Regulation and Expression of Retinoblastoma Proteins \(p107\) and \(p130\) during 3T3-L1 Adipocyte Differentiation*

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During 3T3-L1 adipocyte differentiation, growth-arrested, postconfluent preadipocytes are required to re-enter the cell cycle and proceed through a mitotic clonal expansion phase prior to terminal differentiation. The retinoblastoma proteins (pRB, \(p107\), and \(p130\)) are thought to be critical in controlling cell cycle progression by binding to and regulating the activity of the E2F transcription factors. We show here that \(p130/p107\) protein levels, \(p107\) mRNA levels, and E2F DNA binding complexes are regulated during 3T3-L1 adipogenesis. The predominant E2F binding complex in day 0 preadipocytes was \(p130-E2F\) with no detectable free E2F or \(p107\). On Day 1, during mitotic clonal expansion, there was a distinct switch to free E2F and \(p107-E2F\) complexes associated with increased \(p107\) mRNA and protein along with decreased \(p130\) protein levels. Following differentiation, the day 0 pattern is reestablished. The switch is not just a consequence of reentry into the cell cycle, in that \(p107\) protein levels are both detectable and unchanged in dividing, serum-restricted, or serum-reconstituted preconfluent cells. Interestingly, hormonal stimulation of 3T3-C2 cells, a related nondifferentiating cell line, also induces a mitotic clonal expansion phase that is associated with the \(p130/p107\) switch in a pattern very similar to 3T3-L1 cells, suggesting the block in differentiation observed in 3T3-C2 cells occurs after clonal expansion. Combined, these findings suggest that the regulatory mechanisms of the \(p130/p107\) switch are not specific to differentiation but may play a key role in regulating the mitotic clonal expansion necessary for adipocyte differentiation in 3T3-L1 cells.

Obesity is a major health problem in the United States and is a risk factor for many serious illnesses such as heart disease, arthritis, diabetes, and others. To better understand obesity, we need to have a solid underlying knowledge of the molecular events that trigger the differentiation of adipocytes, the major cellular component of adipose tissue. It is now recognized that animals (including humans) are capable of increasing adipocyte number (hyperplastic development) and that within the adipose tissue there is present throughout life a population of preadipocytes capable of replicative growth as well as differentiation into mature adipocytes (1–3). Fibroblast-like \textit{in vitro} cell lines such as the closely related 3T3-L1 and the F442A lines are well characterized preadipocyte models capable of differentiating into mature adipocytes within 4 or 5 days following appropriate hormonal stimulation (reviewed in Ref. 4). Upon reaching confluence, these cells become contact-inhibited and growth arrest at the G/S boundary, and they begin to express some of the early markers of adipocyte differentiation such as lipoprotein lipase and fatty acid-activated receptor (5). Following hormonal induction of differentiation with IDX\(^1\) (insulin, dexamethasone, and isobutylmethylxanthine), these growth-arrested confluent cells proceed through the G/S boundary, reentering the cell cycle, and undergo several rounds of mitotic clonal expansion that is essential to completing terminal differentiation into mature adipocytes (4). Because reentry into the cell cycle is required for preadipocyte differentiation, we were interested in beginning to identify the cellular mechanisms that trigger this event.

The E2F family of transcription factors are DNA-binding proteins that have been shown to be important in regulating the transcription of many genes associated with controlling cell cycle and differentiation (6, 7). E2F transcription factors bind as a heterodimeric complex, consisting of one of the E2F proteins (E2F1–5) and a DP protein (either DP1 or DP2; Ref. 8). Transcriptional activity regulated by E2F is thought to occur in a cell cycle-dependent manner through interactions with other cellular proteins, including products of the retinoblastoma gene family. Through interactions with the E2F family of transcription factors, the product of the retinoblastoma gene (pRB; Refs. 9 and 10) and related members p107 (11, 12) and p130 (13, 14) have been shown to play a critical role in regulating cell cycle progression. During the G/S phase of the cell cycle, E2F forms a complex with underphosphorylated pRB (pRB-E2F) and serves to inactive E2F-mediated transcription (15, 16). Similarly, p107 binds E2F-4, forming a p107-E2F complex during S phase, and analogous to pRB-E2F, it also inhibits E2F-mediated transactivation (17, 18). The major G/S\(\rightarrow\)G\(_1\) E2F-binding partner is p130 and interacts with E2F-5 in murine fibroblasts (19). To add to the complexity, these complexes are frequently associated with other cell cycle-specific proteins such as the association of p107-E2F with cyclin A and Cdk2 (20) and the association of p130-E2F with cyclin E and Cdk2 in late G\(_1\) (19). Combined, the diversity of potential interactions between these
protein families allow for a high degree of control in regulating cell cycle progression.

We were interested in determining whether members of the retinoblastoma gene family were regulated during the mitotic clonal expansion stage that occurs early in the differentiation of 3T3-L1 preadipocytes into adipocytes. We describe here that the predominant E2F binding complex is p130-E2F in confluent, growth-arrested, day 0 preadipocytes. However, on day 1 of differentiation, when the cells are stimulated to reenter the cell cycle, there is a distinct switch in the major E2F binding complex from p130-E2F to p107-E2F. This switch is associated with a rapid induction in p107 mRNA and protein levels accompanied by a fall in p130 protein levels. Furthermore, by days 3 and 4 of differentiation, as cells withdraw from the cell cycle, there is a reversion back to predominantly p130-E2F complexes. To determine if the switch was specific to the differentiation process, a similar analysis was performed on 3T3-C2 cells, a cell line closely related to 3T3-L1 cells well characterized as not undergoing adipocyte differentiation following hormonal stimulation (21). Following hormonal induction, 3T3-C2 cells also undergo mitotic clonal expansion, and analogous to 3T3-L1 cells, the switch in E2F binding proteins from p130 to p107 also occurs. We also present evidence that the mechanism of regulation of these proteins is not just a result of reentry into the cell cycle. The E2F binding complexes observed following serum stimulation in growth-arrested preconfluent preadipocytes are different than those observed after stimulation of differentiation. Combined, these findings suggest that cell cycle is differentially regulated between preconfluent proliferation and postconfluent, hormonally stimulated clonal expansion. These results also suggest that the stage at which differentiation is blocked in 3T3-C2 cells occurs after clonal expansion. Furthermore, these data suggest that the p107:p130 switch is not differentiation-specific but is specific to the clonal expansion phase and may play a key role in adipocyte development by regulating the mitotic clonal expansion necessary for adipocyte differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture, Adipocyte Differentiation, and 5-bromo-2′-deoxyuridine (BrdUrd) Immunocytochemistry—3T3-L1 cells (American Type Culture Collection, Rockville, MD) or nontumorigenic 3T3-C2 cells (generous gift of Dr. Howard Green) were grown to confluency in standard growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (each from Life Technologies, Inc.) at 37 °C in a 5% CO2 atmosphere and hormonally stimulated (which induces adipocyte differentiation in 3T3-L1 cells) by treatment with IDX (1.7 μM insulin, 0.5 μM dexamethasone, and 0.5 mM isobutylmethylxanthine; each ingredient Sigma) as described previously (22). Briefly, confluent cells (day 0) were treated with standard growth medium described above supplemented with IDX for 3 days. After 3 days, this medium is replaced by replacement medium supplemented with 1.7 μM insulin only. Typically, by day 4 >95% had differentiated into adipocytes as determined by lipid accumulation with oil red O staining and induction of adipocyte-specific mRNAs such as aP2. Immunocytochemistry was performed with the Cell Proliferation kit (Amersham Corp.) as described by the manufacturer. Serum restriction experiments were performed on 50% preconfluent cultures with treatment by DME supplemented with 1% FBS for 24 h. Serum stimulation experiments were performed on 24-h serum-restricted cultures by replacement of restriction medium with standard growth medium (DMEM, 10% FBS).

RNA Isolation and Northern Blot Analysis—20 μg of total cellular RNA was isolated from cells at each of the respective days of differentiation, electrophoresed on 1% agarose, 6% formaldehyde gels, and then transferred to 0.45 μm nitrocellulose membranes (Micron Separations, Inc., Westboro, MA). Following transfer, filters were hybridized at 48 °C to 32P-labeled cDNA probes in 25 mM NaH2PO4 (pH 7.4), 0.1% sodium dodecyl sulfate, 1 mM EDTA, 0.25 M NaCl, 50 μg/ml wheat germ tRNA, 50 μg/ml polyadenylic acid, 100 μg/ml salmon sperm DNA, and 50% formamide as described previously (22). Filters were washed at 65 °C in 0.1 × SSC (1 × SSC: 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0), and exposed at −70 °C for 24–72 h to Kodak X-OMat AR film (Eastman Kodak Co.). Probes utilized were cDNAs encoding the complete coding region for pRB, p107 (generously provided by Drs. M.-H. Lee and E. Harlow), p130 (generously provided by Drs. A. Baldi and A. Giordano), and the adipocyte-specific cDNA encoding aP2. cDNA probes were labeled by the random primer method with α-32PdATP.

Nuclear Extracts, Electrophoretic Mobility Shift Assays, and Western Blot Analysis—Nuclear extracts were prepared from differentiating cells at the indicated times as described previously (22) and used for both electrophoretic mobility shift assays (EMSAs) and Western blot analysis. EMSAs were performed as described previously (12). Briefly, 10 μg of nuclear protein extract was mixed with 20,000 cpm 32P-labeled oligonucleotide probe (or along with unlabeled competitor oligonucleotide in competition experiments) and incubated for 10 min at room temperature. Bound complexes were resolved on 6% nondenaturing polyacrylamide gels, dried, and autoradiographed. For EMSA antibody supershift experiments, 1 μl (−0.25 μg) of antibody (described below) was added to the incubation at room temperature 10 min prior to the addition of the oligonucleotide probe. The following double-stranded oligonucleotides (sense strand shown) were used in these experiments: E2F2/E2F2′, 5′-AGCTTGTTTTTGGCTTAAATTTGAGAAAGGGCGCGAGAACATAGCTCA-3′; mutant E2F2/E2F2′, 5′-AGCTTTGGCTTAAATTTGAGAAAGGGCGCGAGAACATAGCTCA-3′ (23). In EMSA competition experiments, a 100-fold excess of the unlabeled competitor oligonucleotide was added 10 min prior to the addition of the probe oligonucleotide probe. Western blot analysis was performed as described previously (23), with a 1:10,000 dilution of the primary antibody, and the secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) also diluted 1:10,000. Immune complexes were detected with the ECL system (Amersham). Antibodies used included anti-pRB antibody (catalog number 14001) obtained from PharMingen; anti-p107 antibody (SD9, catalog number sc-250, for Western blots) and anti-p130 antibody (C20, catalog number sc017, for EMSA) from Santa Cruz Biotechnology, Inc.; and anti-Cdk2 antibody obtained from Upstate Biotechnology, Inc. (catalog number 06-148). The anti-p107 antibody, SD-15, used for EMSA was generously provided by Drs. N. Dyson and E. Harlow. The anti-p130 antibody, Z11 (that also cross-reacts with p107), was used for Western blots and was the generous gift of Dr. P. Whyte. Negative control antibodies used in EMSA included monoclonal antibody 63031 obtained from PharMingen or preimmune rabbit serum, neither of which exhibits supershift activity.

RESULTS

Induction of 3T3-L1 Adipocyte Differentiation Is Associated with Reentry into the Cell Cycle—3T3-L1 cells plated on two-well chamber slides were hormonally stimulated with IDX to undergo adipocyte differentiation. Cell proliferation and DNA synthesis were assessed in these cultures by monitoring incorporation of BrdUrd using immunocytochemistry with a BrdUrd monoclonal antibody. In day 0, unstimulated postconfluent cultures, cells are quiescent, as is evidenced by the small number of BrdUrd-positive cells (Fig. 1A). However, on day 1, −24 h after induction of differentiation with IDX, almost all of the cells were actively proliferating and stained BrdUrd-positive (Fig. 1B). Following the burst in proliferation observed in day 1, the cells begin to withdraw from the cell cycle and by day 3 of differentiation were essentially quiescent again and arrested at G0 (Fig. 1C), a time when late markers of adipocyte differentiation are beginning to be expressed. Similar results were obtained in four separate experiments.

Alterations in E2F DNA Binding Complexes during 3T3-L1 Adipocyte Differentiation—We were interested in examining whether E2F DNA binding complexes were present during differentiation of 3T3-L1 cells and assessing whether changes in these binding complexes were associated with reentry and withdrawal from the cell cycle. Nuclear extracts were prepared from differentiating cells at days 0, 1, 1.5, and 4 and analyzed by gel mobility shift assay using the E2F2/E2F2′ oligonucleotide (Fig. 2). Only one complex was detected in day 0 extracts, and it contained p130 as determined by antibody supershift analysis (Fig. 2A). Complexes associated with p107 were not detected in day 0 extracts. Interestingly, two main complexes were detected on day 1 of differentiation (Fig. 2A). The lower complex
is the free form of E2F, and the upper complex is associated with p107 as determined by antibody supershift analysis. The lower complex was identified as the free form of E2F based on its ability to interact with bacterially expressed GST-pRB that was added to the gel shift reaction (data not shown). The p130 antibody also caused a supershift of the upper complex, and while we cannot rule out that part of the day 1 complex contains p130, it is important to note that the p130 antibody cross-reacts with p107 (24). The likelihood that the p130 supershift is due to a cross-reactivity to p107 is substantiated by results described below demonstrating very little p130 protein is present in day 1 extracts. By day 3, both free E2F and the amount of p107 in the upper complexes had decreased, with two p130-E2F complexes becoming the major E2F binding complexes (Fig. 2B). On day 4, the free E2F was no longer detected, while the p130-E2F complexes remained similar to the complexes detected on day 3 (Fig. 2B). Throughout the time course of differentiation, E2F-pRB binding complexes were undetectable. Similar results were obtained from at least three different sets of extracts. Both p107-E2F and p130-E2F complexes have previously been shown to contain Cdk2 (20). Supershift analysis with a Cdk2 antibody revealed that Cdk2 is absent in the p130-E2F complex observed at day 0; however, Cdk2 is present in the day 1 p107-E2F complex when the cells were actively proliferating (Fig. 2C). In addition, all of the identified complexes were specific for E2F binding in that 100-fold competition with unlabeled wild type oligonucleotide competed all binding, while a mutant oligonucleotide had no effect (Fig. 2C, lanes 6 and 7 and lanes 12 and 13). The complexes located just above the free probe in each of the three panels represent nonspecific binding in that neither wild type nor mutant oligonucleotides exhibit competition (Fig. 2C).

p107, p130, and pRB mRNA and Protein Levels during 3T3-L1 Adipocyte Differentiation—We next determined whether the changes in p107 and p130 associated with E2F could be explained by changes in the level of their expression. Nuclear extracts were prepared from each day of culture during 3T3-L1 differentiation, and the level of p107, p130, and pRB was determined by Western blotting (Fig. 3). In day 0 preadipocytes, p107 protein levels were undetectable (Fig. 3A). Day 1 of differentiation was accompanied by a dramatic increase in p107 protein levels that decreased to 20% of peak values during differentiation days 2–4 (Fig. 3, A and B). In contrast, high levels of expression of two electrophoretically distinct forms of p130 were observed at day 0 that decreased to 20% of peak values by day 1 followed by a variable but significant increase by day 4 (Fig. 3, A and B). The pRB protein was present in the underphosphorylated state on day 0 and remained in that form through day 4 with little to no change in the level of protein (Fig. 3A). Levels of mRNA encoding these proteins were also determined by Northern blot on total cellular RNA isolated from 3T3-L1 cells at each day of differentiation (Fig. 4). In a pattern very similar to the protein, p107 mRNA, detectable at day 0, was induced 12-fold on day 1 followed by a significant decrease back to near day 0 levels on days 2–4 (Fig. 4, A and B). Both the p130 and pRB mRNA were detectable at day 0, but steady-state levels did not appear to be regulated during differentiation (Fig. 4, A and B). As a control for adipocyte differentiation-specific mRNA expression, blots were stripped and reprobed with a cDNA encoding aP2, a 600-base mRNA well characterized by up-regulation during adipocyte differentiation (Ref. 3; Fig. 4). Levels of aP2 mRNA not present in day 0 preadipocytes were detectable on day 2 and was maximally expressed in day 4 adipocytes.

Proliferation, p107/p130 Protein Levels, and E2F Binding Complexes during Hormonal Stimulation of Nondifferentiating 3T3-C2 Cells—To determine if the previous observations were specific to adipocyte differentiation, similar experiments were performed utilizing 3T3-C2 cells. Like the 3T3-L1 cells, the 3T3-C2 cells were also isolated as clonal derivatives from murine 3T3 cells, but they are resistant to hormonally induced adipocyte differentiation (21). To assess hormonally induced clonal expansion, two-well chamber slides containing postconfluent 3T3-C2 cells were stimulated with IDX, and cell proliferation was determined using BrdUrd immunocytochemistry as described earlier. As expected, in day 0 cultures, cells were quiescent as demonstrated by the lack of BrdUrd immunostaining (Fig. 5A). Surprisingly, on day 1, most of the nondifferentiating 3T3-C2 cells stained BrdUrd-positive and thus were actively proliferating (Fig. 5B). By day 3, similar to 3T3-L1 cells, the cells were again quiescent and had withdrawn from the cell cycle (Fig. 5C). To determine if the p130/p107 switch was intact in this cell
line, nuclear or whole cell extracts were prepared from 3T3-C2 cells on days 0, 1, and 3 following IDX treatment. p130 and p107 protein levels examined by Western blot analysis revealed that both the level and expression pattern of these two proteins were similar between the two cell lines in whole cell extracts isolated at each time point (Fig. 6A). The transient decrease in p130 protein levels observed on day 1 does not occur to the same extent in the 3T3-C2 cells, and there appears to be quantitatively more of the upper form of p130 (thought to be hyperphosphorylated (25)) in day 1 extracts (Fig. 6A, top). In addition, the induction on day 1 in p107 expression is essentially indistinguishable between the two cell lines (Fig. 6A, bottom).

EMSA analysis revealed that during clonal expansion in nondifferentiating 3T3-C2 cells, regulation of E2F binding complexes was also very similar to results described earlier for 3T3-L1 cells. Nuclear extracts were prepared from 3T3-C2 cells on days 0, 1, and 3 following IDX treatment. p130 and p107 protein levels examined by Western blot analysis revealed that both the level and expression pattern of these two proteins were similar between the two cell lines in whole cell extracts isolated at each time point (Fig. 6A). The transient decrease in p130 protein levels observed on day 1 does not occur to the same extent in the 3T3-C2 cells, and there appears to be quantitatively more of the upper form of p130 (thought to be hyperphosphorylated (25)) in day 1 extracts (Fig. 6A, top). In addition, the induction on day 1 in p107 expression is essentially indistinguishable between the two cell lines (Fig. 6A, bottom).

EMSA analysis revealed that during clonal expansion in nondifferentiating 3T3-C2 cells, regulation of E2F binding complexes was also very similar to results described earlier for 3T3-L1 cells. Nuclear extracts were prepared from 3T3-C2 cells on days 0, 1, and 3 following IDX treatment and analyzed by EMSA using the E2F/E2 oligonucleotides as a probe (Fig. 6B). On day 0, only one complex is detected, and supershift analysis demonstrates that it is associated with p130. In addition, there is very little free E2F detectable in day 0 extracts. In day 1 extracts, there are two detectable complexes, the lower being the free form of E2F while the upper complex appears to be predominantly associated with p107. By day 3, the day 0 pattern is reestablished with little detectable free E2F and the major E2F binding complex being associated with p130.

Using anti-Cdk2 antibodies, specificity of binding was tested by competition with a 100-fold excess of unlabeled E2F/E2 wild type (wt) or mutant (mut) oligonucleotide as described under "Experimental Procedures." Arrows to the right of each panel indicate specific complexes.
tained for both EMSA and Western blots from three separate sets of extracts.

**p107/p130 Protein Levels and E2F Binding Complexes during Serum Stimulation of 3T3-L1 Preadipocytes**—We next determined if the p130:p107 switch was specific to the clonal expansion phase observed during differentiation in 3T3-L1 cells (or hormonal stimulation in 3T3-C2 cells) or if it occurred solely as a result of quiescent postconfluent cells having been stimulated to reenter the cell cycle. This was tested by causing logarithmically dividing cells to cell cycle arrest by 24-h serum restriction and subsequently allowing cell cycle reentry by serum stimulation. These culture conditions do not induce adipocyte differentiation. Logarithmically dividing preadipocytes (normally cultured in DMEM supplemented with 10% FBS as described under “Experimental Procedures”) were subjected to serum restriction by treatment with medium supplemented with only 1% FBS for 24 h. This treatment was effective at causing cell cycle arrest as determined by cell proliferation analysis using BrdUrd immunocytochemistry (data not shown). Cells were released from cell cycle arrest by treating serum-restricted cultures with standard medium supplemented with 10% FBS. Nuclear extracts were prepared from either proliferating, 24-h serum-restricted, or serum-stimulated cells and analyzed by gel mobility shift assay using the E2F/E2 oligonucleotide and Western blot. The presence of pocket proteins in the complexes was detected by antibody supershift analysis as described earlier. At least two complexes...
were detected in the preconfluent cells, one containing p107 and the other p130 (Fig. 7A, lanes 1–4). Following 24-h serum restriction, the complexes did not appear to change significantly (Fig. 7A, lanes 5–8). In addition, only a small amount of free E2F binding complexes were detectable in either preconfluent or serum-restricted cells. Upon serum stimulation, both p107-E2F and p130-E2F complexes were still evident; however, the p107-E2F complex appeared to be the most predominant (Fig. 7A, lanes 9–12). There was also an increase in the free E2F shift following serum stimulation. The levels of p130 and p107 in the E2F binding complexes were detected by supershift analysis using the indicated control nonimmune or anti-pRB antibodies as described under “Experimental Procedures.” Arrows to the left of each panel indicate specific complexes and free probe. ns, nonspecific.

DISCUSSION

We report here that the burst in cell proliferation that occurs early in 3T3-L1 adipogenesis is associated with a p130:p107 switch in E2F protein binding complexes and, furthermore, that the subsequent cell cycle withdrawal observed later in differentiation is associated with a reversal of this switch. E2F transcription factors are critical in regulating many genes associated with control of cell cycle and differentiation (6), and protein-protein interactions between members of the retinoblastoma and E2F families have been shown to be important in...
regulating E2F activity (26). In quiescent preadipocytes, the switch is off, and there is no free E2F, presumably because it is associated with p130. Upon stimulation of differentiation (day 1), proliferation is switched on, and there is a conversion in E2F binding complexes from p130 to p107 (as well as the recruitment of Cdk2) along with a concomitant increase in free E2F complexes. Later in adipogenesis, as cells withdraw from the cell cycle and begin terminal differentiation, the switch is again inactivated by a reversion back to predominantly p130-E2F with no free form of E2F evident. Western blot analysis revealed that protein levels of p107 correlate well with the gel shift data in that p107 is induced by day 1 of differentiation. p107 protein and mRNA levels also correlate well, and it is likely that p107 is regulated at the level of transcription, although mRNA stability cannot be excluded. In contrast to p107, it appears that alterations in p130 protein are regulated at the post-transcriptional level, since mRNA levels are unchanged during adipogenesis while the level of p130 protein decreases by day 1 and then reaccumulates by day 4. To our knowledge, this report describes for the first time differential regulation of both protein and mRNA levels for p130/p107 in a model of cellular differentiation. In serum-starved A31 fibroblasts, p107 protein levels were shown to be induced at 15 h following serum restimulation (19). By gel shift analysis, a transition in E2F complexes has also been observed during myogenesis, in which the predominant E2F complex in undifferentiated myoblasts is p107-E2F, followed by a transition to p130-E2F in differentiated myotubes (7, 27). Although protein and mRNA levels have not been determined during myogenesis, it is intriguing that in both myogenesis and adipogenesis, participation in E2F binding by p107 and p130 are differentially regulated and that upon terminal differentiation, the final E2F complexes are p130-E2F. Since myocytes and adipocytes are thought to be derived from the same pluripotent stem cells, it is tempting to speculate that differential regulation of p130 and p107 may be involved in regulating the expression of lineage-specific genes.

The p130:p107 switch in E2F complexes correlates with the ability of 3T3-L1 cells to undergo hormonally induced mitotic clonal expansion rather than differentiation. 3T3-C2 cells do not differentiate in response to hormonal stimulation (21); however, we report here that these cells do undergo mitotic clonal expansion. As the 3T3-C2 cells undergo the mitotic clonal expansion, there is an increase in p107 mRNA and protein accompanied by a shift in E2F binding from predominantly p130-E2F complexes on day 0 to p107-E2F complexes on day 1. This result was very analogous to what was observed in the 3T3-L1 cells following induction of differentiation and could be interpreted to indicate that the p130:p107 switch is necessary but insufficient on its own to induce the adipocyte differentiation program. Interestingly, there is a difference in the regulation of p130 protein levels. In the 3T3-L1 cells, there was a significant decrease in p130 protein by day 1. The decrease in p130 protein by day 1 was not as dramatic in the 3T3-C2 cells, and the p130 protein that was detected was predominantly the slower migrating, highly phosphorylated form. The highly phosphorylated form of p130 has been previously demonstrated to correlate with a loss in the ability of p130 to bind E2F (25), and consistent with that observation, we detect predominantly p107-E2F complexes on day 1. Therefore, in part, inactivation of p130 binding to E2F complexes on day 1 in 3T3-C2 cells appears to be associated with both phosphorylation and down-regulation or loss of the protein, while in differentiating 3T3-L1 cells inactivation correlates with only a loss of p130 protein. We do not know whether this difference in p130 regulation is involved in the differentiation phenotype, although it remains a possibility that the highly phosphorylated form of p130 does possess functional significance that is related to the inability to differentiate.

At this point, an interesting question arose regarding whether the p130:p107 switch was the ordinary mechanism utilized by 3T3-L1 cells under any conditions that lead to cell cycle reentry. The serum restriction/stimulation experiments using preconfluent proliferating cells strongly suggest that the switch is specific to the mitotic clonal expansion phase. The p130 half of the switch remains intact, in that there are high levels of the protein in quiescent serum-restricted cells that decrease 4-fold following serum stimulation, very analogous to what occurs with induction of differentiation. However, when compared with differentiation, regulation of the E2F complexes and p107 protein levels in these experiments were significantly different. For example, p107 protein was detected in quiescent serum-restricted cells with no observed increase in p107 protein levels following serum stimulation. This observation was in contrast to results obtained from cells stimulated to undergo adipocyte differentiation, where levels of p107 protein (undetectable in quiescent day 0 confluent preadipocytes) were dramatically up-regulated by day 1 of differentiation. Also in contrast to what was observed during hormonal stimulation in 3T3-L1 and 3T3-C2 cells, both p130-E2F and p107-E2F complexes were present in proliferating, serum-restricted or serum-stimulated cells with an apparent increase in p107-E2F binding complexes following serum stimulation. The discrepancy between increased p107-E2F binding complexes in the absence of increased immunoreactive protein may be due to the altered stoichiometry between p130 and p107, i.e. the increase in p107-E2F binding complexes might strictly be due to the fall in p130 protein levels or possibly even post-translational modifications such as phosphorylation status. Taken together, these results suggest that within the same cell line, there are two different cell cycles: one associated with hormonally stimulated mitotic clonal expansion and another associated with normal subconfluent proliferation.

The role that members of the E2F and RB families serve during adipogenesis is currently unknown. It has been demonstrated previously that the levels of over 100 different proteins are altered during the first few hours of 3T3-L1 adipogenesis (during the mitotic expansion), and by the time terminal differentiation is achieved this number is increased to over 300 (28). Combined with the known importance of the E2F and RB proteins in other systems and the high level of regulation of p130/p107 coupled to the availability of free E2F complexes for transcriptional regulation, it seems probable that they may also exert an important role in the regulation of mitotic clonal expansion associated with adipocyte-specific gene expression and differentiation.

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