Inhibition of Mitogenesis in Balb/c-3T3 Cells by Trichostatin A

MUTIPLE ALTERATIONS IN THE INDUCTION AND ACTIVATION OF CYCLIN-CYCLIN-DEPENDENT KINASE COMPLEXES*

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Trichostatin A (TSA), a global repressor of histone deacetylase activity, inhibits the proliferation of a number of cell types. However, the identification of the mechanisms underlying TSA-mediated growth arrests has remained elusive. In order to resolve in more detail the cellular process modulated during the growth inhibition induced by TSA, we studied the effect of the drug on G1/G0 traverse in mitogen-stimulated quiescent Balb/c-3T3 cells. Cyclin D1 and retinoblastoma proteins were induced following the mitogenic stimulation of both control and TSA-treated cells, and cyclin D1 formed complexes with Cdk4 under both conditions. However, cyclin D1-associated kinase was not increased in growth-arrested cells. The lack of cyclin D-associated kinase was paralleled by an accumulation of RB in a hypophosphorylated form, as would be expected. In contrast, p130 became partially phosphorylated, accompanied by a marked increase in p130-dependent E2F DNA binding activity and a partial release of free E2F-4. Despite the absence of E2F complexes not bound to pocket proteins, late G1 E2F-dependent gene expression was not observed. The lack of cyclin D1-associated kinase in TSA-treated cultures was potentially due to high levels of the cyclin-dependent inhibitor p27kip1. However, the modulation of p27kip1 levels by the deacetylase inhibitor cannot be responsible for the induction of the cell cycle arrest, since the growth of murine embryo fibroblasts deficient in both p27kip1 and p21cip1 was also inhibited by TSA. These data support a model in which TSA inhibits its very early cell cycle traverse, which, in turn, leads to a decrease in cyclin D1-associated kinase activation and a repression of late cell cycle-dependent events. Alterations in early G1/G0 gene expression accompany the TSA-mediated growth arrest.

Histone acetylation/deacetylation has long been proposed to play a critical role in the regulation of transcription (1). Core histones are acetylated at amino-terminal lysine residues, causing a net neutralization of positive charge and a decrease in affinity for DNA (2). Such changes have been postulated to increase the ability of transcriptional regulators to access regulatory regions in structurally relaxed chromatin. The reverse reaction, the deacetylation of histones, would then be presumed to increase the strength of histone/DNA interactions and decrease the access of transcription complexes to localized regions of DNA (3, 4). Consistent with these hypotheses, histone acetylase activity has been found associated with several transcriptional co-activators (5, 6), and histone deacetylase activity has been found associated with transcriptional repressors (7, 8).

Multiple homologs of mammalian histone deacetylase (HDAC) have been identified. HDAC1 was isolated as a factor that binds to trapoxin, a bacterial product that inhibits HDAC activity (9). HDAC1 is a homolog of RPD3, a yeast gene that plays an important role in the global regulation of transcription. HDAC2 was subsequently identified in a two-hybrid screen as a binding partner for the transcriptional repressor YY1 (7). HDAC3 corresponds to an expressed sequence tag sequence with homology to HDAC1 and HDAC2 (10). Additional HDAC isoforms have been identified based on either homology to the known genes or by functional assays (11, 12). The identification of HDAC cDNAs in several organisms has provided evidence that the biological actions of these enzymes might be more complicated than earlier predicted. RPD3, the yeast gene homologous to HDAC, was originally isolated as a protein required for both positive and negative regulation of transcription (13). In addition, some aspects of telomeric silencing in both yeast and Drosophila are prevented by the expression of RPD3 (14). Inhibitors of deacetylases have also been reported to induce, rather than repress, the expression of specific classes of genes in mammalian cells (15, 16). These results suggest that the acetylation of histones might play both positive and negative roles in gene expression due to variables that have not yet been described.

There have been suggestions that HDAC activity plays a role in the repression of E2F-dependent transcription mediated by members of the RB family of pocket proteins. A direct interaction between HDAC1 and either p130 or RB has been found in both in vitro binding assays and in vivo coimmunoprecipitation experiments (17, 18). Whether HDAC1 also binds p107 has been the subject of some controversy (18). HDAC inhibitors such as trichostatin A (TSA) prevent RB-mediated transcriptional repression of transfected E2F-dependent reporter gene constructs (17). The repression of cyclin A transcription in quiescent NIH 3T3 cells (19) and cdc25A transcription in transforming growth factor-β-treated keratinocytes (20), both mediated through the binding of RB family members to E2F sites in the 5′ regulatory regions, has also been shown to be relieved by TSA. However, if inhibitors of HDAC activity acted only by

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§ The abbreviations used are: HDAC, histone deacetylase; TSA, trichostatin A; PDGF, platelet-derived growth factor; FCS, fetal calf serum; PAI-2, plasminogen activator inhibitor-2; RB, retinoblastoma.
relieving RB-mediated repression of E2F-dependent transcription, they would be predicted to stimulate cell cycle traverse, since expression of E2F in quiescent cells has, at least under some circumstances, been found to be mitogenic (21, 22). HDAC inhibitors instead inhibit the proliferation of a number of cell types (23, 24). In many cases, the inhibition of growth has been accompanied by an increase in the transcription of p21<sup>cip1</sup>. However, in cultured mouse fibroblasts, deletion of p21<sup>kip1</sup> did not affect the ability of HDAC inhibitors to cause a cell cycle arrest, and instead, a repression of cyclin D1 expression was postulated to mediate the growth inhibition (25).

In this paper, we have characterized the ability of TSA to inhibit the proliferation of mitogen-stimulated density-arrested Balb/c-3T3 cells. The TSA-induced growth arrest could not be attributed to the actions of either p21<sup>cip1</sup> or p21<sup>kip1</sup>, or to a repression of cyclin D1 expression. However, cyclin D1-associated kinase activity was repressed in TSA-treated cells, probably through a p21<sup>kip1</sup>-dependent process, leading to a unique and complex alteration in the expression and modification of RB family members.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Stock cultures of Balb/c-3T3 cells were maintained in α-minimal essential medium supplemented with 10% fetal calf serum (FCS), 50 units/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> and were subcultured on a 3-day schedule as described previously (26). Cells to be used in experiments were seeded into 100-mm plates at an initial density of approximately 5 x 10<sup>3</sup> cells/dish. Fresh medium was added 3 days later, and experiments were performed after a further 5–6-day period (2–3 days after the cells reached density-dependent growth arrest). Cultures of murine fibroblasts were established from day 14 embryos derived from a cross of C57BL/6J × 129/SvEv. Lines deficient in both RB and p16<sup>ink4a</sup> were generated by random integration and screened for loss of RB expression by Western analysis. Stock cultures of double knockout cells were analyzed with a Becton Dickinson FACScan by the H. Lee Moffitt Cancer Center Flow Cytometry Core Facility.

**Flow Cytometry**—Cells were removed from plates with PBS containing 0.125% trypsin and 0.5 mM EDTA. The cell slurry was added to an equal volume of Dulbecco’s modified Eagle’s medium containing 10% serum to neutralize the trypsin and centrifuged. The cell pellet was thoroughly resuspended in 1 ml of PBS, and 4 ml of 95% ethanol were slowly added. Cells were stored in the freeze for at least 24 h at 4 °C prior to analysis. Samples were centrifuged, and cell pellets were resuspended in PBS containing 20 and 0.1% bovine serum albumin (BSA) containing 0 µg/ml RNase A and 50 µg/ml propidium iodide. After incubation at 4 °C for a minimum of 4 h, cell cycle distributions were analyzed with a Becton Dickinson FACScan by the H. Lee Moffitt Cancer Center Flow Cytometry Core Facility.

**RNA Quantitation**—RNA was prepared using an RNeasy Mini Kit (Qiagen) and separated in a 1% agarose/formaldehyde gel. For Northern analyses, the RNA was transferred to Gene Screen Plus. Following UV cross-linking, blots were hybridized with a radiolabeled probe, washed, and visualized by autoradiography by standard techniques (27). For a reverse transcription-polymerase chain reaction analysis, the RNA, whose quantitation was verified by the denaturing electrophoresis analysis described above, was transcribed and amplified using a OneStep RT-PCR Kit, also from Qiagen. Preliminary experiments verified that the conditions were chosen to ensure that the size of the amplified products was directly proportional to mRNA levels. As shown in Fig. 1A, at the time of restimulation cells were quiescent, with 95% of the population containing a 2N content of DNA. After treatment with fresh medium supplemented with PDGF and FCS in the absence of TSA, only 20% of the cells remained in G0/G1. The addition of TSA at concentrations greater than 0.3 ng/ml caused a dose-dependent decrease in the entry of cells into S phase, with a complete decrease in the entry of cells into S phase, with a complete

**RESULTS**

**Inhibition of Proliferation by TSA**—The ability of inhibitors of HDAC activity to modulate cellular proliferation has been previously described. However the underlying alterations in cyclin/CDK activity that mediate or reflect this growth inhibition have been difficult to establish, in part because most of these studies have been performed with transformed cells. Such model systems suffer from difficulties in cell cycle synchronization as well as preexisting alterations in cyclin/CDK metabolism characteristic of neoplastic cells. In order to more precisely describe the effects of HDAC inhibitors on the induction and activation of components of the cyclin/CDK pathway, we determined the actions of TSA on the growth properties of Balb/c-3T3 cells, a well characterized model of cell cycle regulation.

Quiescent, density-arrested cultures of Balb/c-3T3 cells were stimulated by the addition of fresh medium containing 5% FCS and 10 ng/ml PDGF-BB together with increasing concentrations of TSA. At 22 h following refeeding, the plates were removed, and DNA distributions were determined by flow cytometry. As shown in Fig. 1A, at the time of restimulation cells were quiescent, with 95% of the population containing a 2N content of DNA. After treatment with fresh medium supplemented with PDGF and FCS in the absence of TSA, only 20% of the cells remained in G0/G1. The addition of TSA at concentrations greater than 0.3 ng/ml caused a dose-dependent decrease in the entry of cells into S phase, with a complete inhibition of growth observed at 10 ng/ml. TSA was, therefore, a potent inhibitor of a process required for the initiation of DNA synthesis in density-arrested Balb/c-3T3 cells stimulated with fresh medium containing PDGF and serum.

**Modulation of Cell Cycle-dependent Gene Expression by
TSA—Since TSA inhibited the ability of stimulated Balb/c-3T3 cells to enter S phase, we determined the pattern of expression of proteins induced in late G1/S, in TSA-treated cells (Fig. 1B). Neither p107 nor cyclin A was detectable in quiescent cultures, although the expression of both proteins was markedly induced 18 h after restimulation. The addition of TSA caused a dose-dependent decrease in the expression of these proteins that paralleled the decrease in DNA synthesis. Only minor decreases in expression were observed at 0.3 ng/ml TSA. The addition of 1 or 3 ng/ml TSA, where there were intermediate decreases in entry into S phase, as shown in Fig. 1A, caused further decreases in the levels of both cyclin A and p107. At 10 ng/ml TSA, where the restimulation of mitogenesis was completely inhibited, the induction of both proteins was completely abolished.

Since TSA has been reported to increase the expression of Stra 13, with a subsequent repression of c-myc expression (29), it was at least possible that the inhibition of entry into S phase seen in Fig. 1A was due to a repression of the PDGF-mediated increase in nmy levels that are required for G1/S traverse (30, 31). As shown in Fig. 2A, c-myc RNA was undetectable in quiescent cells and markedly induced 1 h following the addition of PDGF. By 6 h following stimulation, levels returned almost to those seen prior to the addition of PDGF. The addition of TSA together with PDGF affected neither the increase seen at early times nor the subsequent decrease in c-myc RNA levels, suggesting that there was not a global repression of immediate early gene expression in TSA-treated cells. We next investigated whether the inductions of transcripts that are potentially direct downstream targets of immediate early genes are modulated in growth-inhibited cells. Plasminogen activator inhibitor-2 (PAI-2) mRNA is induced in stimulated quiescent cells in a manner that requires multiple mitogens and continuous protein synthesis, making it an example of a secondary response gene. As shown in Fig. 2A, PAI-2 message was not detected in quiescent Balb/c-3T3 cells and was induced 4 h following the addition of fresh medium supplemented with PDGF and serum. The addition of medium containing only TSA did not cause an increase in PAI-2 expression. Co-addition of TSA together with PDGF and serum did not repress the induction mediated by mitogenic stimulation and instead markedly enhanced expression. Therefore, at least one marker of the exit from G0/G1 was expressed in cells whose growth was inhibited by treatment with TSA.

Modulation of Pocket Proteins in Growth-arrested Cells—Since the RB family of proteins may be involved in the regulation of late G1/S phase traverse, including the modulation of processes involving the expression of cyclins A and p107, we investigated the effects of TSA on the expression and phosphorylation of RB, as shown in Fig. 3A. Low levels of RB protein were seen in quiescent cells. The addition of medium supplemented with PDGF and serum caused an induction of RB expression as well as a predominant shift to the hyperphosphorylated form. While the addition of TSA at concentrations up to 100 ng/ml did not inhibit the increase in RB protein levels after stimulation of quiescent cells, there was a dose-dependent decrease in RB phosphorylation in TSA-treated cells that, in general, paralleled the decrease in the expression of late G1/G1 gene expression as well as entry into S phase.

Another RB family member that is phosphorylated by a mechanism similar to that involved in RB modification and that is thought to be involved in the regulation of G1/S traverse is p130. The effects of TSA on mitogen-induced alterations in p130 phosphorylation are shown in Fig. 3B. In quiescent cells, p130 was found as three isoforms, termed A, B, and C, that have been shown to differ in the extent of phosphorylation (32). Eighteen h following the addition of PDGF and serum, the two fastest migrating forms of p130 were not present, and the amount of the slowest migrating form decreased by over 50%. When cells were restimulated in the presence of 3 or 10 ng/ml TSA, conditions where there were marked decreases in the number of cells induced to enter S phase, p130 accumulated in the intermediate B isoform, with only very low levels of forms A and C remaining. Therefore, the modifications that convert p130 from the intermediate to the slowest migrating form, leading eventually to p130 degradation, are blocked in TSA-treated cells.

The phosphorylation of both p130 and RB lead to modifications in the binding of members of the E2F family of transcription factors. The results of gel shifts using a labeled DNA probe containing the E2F binding site from the dihydrofolate reductase promoter and extracts from TSA-treated cells are shown in Fig. 3C. Quiescent cells contained a predominant single DNA binding complex that was identified as containing p130 by
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Supershifts with a panel of antibodies against RB family members (data not shown), with no evidence of “free” E2F DNA binding complexes. Cells stimulated for 18 h with PDGF and serum contained no residual p130 complexes, although a new high molecular weight complex containing p107 (based on supershifts) appeared, as did a smear of “free” E2F binding activity corresponding to multiple members of the E2F family that are induced as cells progress through G0/G1. Extracts of cells stimulated in the presence of TSA had marked elevations in the level of DNA binding activity associated with p130. In addition, a distinct band of “free” E2F was seen that was identified by comigration and antibody reactivity as E2F-4 (data not shown). Therefore, the processes responsible for the degradation of the p130 isoform with the slowest mobility were not inhibited by TSA, nor was the conversion of form A into form B. However, p130 was modified in some manner in cells stimulated with PDGF and serum in the presence of TSA such that the total DNA binding capacity was increased accompanied by a modest accumulation of “free” E2F DNA binding activity.

Inhibition of Cyclin/CDK Activation by TSA—The lack of phosphorylation of RB and p130 in TSA-treated cultures suggests that the induction of cyclin D-associated kinase activity was modulated by the deacetylase inhibitor. The effects of TSA on the induction of kinase activity associated with cyclin D1-CDK4 complexes are shown in Fig. 4A. Cyclin D1-associated kinase activity measured in an in vitro assay using RB as a substrate was low in quiescent cells and increased between 6 and 9 h following stimulation with medium supplemented with PDGF and serum. Peak activity was seen at 12 h with levels steadily declining toward baseline between 15 and 21 h. The addition of 10 ng/ml TSA to the medium at the time of stimulation with PDGF and serum abolished the induction of kinase activity associated with cyclin D1. These results suggest that the growth arrest invoked by TSA was temporally prior to the mitogen-stimulated cell cycle-dependent events that lead to the activation of the cyclin/CDK cascade.

Based on the results shown above we investigated the mechanism by which TSA inhibits cyclin D1-associated kinase activity, first by determining the ability of TSA to alter the induction of cyclin D1 protein following stimulation with mitogens. Density-arrested Balb/c-3T3 cells were stimulated with fresh medium containing 5% FCS and 10 ng/ml PDGF together with 10 ng/ml TSA. As can be seen in Fig. 4B, quiescent cells did not contain measurable levels of cyclin D1 protein. Following stimulation with medium supplemented with PDGF and serum alone, there was a 4–5-fold increase in the expression of cyclin D1. Co-addition of TSA together with fresh mitogens did not markedly alter the induction of cellular levels of cyclin D1. Therefore, the inhibition of cyclin D1-associated kinase activity in cells stimulated in the presence of TSA could not be accounted for by a repression of cyclin D1 protein expression.

The lack of cyclin D1-associated kinase activity was also not due to a failure of cyclin D1 protein to associate with CDK4. Cultures treated in parallel to those shown in Fig. 4B were harvested, and cyclin D1 (together with associated proteins) was immunoprecipitated. Complexes were disrupted and separated on denaturing gels, and levels of CDK4 were determined by Western analysis. As shown in Fig. 4C, the amount of CDK4 coprecipitated with cyclin D1 was equivalent in cells stimulated with medium containing PDGF and serum alone (that had robust kinase activity) or with mitogens together with TSA (conditions where there was a repression of cyclin D1-associated kinase activity). We next investigated whether alterations in levels of protein inhibitors of cyclin-dependent kinase activity could account for the lack of cyclin D1-associated kinase activity. Density-arrested Balb/c-3T3 cells have been shown to have an elevated level of p27kip1. Following mitogenic stimulation, p27kip1 levels are markedly reduced by a combination of translational repression and increased instability (33, 34). In order to determine the effects of TSA on the reduction in p27kip1 levels following mitogenic stimulation, quiescent cells were treated for 18 h in medium supplemented with 10 ng/ml PDGF, 5% FCS, and various concentrations of TSA. Cellular levels of p27kip1 were determined using immunobots. As shown in Fig. 4D, the elevated levels of protein seen in quiescent cultures were reduced by over 90% following stimulation with medium containing only PDGF and serum. The addition of TSA at concentrations between 0.3 and 3 ng/ml led to the activation of the cyclin/CDK cascade.
stimulation of entry into S phase, p27kip1 levels were stabilized at approximately 25% of those seen in quiescent cells (therefore 5-fold over those in cells stimulated in the absence of TSA). These concentrations of p27kip1 would be predicted to be sufficient to cause the lack of cyclin D1-associated kinase activity (35), independent of any TSA-induced modulation of p21cip1 levels.

In order to determine whether cellular events modulated by TSA directly impinged on p27kip1 expression with a subsequent repression of cyclin D1 activation and cell cycle traverse or whether elevated p27kip1 levels reflected rather than mediated an early cell cycle inhibition, we determined the ability of TSA to inhibit the growth of cells deficient in p27kip1. Murine embryo fibroblasts deficient in both p27kip1 and p21cip1 (determined by a polymerase chain reaction analysis of genomic DNA; data not shown) were plated at low density in medium supplemented with 10% FCS. After a 24-h period to allow for cell attachment, fresh medium containing serum together with increasing concentrations of TSA was added. Five days later, cells were removed from the plates, and total cell number was determined. The number of cell divisions the population underwent during the incubation period in drug was then calculated, as shown in Fig. 5. Control cultures (i.e. cells incubated in the absence of TSA) doubled approximately 5.5 times over the 5-day period. The addition of TSA caused a marked inhibition of cell proliferation with a similar dose dependence to that seen in Fig. 1. These data suggest that the inhibitory actions of TSA on the proliferation of murine fibroblasts were largely independent of any direct modulation of p21cip1 or p27kip1. Therefore, the elevated levels of p27kip1 protein levels reflect, rather than mediate, the growth arrest.

**Pattern of Reversal of TSA-mediated Growth Inhibition**

The data presented above suggest that genes induced early after the mitogenic stimulation of quiescent cells (myc, RB, and cyclin D1 induction) are not affected by TSA. However, later events such as the activation of cyclin D1-associated kinase activity are not observed. In order to more firmly establish the temporal location of the growth inhibition mediated by TSA, cells were stimulated with PDGF and serum in the presence of 10 ng/ml TSA for 24 h. The cultures were then rinsed to remove the TSA, fresh mitogen-supplemented medium was added, and plates were harvested at regular intervals. As shown in Fig. 6, cyclin A protein was absent in quiescent cells and strongly induced following re-stimulation. The co-addition of TSA prevented the induction of cyclin A, similar to the results shown above. When cells were released from the TSA-mediated growth arrest, cyclin A protein was first induced at 12 h, at which time cells also initiated DNA synthesis as measured by flow cytometry (data not shown). This 12-h delay between the removal of the growth inhibitor and the induction of cyclin A and entry into DNA synthesis was similar to the kinetics observed when quiescent cells were stimulated in the absence of TSA, suggesting that the arrest point was temporarily localized early in the G0/G1 period of the cell cycle.

Alterations in p27kip1 protein levels following release from a TSA-induced growth arrest are also shown in Fig. 6. p27kip1 levels were high in quiescent cells and fell by over 90% following the addition of fresh medium supplemented with PDGF and serum. Stimulation with fresh mitogens in the presence of 10 ng/ml TSA caused p27kip1 protein to fall to an intermediate level, similar to the results presented above. When TSA was removed from the medium and fresh PDGF and serum were added, p27kip1 levels remained constant for the first 3 h, after which they fell steadily, reaching levels approximating those seen in control restimulated cells by 9 h.

**DISCUSSION**

TSA, a potent and specific inhibitor of histone deacetylase activity, effectively prevented cell cycle traverse in mitogen-stimulated quiescent Balb/c-3T3 cells. While inhibitors of deacetylase activity have previously been shown to arrest the growth of other cell types, there are several unique aspects to the cell cycle checkpoint described here. In human tumor cells, the growth inhibition mediated by deacetylase inhibitors has been postulated to be due primarily to an induction of p21cip1 (23, 24), with a subsequent inhibition of cyclin/CDK activity. However, mouse embryonal fibroblasts cultured from p21cip1-deficient mice did not have an abrogated arrest following treatment with deacetylase inhibitors (25), suggesting a p21cip1-independent growth inhibition. We also found that mouse embryonal fibroblasts deficient in p21cip1 remained sensitive to a TSA-induced growth arrest. Together, these results directly suggest that the modulation of p21cip1 levels do not mediate the TSA-induced growth arrest in mouse fibroblasts.

Mitogen-regulated modulations in p27kip1 levels are, however, altered in cells stimulated in the presence of TSA. The initial fall in p27kip1 protein seen after mitogenic stimulation, postulated to be mediated by a translational repression (33), was not affected by treatment with TSA, and the protein decreased to an intermediate level similar to that seen in cells stimulated with PDGF alone (36). However, the more complete decrease in p27kip1 protein characteristic of cells stimulated to traverse G0/G1 (35), was not observed in cells treated with TSA. It is likely that the increased p27kip1 levels present in TSA-treated cells are sufficient to prevent the activation of cyclin D1-associated kinase activity even in the absence of any contribution of p21cip1, based on a comparison of the regulation of cyclin D1 activation in quiescent cells treated with PDGF alone (35). However, independent of the mechanism by which cyclin D1 activity is regulated in TSA-treated cells, the resultant growth arrest cannot be explained only by modulation of cyclin dependent inhibitors, since the proliferation of fibroblasts de-
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significant in both p27

kip1

and p21
c

ip2

is efficiently repressed by the deacetylase inhibitor. Therefore, the lack of cyclin D1-associated kinase activity in TSA-treated cells probably reflects rather than mediates the growth arrest at an early point in G0/G1 traverse.

An important substrate of activated cyclin D1 complexes clearly is RB (37). At concentrations of TSA where cell cycle traverse was maximally inhibited, we observed an induction of RB protein levels. However, the phosphorylation of RB measured by electrophoretic shifts was not observed, an expected result, since there was no activation of cyclin D1-associated kinase activity. More surprisingly, at intermediate concentrations of TSA, where neither cell cycle traverse nor the induction of E2F dependent gene activity was inhibited, RB remained in a hypophosphorylated form. This result suggests that full phosphorylation of RB is not required for G0/G1 traverse and that the TSA-induced cell cycle checkpoint might be a useful paradigm for the study of the relationship between the phosphorylation of RB at specific residues by upstream modulators and the downstream activation of E2F-dependent transcription and subsequent cell cycle traverse.

When added at the time of stimulation of quiescent Balb/c-3T3 cells, TSA completely inhibits the induction of cyclin A protein, almost certainly by preventing the activation of cyclin D1-CDK4 and the phosphorylation of RB coincident with a complete inhibition of entry into S phase. These results, which we also obtained in restimulated human fibroblasts (data not shown) are different from those reported recently (19), where an induction of cyclin A protein was observed in serum-starved NIH 3T3 cells treated with TSA in the absence of mitogenes. The basis of the differences in these results is not presently understood, but they might be due to differences in cell types.

E2F-dependent DNA binding activity is also modulated in TSA-treated cells. In cultures stimulated in the presence of the inhibitor, p130 accumulates in a form with intermediate phosphorylation, suggesting that the processes involved in the degradation of hyperphosphorylated p130 and the phosphorylation of hypophosphorylated p130 are induced in TSA-treated cells. However, the DNA binding capacity of the p130 complexes is increased approximately 4-fold in cells stimulated in the presence of TSA, with no detectable difference in the intermediate p130 band. These results suggest that the ability of p130 to interact with DNA is modulated by processes not reflected by alterations in electrophoretic mobility, although the biological implications of these alterations are not known. In addition, we observed an increase in "free," presumably transcriptionally active, E2F-4 complexes in TSA-treated cells, although E2F-dependent genes such as cyclin A were not induced. It is possible that E2F family members regulate the expression of discrete families of genes (37), potentially through binding to discrete recognition sequences (38). In addition, the regulation of E2F-4 by alterations in nuclear/cytoplasmic transport has been described (39). It is therefore possible that E2F-dependent activity interdependently cascades into the full transcriptional activation seen in late G0/G1 through a pathway that is blocked at an early/intermediate stage in TSA-inhibited cells.

The kinetics of cell cycle resumption following release from a TSA-induced growth arrest suggested that cultures were blocked early in G0/G1. Because of the recent report (29) that Stra13, a repressor of myc transactivation, is induced following the addition of TSA, we investigated whether the PDGF-mediated induction of myc expression was modulated in growth-arrested cells. Interestingly, neither the initial increase in myc mRNA, nor the subsequent return to basal levels of expression was affected by the addition of the inhibitor. These results suggest that TSA did not cause a global repression of either early secondary messenger activation or immediate early gene transcription, although it remains possible that there was a selective inhibition of specific yet unidentified pathways.

The regulation of PAI-2 expression in TSA-inhibited cells was investigated as a reflection of the induction of secondary response genes in growth-arrested cultures. The addition of TSA alone did not cause an increase in PAI-2 mRNA levels. In addition, the mitogen-mediated increase in expression was enhanced rather than repressed by co-addition of the deacetylase inhibitor. This observation is interesting in light of the generally accepted model in which the inhibition of histone deacetylase activity leads to a loss of transcriptional repression. Although the molecular mechanisms that regulate the expression of PAI-2 have not been described, it appears that if TSA is acting by inhibiting a transcriptional repression, such relief alone is not sufficient to cause an increase in expression. Rather some mitogen-dependent initiating event would be predicted to be further modulated by a TSA-dependent step. Independent of the pathways involved in enhanced PAI-2 expression, it appears that some aspects of secondary response gene induction remain intact in restimulated TSA-treated cells.

These data do suggest, however, that the growth-inhibitory actions of TSA may well involve modulation of mitogen-regulated transcription pathways. We are currently utilizing a number of approaches, including the use of microarrays, to identify specific gene products whose expressions are affected by TSA in an effort to determine the molecular pathways that underlie the dramatic inhibition of cellular proliferation.

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kip1

/p21
c

ip2

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