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REGULATION OF CYCLINS AND p34^{CDC2} EXPRESSION DURING TERMINAL DIFFERENTIATION OF C2C12 MYOCYTES

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SUMMARY : Little is known about the expression of cell cycle regulatory genes upon terminal differentiation of skeletal muscle cells. In this report, we demonstrate that the expressions of cyclin A, cyclin D1 and p34^{cdc2} are downregulated upon C2C12 myocytes differentiation and are not inducible in differentiated myotubes. SV40 large T antigen can induce cell cycle entry of myotubes through its induction of these genes' expressions and pRB phosphorylation as well as its suppression of Rb expression. These results provide the first direct evidence that the irreversible downregulation of cyclins and cyclin-dependent kinases is one mechanism for the permanent cell cycle withdrawal of myotubes.

INTRODUCTION : Upon terminal differentiation, cultured skeletal muscle cells withdraw irreversibly from the cell cycle (1) and express a set of muscle specific structural proteins (2). Differentiated myotubes will not re-enter the cell cycle upon serum stimulation. The mechanism for this permanent cell cycle withdrawal has not been well understood. Recent works from our group have shown that SV40 large T antigen (SV40-T) can reverse the terminal differentiation skeletal muscle cells (3, 4, 5). In those studies, the mouse myoblast cell line C2C12 (C2) has been stably transfected with a temperature sensitive mutant of SV40-T gene under the control of mouse metallothionein-I (Mt-I) promoter. The resulting cell line (Ts11) will only express an active SV40-T at the permissive temperature (33°C) and in the presence of Zinc. Ts11 cells can undergo terminal differentiation at non-permissive temperature (39°C). However, when SV40-T was induced, Ts11 myotubes could re-enter the cell cycle in high

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serum medium. This cell line hence provids a useful model for studying the mechanisms by which myotubes permanently withdraw from the cell cycle. With this purpose, we investigated the expression patterns of cyclin A, cyclin D1 and $p34^{cdc2}$ during C2 and Ts11 myoblasts differentiaiton. The data obtained from these two cell lines were compared. Our results provide important clues on the molecular mechanisms for both terminal differentiation and SV40-T function.

MATERIALS AND METHODS

- **Cell cultures** : Mouse myoblast cell line C2 and Ts11 were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Growth medium). Cell differentiation was initiated by shifting 70% confluent cells to DMEM medium supplemented with 2% horse serum (Differentiation medium) for 2 days at non-permissive temperature (39°C). At this point, the cells were changed to growth medium with 6µg/ml Cytosine ß-D-arabino-furanoside (Ara-C, Sigma) and cultured at 39°C for 2 days to kill uncommitted myoblasts. The cells were then cultured for an additional 3 days in differentiation medium at 39°C for the cells to fully differentiate and fuse. The resulting pure myotubes were then shifted to growth medium with 100µM of $Zn^{++}SO4$ and cultured at permissive temperature (33°C). Cells were collected at different time points during this process and subjected to Northern and immunoblotting analysis.
- **RNA preparation and analysis** : Total cytoplasmic RNAs were isolated from cultured cells by guanidinium isothiocyanate/CsCl procedure (6). Twenty µg RNA from each sample was separated on 1% agarose-formaldehyde gel and transferred to nylon membrane (Micron Separations Inc.). The membranes were hybridized with [^{32}p]CTP radio-labeled cDNA fragments. The cDNA fragments used for probes are as following: the 1.6 kb EcoRI fragment of human cyclin A cDNA (7); the 1.0 kb BamHI fragment of mouse cyclin D1 cDNA (8); the 1.1 kb EcoRI fragment of mouse cdc2 cDNA (9); the 4.7 kb BamHI fragment of human Rb cDNA (10).
- **Immunoblotting analysis** : Whole cell extract was prepared as described (11). Briefly, cells were incubated for 2 hours in 1.5 volume of lysis buffer (50mM This, pH7.6, 250mM NaCl, 5mM EDTA, 0.1% Nonidet P-40) at 4°C. After 15 minutes centrifugation at 13,000, The clear lysate was transferred to a new eppendhorf tube and protein concentration was determined by Bradford method (Bio-Rad). For Western analysis, 40µg cell extract from each sample was separated on SDS/PAGE gel and transferred to Immobilom membrane (Millipore) by Semiphor transfer (Hoefer). The proteins were detected by the enhanced chemiluminescence (ECL) system with procedure recommended by the manufacturer (Amersham). Purified monoclonal antibody against the human Rb protein was purchased from Pharmigen (clone PMG3-245, 14001A).
- **Histone H1 kinase assay** : H1 kinase assays were performed as described (11, 12). Briefly, 5µg cell extract was rocked with p13-agarose

beads (Oncogene Science) at 4°C for 2 hours. The beads were then washed twice with 1 ml cell lysis buffer and twice with 1 ml kinase buffer (50mM Tris, pH8.0, 10mM $MgCl_2$ and 1mM dithiothreitol), and used immediately for H1 kinase assay. The kinase reactions were carried out in 50µl of kinase buffer with 100µg/ml of histone H1, 50µM ATP and 10µCi of [^{32}p]ATP. After 30 minutes incubation at 30°C, the reactions were stopped by adding an equal volume of 2x sample buffer and aliquots were analyzed by 12% SDS-PAGE electrophoresis and autoradiography. After autoradiography, gel slices were excised and their radioactivities were measured by Liquid Scintillation Counting in the presence of Econofluor-2 (Du Pont).

RESULTS : We investigated the expression pattern of cyclin A, cyclin D1 and cdc2 during C2 and Ts11 myoblasts differentiation by Northern blot analysis (Fig.1). The mRNA level for cyclin A, cyclin D1 and p34^{cdc2} genes decreased slowly upon myogenesis in both C2 and Ts11 cells (Fig. 1, lane 1,2,3 and 8,9,10). Myotubes contained very low level mRNA for these genes (Fig. 1, lane 4 and 11). When C2 myotubes were shifted to high serum medium, the mRNA levels of these genes were not significantly changed (Fig. 1, lane 5, 6 and 7). However, when Ts11 myotubes were induced for SV40-T expression and stimulated with serum, the expression of cyclin A, cyclin D1 and cdc2 was induced. The increase of cyclin D1 mRNA level appeared first, reaching its peak at 12 hours after the induction of SV 40 T antigen (Fig. 1, lane 12). This is consistence with cyclin D1's role in early G1 phase of the cell cycle. The mRNA levels of cyclin A and cdc2 were also increased at 12 hours, but the maximum expression appeared at 20 hours after SV40-T induction (lane 13 and 14 of Fig. 1). The maximum induction of cyclin A and cdc2 mRNA correlates with the time of S phase entry in Ts11 myotubes (4).

To determine if the changes in cyclin and cdc2 mRNA levels reflecting the fluctuation of cyclin-dependent kinase activity, we measured the p13-associated H1 kinase activity in C2 and Ts11 cells (Fig. 2). P13^{suc1} is a yeast protein which specifically binds to most cdk proteins. Thus, p13-associated H1 kinase can be served as an indicator for overall cyclin-dependent kinase activity (13). P13-associated H1 kinase activity was very low in myotubes. This enzyme activity was not increased in C2 myotubes stimulated by serum. In Ts11 myotubes, however, the p13 associated H1 kinase activity was stimulated after addition of serum and the induction of SV40-T. The enzyme activity reached the highest level at 20 hours after large T induction and remained the same at 30 hours. Again, the time period with highest p13 associated H1 kinase activity correlates well with the time of S phase entry in Ts11 myotubes after SV40-T expression and serum stimulation (4, 5).

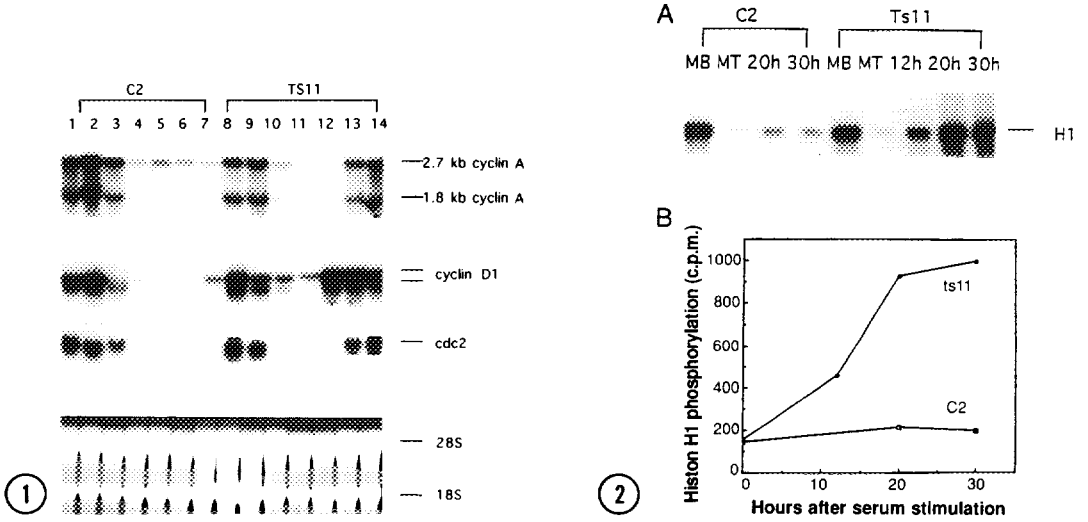

Figure 1. Cyclin A, cyclin D1 and cdc2 mRNA levels in C2 and Ts11 cells. Total RNA (20μg) from each sample was separated on 1% agarose-formadehyde gel and analyzed by Northern blot with probes to cyclin A, cyclin D1 and cdc2. Ethidium bromide stain of RNA loading is shown at the bottom. 1 and 8: myoblasts; 2 and 9: 12 hours in differentiation medium; 3 and 10: 24 hours in differentiation medium; 4 and 11: Myotubes; 5 and 12: myotubes stimulated with serum and large T expression for 12 hours; 6 and 13: myotubes stimulated with serum and T expression for 20 hours; 7 and 14: myotubes stimulated with serum and large T expression for 30 hours.

Figure 2. P13-associated H1 kinase activities. (A) p13-associated Histon H1 kinase assay was performed with whole cell extracts from different time points as indicated. MB: myoblasts; MT: myotubes; 12h, 20h and 30h: myotubes stimulated with serum and induced for SV40-T expression for 12 hours, 20 hours and 30 hours, respectively. The kinase reaction products were separated on 12% SDS-acrylamide gel and histon H1 phosphorylation was visualized by autoradiography. (B) ^{32}p incorporation into histone H1 was estimated by cutting each band from the gel and counting on a liquid scintilater.

The retinoblastoma gene (Rb) product functions as a repressor for cell cycle progress at G1 phase (14). It is also required for skeletal muscle cell differentiation (4). Thus, we investigated Rb expression pattern in C2 and Ts11 cells by Northern blot (Fig. 3A) and immunoblotting analysis (Fig. 3B). Rb mRNA level was dramatically induced upon C2 and Ts11 myoblasts differentiation. This result is consistent with its importance in muscle cell differentiation(4, 15). Serum stimulation of C2 myotubes did not change Rb mRNA level. In Ts11 myotubes, however, Rb mRNA level was reduced after the induction of SV40 large T antigen.

On immunoblot analysis, Rb protein levels did not change during muscle cell differentiation (Fig. 3B). However, the phosphorylation status of

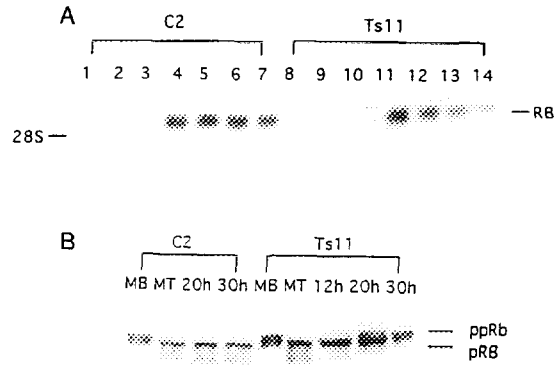

Figure 3. (A) Northern blot analysis of Rb mRNA levels in C2 and Ts11 cells. Total RNAs from each time point as in Fig. 1 were subjected to Northern analysis for Rb expression. SV40 large T antigen expression in Ts11 myotubes repressed Rb transcription. **(B) Immunoblotting analysis of pRb protein.** Whole cell extracts of C2 and Ts11 cells from each time point as in Fig. 2 were subjected to immunoblotting analysis with anti-pRb antibody. pRb phosphorylation was induced by the expression of SV40 large T antigen.

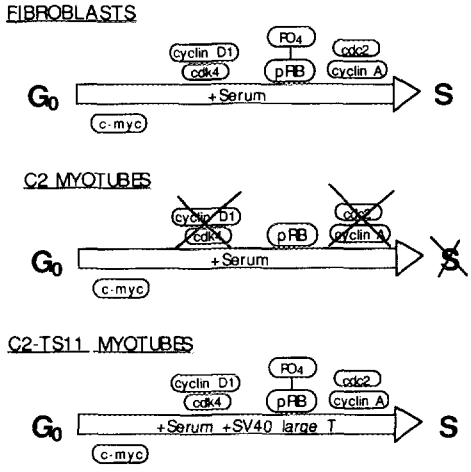

Figure 4. Summary of the cell cycle events in C2 and Ts11 myotubes. Serum stimulation of quiescent fibroblasts induces cyclins and cdk's expression and cell cycle entry. C-myc is still inducible in terminally differentiated myotubes. However, serum stimulation does not induce the expression of cyclin A, cyclin D1 and $p34^{cdc2}$ in myotubes, pRb protein remains unphosphorylated and DNA synthesis will not be re-initiated. The expression of SV40 large T antigen in Ts11 myotubes can induce cyclins and cdk's expression and pRb phosphorylation and also repress Rb expression. It can thus induce cell cycle entry in myotubes.

pRb changed. In replicating myoblasts, the majority of Rb proteins were in phosphorylated state. In differentiated myotubes, Rb proteins remained largely unphosphorylated. Serum stimulation of C2 myotubes did not cause phosphorylation of Rb proteins. In Ts11 myotubes, pRb was gradually phosphorylated after SV40-T induction. Taken together, these results demonstrated that SV40 large T antigen can suppress Rb expression and induce pRb phosphorylation in myotubes, probably by the induction of cyclin-dependent kinase activities. It is thus able induce cell cycle entry in terminally differentiated myotubes.

DISCUSSION : In this study, we compared the expression patterns of cyclin A, cyclin D1 and $p34^{cdc2}$ in C2 myotubes and C2 myotubes expressing SV40 large T antigen. Our data indicate that the inability to reexpress these cell cycle regulatory genes correlates with the permanent cell cycle withdrawal in terminal differentiated myotubes. Figure 4 summarizes these results. Serum stimulation of quiescent fibroblasts induces the expression of a set of growth related proteins, including c-myc, ras, cyclins and cdk's. Rb protein is then phosphorylated and the cells enter the cell cycle. In terminally differentiated myotubes, *c-myc* is still serum inducible (16), implicating that the signal transduction passway of mitogen stimulation remains functional. However, the expression of cyclin A, cyclin D1 and cdc2 is not induced by serum in myotubes. Rb protein remains unphosphorylated in serum stimulated myotubes and the cells will not enter the cell cycle. These data indicate that the failure to reexpress cyclins and cdk's represents one of the mechanisms for the permanent cell cycle withdrawal of myotubes. This situation is very similar to that observed in senescent fibroblasts (17, 18, 19). Thus, our results suggest a common mechanism of cell cycle regulation between the states of terminal differentiation and cellular senescent.

While this manuscript is in preparation, Jahn et al., reported that cyclin D1 and cdc2 are still inducible in differentiated myotubes (20). In that experiment, however, the C2C12 cells were cultured in differentiation medium for only 48 hours and were not treated with Ara-c. Our experiences indicate that undifferentiated myoblasts are abundant (30 to 50%) in that culture condition. Therefor, we believe our results represent the situation in terminally differentiated myotubes.

It has been shown that SV40 large T antigen can activate cyclin A and cdc2 expression in fibroblasts (21). We demonstrate here that it can also induce the expression of cyclinA, cyclin D1 and $p34^{cdc2}$ in myotubes. This indicates that the gene structures of cyclin A, cyclin D1 and $p34^{cdc2}$ are not altered upon skeletal muscle cell differentiation. Thus, defects in

the transcription initiation machinery may be responsible for the failure to re-express these genes in myotubes. We have shown that SV40-T can repress Rb expression and induce pRb phsophorylation in myotubes. Thus, our results provide insight on the mechanisms by which SV40-T reverse the terminal differentiation of myotubes.

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