentiation medium and subjected to Northern analysis. Data presented in Fig. 1B clearly illustrate the mutually exclusive nature of growth and differentiation with a clear switch in gene expression associated with these independent processes occurring approximately 3 days following induction of differentiation. Of particular interest, the onset of C/EBPα and PPARγ coincided with the switch in gene expression, suggesting that these transcription factors may play a role in coupling growth arrest and adipocyte differentiation. It is also important to note that the same chemical agents responsible for the induction of differentiation were also responsible for activation of clonal expansion. Thus, the decision to switch between growth and differentiation pathways, although continually in the presence of abundant mitogens, is made at the cellular level and not by the investigator. This is in contrast to other differentiating systems (e.g. skeletal muscle), where induction of differentiation typically requires technical manipulations necessary to ensure a prerequisite state of growth arrest. Considering this and the synchrony of clonal expansion, we propose, as have others (29), that differentiation of preadipocyte cell lines provides an excellent model for mechanistic studies concerning the coupling of growth arrest and cell differentiation.

A Cascade Expression of Cyclin-dependent Kinase Inhibitors That Define Distinct States of Growth Arrest during Adipocyte Differentiation—It is well accepted that cell cycle progression is controlled by cyclin/cdk protein kinases where phosphorylating activity can be modulated by functionally and structurally distinct CKIs. To assess the involvement of CKIs in coupling of growth arrest and adipocyte differentiation, the gene expression of the seven known members of the INK4 and CIP/KIP families of CKIs was examined by Northern analysis, where it was determined that terminal differentiation was marked only by elevated levels p18 and p21 mRNA (data not shown). Based on this screen, p18 and p21 were further examined at the level of protein expression during the time course that entailed the switch between growth and differentiation. Albeit modestly regulated at the level of gene expression, p27 was also examined due to numerous reports linking this CKI to density arrest and the well documented post-transcriptional regulation of its protein expression (30, 31). To carefully evaluate the kinetics of CKI expression during exit from clonal expansion and the onset of irreversible growth arrest, whole cell lysate proteins were harvested following a change to differentiation medium and subjected to Western analysis during two independent time courses. The first examined protein expression every 24 h for 6 days (Fig. 2A) and the second every 6 h during the first 48 h and every 12 h thereafter for 96 h (Fig. 2B). For comparison, total RNA was isolated from the same experiment depicted in Fig.
cycle as indicated by a significant decrease in the number of cells entering S phase (i.e. decreased histone expression).

Interestingly, the protein expression of proliferating cell nuclear antigen, which began to accumulate during early G1, was maintained during this period, suggesting that the cells had entered a transient state of growth arrest that was unique from that observed during density arrest (day 0) or terminal differentiation (day 6). The expression of p18 appeared to kinetically succeed exit from the cell cycle and the early onset of adipocyte gene expression with significant protein levels accumulating only during later stages of terminal differentiation. Unlike p21 and p27, p18 protein was not significantly expressed in proliferating or density-arrested preadipocytes.

Numerous reports have indicated that CKI expression, in particular p21, can be directly modulated by various mitogens and hormonal agents. To confirm that the cascade regulation of CKI expression was linked to molecular processes of differentiation and not simply due to exposure to the chemical inducers, density-arrested preadipocytes were chemically induced to differentiate in the presence and absence of tumor necrosis factor α (TNF-α). This cytokine has been shown to completely block the development of the fat-laden phenotype and associated gene expression when applied to cells during the induction of preadipocyte differentiation. Whole cell lysates were harvested on days 3 and 6 following chemical induction in the presence and absence of TNF-α and subjected to Western analysis for CKI expression. Protein expression of Glut4 was also examined to confirm the state of adipocyte differentiation. As shown in Fig. 3A, the increase in p21 and p18 protein expression, observed on days 3 and 6, respectively, was prevented in the presence of TNF-α suggesting that the expression of these CKIs was dependent upon adipocyte differentiation and not due to secondary effects of the mitogen and hormonal agents necessary to induce differentiation. This conclusion was further supported by the lack of effect of TNF-α on p27 protein, suggesting that the inhibitory effect on differentiation was not due to toxicity of this potent cytokine.

A Role for Adipogenic Transcription Factors in Regulating CKI Expression—Considerable evidence implicates C/EBPs and PPARγ as major transcription factors responsible for development of the mature adipocyte. The next objective was to determine if these adipogenic transcription factors play a role in regulating the cascade expression of CKIs during adipogenesis. Initially, the mRNA accumulation of p18, p21, and p27 was kinetically compared with the expression of C/EBPα and PPARγ over the time course of 3T3-L1 preadipocyte differentiation. Total RNA was harvested every 24 h for 6 days following chemical induction and subjected to Northern analysis. As shown in Fig. 4, the mRNA expression of p27, highest in density-arrested preadipocytes (0 day), declined following chemical
induction and remained at low levels throughout the course of differentiation. Although the precipitous decline in protein that immediately precedes S phase of clonal expansion was accompanied by a moderate fall in mRNA accumulation (compare Figs. 2 and 4), the return of p27 protein to predifferentiation values equivalent to those observed in fully differentiated 3T3-L1 adipocytes. Interestingly, CKI mRNA accumulation occurred in the absence of C/EBPα, which has been shown to be repressed in NIH3T3 fibroblasts (9, 10). It is important to note, however, that the role of C/EBPα as an adipogenic transcription factor may, in part, be played by the ectopic expression of C/EBPβ and/or C/EBPδ. To continue to dissect the molecular mechanism responsible for CKI mRNA accumulation, these engineered fibroblasts were cultured under various diagnostic conditions to alter gene expression and exposure to chemical inducers and TZDs. Total RNA was harvested following 6 days of conditional treatment and the results of Northern analysis depicted in Fig. 5B. Exposing the NIH-3T3 fibroblasts, not expressing ectopic C/EBPβ and C/EBPδ (i.e. in the presence of tetracycline), to chemical inducers and TZD (lane 1) resulted in a modest increase in p21 mRNA that was not observed in cells ectopically expressing the C/EBPs in the absence of differentiation mixture (lane 2). Although chemical induction of cells expressing the C/EBPs resulted in PPARγ and adipocyte-specific (e.g. adipinsin)

**Coupling Growth Arrest and Adipocyte Differentiation**

![Fig. 3. Dependence of p18, p21, and p27 protein expression on adipocyte differentiation. A, 3T3-L1 preadipocytes were differentiated as described under “Experimental Procedures” in the presence and absence 500 μM TNPα. Whole cell lysates were collected at 3 and 6 days following chemical induction of differentiation. One hundred μg of protein was examined by Western analysis. B, protein from cytosolic (C), nuclear (N), and whole cell lysates (W) was prepared from differentiated adipocytes isolated from epididymal fat pads collected from 200-g male Sprague-Dawley rats as described under “Experimental Procedures.” Whole cell lysates were also isolated from 3T3-L1 cultured adipocytes at 0, 3, and 6 days of differentiation. Fifty μg of protein was examined by Western analysis. The 42-kDa band of C/EBPα was illustrated.**

![Fig. 4. Correlation of p18, p21, and p27 mRNA accumulation with the expression of adipogenic transcription factors during 3T3-L1 preadipocyte differentiation. Cultured preadipocytes were differentiated for as described under “Experimental Procedures.” Total RNA was collected every 24 h for 6 days following chemical induction of differentiation, and 20 μg of RNA was examined by Northern analysis. The 1.2- and 2.4-kb transcripts of p18 were illustrated.](image)
gene expression (lane 3), significant p18 and p21 mRNA accumulation and acquisition of the adipocyte phenotype was observed only when cells where cultured under conditions leading to PPARγ gene expression in the presence of an exogenously supplied ligand specific for PPARγ activation (lane 4).

As accumulation of both p18 and p21 mRNAs correlated with ligand-activated PPARγ and not C/EBPβ expression, we continued to the explore the relationship between this adipogenic transcription factor and CKI gene expression by utilizing a retroviral system to produce fibroblastic cell lines ectopically expressing PPARγ and C/EBPβ in a tetracycline-repressive manner (βδ cells) were differentiated as described under “Experimental Procedures.” Total RNA was collected at 0 and 6 days following chemical induction of differentiation, and 20 μg of RNA was examined by Northern analysis. The 1.2-kb transcript of p18 was illustrated. For comparison, RNA from 3T3-L1 adipocytes (L1) differentiated for 6 days was included. β cells were differentiated for 6 days under various conditions depicted below the illustrated data. The absence of ectopic C/EBPβ and C/EBPδ expression (βδ) was accomplished by supplement of tetracycline (1 μg/mL) to the differentiation medium. Cells expressing βδ only were cultured for an identical length of time as other conditions but in the absence of chemical inducers (MDI) and T2D supplement (citrilazine; 10 μM). Twenty μg of total RNA was examined by Northern analysis.

![Diagram](https://via.placeholder.com/150)

**Fig. 5.** Inducible ectopic expression of C/EBPβ and C/EBPδ in NIH-3T3 fibroblasts induces adipogenesis and accumulation of p18 and p21 mRNA in a PPARγ ligand-dependent manner. A, NIH-3T3 fibroblasts, ectopically expressing C/EBPβ and C/EBPδ in a tetracycline-repressive manner (βδ cells) were differentiated as described under “Experimental Procedures.” Total RNA was collected at 0 and 6 days following chemical induction of differentiation, and 20 μg of RNA was examined by Northern analysis. The 1.2-kb transcript of p18 was illustrated. For comparison, RNA from 3T3-L1 adipocytes (L1) differentiated for 6 days was included. B, βδ cells were differentiated for 6 days under various conditions depicted below the illustrated data. The absence of ectopic C/EBPβ and C/EBPδ expression (βδ) was accomplished by supplement of tetracycline (1 μg/mL) to the differentiation medium. Cells expressing βδ only were cultured for an identical length of time as other conditions but in the absence of chemical inducers (MDI) and T2D supplement (citrilazine; 10 μM). Twenty μg of total RNA was examined by Northern analysis.
functions of these CKIs during adipogenesis has yet to be
determined, it would not be unexpected to find that one of their
roles is to inhibit cell proliferation prior to S phase transition
during the onset of terminal differentiation.

DISCUSSION

This investigation presents a molecular mechanism coupling
growth arrest and adipocyte differentiation. First, we demon-
strate a clear switch in gene expression mediating the proc-
esses of growth and differentiation and that growth arrest
following clonal expansion correlates closely with the expres-
sion of adipogenic transcription factors, C/EBPα and PPARγ.
Second, data are presented documenting a cascade of CKI
expression that mark distinct states of growth arrest associ-
ated with adipogenesis. Third, the differentiation-dependent
up-regulation of p18 and p21 is regulated at the level of mRNA
and protein expression when non-precursor fibroblasts are con-
verted to adipocytes by the expression of PPARγ in a ligand-
associated fashion. Collectively, these data demonstrate that
transcription factors that mediate adipogenesis also regulate
the expression of cell cycle inhibitors providing a molecular
mechanism coupling these processes during exit from the cell
cycle and ensuring the irreversible growth arrest of terminal
differentiation.

Data presented in this investigation confirm and extend in

FIG. 6. Retroviral expression of PPARγ in Swiss and Balb/c fibroblasts results in conversion to adipocytes and a coordinate regulation of p18 and p21 at the level of mRNA and protein expression. A, retroviral infection was used to generate stable cell lines with ectopic expression of PPARγ2 in Swiss (SPγ) and Balb/c (BPγ) fibroblasts as described under “Experimental Procedures.” Following puromycin selection, cells were differentiated in the presence of troglitazone (10 μM). Total RNA was examined by Northern analysis. The 1.2-kb transcript of p18 was illustrated. For comparison, RNA from 3T3-L1 adipocytes (L1) differentiated for 6 days was included. B, Swiss and Balb/c fibroblasts ectopically expressing PPARγ (Pγ) or empty vector (V) were differentiated in the presence or absence of troglitazone (10 μM). Total RNA was collected following 6 days of differentiation and 20 μg of RNA was examined by Northern analysis. For comparison, RNA from 3T3-L1 adipocytes (L1) differentiated for 6 days was included. C, Balb/c fibroblasts ectopically expressing PPARγ (Pγ) or empty vector (V) were differentiated in the presence and absence of troglitazone (10 μM), respectively. Whole cell lysates were collected following 6 days of differentiation and 100 μg of protein was examined by Western analysis. Protein from differentiated 3T3-L1 adipocytes was included for comparison.
greater detail a recent report (33) documenting a cascade expression of members of two independent families of CKIs during the course of adipogenesis. As summarized in Fig. 7A, the protein expression of p27, p21, and p18 defines three unique states of growth arrest associated with saturation density, post-mitotic growth arrest, and the onset of terminal differentiation, respectively. The complexity and timing of expression suggest that multiple CKIs may play specific and diverse roles in coupling growth arrest and cell differentiation. It is interesting to note the synchrony of the inverse relationship between p27 protein and histone gene expression during clonal expansion. Other reports have indicated that p27 protein accumulates under quiescent conditions of serum deprivation and density arrest, decays rapidly with the onset of cell cycle progression, remains low during subsequent cell cycles and returns to high levels concomitant with the onset of growth arrest (35). The high levels of p27 immediately juxtaposed to S-phase presented in this investigation attest to a potential function of this cell cycle inhibitor and to the synchrony of entry into and exit from the cell cycle associated with clonal expansion. Although the increase in p27 following clonal expansion is consistent with establishment of a new saturation density, the combined expression of p27 with p21 during exit from the cell cycle and p27 with p18 during terminal differentiation may represent a synergistic role for multiple CKIs during distinct states of growth arrest. In this regard, the expression of p21 and p27 simultaneously may attribute to the synchrony and rapidity of growth arrest following clonal expansion. The possibility also exists that combined up-regulation of p21 and p27 following clonal expansion may play a permissive and/or regulatory role for subsequent adipocyte differentiation. In support of this notion, the highest degree of myelomonocytic cell differentiation has been shown to occur independent of chemical inducers when p21 and p27 were ectopically expressed together, suggesting that multiple CKI expression may be required for complete cell differentiation (36). Moreover, the observation that ectopic expression of p21 or p27, but not of p16, leads to megakaryocytic differentiation suggests the possibility of CKI specificity in regulating cell differentiation independent of growth arrest (37). Therefore, the timing and overlapping nature of CKI expression during adipogenesis may impart synergistic and specific functions specific to distinct states of growth arrest and different stages of adipocyte differentiation.

Although absent in fully differentiated adipocytes in vitro and in vivo, it should be emphasized that the expression of p21 dramatically increases twice during the course of cultured adipocyte differentiation. The initial up-regulation of p21, which coincides with the G1 phase of clonal expansion, is consistent with reported regulation and function of p21 during early phases of cell cycle progression (38). In contrast, the subsequent up-regulation of p21 correlates directly with “exit” from clonal expansion and is dependent on the differentiation program for expression. Thus, it appears that one mixture of mitogen and hormonal agents utilized in the induction of differentiation activates two kinetically independent peaks of p21 protein expression, suggesting the possibility of two independent regulatory mechanisms based on the progression of proliferation versus differentiation. The decay in p21 in the presence of other CKIs during advanced stages of adipogenesis is consistent with other reports indicating a transient expression of p21 during myocyte differentiation in vitro (39) and during cardiac development in vivo (40). The association of p18, p21, and p27 with other proteins mediating both growth and differentiation is currently being investigated to ascribe specific functions of these CKIs during adipogenesis.

This investigation also presents data, generated from three fibroblastic cell lines regulating the expression of PPARγ by two independent mechanisms and supplement with two pharmacologically different ligands for PPARγ, that provide direct evidence for a role of adipogenic transcription factors in regulating CKIs at the level of mRNA and protein expression. As summarized in Fig. 7B, cell lines that were PPARγ ligand-dependent for adipogenic gene expression were also ligand-dependent for regulation of p18 and p21, suggesting a regulatory role for PPARγ at some point upstream during the course of adipogenesis. The proximity of PPARγ in the differentiation paradigm to the regulation of p18 and p21 has yet to be determined. It is predicted, however, that regulation of p18 during adipogenesis occurs primarily at the level of gene expression, as changes in protein expression correlated with equivalent changes in mRNA accumulation. In preliminary experiments, inhibition of protein synthesis by cycloheximide in cells expressing PPARγ prevented p18 mRNA accumulation (data not shown), suggesting that other proteins downstream of PPARγ are likely to be involved in mediating p18 gene expression.

The notion of an intermediate transcription factor is consistent with the observed delay between the expression of PPARγ and p18. Preliminary cycloheximide studies with p21 were not interpretable, inasmuch as inhibition of protein synthesis, independent of PPARγ expression, led to a dramatic increase in p21 mRNA accumulation. However, based on a potential conserved consensus sequence in the promoter of p21 and the coordinate kinetics of p21 and PPARγ expression, it is possible that this CKI is directly regulated at the level of gene expression by PPARγ. Regulation of p21 protein expression during adipogenesis, however, is likely to be complex with changes in the magnitude and kinetics of protein expression occurring without coordinate changes in mRNA accumulation.

Although the data presented here demonstrate an upstream regulatory role for PPARγ in the regulation of p21 during adipocyte differentiation, it is important to note that p21 protein expression presented in this investigation correlated closely with the expression of C/EBPα. Interestingly, a role for C/EBPα has recently been shown in the regulation of p21 in hepatocytes and fibrosarcoma cells at the level of protein stability (41, 42). Thus, the possibility exists that post-transcriptional regulation of p21 protein may, in fact, be regulated by C/EBPα during adipogenesis. The observation that ectopic expression of PPARγ also results in the expression of C/EBPα shown here and elsewhere (24) suggests the possibility of a cascade effect of these transcription factors in the regulation of p21 at the level of gene expression and protein stability, respectively. As numerous reports have emerged demonstrating a synergy of these transcription factors in the regulation of many aspects of the mature adipocyte, it would not be surprising to find that the complex regulation of CKI expression during adipogenesis also involves the combined efforts of both PPARγ and C/EBPα. Experiments addressing the direct and indirect mechanisms of CKI expression by these and other transcription factors during adipogenesis are currently under investigation.

Others have reported that both C/EBPα (43) and PPARγ (44), when ectopically expressed, suppress the growth of various subconfluent, proliferating fibroblastic cell lines. Although the data presented here suggest that growth arrest is coupled to adipocyte differentiation through the expression of CKIs, it should be noted that other growth arrest mechanisms independent of these cell cycle inhibitors may also be imparted by adipogenic transcription factors under conditions that may not support adipogenesis. For example, it has been reported that C/EBPα regulates the growth-arrest-associated gene, gadd45 (45), and that PPARγ can induce cell cycle withdrawal by
inhibition of E2F binding activity via down-regulation of the protein phosphatase, PP2A (44). Thus, it is likely that various mediators of adipocyte gene expression may regulate independent and/or synergistic growth arrest mechanisms as a process to ensure terminal differentiation. Determining the function of multiple CKIs during adipogenesis and deciphering the complex interactions of numerous adipogenic transcription factors in regulating their expression will provide a better understanding of the physiological control of adipocyte proliferation through coupling of growth arrest and cell differentiation.

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