Adenovirus-5 E1A Suppresses Differentiation of 3T3 L1 Preadipocytes at Lower Levels Than Required for Induction of Apoptosis

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To investigate the functional relationship between the ability of the adenovirus-5 E1A oncogene product to transform with its ability to block adipocytic differentiation and induce apoptosis, we expressed E1A in the 3T3 L1 preadipocytic cell line. The results demonstrate a dramatic, quantitative reciprocal regulation of differentiation and several transformation-associated properties in response to graded levels of E1A expression, with the suppression of differentiative capacity, focus formation, and anchorage-independent proliferation requiring increasing levels of E1A. Progressively higher E1A levels were accompanied by apoptosis induction. The effect of E1A upon adipocytic differentiation as well as transformation and apoptosis required binding to the retinoblastoma-susceptibility gene product. These data reveal a dissociation between E1A signals leading to transformation, suppression of differentiation and induction of apoptosis, based on levels of expression. © 2005 Wiley-Liss, Inc.

Key words: adipocytes; adenovirus E1A proteins; E2F proteins; Akt-1 protein kinase; cell differentiation

INTRODUCTION

The adenovirus-5 E1 oncogene (E1A) plays an essential role in establishing a productive virus infection in human cells, by triggering entry into S phase to create the appropriate environment for the replication of viral DNA. The same growth stimulatory function of E1A is also essential for the transformation of primary rodent cells. E1A accomplishes cell cycle deregulation by binding to and perturbing the normal function of key negative regulators of cell growth, including the retinoblastoma protein (Rb) and the transcriptional coactivator CBP/p300 [reviewed in 1,2]. Ironically, the same activities of E1A that are required for oncogenic transformation also stimulate programmed cell death (apoptosis). In fact, the induction of apoptosis by E1A nearly abrogates E1A’s oncogenic activity and necessitates that E1A be coexpressed with an inhibitor of apoptosis, such as Ras or E1B for transformation to occur [1].

Proliferation and differentiation are often mutually exclusive processes. A host of experimental data have indicated that the effect of adenovirus E1A upon cell differentiation is complex. E1A can suppress neuron-like differentiation in rat PC12 cells and muscle differentiation in rat and mouse myoblasts [3]. In the latter, E1A represses transcription from muscle-specific genes, perhaps through its interaction with CBP/p300, which is a required coactivator of members of the MyoD family of transcription factors which control muscle differentiation [2]. Regarding epithelial cell differentiation on the other hand, E1A can actually induce an epithelial phenotype into various human carcinoma lines. This constitutes a nearly unprecedented mesenchymal to epithelial conversion, a fact that may be clinically exploited [4,5].

The effect of E1A upon adipocytic differentiation has not been extensively investigated. Early work indicated that adenovirus-12 E1A promotes adipocytic differentiation [6] while later data showed that infection with wild-type (wt) adenovirus-5 blocks terminal differentiation of adipocytes [7]. Since E1A affects transformation, as well as differentiation and apoptosis, in an attempt to resolve this apparent

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controversy, we sought to examine the interrelatedness between these three processes. A cellular system, such as the 3T3 L1 preadipocytes, in which an oncogenic signal can be variably interpreted as differentiative, proliferative or apoptotic has the potential (i.e., possesses the molecular machinery) to respond to all three types of signals, thus offering this opportunity. In addition, since the effect of a variety of oncogenes was previously shown to depend upon their levels of expression [8, 9], we examined the consequences of expression of different levels of E1A upon the phenotype of these cells [9]. The results demonstrate that E1A causes a quantitative reciprocal regulation of differentiation and several transformation-associated properties in response to graded levels of expression. The loss of differentiative capacity and morphologival transformation, anchorage-independent proliferation and apoptosis induction required progressively increasing levels of E1A; while just ~5% of the levels present in the prototype 293 line were sufficient to cause a dramatic reduction in differentiation efficiency, anchorage independent proliferation and apoptosis induction required the highest E1A levels. In addition, our results indicate that binding to and inactivation of Rb is required for the E1A-mediated block of adipocytic differentiation, as well as for induction of transformation and apoptosis.

MATERIALS AND METHODS

Materials

Tissue culture media and sera were from Flow Labs, Carlsbad, CA, Inc. Hygromycin B was from Boehringer Mannheim Biochemicals (Indianapolis, IN), dexamethasone (Dex), insulin, Oil Red-O, hematoxylin and most other chemicals were from Sigma, St. Louis, MI.

Cell Lines and Culture Techniques

3T3 L1 is a preadipocytic cell line which was obtained from American Type culture collection (ATCC) (CCL92.1). Cell cultures were maintained at 37°C in a humidified, 5% CO2 incubator. Standard tissue culture medium consisted of 10% heat-inactivated fetal calf serum (FCS) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM l-glutamine and antibiotics. Medium was changed every 2–3 days unless otherwise specified.

Constitutive Expression of Adenovirus E1A

For the expression of the 243R or 289R adenovirus proteins, sequences coding for these genes were cut out from the rv12S or rv13S vectors [10] and cloned at the BamH1 site of the pBabe-Hygro retroviral vector [11]. Psi-2 packaging cells were transfected with these plasmids, selected for hygromycin resistance and 3T3 L1 preadipocytes infected with the culture supernatants as described [12]. A similar approach was used to express a substitution mutant (glycine (gly124)). This mutant is unable to bind pRb, while binding to p130 is partially impaired and binding to p107 is unaffected, at least in a lytic infection [13] (a gift of Dr. Jon Horowitz, North Carolina State University). Following infection, cells were selected for hygromycin resistance for 3–4 weeks, individual colonies picked, expanded into lines and tested for levels of E1A expression. The E1A genes were also expressed through plasmid transfection, under control of the strong CMV promoter (a gift from Dr. J. DiCaprio, Harvard Medical School). For all lines, a large number of vials were frozen in liquid nitrogen as soon as possible after isolation and cells used for a maximum of six passages after thawing.

E1A protein was quantitated by immunoblotting with the monoclonal anti-E1A antibody M73 (Oncogene Science, San Diego, CA). Proteins were extracted with 1% NP-40 essentially as described [12] and protein concentration carefully determined using the BCA protein assay kit (Sigma). Forty micrograms of lysate were resolved by SDS-PAGE and electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL). The membrane was probed with the monoclonal antibody M73, followed by an alkaline phosphatase-conjugated secondary goat anti-mouse antibody and an enhanced chemiluminescence (ECL) detection system according to the manufacturer’s instructions (PerkinElmer Life Sciences, Boston, MA). Quantitation of all bands corresponding to E1A was achieved by fluorimager analysis using the FluorChem program (AlphaInnotech Corp., San Leandro, CA), with the levels in the highest expressor, L1-e1a-1, taken as 100%. As a control for protein loading, parallel blots were routinely probed for the abundant protein, 90 kDa, heat-shock protein (Hsp90), using a mouse monoclonal antibody (Stressgen, Victoria, BC). To ensure uniformity of expression throughout the culture, fixed cells were also stained with the anti-E1A antibody as described [12].

For Akt analysis, cells were serum-starved for 0, 24, 48, or 72 h. Extracts prepared as above were blotted and probed with an antibody specific for the ser-473 phosphorylated, i.e., activated form of Akt (Biosource, Worcester, MA). Parallel blots were probed for Hsp90.

Induction and Quantitation of Cell Differentiation

Approximately 1000 cells were seeded into each well of a 24-well plate (Corning, Acton, MA) in 10% FCS. A number of sera were tested for their ability to sustain differentiation in the presence of insulin and low background in its absence. At confluence, cultures were treated with differentiation medium containing 10 µg/mL insulin, 0.25 µM Dex, and 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) for 48 h. At this time the medium was changed to DMEM with FCS and insulin only. Lipid droplets appeared approximately 10 days later.
To increase the attachment of transformed cells to the culture plates, cells were plated onto Cell-Tak™, a polyphenolic protein extracted from a marine mussel, according to the manufacturer’s instructions (Collaborative Research, Bedford, MA).

Oil Red-O staining for the assessment of adipose conversion frequency was performed as described [9]. To quantitate the degree of differentiation, levels of RNA for the adipocyte-specific genes adipin and activating enhancer binding protein-2 (aP2) were assessed by Northern blotting of total cellular RNA. RNA was extracted with Trizol (Sigma) and blots probed with complementary RNA to the adipocyte-specific genes, aP2 and adipin [12]. Briefly, a sequence coding for aP2 was cloned in the PstI site of the pBluescript plasmid and in vitro transcription carried out with the T7 RNA polymerase and digoxigenin-coupled UTP following linearization with the HindIII restriction enzyme. Similarly, adipin was cloned in the BamH1 site of pBluescript and transcribed in vitro after cutting with EcoR1. As a control, the probe was stripped and the same membrane reprobed for β2-microglobulin using a similar approach [9]. Following overnight hybridization in DIG Easy Hyb solution at 68°C, bound RNA was detected by probing with an alkaline phosphatase-coupled, anti-digoxigenin antibody and Chemiluminescence reagents according to the manufacturer’s protocol (Roche, Indianapolis, IN, cat. #1585614). Blots were exposed to Kodak Biomax film. Bands were quantitated by fluorimager analysis using the FluorChem program (AlphaInnotech Corp.), with the levels in differentiated, 3T3 L1 cells taken as 100%. In addition, Western blots were probed for CCAAT enhancer binding protein (C/EBPα) expression levels using the 14AA antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), adipocyte determination and differentiation factor (ADD1) levels by probing with the K-10 and peroxisome proliferator-activated receptor-γ (PPARγ) levels by probing with the E-8 antibodies, respectively (Santa Cruz). Anchorage-Independent Proliferation Assays

Approximately 10^4 cells were suspended in 2 mL of 0.32% agarose (Sigma) in Dulbecco-modified Eagle’s medium supplemented with 15% FCS, on top of a feeder layer of the same medium containing 0.7% agarose, in 6 cm petri dishes. Growth was recorded 20 days later. For anchorage-independent growth quantitiation, cells were plated in Methocel (Sigma) in DMEM with 15% FCS and 20 μCi/mL [3H]thymidine [14]. Twenty days later, cells were washed from the water-soluble methocel and the trichloroacetic acid (TCA)-precipitable radioactivity determined. Levels in the highest expressor, L1-e1a-1 were taken as 100%.

Induction and Quantitation of Apoptosis

Cells were starved of serum for 0, 24, 48, or 72 h. Apoptosis was assessed through DNA degradation measurements using a modified Hirt procedure. Briefly, cells were lysed in 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and NaCl added to 1M. Following centrifugation at 10000g for 30 min, the supernatant containing small molecular weight nucleic acids was treated with 1 μg/mL RNase for 20 min at room temperature, precipitated with ethanol, and analyzed by electrophoresis in 1% agarose gels. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was also performed using FITC-coupled nucleotides, according to the manufacturer’s instructions (Roche, #1684809), and cells with apoptotic nuclei were visualized under fluorescence and phase-contrast illumination. For a more precise quantitation, cells growing in a 6 cm plate were fixed with ethanol, stained with propidium iodide, treated with RNase, and the percentage of cells with a sub-G1 DNA content examined by fluorescence-activated cell sorter (FACS) analysis [15]. The percentage of cells at each stage of the cell cycle was determined at the same time.

RESULTS

Production of Preadipocytic Lines Expressing Varying Levels of wt or Mutant E1A

To examine the relationship between the effect of E1A upon adipocytic differentiation, neoplastic transformation and apoptosis, we introduced the wt 243R and 289R (12S or 13S) E1A gene products in the 3T3 L1 preadipocytic cell line (ATCC CCL92.1). Data with the 243R gene are presented, but both genes gave indistinguishable results. To avoid potential problems associated with inducers of commonly used regulatable promoters which might affect differentiation [16], the E1A genes were introduced under control of the strong constitutive MoMLV promoter, through infection with the culture supernatant from retrovirus packaging lines which secrete virus carrying these genes [10]. E1A was also expressed by transfection with a plasmid carrying E1A under control of the CMV promoter (see “Materials and Methods”), with very similar results. Following selection in hygromycin-containing medium, a number of individual colonies from each infected culture were picked without regard to morphology, propagated and screened for levels of E1A expression by Western blotting (Figure 1). To examine the effect of different levels of E1A, and to eliminate the problem of clonal variability, we analyzed a total of 35 clones expressing different levels of the E1A genes (clones L1-e1a-1 to L1-e1a-35, by order of decreasing E1A levels, Figure 1 and Table 1). The clone expressing the highest E1A levels (L1-e1a-1) had approximately 80% the levels present in the human line, 293. In addition, since the ability to bind the Rb family proteins has been shown to be important for E1A transformation, we also produced lines expressing an E1A mutant with a point substitution at aa
124 (C124G), which abrogates binding to pRb [13]. As a control, the same retroviral vector lacking an E1A insert was used and found to have no effect (line L1-Hygro, Table 1).

Adipocytic differentiation is a stochastic event, with individual cells exhibiting different amounts of lipid within the same dish [17]. For this reason, to investigate the effect of an oncogene upon differentiation, it is important to ensure uniformity of gene expression throughout the culture. This was examined by immunostaining with the same anti-E1A antibody, as described in “Materials and Methods.” As shown in Figure 1B, the level of expression in line L1-e1a-1 (a), or the L1-ARb line expressing the gly124 mutant (b) was uniform throughout the culture, while no staining was detectable with the control L1-Hygro (c). Similar results were obtained with all lines used for further experiments (not shown).

Reciprocal Regulation of Adipocytic Differentiation, Transformation, and Apoptosis by Adenovirus E1A

Previous results demonstrate a quantitative hierarchy in the manifestation of transformation-related properties in response to graded levels of expression of oncogenes such as the middle tumor antigen of polyoma virus, v-Src or v-Ras in murine fibroblasts [8,9,18]. Therefore, we examined the dependence of various phenotypic parameters upon levels of E1A expression using the series of E1A-expressing 3T3 L1 preadipocytes described above.

Loss of differentiative capacity and morphological transformation have lower requirements for E1A gene expression levels than focal formation and anchorage independent proliferation

A change in cell morphology is a characteristic feature of neoplastically transformed or differentiating
To examine the effect of E1A upon differentiation, cells were grown to confluence in 24-well plates and adipocytic differentiation induced by the addition of insulin, Dex, and IBMX (see “Materials and Methods”). The degree of adipocytic differentiation was quantitated using three criteria [9]: (i) morphological changes such as cell rounding and accumulation of intracellular lipid, visible by phase contrast microscopy of live cultures, or after staining of fixed cells for lipid with Oil Red-O (Figure 2A). (ii) Northern blotting analysis of several mRNA’s known to be adipocytic differentiation-specific markers, including those encoding the fatty acid binding protein aP2 and adipsin (Figure 2B). Hybridization signals were normalized to the amount of β2-microglobulin mRNA, which does not change with differentiation. (iii) Western blotting analysis for levels of the adipocyte-specific, transcription factors C/EBPa, PPARg, and ADD1 (Figure 3A, 3B and 3C) [19]. As a control, we tested cells infected with the same vector lacking an E1A insert and selected for hygromycin resistance (line L1-Hygro). As observed before [9] there was no decrease in differentiation ability in L1-Hygro cells, compared to uninfected 3T3 L1.

The results using all of the above criteria showed that the block of differentiation and acquisition of a transformed morphology are remarkably sensitive to E1A expression; E1A levels as low as 6% the levels present in the highest expressor (e.g., line L1-e1a-100).

### Table 1. Effects of Increasing E1A Levels Upon Transformation, Differentiation and Apoptosis of 3T3 L1 Preadipocytes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>E1A %a</th>
<th>Transformation</th>
<th>Differentiationb (%)</th>
<th>Apoptosisc (%)</th>
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<td>26.4</td>
<td>100 ± 4</td>
<td>100 ± 3</td>
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<td>E1A-expressing, 3T3 L1 lines9</td>
<td></td>
<td></td>
<td>100 ± 3</td>
<td>100 ± 3</td>
</tr>
<tr>
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<td>100 ± 4</td>
<td>100 ± 3</td>
<td>100 ± 3</td>
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<tr>
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<td>L1-AΔRb9</td>
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<tr>
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<td>—</td>
<td>20 ± 4</td>
<td>20 ± 4</td>
<td>20 ± 4</td>
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<tr>
<td>130—/—</td>
<td>100</td>
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<td>0 ± 0</td>
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<tr>
<td>L1—e1a</td>
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<td>15 ± 3</td>
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<td>L1-ΔRb</td>
<td>140</td>
<td>290 ± 19</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
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</table>

The 243R adenovirus E1A gene or activated Ras<sup>αmut1</sup> were introduced in 3T3 L1 preadipocytes and their phenotype regarding differentiation and transformation examined.

#### Notes:

- aE1A levels were measured by Western blotting and Fluorimager scanning, with the levels in the highest expressor of wtE1A, L1-e1a-1, taken as 100%.
- bAnchorage-independent growth was quantitated by suspending 50 000 cells in 2 mL Methocel containing 10 μCi/mL [3H]-thymidine and measuring incorporation 20 d later (see “Materials and Methods”). Numbers represent percentages, with the levels of the highest expressor, L1-e1a-1 defined as 100%.
- cApproximately 200 cells were seeded together with 10 000 normal 3T3 L1 cells in 6 cm plates. Foci were scored 10 d later.
- dNorthern blotting analysis was quantitated using three criteria [9]: (i) morphological changes such as cell rounding and accumulation of intracellular lipid, visible by phase contrast microscopy of live cultures, or after staining of fixed cells for lipid with Oil Red-O (Figure 2A). (ii) Hybridization signals were normalized to the amount of β2-microglobulin mRNA, which does not change with differentiation. (iii) Western blotting analysis for levels of the adipocyte-specific, transcription factors C/EBPa, PPARg, and ADD1 (Figure 3A, 3B and 3C) [19].

For illustration purposes, only some of the clones are presented.

3T3 L1 cells constitutively expressing activated Ras<sup>αmut1</sup> [9].
28), caused a dramatic reduction in differentiation efficiency, while levels of 20% or greater resulted in a total loss of differentiative capacity (e.g., line L1-e1a-24, Figure 2B and Table 1). Examination of the adipocyte-specific factors revealed a dramatic reduction in C/EBPα levels upon E1A expression (Figure 3A).

In addition, we noted a transient increase in PPARγ levels with differentiation of the control L1-Hygro preadipocytes, which gradually waned at terminal stages (Figure 3B, top panel), as observed before [20]. Just as C/EBPα, E1A expression in 3T3 L1 cells caused a dramatic decrease in PPARγ levels (e.g., line L1-e1a-1, Figure 3B, bottom panel). Similarly, the levels of ADD1 increased during differentiation levels with differentiation of the control L1-Hygro preadipocytes, which gradually waned at terminal stages (Figure 3B, top panel), as observed before [20]. Just as C/EBPα, E1A expression in 3T3 L1 cells caused a dramatic decrease in PPARγ levels (e.g., line L1-e1a-1, Figure 3B, bottom panel). Similarly, the levels of ADD1 increased during differentiation
of the control L1-Hygro line, although the differences observed were less pronounced than PPARγ (Figure 3C, top panel). Still, E1A expression (L1-e1a-1 line) caused a substantial reduction in ADD1 levels (Figure 3C, bottom panel). Examination of the cell cycle profile by FACS analysis following induction of differentiation through the addition of insulin, Dex, and IBMX at confluence indicated that the majority of the cells of the control L1-Hygro line were arrested at the G1 phase at points beyond 3 days postinduction, while the percentage of cells in G1 was substantially reduced by E1A expression (Table 2). These data point to the possibility that E1A represses the cell's ability to exit the cell cycle upon confluence, which is a prerequisite for terminal adipocytic differentiation.

The ability of E1A-expressing cells to grow as dense foci overgrowing a monolayer of normal cells was examined next as described [21]. Focus formation was detectable at ~65%, and it increased with E1A levels, up to 100%, although focus forming ability was not as high as in 3T3 L1 cells transformed by vRas
t17 (Figure 2A, panels i–k and Table 1). Foci formed by lines with lower E1A levels were also
smaller and appeared flatter than foci formed by L1-e1a-1.

For the examination of the degree of anchorage-independent proliferation as a function of E1A levels, cells were plated in soft agarose and their growth visually inspected. Quantitation was achieved by plating in Methocel supplemented with 20 μCi/mL [3H]-thymidine [14], see “Materials and Methods”). As shown in Table 1, 3T3 L1 cells expressing maximal E1A levels (line L1-e1a-1, Figure 1A), grew in semisolid nutrient methocel, although not as efficiently as cells transformed by other oncogenes, such as v-Ras, while no growth was observed in the control L1-Hygro line (Table 1). Lines expressing intermediate E1A levels were used to examine this dependence further. As shown in Table 1, in sharp contrast to the differentiation block, little anchorage-independent growth was observed when E1A levels were ~88% the levels in the highest expressor, and the growth response increased roughly proportionally to the amount of E1A present, up to 100%. Similarly, their growth rate on plastic increased with increasing E1A levels, with doubling times from 26.4 to 19.5 h, in a manner which paralleled their acquisition of anchorage-independent proliferation and formation of foci (Table 1). The above results taken together suggest that in the dominance hierarchy between adipocytic differentiation and transformation, it is the level of E1A expression which determines the outcome (Figure 5).

Table 2. Effects of Increasing E1A Levels Upon Apoptosis of 3T3 L1 Preadipocytes at Different Times Following Differentiation Induction

<table>
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<th>Cell line</th>
<th>Days postinduction</th>
<th>Sub-G1</th>
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N/D, not determined, due to cell death.

Differentiation was induced by the addition of 10 μg/mL insulin, 0.25 μM dexamethasone, and 0.5 mM IBMX added to all lines at confluence (d 0). At the indicated days post differentiation induction, cells were stained with propidium iodide and their cell cycle profile analyzed by fluorescence activated cell sorting [15]. The results are averages of three independent experiments.

Induction of apoptosis requires maximal levels of E1A gene expression

Results from a number of labs have indicated that E1A can induce apoptosis in a number of systems, which is evident under conditions of growth arrest such as serum starvation or confluence [22]. Therefore, cells expressing different E1A levels were starved at subconfluence for 0, 24, or 48 h and apoptosis was assessed by in situ TUNEL staining as well as by FACS analysis of the cellular sub-G1 profile and DNA degradation assays [15] (see “Materials and Methods”). As shown in Figure 4A, little apoptosis was detected in the control L1-Hygro line (panels c and f), while expression of high E1A levels resulted in intense TUNEL staining following a 48 h starvation (panels b and e), similar to the prototype apoptosis inducer, v-myc oncogene (panels a and d). Further quantitation of apoptosis by DNA degradation analysis revealed that while very low levels of apoptosis were detected in cells expressing ~88% the E1A levels present in the highest expressor, apoptosis gradually increased with E1A, to a degree similar to 3T3 L1 cells expressing v-myc (Figure 4B). Examination of the
sub-G1 profile by FACS analysis gave similar results (Figure 4C and Table 1).

It was previously shown that E1A can also induce apoptosis following growth arrest through confluence [22]. Since confluence-induced growth arrest is a requirement for terminal adipocytic differentiation [20], to investigate the effect of E1A upon apoptosis we examined the degree of apoptosis at different times following induction of adipocytic differentiation with the addition of IBMX, Dex, and insulin at confluence. As shown in Table 2, the percentage of cells with sub-G1 DNA content was dramatically increased in L1-e1a-1 cells compared to the control L1-Hygro at time-points beyond 5 days following treatment. These data indicate that E1A can induce apoptosis in 3T3 L1 cells following induction of differentiation, despite the presence of 10% FCS and insulin, known apoptosis inhibitors. However, the above results taken together also show that high levels of E1A expression are required for apoptosis.

![Figure 4. High levels of E1A induce apoptosis of 3T3 L1 preadipocytes.](image)

A

B

C

D

Figure 4. High levels of E1A induce apoptosis of 3T3 L1 preadipocytes. (A) TUNEL staining L1-myc (a and d), L1-e1a-1 (b and e), or L1-Hygro (c and f) cells were grown to confluence and starved for 48 h. Apoptosis was determined by TUNEL staining, using FITC-coupled nucleotides (see "Materials and Methods"). Pictures were taken under fluorescence (a–c) or phase-contrast (d–f) illumination. (B) DNA degradation. Control L1-Hygro (lanes 1–3), L1-e1a-3 (lanes 4–6), L1-e1a-2 (lanes 7–9), or L1-e1a-1 (lanes 10–12) cells were grown to confluence and starved for 0, 24, or 48 h, as indicated. DNA was extracted following a modified Hirt procedure (see "Materials and Methods") and DNA degradation assessed by agarose gel electrophoresis. Lanes M: Molecular weight markers, of the indicated molecular weights (left, 1 kb DNA ladder, right, 100 bp ladder, New England Biolabs). (C) FACS analysis Control L1-Hygro or L1-myc cells, or 3T3 L1 lines expressing high E1A levels, or L1-ΔRb cells as indicated, were grown to confluence and starved for 24 or 48 h, then stained with propidium iodide and examined for apoptosis induction through sub-G1 profile analysis using a fluorescence activated cell sorter. The results are averages ±SEM of at least three independent experiments. (D) E1A expression downregulates Akt in serum-starved cells. Control L1-Hygro cells (lanes 1, 5, 8, and 11), or their counterparts expressing progressively increasing E1A levels, were grown in 10% FCS (lanes 1–4) or starved for 24 (lanes 5–7), 48 (lanes 8–10), or 72 h (lanes 11 and 12), as indicated. Detergent cell extracts were resolved by gel electrophoresis, blotted and probed for the ser-473-phosphorylated form of Akt (top panel) or Hsp90 (bottom panel), see "Materials and Methods"). Arrow points to the position of the 60 kDa Akt protein.
induction following growth arrest by either serum starvation, or confluence in the presence of differentiation inducers.

The mechanism of the E1A-mediated induction of apoptosis was investigated further. Most of the cellular proteins that have been identified as targets of E1A are located in the nucleus, yet only half of the E1A is nuclear [23–25]. However, E1A is also known to modulate the phosphatidylinositol-3 kinase (PI3-ki)/Akt pathway at least in human cells [26]. Since Akt inhibition could lead to apoptosis, we examined Akt phosphorylation levels following E1A expression by probing Western blots with an antibody specific for the ser-473 phosphorylated form of this protein, which correlates with activation [27]. As shown in Figure 4D, the levels of Akt phosphorylation were high in cells growing in 10% serum (lanes 1–4), and E1A expression did not have a significant effect. However, serum starvation for 24 h caused a decrease in p-Akt levels especially in the highest E1A expressor, L1-e1a-1, which became more pronounced upon starvation for 48 or 72 h (Figure 4D, lanes 5, 8, 11 vs. 7, 10, 12). These results indicate that E1A expression downregulates Akt in 3T3 L1 preadipocytes, which may contribute to E1A’s effect in inducing apoptosis in this system.

Effect of E1A Upon Adipocytic Differentiation, Transformation, and Apoptosis of 3T3 L1 Cells Requires E1A Interaction With Rb

One of the important targets of E1A is the pocket proteins, pRb, p107, p130 [2]. Genetic evidence using a number of substitution or deletion mutants previously demonstrated that binding to Rb is not necessarily required for suppression of differentiation of myoblasts by E1A [13]. Since, as shown above, adipocytic differentiation is very sensitive to wtE1A expression, to examine the importance of binding to Rb family proteins upon the differentiation-inhibiting effect of E1A, we expressed a mutant carrying a point substitution at amino acid 124 (gly124), which impairs binding to Rb and to a lesser degree to p130 [13]. As shown in Figure 2A (panel f), cells expressing this mutant even to levels similar to clone L1-e1a-1, with the highest levels of wtE1A (e.g., line L1-ARb), were able to accumulate triglycerides to a degree...
Table 1. Expression of wtE1A but not the gly124 mutant blocked differentiation of 3T3 L1/C0 adipocytes, although to a lower degree than 3T3 L1/C0. Differentiation of p130/+/+ cells is unable to block the differentiation of 3T3 L1/C0 cells. As shown before [19], p130−/− cells are able to differentiate into adipocytes, although to a lower degree than 3T3 L1/C0 (Table 1). Expression of wtE1A but not the gly124 mutant blocked differentiation indicating that, in this system at least, it is the E1A binding to Rb which is responsible for the block of adipocytic differentiation (Table 1). Apparently, although p300/CBP binding by E1A may also play a role, it cannot substitute for E1A’s effect upon Rb inactivation. These results taken together indicate that, unlike the myoblast system, Rb binding is important in determining E1A’s effect upon adipocytic differentiation.

The ability of the gly124 mutant to induce apoptosis was next investigated by examining the sub-G1 profile of L1-ΔRb cells following serum starvation, or following differentiation induction by FACS analysis as above. As shown in Table 1, the sub-G1 profile of L1-ΔRb cells following serum starvation was similar to the control 3T3 L1, indicating the absence of apoptosis. Similar results were obtained following induction of differentiation (Table 2), indicating that Rb binding by E1A plays an important role for apoptosis induction. In support of this conclusion, cells with a targeted disruption of the Rb gene (Rb−/−) entered apoptosis upon serum starvation (Table 1), or differentiation induction (Table 2). Moreover, examination of the cell cycle profile of cells in the process of terminal differentiation revealed that, same as the parental 3T3 L1, the L1-ΔRb were arrested in G0/G1 at time-points beyond the 2nd day postinduction, indicating that Rb binding by E1A is required to overcome the growth arrest, which is a prerequisite for adipocytic differentiation (Table 2). The above results taken together indicate that E1A function is dependent upon its ability to bind Rb, even in order to inhibit adipocytic differentiation, which can be blocked by very low levels of wtE1A.

DISCUSSION

The transition between cell proliferation and adipocytic differentiation is a tightly regulated process where cell cycle regulators and differentiation factors interact. Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. Following hormonal induction, growth arrested cells re-enter cell cycle, a phase termed clonal expansion, which is followed by a final proliferation arrest and terminal differentiation [reviewed in 28]. Current hypotheses postulate that terminal adipocytic differentiation requires the interplay between the nuclear receptor, PPARγ, and the C/EBP, whose function is affected by Rb, a cell cycle regulator of the retinoblastoma protein family. During clonal expansion, Rb is inactivated through phosphorylation, consistent with an active cell cycle [29], but Rb protein function is required for the growth arrest preceding terminal differentiation [30]. In addition to its growth arresting function, Rb enhances the transactivation capability of C/EBP via direct protein-protein interaction, thereby inducing the transcription of adipocyte-specific genes and terminal differentiation. As a result, fibroblasts from Rb knockout mice fail to differentiate when properly stimulated [30]. Contrary to Rb however, the other members of the family (p107, p130) do not
bind the C/EBPα promoter and they have been reported to negatively regulate adipogenesis, although their precise role is presently unknown [19].

A variety of oncogenes have been shown to block differentiation, but whether this is simply a consequence of their growth promoting capacity is currently under investigation. In the case of E1A, the functions required to suppress adipocytic differentiation have not been established. Concerning myoblast differentiation, a genetic analysis using adenovirus mutants indicated that, although binding to either p300/CBP or to Rb family members was sufficient for the downregulation of muscle regulatory genes, the capacity to reactivate the cell cycle in quiescent cells segregated with the capacity to bind Rb, while binding to p300/CBP was not required. These data indicate that the suppression of the differentiation program is not merely a consequence of cell cycle reactivation in this system [13]. On the other hand, induction of apoptosis, as well as induction of DNA synthesis in quiescent baby rat kidney cells required binding to both p300 and Rb, indicating that these two functions may be connected [22,31]. Our results concerning adipocytic differentiation revealed that E1A binding to Rb is indispensable for both suppression of differentiation, as well as induction of transformation and apoptosis; a mutant which is defective in Rb binding was unable to block adipocytic differentiation, transform or induce apoptosis, even when expressed to high levels. However, our results revealed a profound dissociation between E1A signals leading to transformation, differentiation suppression and apoptosis induction, based on levels of expression. The differentiative process was found to be exquisitely sensitive to low levels of E1A, while progressively higher levels induced transformation and apoptosis in a dose-dependent manner. These data are consistent with previous observations which established a quantitative hierarchy in the manifestation of certain transformation-related properties and differentiation ability of 3T3 L1 cells following expression of oncogenes such as Ras or the polyoma middle tumor antigen [8,9]. The present study incorporates apoptosis induction into the gamut of parameters which can be regulated by graded levels of expression of E1A, one of the archetypal oncogenes with this capacity. The fact that E1A expression can downregulate Akt activity, further points to the possibility that the inhibition of PI3-k/Akt pathway may be exploited by E1A to induce apoptosis in this system.

The reasons why E1A requires higher levels of expression to induce apoptosis than to block differentiation are not clear. It is known that Rb sequestration by E1A would release free E2F, which can activate promoters of genes involved in cell division, thus forcing cells to continue cycling, precluding cell entry into the differentiation-permissive state of growth arrest [2]. In addition, it is known that Rb inactivation can activate Ras [32], which could also lead to E2F activation [33]. In fact, current models of E2F function postulate that higher free E2F levels are required for apoptosis induction than for neoplastic transformation [34], which is consistent with our findings. In addition to freeing E2F transcription factors from Rb, E1A brings together p300/CBP and Rb, each binding on a different site of E1A, and this results in Rb activation through acetylation by the p300/CBP acetyltransferase. Rb acetylation, in turn, is known to promote its association with the MDM2 oncoprotein. Rb and MDM2 form a complex with the tumor suppressor p53, and this interaction was shown to block p53 degradation and promote apoptosis [35,36]. Although the requirement for high E1A levels for p53 activation has not been conclusively demonstrated, such a mechanism could explain the dramatic differences in E1A levels required for differentiation suppression as opposed to apoptosis induction.

The system of E1A expressing 3T3 L1 preadipocytes on which the present study was based, permits the investigation of the functional integration of transforming and differentiative cues with signals inducing apoptosis. Our data suggest that in the dominance hierarchy between transforming, differentiative and apoptotic signals, the level of oncogene expression is critical in determining which signal prevails.

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