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Induction of Cyclins E and A in Response to Mitogen Removal: a Basic Alteration Associated with the Arrest of Differentiation of C2 Myoblasts Transformed by Simian Virus 40 Large T Antigen

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We previously showed that C2 myoblasts transformed by simian virus 40 large T antigen (SVLT) stop the myogenic process after the induction of myogenin and of high Rb levels; the induced Rb, however, becomes notably phosphorylated. We have analyzed the protein levels and activities of cyclin-dependent kinases (cdks) in untransformed C2 cells and in transformants of either SVLT or the cytoplasmic mutant NKT1 (which permits differentiation) upon a shift from growth medium (GM) to mitogen-poor differentiation medium (DM). After the shift, cdk4 levels remained constant and cdk6 levels decreased in all cell types; cdk2 minimally increased only in SVLT cells. Cyclin D1 was downregulated in DM in all cell types, and cyclin D3 was upregulated (albeit less strongly in SVLT cells than in the others). In contrast, a dramatic difference between SVLT cells and the other cells was observed for cyclins E and A, which essentially disappeared (as protein and RNA) in normal C2 and NKT1 cells upon the shift from GM to DM, whereas they increased in SVLT cells. Concurrently, cdk2 activity ceased in C2 and NKT1 cells in DM, whereas it persisted at 20% of the GM level in SVLT cells. cdk4 activity was detectable in all cells only in GM. Cyclin E and A induction thus appeared to sustain enough Rb phosphorylation to interfere with tissue-specific expression, with cdk activity not high enough to activate cyclin self-regulation. In DM, cdk2 complexed to D3 was underphosphorylated in all cells, and SVLT allowed strong inductions of p21 and p27 without affecting their complexes with cdks.

Egress from the cell cycle is a prerequisite for the terminal differentiation of most cell types; thus, almost inevitably, the growth-promoting activity of oncogenes creates regulatory conflicts in differentiating cells, usually resulting in an early blockade of differentiation (2, 31, 52). The C2 line of mouse myoblasts (86) can be induced to differentiate in culture, with the formation of long multinucleated myotubes, when the cells, after reaching contact, are shifted to a medium with low concentrations of mitogens. In such cells, with the onset of differentiation, the control of cellular events is transferred from growth regulators to muscle-specific regulators (8, 52, 54, 82). Among these, the basic helix-loop-helix transcription factor MyoD (12) is of central importance: its functioning is needed for the induction of Rb (45), which increases in many cell types during differentiation (11, 72), for the induction of the cyclin-dependent kinase (cdk) inhibitor p21 (24, 26, 56), and for the induction of myogenin, a factor which controls the expression of myosin and other tissue-specific genes (28, 30).

The simian virus 40 large T antigen (SVLT), like many other oncogene-encoded proteins (see references 2 and 44 for reviews), when expressed in C2 myoblasts causes the arrest of differentiation (18, 23, 25, 50, 76). We have shown that this arrest occurs only after the MyoD-dependent induction of myogenin and high Rb levels; an important difference from normal C2 cells, however, is that a large part of this increased Rb is hyperphosphorylated (76). Among SVLT mutants, the ability to inhibit myogenesis correlates with capability of promoting Rb hyperphosphorylation but not with that of physically complexing Rb (76).

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Rb is known to be phosphorylated by cdks, whose activities and regulation during the cell cycle are fairly well understood (3, 5, 13, 22, 48, 64, 69, 81). One important effect of Rb hyperphosphorylation is the release of the E2F transcription factors, whose activity is blocked when they are complexed with Rb (9, 16, 47, 66, 68, 74). E2F factors activate the expression of genes necessary for cell proliferation, including genes encoding cyclins and those implicated in DNA synthesis (14, 20, 40, 49, 51, 65). Thus, cdk activity is initially able to upregulate itself, by modifying Rb. Eventually, E2F1 itself becomes a substrate for the high cyclin A-cdk activity (15, 37, 38, 85), and the inactivation of this transcription factor, with the consequent downregulation of cyclins E and A, closes a regulatory loop. cdk inhibitors (p21, p27, and p16) also help to control cdk activity, generally by a mechanism involving the sequestration and release of the inhibitors by different cdks (27, 55, 59, 60, 70, 75, 78, 84).

In C2 transformants, as mentioned above, the growth-stimulating functions of SVLT must counteract the antagonistic effect of a strong Rb induction (76); this appears to involve a mechanism able to hyperphosphorylate Rb even under differentiating conditions. Analysis of the critical components of this mechanism could help to elucidate the balance of controls on which myogenic differentiation depends as well as the mode of SVLT action in other cell types. We thus analyzed the cell levels and activities of the cyclins and cdks known to be active in the G_1 and S phases of the cell cycle and compared them in three cell types: normal C2 cells, C2 cells transformed by wild-type (wt) SVLT (WTSV cells), and C2 cells transformed by SVLT mutant NKT1 (defective for transport to the nucleus), which does not arrest terminal differentiation (NKT1 cells). Both transformants, unlike normal C2 cells, are able to form colonies when plated in soft agar (76).

The principal finding of this study was the strong induction of cyclins E and A that occurred in WTSV cells upon a shift to mitogen-poor medium. Levels of these cyclins actually rose in WTSV cells above the growing-cell level, under conditions causing their disappearance in untransformed C2 and NKT1 cells. This unusual response of WTSV cells, in which an increase of cyclins E and A followed a removal of growth stimuli, was accompanied by partial conservation of cdk2 activity, which did not occur in C2 and NKT1 cells. Thus, Rb phosphorylation could persist (as found), with cdk2 activity presumably not high enough to modify also E2F (and trigger cyclin repression). This potentially self-sustaining regulatory loop in WTSV cells coexisted with minimal or no differences, compared to normal C2 and NKT1 cells, in the behavior of other regulators. In particular, the variations occurring in WTSV cells upon a shift from growing to differentiating conditions, with respect to the cdk2, cdk4, cdk6, and cyclin D1 protein levels, cdk4 activity, and the induction and complexing of p21 and p27, were not significantly different from those in C2 and NKT1 cells; only cyclin D3 was less upregulated in WTSV cells placed in differentiation medium (DM) than in the other cells.

MATERIALS AND METHODS

Cells and cell cultures. All cells were cultured in Dulbecco's medium with 20% fetal calf serum (growth medium [GM]). Cell stocks were carefully passaged before reaching cell-cell contact, to avoid selection. To induce differentiation, 2×10^5 cells seeded in 10-cm-diameter dishes and allowed to grow for 3 days were exposed for 2 days to DM (Dulbecco's medium containing 2% newborn calf serum).

Clone 7 of the C2 line of mouse myoblasts (86) was originally obtained from M. Buckingham and donated to us by P. Amati. The WTSV and NKT1 transformants derived from C2 cells have been previously described (76). Briefly, the WTSV clone expresses normal SVLT (and small t) under the control of the wt viral regulatory elements. The NKT1 clone is transformed by a mutant which differs from wt SVLT only in that it carries five amino acid substitutions in the nuclear localization signal (77). Both clones efficiently promote the formation of colonies in soft agar.

Immunoreactions and Western blotting. The following antibodies were used: anti-T PAb 101 (ATTC culture TIB-117), anti-Rb PMG3-245 (Pharmingen, San Diego, Calif.), anti-myosin heavy chain (MHC) MF20 (4), anti-myogenin IF5D (83), anti-p27 K25020 (Transduction Laboratories, Lexington, Ky.), anti-p27 sc528, anti-MEF2 sc313, anti-cdk2 sc163, anti-cdk4 sc260, anti-cdk6 sc177, anti-cyclin E sc481, anti-cyclin A sc596, anti-cyclin D1 sc450, anti-cyclin D2 sc452, anti-cyclin D3 sc453, anti-p107 sc318 (Santa Cruz Biotechnologies, Santa Cruz, Calif.), and anti-p21 and anti-p130 (kind gifts of C. Schneider and A. Giordano, respectively).

Cell extracts (those not to be immunoprecipitated) were prepared by lysing cells with 0.1% sodium dodecyl sulfate (SDS)–8 M urea–5 mM EDTA–50 mM Tris (pH 6.8), followed by 10-s sonication. Protein concentration was determined by the Bradford quantitation assay, and 200-μg aliquots were then made 2% in SDS and 1% in 2-mercaptoethanol, boiled, and electrophoresed in SDS–6.5 or 10% Laemmli polyacrylamide gels. For immunoprecipitations, extracts were prepared by lysis in a mixture containing 0.1% Tween 20, 5 mM EDTA, 150 mM NaCl, 10 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM β-glycerophosphate, and 20 mM HEPES (pH 7.5), followed by 10-s sonication. After clearing by centrifugation at 20,000 × *g* for 5 min, extracts were assayed for protein concentration as described above; then 500-μg aliquots were incubated (with agitation in the cold) for 3 h with the indicated antibodies and either protein A- or protein G-Sepharose. Pellets were washed three times with lysis buffer; samples were eluted by 2 min of boiling in buffer containing 2% SDS, 7 M urea, 1% 2-mercaptoethanol, and 50 mM Tris (pH 6.8) and electrophoresed as described above.

Western blotting was carried out by semidry electric transfer of gel proteins to polyvinylidene difluoride filters (Amersham), monitored by use of prestained protein markers (Sigma). Filters blocked and exposed to the proper antibodies were then processed for enhanced chemiluminescence (ECL) detection (Boehringer). Records were made on Kodak X-OmatS films.

Indirect immunofluorescence on cultured cells was carried out as previously described (77).

Kinase assays. Kinase activity was assayed with a synthetic peptide as the substrate. This peptide had the sequence NIYISPLKSPYKISE, corresponding to residues 796 to 810 of the murine Rb sequence (6) (residues 803 to 817 of human Rb). The two serines followed by prolines are known to be phosphorylated in vivo (39). Samples were prepared by lysing cells in cold buffer (0.1% Tween 20, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 μg of leupeptin per ml, 50 mM HEPES [pH 7.5], 0.1 mM phenylmethylsulfonyl fluoride), followed by a 10-s sonication and clearing by 2 min of centrifugation at 20,000 × *g*. Supernatants were assayed for protein concentrations as described above; protein samples of 1 mg from growing cultures and 4 mg from cultures kept for 48 h in DM were then immunoprecipitated with either anti-cdk2 or anti-cdk4 antibody and protein A-Sepharose (3 h under agitation in the cold). Samples were washed three times with the buffer described above and once with 100 μl of kinase buffer (2 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 50 mM HEPES [pH 7.5]) and then resuspended with 25 μl of kinase buffer containing 5 μg of substrate peptide and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). Samples were incubated for 60 min at 37°C for cdk2 assays and at 30°C for cdk4 assays, with agitation after 30 min. The reaction was stopped by adding 80 μl of loading buffer (8 M urea, 20 mM EDTA, 0.02% bromophenol blue, 50 mM $NaHCO_3$ [pH 9.8]). The exclusion of ionic detergents and the use of high pH are important to ensure that peptide migration (toward the positive pole) depends on the charge acquired by phosphorylation.

After the resin was removed by centrifugation, half samples were electrophoresed in 1.5-mm-thick, 15-cm-long 20% polyacrylamide gels in 50 mM $NaHCO_3$ (pH 9.8). In this buffer, the gels were also run, at 200 V, until the dye and the nearly comigrating phosphopeptide were at about half length (by this time, all radioactive ATP had segregated to the bottom reservoir, as signaled by a Geiger counter). Gels were fixed for 30 min with 10% glutaraldehyde in 100 mM Na_2HPO_4 (pH 7.2), washed once with 30% isopropanol, left in 30% isopropanol for another 15 min, sealed in plastic envelopes, and exposed to a PhosphorImager apparatus (Molecular Dynamics).

Northern blotting. Northern blotting was carried out by electrophoresing, for each sample, 0.02 mg of total cell RNA, extracted by the guanidinium-phenol method, in 1% agarose–10% formaldehyde gels. The RNA was vacuum blotted to Hybond N+ filters (Amersham) and probed with specific DNA ^{32}P labeled by random priming. Ethidium bromide staining on filters was photographed to monitor the presence of equal rRNA bands. DNA probes were kindly donated for cyclin A mRNA by A. Giordano (21), for cyclin E mRNA by M. Eilers (73), and for cyclin D3 mRNA by C. J. Sherr (34). Hybridization was overnight at 42°C in 50% formamide–5× SSPE (1× SSPE is 180 mM NaCl, 1 mM EDTA, and 10 mM sodium phosphate [pH 7.5]–10× Denhardt solution–2% SDS–0.1 mg of herring sperm DNA per ml–10^6 cpm (10^8 cpm/μg) of labeled probe per ml. Washing was done three times in 0.5× SSPE–0.1% SDS for 20 min at 65°C. Radioactivity was detected with a PhosphorImager.

RESULTS

Properties of the transformants. Some basic properties of the cells analyzed are illustrated in Fig. 1. WTSV and NKT1 cells have transformed phenotypes enabling them to grow in semisolid medium (76). As shown in Fig. 1A, however, WTSV cells exposed to DM cannot differentiate morphologically, whereas NKT1 cells can. From a biochemical point of view, the myogenic process in WTSV cells appears to be arrested after expression of the muscle-specific factor myogenin. Figure 1B shows that (i) in both transformants, the SVLT cell level does not decrease under differentiating conditions; (ii) in both transformants, the induction of Rb, myogenin, and Mef2 is very similar to that occurring in normal C2 cells (an important difference, however, is that WTSV cells in DM hyperphosphorylate a significant fraction of the induced Rb); and (iii) the induction of MHC in WTSV cells occurs at only minimal levels. The incorporation of bromodeoxyuridine in WTSV cells drops after 24 to 48 h in DM (as it does in C2 and NKT1 cells) and becomes negligible thereafter; at least 90% of the cells remain viable, however, as indicated by cell replating and trypan blue exclusion (data not shown).

Cell levels of cyclins and cdks. Searching for key deviations from the normal processes in WTSV cells leading to the differentiative arrest, we examined the levels and activities of cdks. Attention was focused on cyclins and cdks involved in the G_1-S transition of the cell cycle, normally inactivated after myoblasts exit from the cycle and the onset of differentiation (26, 33, 50, 80). Figure 2A shows the changes in cdk2 levels observed in untransformed C2, WTSV, and NKT1 cells when they were shifted from growing to differentiation conditions. It can be seen that under growth conditions, the cdk2 content was very similar in the three cell types and about equally divided between the active (fast-migrating) and inactive (slow-migrat-

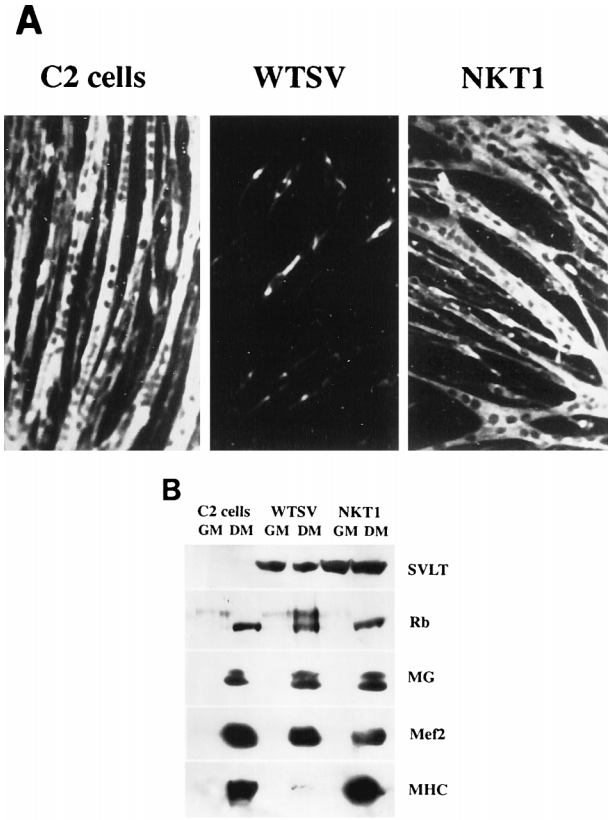

FIG. 1. (A) Immunofluorescent staining with anti-MHC antibody of cell cultures 2 days after transfer to DM. Magnification, ×160. (B) Western blots of the indicated proteins were obtained by electrophoresing in SDS–6.5% polyacrylamide gels equal protein samples of total extracts from cells harvested either during active growth (GM) or after exposure for 2 days to DM. ECL detection was performed with the specific antibodies listed in Materials and Methods. MG, myogenin.

ing) forms (61). After 48 h in DM, the overall cdk2 level appeared to be slightly decreased in both C2 and NKT1 cells and moderately increased in WTSV cells; more significantly, in C2 and NKT1 cells only the inactive form of cdk2 was present, whereas in WTSV cells the active form persisted.

Cyclins E and A, the main regulatory subunits of cdk2, behaved very similarly to one another. Shifting the cells from GM to DM resulted in the disappearance of both cyclins in C2 cells and in their near-disappearance in NKT1 cells. In striking contrast, when WTSV cells were shifted to DM, levels of cyclins E and A actually increased over the growing-cell level (Fig. 2A). As shown below, the augmented protein content was also accompanied by an augmented mRNA content.

Figure 2B illustrates the cell levels of cdk4, cdk6, cyclin D1, and cyclin D3. In all cell types, after the shift to DM, cdk4 remained essentially unchanged and cdk6 decreased; cyclin D1 was downregulated, and cyclin D3 was induced. The cyclin D1 level in WTSV cells was somewhat lower than in the other cells even during growth (Fig. 2B); presumably this was due to a repression of cyclin D1 by SVLT (41, 58). Cyclin D3 strongly increased when C2 cells were exposed to DM, as observed by others (36, 62, 63). The cyclin D3 increase was smaller in WTSV cells (and also, though not to as great an extent, in NKT1 cells), especially after 24 h in DM (Fig. 2B). This, however, did not reflect a general delay of induction in WTSV cells, since parallel examination of myogenin induction showed that it did not differ in the three cell types. Conceivably, SVLT may antagonize some auxiliary role played by cyclin D3 in differentiating C2 cells; a possible role in arresting cdk2 activity is suggested by the exclusively unphosphorylated form of cdk2 found associated with cyclin D3 (see below).

We investigated the possibility that the increase of cyclins E and A occurring in WTSV cells in DM, and that of cyclin D3 occurring in all cells in DM, might be due solely to diminished protein turnover, without an increase of transcripts. The Northern analysis clearly indicated, however, that also mRNA was augmented in these three cases (Fig. 3).

cdk2 and cdk4 activities. Figure 4 shows the activities of cdk2 and cdk4, whose protein levels did not decline during differentiation. After the shift from growing to differentiating conditions, the cdk2 activity in C2 and NKT1 cells almost totally disappeared. In contrast, the activity in WTSV cells persisted, at a level about one-fifth of that of cells in growing conditions. These results strongly suggested that the large induction of cyclins E and A shown above was instrumental in preventing the arrest of cdk2 activity in WTSV cells under differentiating conditions, despite the normal induction of cdk inhibitors (shown below). Cyclin binding is known to facilitate phosphorylation and hinder dephosphorylation of cdk2-activating sites (48, 61).

cdk4 did not appear to contribute to the altered phosphorylating activity of WTSV cells in DM, since cdk4 activity could be detected only under growing conditions, with no difference in DM among the three cell types.

Levels and complexes of p21 and p27. We also examined the cell levels and complexes of cdks inhibitors p21 and p27. Figure

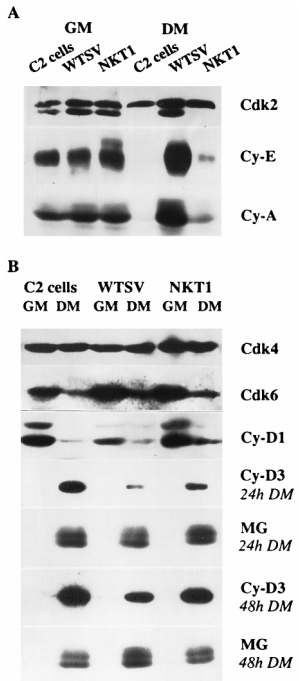

FIG. 2. Cell levels of cyclins and cdks. (A) Western blots of the indicated proteins, extracted from cells either growing (GM) or exposed for 48 h to DM. Equal protein samples (see Materials and Methods) were electrophoresed in 10% polyacrylamide gels; those of cyclin A were aliquots directly from extracts; those of cdk2 and cyclin E were first immunoprecipitated with specific antibodies as detailed in Materials and Methods. (B) Western blots of the indicated proteins, obtained by direct electrophoresis of extract aliquots as described above. Cyclin D3 and myogenin inductions were also examined after 24 h in DM. Blotted filters were processed for ECL. Cy-E, -A, -D1, and -D3, cyclins E, A, D1, and D3, respectively; MG, myogenin.

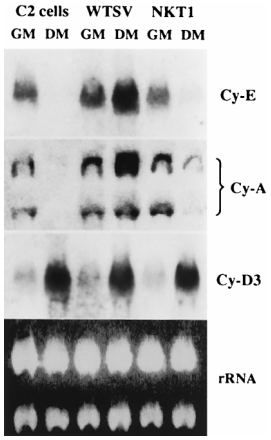

FIG. 3. Northern blots of cyclin E, A, and D3 mRNAs. Twenty micrograms of total cell RNA was extracted, electrophoresed, blotted, and hybridized with ^{32}P-labeled specific probes as detailed in Materials and Methods. Radioactivity was detected with a PhosphorImager. Ethidium bromide staining of rRNA on filters was photographed with UV light. Shown are results for growing cells (GM) and cells kept for 48 h in DM.

5A shows that both p21 (as already reported by others [24, 26, 56]) and p27 were clearly induced under differentiating conditions, without significant differences between the WTSV cell level and the C2 and NKT1 cell levels. An analysis of the association of p21 and p27 with cdk2 and cdk4 was carried out, since the physical binding is known to be critical for regulation by these inhibitors. In particular, we wanted to establish whether the cdk2 activity observed to persist in WTSV cells in DM was facilitated by a hindrance of the association with the inhibitors. The results displayed in Fig. 5B showed that this was not the case. cdk2 from WTSV cells appeared to be complexed with somewhat less p27 than the cdk2 from the other cells but also to be complexed with notably more p21 than the cdk2 from the other cells. This imbalance might only reflect a competition by the two inhibitors for the same sites; it can also be noted that cdk4 exhibited a similar pattern of association with p21 and p27.

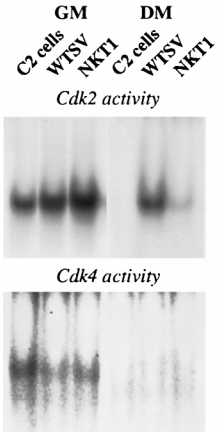

FIG. 4. cdk2 and cdk4 activity assays. The assays were performed with the kinases immunoprecipitated by specific antibodies as detailed in Materials and Methods, using a synthetic peptide (amino acids 796 to 810 of mouse Rb) as a substrate. The ^{32}P-labeled peptide was then separated in a 20% polyacrylamide gel, and radioactivity was detected with a PhosphorImager. Bands quantitation, by the same apparatus, was as follows (in arbitrary units): cdk2 activity, 10,171, 12,224, and 15,928 in GM and 135, 10,056, and 3,225 in DM; cdk4 activity, 1,812, 981, and 1,254 in GM and 15, 27, and 64 in DM. The DM samples were obtained from cell extracts fourfold more concentrated than GM samples, as detailed in Materials and Methods.

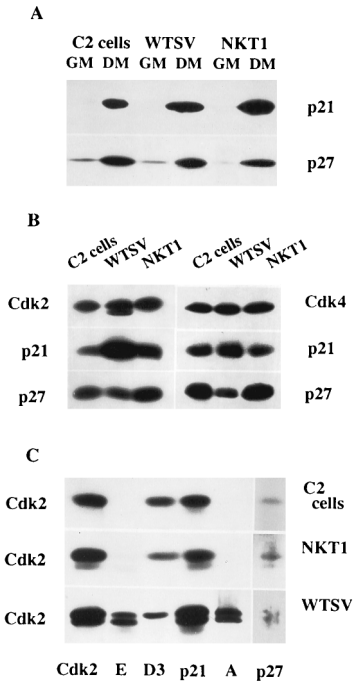

FIG. 5. (A) Total cell levels of cdk inhibitors p21 and p27. Western blots (after electrophoresis in 10% polyacrylamide gels) of extracts from cultures either growing (GM) or exposed for 48 h to DM were probed with specific antibodies and processed for ECL as detailed in Materials and Methods. (B) Complexes of p21 and p27 with cdk2 and cdk4. Anti-cdk2 and anti-cdk4 immunoprecipitates from the indicated cells kept for 48 h in DM were electrophoresed and blotted as described above; the filters were then sequentially probed with anti-cdk2, anti-p21, and anti-p27 (left panel) or anti-cdk4, anti-p21, and anti-p27 (right panel) and processed for ECL. (C) cdk2 forms coimmunoprecipitated by different complexes. Extracts from the three cell types (indicated on the right), kept for 48 h in DM, were immunoprecipitated with antibodies against the proteins indicated at the bottom; Western blots were then probed with anti-cdk2 antibodies and processed for ECL. The faster-migrating band is considered the active form (see the text).

Active and inactive cdk2 forms in complexes. The active and inactive forms of cdk2 can be electrophoretically distinguished (61); therefore, we investigated whether the proportion between the two forms changed in different complexes formed by cdk2 in cells in DM. Equal amounts of cell extracts were immunoprecipitated with antibodies specific for cdk2, cyclin E, cyclin A, cyclin D3, p21, and p27; Western blots were then probed for cdk2. The results are displayed in Fig. 5C. In C2 cells, cdk2 (which, as expected, was not precipitated by anti-cyclin E and A antibodies) was essentially all in the slow-migrating inactive form; only trace amounts of the fast-migrating active form were visible in the immunoprecipitates with anti-cdk2 and anti-p21. A similar pattern, except for slightly higher amounts of the active form, was observed in NKT1 cells. In contrast, in WTSV cells, the coprecipitates obtained with anti-cyclin E, anti-cyclin A, and anti-p21 antibodies contained the two cdk2 bands in about equal proportions, as in the precipitate with anti-cdk2 itself. Two equal bands could also be detected in the sample treated with anti-p27, though small amounts of cdk2 were coprecipitated by this antibody in all three cell types. Since not all antibodies work with equal efficiency in various applications, this did not necessarily indicate a special rarity of the p27-cdk2 complexes (which were easily

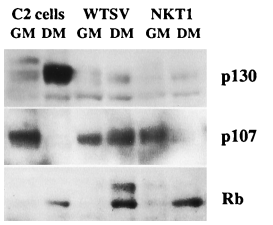

FIG. 6. Cell level variations of the Rb family proteins upon a shift to differentiating conditions. Equal protein samples (see Materials and Methods) from extracts of cells either growing (GM) or placed for 48 h in DM were electrophoresed in SDS–6.5% polyacrylamide gels, blotted, and processed for ECL detection with antibodies specific for the indicated proteins.

detected by coprecipitating p27 with an anti-cdk2 antibody, as shown in Fig. 5B).

Only one of the coprecipitates in Fig. 5C exhibited a single form of cdk2 even in WTSV cells (the hypophosphorylated inactive form); this form was obtained with anti-cyclin D3. This result suggests that the cyclin D3 accumulation occurring in normal cells in DM may facilitate the arrest of cdk2 activity, by hindering phosphorylation of this protein.

p130 and p107. Figure 6 illustrates the changes occurring during differentiation in the cell levels of the Rb family members p130 and p107. Levels of p130 increased dramatically in normal C2 cells shifted to DM but not in either WTSV or NKT1 cells. In view of the normal myotube formation by NKT1 cells, this result suggests that p130 induction is not essential for terminal C2 differentiation. p107 was downregulated in both normal C2 and NKT1 cells in DM but not in WTSV cells. A likely possibility is that the Rb neutralization by phosphorylation occurring in WTSV cells in DM had an effect similar to that of Rb deletions in preventing p107 repression (67).

DISCUSSION

We looked for early alterations caused by SVLT in the series of events leading myoblasts from cycling arrest to full differentiation. We had previously shown that the inhibition of myogenic differentiation by SVLT is not as severe as that by many other oncogene-encoded proteins; neither the expression nor some basic functions of MyoD are blocked, myogenin expression is normal, and the rise of the Rb cell level, typical of myoblasts and other cells during differentiation, is not prevented. We had also found that a notable portion of this induced Rb is hyperphosphorylated (76). This finding suggested that a target of SVLT interference with C2 differentiation might be the regulatory loop in which the Rb family proteins control E2F activity, which regulates cyclins E and A expression, on which Rb phosphorylation depends.

cdk2 activity can be self-sustaining through the induction of cyclins E and A. The present results show that in myoblasts transformed by wt SVLT, part of the cdk2 activity persists under differentiating conditions, unlike in normal myoblasts. Whereas the cdk2 cell level is not significantly augmented, a large increase of cyclin E and A levels occurs upon mitogen removal, and this represents the most important alteration observed in WTSV cells. The striking aspect is that in DM, the level of these cyclins in WTSV cells is not only higher than that in untransformed C2 cells (or NKT1 transformants), in which levels fall near zero, but notably higher than the WTSV cell level under growing conditions.

Transcription of the cyclin E gene is known to depend on E2F1 activity (51); cyclin A (14) has also been found to be transactivated by E2F1, whose activity is tightly controlled by two mechanisms. The first is the formation of inactive E2F1-underphosphorylated Rb complexes; recently, these complexes have been observed on the cyclin E promoter itself (20). The second is E2F1 phosphorylation by cyclin A-cdk2, which downregulates both the DNA-binding and the transactivating abilities of E2F1 (15, 37, 57, 85). Since cyclins E- and A-cdk2 are preeminent Rb-phosphorylating kinases, a self-controlling loop is thus established. Our WTSV cells, after 48 h in DM, display an increased but partially phosphorylated Rb, a normal rise of cdk inhibitors, a strong induction of cyclins E and A, and a partial activity of cdk2. These findings suggest that a new, self-sustaining loop has taken place, with cdk2 active enough to keep a sizable fraction of Rb hyperphosphorylated but not so active as to directly inactivate E2F. In the absence of these two negative controls, cyclins E and A should be allowed to strongly increase, as observed in WTSV cells in DM, thus ensuring some cdk2 activity even in the presence of cdk inhibitors. Before cells reach this steady-state situation, it is necessary that just after the shift to DM, some SVLT function alter normal C2 cell events, preventing the complete loss of cdk2 activity. No transactivation of cyclin genes by SVLT was detected in growing myoblasts by our Western and Northern blot analyses, but an effect too small to clearly emerge cannot be excluded. SVLT can complex with, besides Rb, cyclin-cdk2 (reference 1 and our unpublished data), with an unknown but likely positive effect on its activity. The important consideration is that any mode of initially preventing the disappearance of cdk2 activity (the mechanism to counter the effect of the increase in the level of Rb) would then make it possible to establish the observed steady-state situation. It seems also worth noting that the alterations found in $Rb^{-/-}$ cells include an overexpression of cyclin E (29).

Negative cdk regulation during differentiation by cyclin D3 and cdk inhibitors. As reported by others (36, 62, 63), the level of cyclin D3 increases considerably in untransformed C2 cells upon exposure to DM. The function of this increase, partially inhibited in WTSV cells, remains unclear, though its occurrence during normal myogenesis suggests that it has some antiproliferative role. Interestingly, one of the findings of the present study was that the cyclin D3-cdk2 complexes of WTSV cells in DM contain only the inactive (unphosphorylated) form of cdk2. This is not likely to have a large effect on the overall cdk2 activity of WTSV cells, because the high levels of cyclins E and A should help to keep this protein phosphorylated (48, 61). It can be a clue, however, to the role of cyclin D3 induction in C2 cells: if cdk2 molecules bound to D3 remain unphosphorylated, this could hasten the arrest of cdk2 activity when normal cells begin to differentiate. Minimal or no activity has been found in D3-cdk complexes from L6 myotubes; potentially, D3 binding to Rb family proteins might also hinder their phosphorylation (35, 36).

Previous reports have shown that cyclin D1 is downregulated in cells expressing SVLT (41, 58). This is probably why the cyclin D1 level was somewhat lower also in growing WTSV cells than in the other cells. The finding of a further decrease of the cyclin D1 content under differentiation conditions, together with undetectable cdk4 activity, suggests that cyclin D1 plays no important role in the inhibition of C2 differentiation by SVLT. Cyclin D2 was not detected at any significant levels in either normal or transformed C2 cells (our unpublished observations).

The cdk inhibitor p21 is known to be induced during myoblast differentiation (24, 26, 56); the present results show that also the related inhibitor p27 is induced in differentiating C2 cells. We found no evidence, however, that the p21 and p27 inhibitors were critical in the mechanism of SVLT interference

with C2 differentiation. The results showed that p21 and p27 were induced in WTSV cells upon a shift to DM just as they were in the other cell types. Both inhibitors were also found associated with cdk2 and cdk4, with rather similar patterns, except that WTSV cells exhibited more complexes of p21, and less of p27, with both cdks than the two other cell types. The binding of p21 to cdks is facilitated when the cdks are complexed with cyclins (27), which have higher concentration in WTSV cells than in the other cells. The structurally related p27 (59) probably binds to the same sites as p21; therefore, an effect of mere competition is the simplest way of interpreting this high p21/p27 ratio. More direct destabilizing effects by SVLT on p27 complexes are not excluded, however, by analogy with an ability of this type recently described for E1A (43).

Phenotypic variations among myoblast transformants. Other studies have recently investigated the interference of SVLT with myoblast differentiation. Primary rat myoblasts immortalized by SVLT were still able to form myotubes (32), whereas SVLT in an L8 rat cell clone inhibited (to different degrees) the expression of muscle-specific genes (25). Several investigations of mouse myoblasts have made use of the cell clone C2Ts11 (a C2C12 transformant containing an inducible, thermosensitive SVLT mutant) and have studied the effect of SVLT induction in myotubes preformed in the absence of SVLT expression (18, 19, 23, 50, 80). In such myotubes, SVLT induction and a shift to GM, though neither one alone, repressed expression of MyoD, myogenin, and Rb (18, 19, 80) and induced several genes indicating a reentry in the cell cycle: c-*jun*, *cdc2*, *cdk2*, and the genes encoding cyclin A, cyclin B, and cyclin D1. The basic difference between C2Ts11 cells and our WTSV cells is that MyoD expression is repressed by SVLT in the former but not in the latter. Since the inductions of myogenin, Rb, and p21 in C2 cells depend on MyoD activity (26, 30, 45, 54, 56), the properties of these two cell types are not directly comparable. Unequal sensitivity of MyoD expression to SVLT in different clones should not be surprising: we have observed that among C2 (clone 7) transformants of SVLT, all colonizing in soft agar, MyoD repression occurred in about 1 of 10 clones, which suggests that phenotypes obtainable upon clone selection are not exclusively dependent on the oncogene.

In C2 myoblasts transformed by polyomavirus LT antigen, the expression of MyoD has been found to be unaltered and that of myogenin to be partially inhibited (42). In such cells, the levels of cyclins E and A remain elevated in DM; interestingly, however, considerable apoptosis is induced (41a).

Different roles of Rb family proteins in myoblast differentiation. During growth, Rb acts as a signal integrator, coordinating the expression of particular sets of genes with the phases of the cell cycle (81). In cells undergoing differentiation, the generally observed Rb increase (11, 72) appears to have a role not only in strong negative regulation of cell cycle genes but also in positive regulation. This is suggested during myogenesis by specific Rb properties: there is some evidence that the association of MyoD and myogenin with underphosphorylated Rb helps to stabilize these factors on E boxes (23). On the other hand, not all muscle-specific genes primarily depend on E boxes for expression; in particular, the transcription of myogenin (which is not altered in WTSV cells) has been shown to be mainly Mef2 site dependent in C2 myoblasts (7, 17, 53).

Among the other Rb family members, p107 has been shown to be repressed during the differentiation of both murine C2 cells (67) and L6 rat myoblasts (35). p130 (10, 46, 79), however, exhibited in differentiating L6 cells an increase fourfold larger than that of Rb and properties expected for a differentiation-specific regulator of E2F activity (35, 71). In our cells exposed to DM, p107 decreased in C2 and NKT1 cells but not in WTSV cells. Since a similar p107 persistence in DM has been observed in Rb-deficient mouse myoblasts (67), a likely possibility is that in WTSV cells, the Rb-neutralizing effect due to Rb phosphorylation blocked the mechanism of p107 repression. A marked p130 induction was observed in C2 cells during differentiation, which was absent in both WTSV and NKT1 cells. In view of the fact that NKT1 cells are able to form myotubes, the induction of p130 does not appear to be absolutely required by C2 cells for terminal differentiation.

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