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The Block of Adipocyte Differentiation by a C-Terminally Truncated, but Not by Full-Length, Simian Virus 40 Large Tumor Antigen Is Dependent on an Intact Retinoblastoma Susceptibility Protein Family Binding Domain

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Simian virus 40 (SV40) can promote cell transformation and suppress differentiation. It does this partly by targeting tumor suppressors such as p53 and members of the retinoblastoma susceptibility protein (Rb) family. This work concentrates on mechanisms by which SV40 large tumor antigen (SVLT) suppresses adipocyte differentiation. We created cell lines derived from murine 3T3-L1 preadipocytes expressing different versions of SV40 early-region sequences. SVLT-expressing cells failed to exhibit adipocyte morphology, to induce glycerophosphate dehydrogenase activity, and to induce differentiation-dependent mRNA for adipocyte P2. SVLT alone was sufficient, in the absence of SV40 small tumor antigen, to inhibit differentiation. A truncated SVLT containing only the N-terminal 121 amino acids (SVLT1-121) blocked differentiation, thus mapping at least one differentiation blocking function to the N-terminal region. K1 (Glu-107→Lys) point mutants of SVLT, which are unable to bind to the Rb protein family or induce neoplastic transformation, are defective for blocking differentiation in the case of SVLT1-121 but retain the ability to block differentiation in the case of full-length SVLT. This finding demonstrates that Rb family proteins are important in regulating adipocyte differentiation but that other functions of full-length SVLT can block adipocyte differentiation independently of RB family binding and transformation.

Simian virus 40 (SV40) is a small DNA tumor virus which utilizes host cell gene products for both virus replication and neoplastic transformation. This property has made this virus a useful model for the study of cell regulation. The early proteins of SV40, the large and small tumor antigens (SVLT and SVst) are involved in viral oncogenic transformation in vitro and in vivo (41). The principal transforming protein of SV40 is SVLT, a 708-amino-acid, multifunctional, predominantly nuclear phosphoprotein. SVLT is capable of inducing cellular DNA synthesis and transforming cells (reviewed by Fanning and Knippers [16]).

SV40 has been shown to affect differentiation in a number of different cell systems, including preadipocytes and myoblasts (5, 12, 13, 23, 26, 40). Furthermore the effects are complex, and interference occurs not only at the initiation of differentiation but also during maintenance. In adipocytes at least, the ability to prevent differentiation can be genetically separated from the ability to transform (5).

The purpose of this work is to dissect the mechanisms by which SV40 affects adipocyte differentiation. Terminal differentiation of adipocytes is characterized by withdrawal from the cell cycle (reviewed by Cornelius et al. [8]). It is therefore natural to consider SV40 functions that affect cell growth, while remaining open to the possibility that novel functions, silent in growth assays, contribute to preventing differentiation. A genetic approach to this question has been taken in this work. Different SV40 early-region and mutant protein constructs were tested for the ability to block the differentiation of 3T3-L1 cells into adipocytes. The results clearly implicate retinoblastoma susceptibility protein (Rb) family interactions as a mechanism by which SVLT blocks differentiation and induces neoplastic transformation. Other SVLT functions, present in the full-length molecule but not in the 121-amino-acid fragment, prevent adipocyte differentiation without transformation or the Rb family binding site. Thus, one or more SVLT functions present in the full-length protein complement the differentiation-blocking but not the transforming effect of Rb family binding.

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MATERIALS AND METHODS

Cell culture. The preadipocyte cell line 3T3-L1 has been described previously (19–21). Stock cultures were maintained at subconfluence in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (DMEM) containing 10% bovine calf serum (CS).

Differentiation was induced by growing cells to confluence in differentiation medium (DMEM containing 10% fetal calf serum and 5 μg of porcine insulin per ml). Dishes (35-mm diameter) were seeded at 7×10^4 to 1×10^5 cells per plate in 2 ml of medium. Dishes became confluent within 3 to 5 days and at confluence were induced by treatment overnight with fresh differentiation medium containing 250 nM dexamethasone and 500 μM isobutylmethylxanthine (DEX-IBMX). The following day, the medium was replaced with differentiation medium lacking DEX-IBMX. Differentiation medium was replaced with fresh every 3 to 4 days. Differentiation-competent cultures exhibit 75 to 100% lipid-containing cells and express glycerophosphate dehydrogenase (GPD) activity and adipocyte P2 (aP2) mRNA within 7 days after DEX-IBMX treatment.

Anchorage independence was assayed in agar suspension cultures. Cells were trypsinized and suspended at 5.0×10^5/35-mm-diameter well in 1 ml of DMEM–10% CS–0.36% agar (Bacto Agar; Difco) layered over 2 ml of DMEM–10% CS–0.6% agar. Cultures were fed every 3 days by overlaying with medium containing 0.36% agar. Growth was scored as positive if colonies containing more than two cells were present in cultures 2 weeks after seeding.

Density-dependent growth arrest was assayed by determining the percentage of cells able to incorporate bromodeoxyuridine (BrdU) over a 24-h period when at high density. Cells were plated at 7×10^4/35-mm-diameter well and incubated for 5 days. At day 5, approximately 2 days after reaching confluence, BrdU (10 μM) was added for 24 h, after which BrdU uptake was determined as described by Tischler et al. (39). Briefly, cells were rinsed in Hanks buffered salt solution, fixed in 90% ethanol for 10 min and 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, and reacted with a mouse monoclonal anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), and detection was performed by using a biotinylated horse anti-mouse antibody and strepavidin-alkaline phosphatase detection system (Vector Laboratories, Inc., Burlingame, Calif.). The proportion of a cell culture incorporating BrdU was determined by counting at least 300 cells per culture.

Plasmids and mutagenesis. PVU0, which contains the SV40 early promoter and early region encoding SVLT and SVst (28), *dl*1137Δt, which contains a deletion mutant SV40 early region encoding a truncated SVLT (amino acids 1 to 121 [SVLT1-121]) (30) and no SVst (29, 36), and HSV-neo (1), which utilizes the murine Harvey sarcoma virus long terminal repeat to direct expression of bacterial aminoglycoside phosphotransferase, thus conferring resistance to G418 (Life Technologies, Inc., Grand Island, N.Y.), have been described previously.

The K1 mutation was introduced into the SVLT DNA in plasmid *dl*1137Δt by using PCR mutagenesis and standard procedures (7). SV40 DNA was amplified with primers that contained the K1 mutation (SV40 nucleotide 4499 changed from a G to an A changes amino acid 107 from a glutamate to a lysine) along with nucleotide changes that created a new *Nsp*V restriction endonuclease site while keeping the encoded amino acids, other than amino acid 107, unchanged. The following four primers were used: primer 1, 5′-ATGCTCT**AGATGGATAAAGT TTTAAACAGAGAGGA**-3′ (underlined is an introduced *Xba*I site, and the boldface region corresponds to SV40 nucleotides 5165 to 5138); primer 2, 5′-TCTT TCGAACAAAACAGGTTTTCCTCATTAAAGGC-3′ (underlined are changes to SV40 to make a new *Nsp*V site, and the K1 point mutation is in boldface; this primer anneals to SV40 nucleotides 4495 to 4529); primer 3, 5′-TTTGTTCG**A** AAGAAATGCCATCTAGTGATGATGAG-3′ (underlined are changes made to introduce an *Nsp*V site, and the K1 mutation is in boldface; this primer anneals to SV40 nucleotides 4507 to 4473); and primer 4, 5′-ATGCGGATCCA GACATGATAAGATACATTGATGAG-3′ (underlined are SV40 nucleotides 2533 to 2563). The target sequence was SV40 nucleotides 5190 to 2533 (*Stu*I to *Bam*HI) of plasmid *dl*1137Δt or PVU0. Primers 1 and 2 were used to amplify sequences in *dl*1137Δt containing the SVst deletion (amplificant A). Primers 3 and 4 were used to amplify the region in *dl*1137Δt containing the deletion creating the truncated protein SVLT1-121 and simultaneously introducing the K1 mutation and *Nsp*V site (amplificant B) or to amplify the corresponding region in PVU0 containing wild-type sequence (amplificant C), creating full-length SVLT and simultaneously introducing the K1 mutation and the *Nsp*V site.

PCR conditions were as follows: 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.01% gelatin), deoxynucleoside triphosphates (200 μM each), 50 pmol of each primer, 0.1 pg of target DNA, and 2.5 U of *Taq* polymerase (Life Technologies, Inc., Gaithersburg, Md.) in a total of 100 μl with 1 cycle of 94°C for 1 min, 35 cycles of 55°C for 1 min, 72°C for 1.5 min, and 94°C for 1 min and 1 cycle at 55°C for 1 min and 72°C for 5 min. The mixture was held at 4°C until purified (Perkin-Elmer DNA Thermal Cycler, Perkin-Elmer Corporation, Norwalk, Conn.). Each of these amplificants was isolated by separation on low-melting-point agarose gels and then purified by using a Geneclean kit (Bio 101, Inc., La Jolla, Calif.). Amplificant A was cloned directly into the *Hin*cII site of pUC19. Amplificant B was cut with *Pst*I, and the 5′ fragment was cloned between the *Hin*cII and *Pst*I sites of pUC19.

A *Bam*HI-to-*Pst*I fragment containing amplificants A and B or A and C was constructed by cloning the *Xba*I-to-*Nsp*V fragment of A and the *Nsp*V-to-*Pst*I fragment of B or C between the *Xba*I and *Pst*I sites of pUC19. ZSVLT1-121K1 and ZSVLT1-708K1 were constructed by using the *Bam*HI-to-*Pst*I fragment from the resulting plasmids, cloned together with the *Pst*I-to-*Bam*HI fragment from PVU0 (SV40 nucleotides 3204 to 2533) into the *Bam*HI site of vector ZIPNeo (3). Proper orientation was determined by restriction enzyme analysis. This created two plasmids which lack SVst and which contain the K1 mutation: ZSVLT1-121K1, which expresses the first 121 amino acids of SVLT, and ZSVLT1-708K1, which expresses the entire 708 amino acids of SVLT.

To create a vector expressing wild-type SVLT without SVst (ZSVLT1-708), the *Bam*HI-to-*Pfl*MI fragment from pUC19 carrying amplificant A was ligated together with the *Pfl*MI-to-*Bam*HI fragment of PVU0 (SV40 nucleotides 4558 to 2533) into ZIPNeo. The ZIPNeo vector also encodes neomycin resistance. This is a retroviral vector, but to make the cell lines described in this work, it was used as a plasmid and directly transfected.

Production of preadipocyte cell lines expressing SVLT. The 3T3-L1 preadipocytes expressing wild-type SVLT without SVst were prepared by using virus produced by the ψ2TEXs6 retrovirus-producing line (2) or by transfecting plasmid ZSVLT1-708, described above. Cell lines expressing other SVLTs were created by cotransfecting PVU0 or *dl*1137Δt and HSV-neo in a 10:1 ratio or by transfecting ZSVLT1-121K1 or ZSVLT1-708K1. All transfections were performed by the calcium phosphate precipitate technique, using the Transfinity System as instructed by the manufacturer (Life Technologies). The cells were exposed to the calcium phosphate precipitate overnight, the precipitate was washed away with fresh medium on the following day, and on the subsequent day, cells were trypsinized and plated in medium containing the neomycin analog G418 at 400 μg/ml. G418-resistant colonies of transfected preadipocytes were pooled (>50 colonies per transfection) and maintained as subconfluent growing stocks. When necessary, random G418-resistant colonies of transfected preadipocytes were isolated with cloning cylinders and expanded as growing subconfluent stocks. Those colonies producing SVLT, as determined by immunoblot analysis or by immunocytochemistry, were used for further studies.

Immunocytochemistry. The percentage of cells producing SVLT protein was assessed by indirect immunostaining. Cells were plated at subconfluency into 35-mm-diameter wells, grown overnight, fixed for 10 min in 4% paraformaldehyde in PBS, and then permeabilized in 90% ethanol for 10 min. Cells were then rinsed three times in PBS and blocked 20 min in 10% normal goat serum (NGS) in PBS. Alternatively TS (50 mM Tris-HCl [pH 7.6], 150 mM sodium chloride) was used instead of PBS, and permeabilization was with 100% methanol chilled to −20°C (variation of the fixation method used by Chen et al. [4]). Primary antibody pAB108 (24), 1:5 in 10% NGS in PBS, was applied for 1 h at room temperature, the mixture was rinsed three times in PBS, a second antibody (goat anti-mouse alkaline phosphatase conjugate; 1:250 in 10% NGS in PBS) was applied for 1 h at room temperature, the mixture was rinsed three times with PBS, and detection was carried out with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium chloride, *p*-toluidine salt, as instructed by the manufacturer (Life Technologies).

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Soluble extracts were made from proliferating cultures of preadipose cells by rinsing in wash buffer (137 mM NaCl, 20 mM Tris [pH 8.0], 1 mM $MgCl_2$, 1 mM $CaCl_2$), extracting in wash buffer plus 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, and 1 μg of leupeptin per ml, and rocking at 4°C for 20 min. Extracts were clarified by centrifugation (12,000 × *g*, 10 min) to remove insoluble material.

The total soluble protein concentration of unlabeled extracts for Western blot (immunoblot) analysis was determined (Bio-Rad protein assay reagent) according to the manufacturer's instructions, using bovine serum albumin as the standard. Extracts were stored at −70°C until used. Proteins were resolved by sodium dodecyl sulfate (SDS)-PAGE and transferred onto Immobilon-P (0.45-μm-pore-size polyvinylidene fluoride membrane; Millipore), using a tank electrotransfer unit (25 mM Tris [pH 8.0], 200 mM glycine, 20% methanol transfer buffer) or by semidry blotting (12.5 mM Tris [pH 8.0], 100 mM glycine, 10% methanol transfer buffer). The membrane was blocked in 1% gelatin (enzyme immunoassay grade; Bio-Rad) in Tris-buffered saline (10 mM Tris [pH 8.0], 150 mM NaCl) for 1 h at room temperature and probed overnight with mouse monoclonal anti-SVLT pAB108 (24) (hybridoma conditioned medium was diluted 1:100 in Tris-buffered saline plus 0.05% Tween 20). The secondary antibody was goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Boehringer Mannheim), detected by using enhanced chemiluminescence (Dupont) according to the manufacturer's instructions. Prestained protein standards (Life Technologies) were used as molecular weight markers.

RNA analysis. Total cellular RNA was extracted in 4 M guanidinium isothiocyanate (Research Organics, Inc.)–25 mM sodium citrate (pH 7)–0.5% *N*-lauroylsarcosine–0.1 M 2-mercaptoethanol (6). Equal amounts of total RNA were resolved by electrophoresis on 1.5% agarose–6% formaldehyde gels. The 28S and 18S rRNAs were visualized as size markers by ethidium bromide staining prior to transfer of RNA to nitrocellulose. Complementary DNA probes for murine actin and adipocyte P2 (aP2) mRNAs (35) were labeled by random priming (17) with [α-^{32}P]dCTP (3,000 Ci/mmol), hybridized to blots overnight at 45°C in hybridization buffer (10× Denhardt's solution, 50% formamide, 150 mM Tris [pH 7.4], 5 mM EDTA, 750 mM NaCl, 0.1% sodium pyrophosphate, 100 mM Na_2HPO_4, 0.1% SDS, 100 μg of herring sperm DNA per ml), and washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 55°C prior to exposure to X-ray film at −70°C using intensifying screens.

GPD assays. GPD assays were performed by using a modification of the assay described by Wise and Green (42). Cell monolayers were rinsed with PBS and scraped directly into 0.5 to 1.0 ml of 20 mM Tris (pH 7.4)–1 mM EDTA on ice. Cells were disrupted by sonication (25 W for 10 s at 4°C, using a microtip on a Branson model 250 Sonifier), and insoluble material was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Total soluble protein concentration was determined (Bio-Rad protein assay reagent) according to the manufacturer's instructions, using bovine serum albumin as the standard. GPD activity in extracts containing 5 to 100 μg of total protein was assayed spectrophotometrically at 340 nm in 50 mM triethanolamine (pH 7.5)–1 mM EDTA–225 μM NADH–216 μM dihydroxyacetone phosphate–1 mM 2-mercaptoethanol. A unit of GPD activity is defined as 1 nM NADH oxidized per min.

Materials. All reagents were purchased from Sigma except as noted otherwise. Radioactively labeled reagents were purchased from either New England Nuclear or ICN. Tissue culture medium and supplements were purchased from Life Technologies. Tissue culture sera were purchased from JRH Biosciences. Restriction enzymes were purchased from Life Technologies or New England Biolabs, Inc., Beverly, Mass.

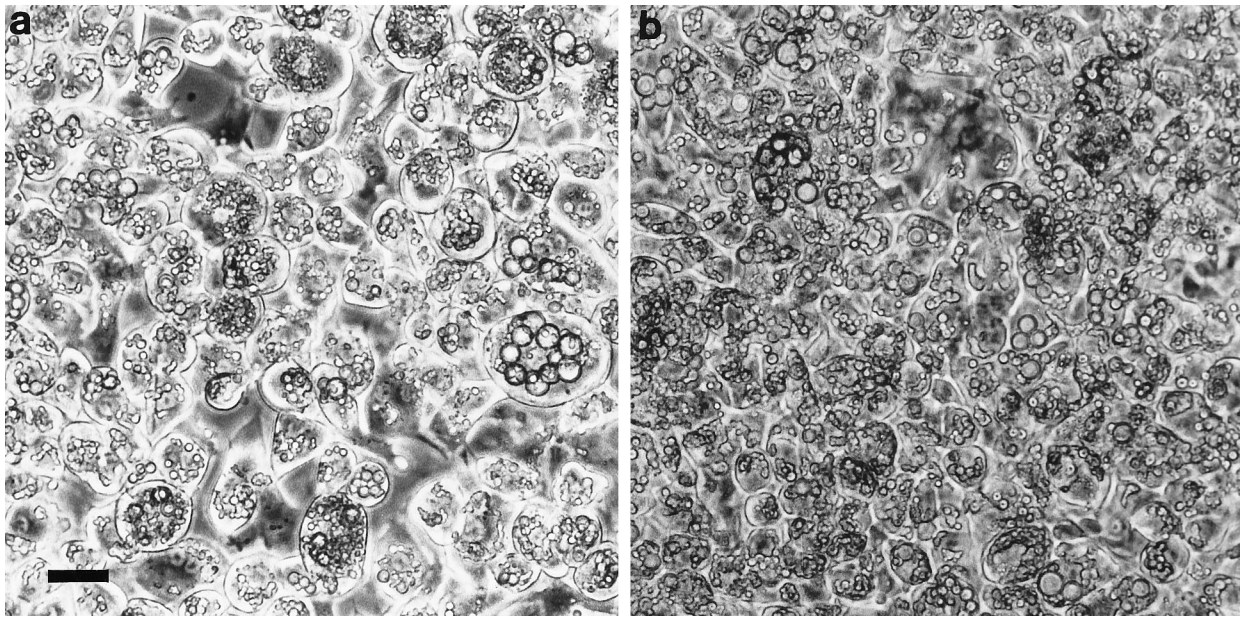

FIG. 1. Morphologies of control, G418-resistant 3T3-L1 preadipocytes when induced to differentiate. G418-resistant 3T3-L1 preadipocyte cell lines were created, as controls, by transfection with plasmid HSV-neo (a) or ZIPNeo (b). Cells were cultured in differentiation medium and photographed 7 days after induction of differentiation. Bar = 35 μm.

RESULTS

A wild-type SV40 early-region function blocks the differentiation of 3T3-L1 preadipocytes. The genetic analysis of the SV40 block of adipocyte differentiation in our earlier studies (5) has been extended by using 3T3-L1 preadipocytes. This cell line is a well-characterized model for adipocyte differentiation (19–21). Three assays can be used to determine the differentiation of adipocytes. The differentiation-dependent mRNA for aP2 can be detected by Northern (RNA) blotting as early as 2 days after inducing differentiation. This assay is used to determine if blocked cells are prevented from differentiating at an early point. Endpoint assays include the measurement of GPD activity, which is elevated in differentiated cells, and the accumulation of cytoplasmic lipid that occurs in adipocytes. If endpoint characteristics appear, it can be assumed that the aP2 mRNA has appeared also. A technical advantage of 3T3-L1 over 3T3-F442A preadipocytes is that they incorporate DNA with greater efficiency in transfections and remain more readily adherent when transformed.

We confirmed that the SV40 early region (capable of expressing both SVLT and SVst) blocks 3T3-L1 differentiation by creating a cell line (L1-PVU0) that expresses SVLT from a plasmid carrying an intact early region of the SV40 genome (PVU0 [28]). 3T3-L1 preadipocytes transfected only with the neomycin-resistant marker (L1-HNeo) served as a control. L1-HNeo and L1-PVU0 cells were cultured under differentiation-inducing conditions and then analyzed for their differentiated characteristics at 7 days postinduction. Control cells, as expected, accumulated cytoplasmic lipid droplets (Fig. 1a) and expressed high levels of GPD enzyme activity (Table 1), which are both indicative of adipocyte differentiation. Conversely, the cell line expressing the SV40 early region, L1-PVU0, did not accumulate cytoplasmic lipid droplets (Fig. 2a) and had reduced GPD levels (Table 1), demonstrating an undifferentiated phenotype.

SVLT alone is sufficient to block adipocyte differentiation. To determine if SVLT, independent of SVst, was sufficient to prevent adipocyte differentiation, we created a 3T3-L1 cell line that expressed only SVLT (amino acids 1 to 708; L1-SVLT1-

TABLE 1. Abilities of preadipocyte lines expressing SVLT to differentiate and regulate growth in soft agar

Vector	Cell line	Region of SVLT expressed	GPD activity[a] (U/mg of protein)	Soft agar growth[b]
None	L1		2,063, 1,599	−
HSV-neo	L1-HNeo		1,519, 1,133	−
ZIPNeo	L1-ZNeo		1,155, 1,123	−
PVU0	L1-PVU0	Early region, both SVLT and SVst	47, 25	+
ZSVLT1-708	L1-SVLT1-708	aa[c] 1–708	42, 60	+
ψ2TEXs6	L1-TEX	aa 1-708	200, 176	ND
*d*l1137Δt	L1-SVLT1-121	aa 1-121	25, 21	+
ZSVLT1-121K1	L1-SVLT1-121K1	aa 1-121, Glu-107→lys	1,020, 1,012	−
ZSVLT1-708K1	L1-SVLT1-708K1	aa 1-708, Glu-107→lys	121, 119	−

[a] Data obtained from duplicate cultures.
[b] +, colony formation; −, no colony formation, ND, not done.
[c] aa, amino acids.

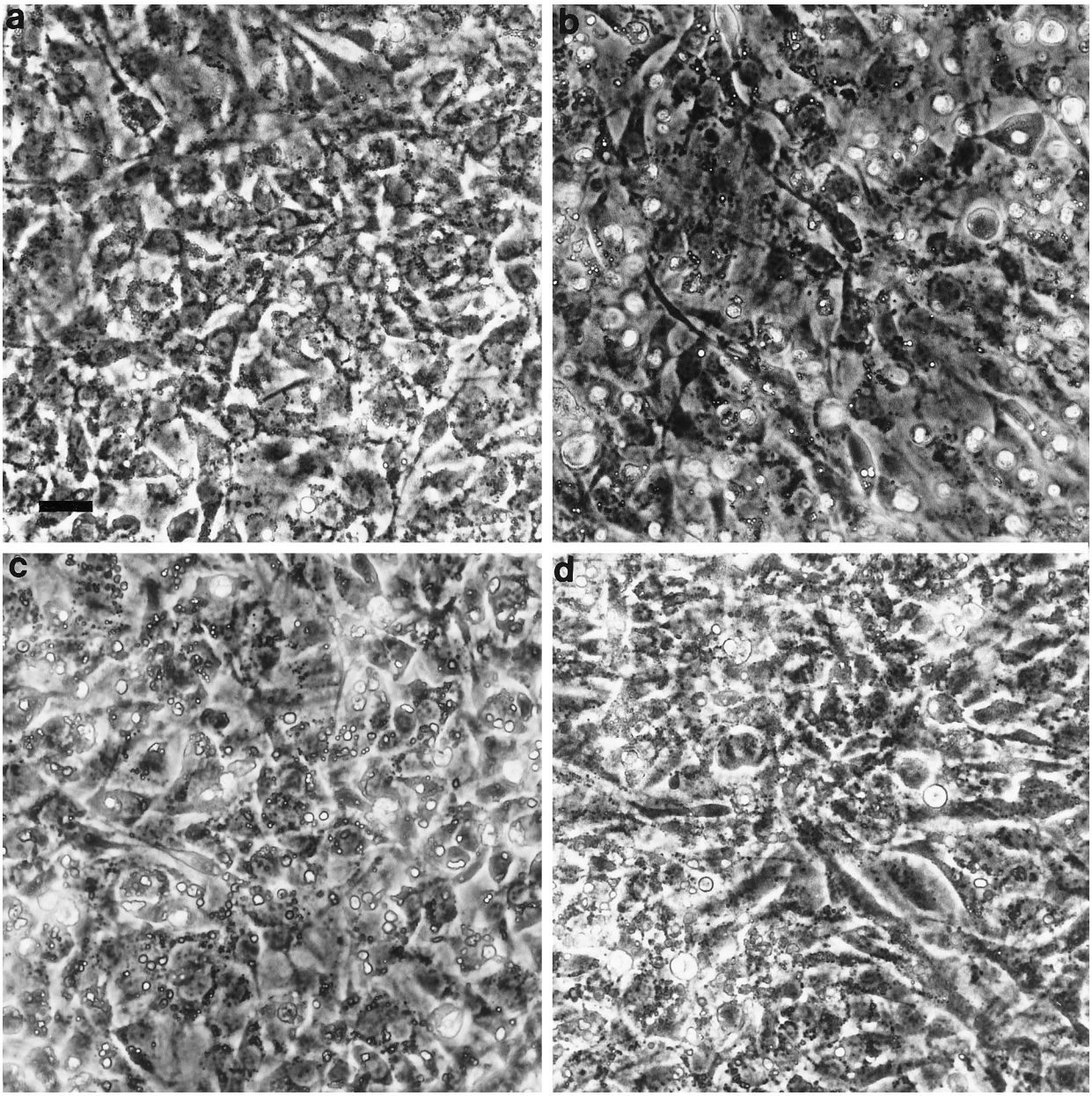

FIG. 2. Morphologies of cells blocked by wild-type and mutant SVLT. Cells were cultured as for Fig. 1. (a) L1-PVU0; (b) L1-SVLT1-708; (c) L1-TEX; (d) L1-SVLT1-121. Bar = 35 μm.

708). When cultured under conditions that induce differentiation, L1-SVLT1-708 cells did not accumulate cytoplasmic lipid droplets (Fig. 2b) and had low GPD levels (Table 1). These observations indicate that L1-SVLT1-708 cells did not differentiate and therefore SVLT expression alone is sufficient to block adipocyte differentiation.

To be sure that the SVst-deficient construct created for this study functioned similarly to a previously published SVst-deficient SVLT expression vector, another 3T3-L1 cell line expressing SVLT alone was established by using the ψTEXs6 retroviral vector (2). The cell line produced (L1-TEX) was also not able to differentiate, as determined by morphology and GPD assays, verifying the result obtained by using the SVLT expression vector created for this study (Fig. 2c and Table 1).

The amino terminus of SVLT is sufficient to block adipocyte differentiation. Since SVLT is a multifunctional protein, one genetic approach to study individual functions has been to express truncated versions of SVLT. To identify a minimal domain of SVLT which retains function in the adipocyte differentiation assay, a plasmid expression vector (*d*l1137Δt [29]) encoding a truncated SVLT was introduced into 3T3-L1 preadipocytes. This plasmid expresses SVLT1-121 and does not express SVst. Cell lines expressing the truncated SVLT product, as confirmed by immunocytochemistry (not shown) and on Western blots (Fig. 3), failed to differentiate. L1-SVLT1-121 cells have lower GPD levels than do 3T3-L1 or neomycin-resistant 3T3-L1 cells (Table 1) following induction of differentiation. The SVLT1-121 cell line retains a fibroblast-like morphology at confluence under differentiation conditions

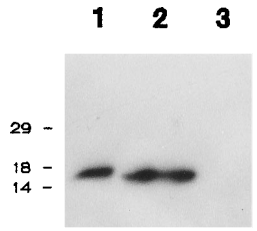

FIG. 3. SVLT1-121 and SVLT1-121K1 protein expression. SVLT1-121 and SVLT1-121K1 migrate on SDS-PAGE at approximately 16 kDa. Cell extracts (10 μg per lane) were separated on a 15% polyacrylamide gel, blotted onto an Immobilon-P membrane, probed with anti-SVLT monoclonal pAb108, and detected by using chemiluminesence. Lanes: 1, L1-SVLT1-121; 2, L1-SVLT1-121K1; 3, L1-ZNeo as a negative control. Sizes are indicated in kilodaltons.

(Fig. 2d), unlike control cells, which convert to a spherical adipocyte morphology and contain many large cytoplasmic lipid droplets (Fig. 1b).

We established that induction of differentiation-dependent mRNA, which precedes GPD and triglyceride production, is also suppressed in 3T3-L1 cells expressing the SV40 early region or SVLT1-121. aP2 mRNA is expressed in the differentiated controls but not in the cells expressing SV40 early proteins (L1-PVU0) or SVLT1-121 (L1-SVLT1-121) (Fig. 4). Therefore, expression of either SVLT or SVLT1-121 prevents expression of any of the adipocyte markers tested. This observation indicates that the block by both proteins is at an early point, prior to induction of aP2 mRNA, and is consistent with our earlier work with 3T3-F442A preadipocytes, which were also blocked by SV40 early proteins (5). Our current results also show that prevention of adipocyte differentiation by the N terminus of SVLT is independent of SVst expression.

SVLT1-121 depends on the Rb family binding domain for blocking adipocyte differentiation, but full-length SVLT does not. A critical SVLT function residing in the amino terminus is the ability to bind to cellular Rb family members (p110 Rb, p107, and p130) (9, 11, 14, 15, 43). To determine if the Rb binding domain contributes to SVLT1-121's ability to prevent differentiation, we created L1-SVLT1-121K1, a 3T3-L1-derived cell line that expresses SVLT1-121 with the K1 mutation introduced (Glu-107 changed to Lys [28]). We isolated a cell line that expressed the expected protein at levels equivalent to those of SVLT1-121 as determined by immunoblotting (Fig. 3) and in essentially 100% of the cells as determined by immunocytochemistry (Fig. 5b). This cell line was able to differentiate into adipocytes. When cultured under differentiation inducing conditions, this line had high levels of GPD activity (Table 1) and accumulated lipid (Fig. 5a). It is important that 100% of the cells express SVLT1-121, because this result shows that it is not lack of expression or nonuniform expression that can account for the inability of this protein to block differentiation. The release of the differentiation block by the K1 mutation demonstrates that the ability of SVLT1-121 to bind to the Rb family of proteins is necessary for the differentiation block by the amino terminus of SVLT.

The dependence of the differentiation-blocking function upon the Rb family binding site is in apparent contrast to our previous result (5) that an Rb-binding and transformation-deficient SV40 early region blocked adipocyte differentiation. However, these previous studies did not determine whether other SVLT functions besides Rb binding and transformation were responsible for preventing differentiation or whether SVst was complementing the Rb-binding defect. To distinguish between these two possibilities, we created a 3T3-L1 cell line that expressed SVLT1-708 with the K1 mutation in the absence of SVst (SVLT1-708K1). 3T3-L1 cells expressing SVLT1-708K1 do not accumulate lipid (not shown), do not have high GPD activity (Table 1), and do not express aP2 mRNA (Fig. 6) under culture conditions used to promote differentiation. Therefore, SVLT1-708K1 prevents adipocyte differentiation, demonstrating that a function of full-length SVLT, independent of SVst and Rb binding, blocks differentiation.

SVLT and SVLT1-121 transform 3T3-L1 preadipocytes to anchorage and density independence, but Rb family binding deficient mutants do not. In 3T3-L1 cells, the ability of the N terminus of SVLT to prevent adipocyte differentiation correlates with its ability to transform these cells. SVLT transforms most cell lines to anchorage- and density-independent growth, but transformation by SVLT1-121 is cell line dependent (36). In addition, the ability of a cell to maintain SVLT1-121 expression above a critical threshold may determine whether it will be transformed by this protein (33). To determine whether the ability of SVLT1-121 to block differentiation was related to a transformation function, we determined whether 3T3-L1 cells expressing SVLT1-121 were transformed to anchorage- or density-independent growth.

Both SVLT1-708 and SVLT1-121 transformed 3T3-L1 preadipocytes to anchorage independence. When plated in soft agar suspension in growth medium, L1-PVU0, L1-SVLT1-708, and L1-SVLT1-121 cells formed colonies (Table 1), whereas the L1-Neo control cells did not. In addition, the L1-SVLT1-708 and L1-SVLT1-121 lines did not exhibit the density-dependent growth arrest exhibited by control cells. Five percent or fewer control, neomycin-resistant 3T3-L1 cells cycled through S phase as confluent cultures, as determined by BrdU uptake over 24 h (Table 2). However, 20, 28, and 64% of cells expressing SV40 early proteins, SVLT1-121, and SVLT1-708, respectively, were cycling as confluent cultures in medium containing CS (growth medium). It is curious that a higher percentage of L1-SVLT1-708 than L1-PVU0 cells was labeled in growth medium. The basis for this difference is not clear from these experiments, but a simple explanation is that these were two different experiments using different batches of serum. Higher proportions 61, 68, and 66%, of cells expressing SV40 early proteins, SVLT1-121, and SVLT1-708, respectively, were cycling at confluence in differentiation medium, conditions identical to those immediately prior to induction of differentiation. Therefore, both full-length and truncated SVLT relax anchorage- and density-dependent growth regulation in 3T3-L1 cells.

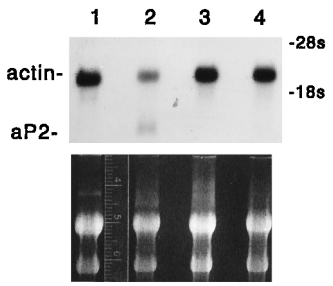

FIG. 4. Wild-type and truncated SVLT block aP2 mRNA expression in 3T3-L1 cells. Total cellular RNA was analyzed by Northern analysis (20 μg per lane) for expression of actin and differentiation-dependent aP2 mRNA. Lanes: 1, RNA prepared from L1-HNeo control preadipocytes prior to induction of differentiation; 2 to 4, cells 7 days after attempted induction of differentiation. Lane 2, L1-HNeo; lane 3, L1-PVU0; lane 4, L1-SVLT1-121. Top, blot probed with actin and aP2 probes, with positions indicated at the left. Migration of 28S and 18S rRNAs is indicated at the right. Bottom, ethidium bromide staining of the gel before transfer to indicate equal loading of lanes.

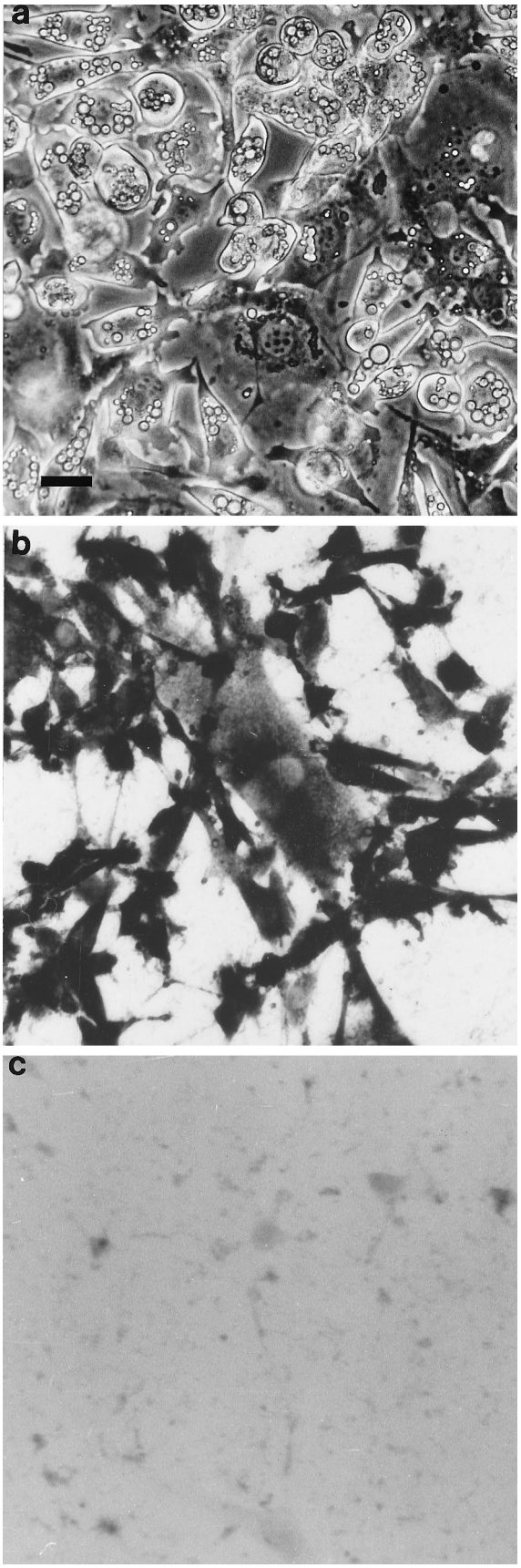

FIG. 5. SVLT1-121K1 does not block adipocyte differentiation despite expression in 100% of the cells. (a) Morphology of L1-SVLT1-121K1 cells after the induction of differentiation. Cells were cultured as for Fig. 1. (b) SVLT1-121K1 expression. Immunocytochemistry using anti-SVLT monoclonal antibody pAB108 followed by alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G was used to detect protein expression. This cell line shows the typical immunostaining pattern for this truncated SVLT, which is throughout the cytoplasm and nucleus (30). (c) L1-ZNeo negative control for immunocytochemistry. Bar = 35 μm.

The Rb-binding mutants of both SVLT1-708 and SVLT1-121 failed to transform 3T3-L1 cells to anchorage- or density-independent growth. L1-SVLT1-708K1 and L1-SVLT1-121K1 cells, like neomycin-resistant controls, did not form colonies in soft agar, consistent with a failure of the K1 mutants to induce anchorage-independent growth (Table 1). Cells expressing SVLT1-708K1 or SVLT1-121K1 arrested growth as efficiently as control cells at high cell density. Only 2 and 7% of L1-SVLT1-121K1 cells and 2 and 0% of L1-SVLT1-708K1 cells incorporated BrdU in growth medium and differentiation medium, respectively, indicating efficient density-dependent growth arrest (Table 2). Thus, the K1 mutation ablated both the anchorage- and density-independent growth phenotype exhibited in 3T3-L1 by SVLT1-708 and by SVLT1-121. This finding indicates that binding to the Rb family of proteins by SVLT is necessary to keep 3T3-L1 cells in the cell division cycle under conditions such that control cells enter G_0. These results also show that the suppression of differentiation by SVLT1-708K1 is independent of transformation.

DISCUSSION

Differentiation in the adipocyte system is blocked by expression of the SV40 early genes (5, 13, 26, 40), but the functions which block differentiation have not been identified. We have expressed mutant SV40 early regions in murine preadipocytes with the long-term goal of identifying individual functions of SVLT which block differentiation. In this work, we demonstrate (i) that one function residing within the N-terminal 121 amino acids of SVLT which blocks differentiation depends upon Rb family binding, (ii) that one or more additional functions of SVLT block differentiation independently of Rb family binding and transformation, and (iii) that all of these functions block differentiation independently of SVst action.

One conclusion that we draw from this work is that multiple SVLT functions independently block adipocyte differentiation. Multiple SVLT functions contribute to tumorigenesis (37),

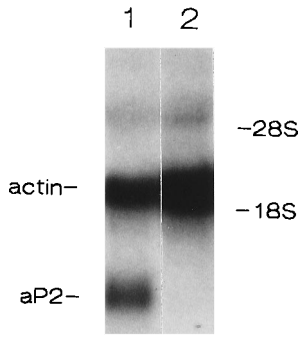

FIG. 6. 3T3-L1 preadipocytes expressing SVLT1-708K1 do not express differentiation-dependent aP2 mRNA. RNA (10 μg per lane) was prepared 7 days after attempted induction of differentiation. Lanes: 1, L1-ZNeo; 2, L1-SVLT1-708K1.

TABLE 2. Density-dependent growth arrest is relaxed in 3T3-L1 cells expressing full-length SVLT and C-terminally truncated SVLT but not in cells expressing Rb-binding-deficient mutants

SVLT expressed	% of cells incorporating BrdU[a]	
	DMEM–10% CS (growth medium)	DMEM–10% FCS–insulin (differentiation medium)
Expt 1		
None (neomycin resistant)	5	3
SV40 early proteins	20	61
SVLT1-121	28	68
Expt 2		
None (neomycin resistant)	2	5
SVLT1-708	64	66
SVLT1-121K1	2	7
Expt 3		
SVLT1-708K1	2	0

[a] Determined by counting at least 300 cells per culture. FCS, fetal calf serum.

transformation (38), and cell cycle progression (10), although the functions required for proliferation and transformation of tissue culture cells are cell line dependent (4, 10, 25, 33, 36). Since interference with differentiation is an important step in multistage carcinogenesis, it is conceivable that the SVLT differentiation-blocking functions which we score in the adipocyte assay correspond to functions which contribute to tumorigenesis or to transformation of tissue culture cells. However, the independent action of the SVLT differentiation-blocking functions are in contrast to the interdependence of functions contributing to transformation and tumorigenesis. By identifying SVLT functions which block differentiation, we will determine whether they correspond to functions which act synergistically in neoplastic transformation and tumorigenesis.

Rb family binding is one of the SVLT functions which block adipocyte differentiation. This is consistent with myogenic differentiation in which SVLT binding to Rb also inhibits differentiation (23). Since SVLT can interact with and alter the functions of at least three Rb family members, Rb, p107, and p130 (9, 11, 14, 43), any one or a combination of these interactions may result in the suppression of differentiation. The identity of the Rb family protein which is the critical SVLT target for blocking differentiation remains to be established. Recent work by Wolf et al. (43) shows that expression of the SV40 early region disrupts E2F-p130 complexes in 3T3-L1 preadipocytes, whereas the K1 SV40 mutant does not. Because the K1 mutant is transformation defective in 3T3-F442A preadipocytes (5) and in 3T3-L1 cells (this work and reference 43), the p130 Rb-related protein may be a critical target of SVLT in preadipocytes for neoplastic transformation. The p130 protein appears to have a differentiation-specific function as well. Shin et al. (32) have shown that p130 forms a muscle differentiation-specific complex with E2F, whereas in undifferentiated cells, E2F complexes with other Rb-related proteins. Thus, p130 may also be a critical target of SVLT in preventing differentiation.

Ablation or alteration of Rb family protein function by SVLT leads to the failure of cells to arrest growth at high density as we have shown in 3T3-L1 preadipocytes. This effect alone may be sufficient to block differentiation, since differentiation and growth arrest are coupled (18, 31). However, at least one additional mechanism blocks differentiation independently of growth arrest, since transformation-defective, full-length SVLT blocks differentiation. Current models of adipocyte differentiation predict not only that differentiation and growth arrest are coupled but also that only adipocyte precursors in a distinct growth arrested state, called G_D by Scott et al. (31), are differentiation competent. Therefore, while an SVLT function which is dependent on Rb family binding may block differentiation by preventing growth arrest, we propose that one or more additional functions, expressed by full-length SVLT but not SVLT1-121, block adipocyte differentiation by preventing entry into a differentiation competent, growth-arrested state. Type beta transforming growth factor, like SVLT1-708K1, also blocks adipocyte differentiation of 3T3-L1 without altering density-dependent growth control (27), suggesting that common mechanisms may be involved.

What SVLT functions block differentiation independently of Rb family binding? Because this differentiation-blocking function is expressed by full-length SVLT but not by SVLT1-121, this function is likely to map outside the N-terminal 121 amino acids, although our experiments to date do not rule out a dependence on N-terminal sites for this Rb-binding-independent function. Our attempts to express the C-terminal portion of SVLT independently of the N terminus in order to directly examine C-terminus-specific functions in preadipocytes have thus far been unsuccessful.

A range of SVLT functions have been mapped to domains outside amino acids 1 to 121, whereas others map to regions overlapping SVLT1-121. Functions which map to regions beyond amino acids 1 to 121 include the adenovirus helper function, ATPase, helicase, viral origin DNA binding, DNA polymerase α binding, p53 binding (reviewed by Fanning and Knippers [16]), and in vitro binding to the transcription-enhancing factor TEF-1 (22). The differentiation-blocking activity of these domains has not been directly addressed except for viral DNA binding. A mutant defective for origin binding was still able to block adipocyte differentiation (5). SVLT complementation of adenovirus E1A defective for p300 binding in Ras cotransformation assays (44) maps to amino acids 17 to 27. TATA-binding protein has been shown to bind in vitro to amino acids 5 to 172 of SVLT (22), and amino acids 1 to 259 (25) or 1 to 147 (34) possess functions capable of inducing DNA synthesis in quiescent cells. Therefore, these functions may be contained within amino acids 1 to 121 and contribute to blocking differentiation.

SVLT functions besides Rb family binding which have been shown to contribute to transformation or tumorigenesis are logical candidates for a differentiation-blocking function. At least one of these functions maps to the C-terminal portion of SVLT. Symonds et al. (37) have shown that full-length SVLT induces more aggressive tumors in transgenic animals than SVLT1-121 as a result of C-terminal functions, one of which is p53 binding. A candidate function which maps within SVLT1-121 but which maps separately from Rb family binding includes complementation of adenovirus E1A defective for p300 binding in Ras cotransformation assays (44). One or both of these SVLT functions may interfere with differentiation without preventing growth arrest.

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