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Mechanism of Cell Cycle Arrest Caused by Histone Deacetylase Inhibitors in Human Carcinoma Cells

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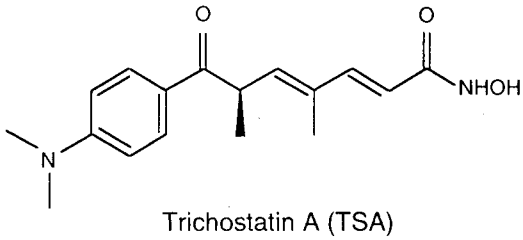
Inhibitors of histone deacetylase (HDAC) block cell cycle progression at G1 in many cell types. We investigated the mechanism by which trichostatin A (TSA), a specific inhibitor of HDAC, induces G1 arrest in human cervix carcinoma HeLa cells. TSA treatment induced histone hyperacetylation followed by growth arrest in G1 as well as hypophosphorylation of pRb. The Cdk4 kinase activity was essentially unchanged during the TSA-induced G1 arrest. On the other hand, the arrest was accompanied by down-regulation of kinase activity of Cdk2, although the total protein levels of Cdk2 and its activator Cdc25A were unaffected. Upon TSA treatment, amounts of cyclin E and the CDK inhibitor $p21^{WAF1/Cip1}$ were markedly increased, while that of cyclin A was reduced. The induction of p21 and down-regulation of cyclin A correlated well with the decreased Cdk2 activity and cell cycle arrest. Furthermore, gel filtration chromatography showed the association of p21 with the cyclin E-Cdk2 complex, suggesting that the activation of Cdk2 by the enhanced expression of cyclin E is blocked by the increased p21. The elevated expression of p21 is also observed in cells treated with trapoxin and FR901228, structurally unrelated histone deacetylase inhibitors. A human colorectal carcinoma cell line lacking both alleles of the *p21* gene (p21−/−) was resistant to TSA several times more than the parental line (p21+/+). These results suggest that the suppression of Cdk2 kinase activity due to p21 overexpression play a critical role in HDAC inhibitor-induced growth inhibition.

A large number of natural products and synthetic compounds were extensively screened for inhibitors of eukaryotic cell cycle. We previously reported that trichostatin A (TSA)[1,2], trapoxin[3], and FR901228[4] (Fig. 1), which had been described to cause cell cycle arrest in various cultured cell lines, were inhibitors of histone deacetylase (HDAC). All of these inhibitors cause arrest of the cell cycle in G1 and/or G2, suggesting that histone acetylation is involved in cell cycle control[4,5]. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase (HAT) and deacetylase, respectively. The net level of acetylation is determined by an equilibrium between these two enzymes[6]. Inhibition of HDAC therefore causes hyperacetylation of chromatin. Recently, a number of transcriptional repressors were shown to recruit the HDAC complex to the promoter regions. For example, hypophosphorylated pRb recruits HDAC to repress the E2F-dependent transcription[7,8]. TSA and trapoxin can abrogate transcriptional repression caused by these repressor-corepressor complexes. These findings suggest that histone acetylation plays an important role in the regulation of the cell cycle through an alteration of gene expression. Since some of the HDAC inhibitors show strong antitumor activity[4,9], a better understanding of the precise mechanism by which HDAC inhibitors cause cell cycle arrest may provide new insights into cancer chemotherapy.

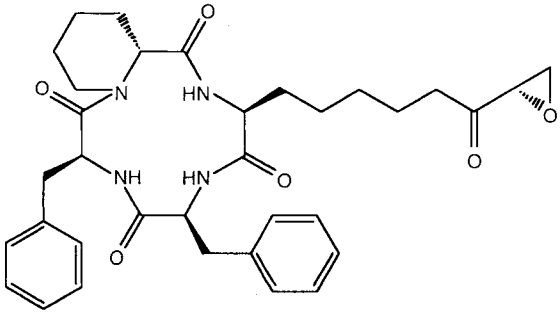
In the present study, we investigated the effects of TSA on the cell cycle progression in HeLa, and showed that TSA caused up-regulation of p21 and inhibition of Cdk2 activity

This paper is dedicated to Late Sir EDWARD P. ABRAHAM in memory of his great contribution to the field of antibiotics.

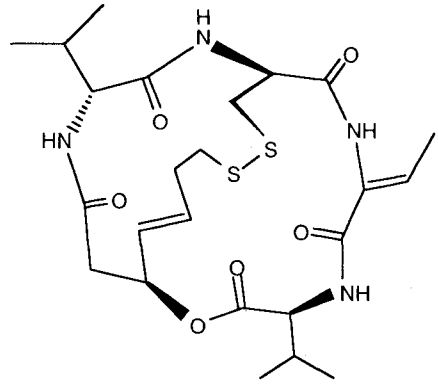
Fig. 1. Chemical structures of microbial metabolites inhibiting HDAC.



Trichostatin A (TSA)



Trapoxin A (TPX)



FR901228 (FK228)

leading to hypophosphorylation of pRb. Experiments with a p21-null cell line suggest an important but insufficient role of p21 in HDAC-induced growth inhibition.

Materials and Methods

Culture Conditions and Analysis of Cell Proliferation

The cell lines used in this study were maintained in DULBECCO's modified EAGLE's medium (DMEM) (Nissui Pharmaceutical Co. Ltd.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Effects of TSA on cell proliferation were determined using a MTT assay[10] and BrdU incorporation.

Cell Labeling and Immunoprecipitation

HeLa cells that had been grown in 100-mm tissue culture dishes were treated with or without 500 ng/ml of TSA for 18 hours in DMEM complemented with 10% FCS. After cells had been washed twice with methionine- and cysteine-free DMEM (Sigma Chemical Co., St. Louis, MO), the cells were preincubated in the starved medium for 20 minutes. The cells were then labeled for 6 hours with 1 mCi of Pro-mix L-[^{35}S] *in vitro* cell labeling mix (Amersham Life Science, Buckinghamshire, UK) per 100-mm dish in the presence or absence 500 ng/ml of TSA. The cells were washed with cold PBS and lyzed in IP buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4, 10 μg/ml aprotinin and 10 mM leupeptin, pH 7.5). Labeled pRb was immunoprecipitated with an anti-pRb antibody (Clone C36, Oncogene Research Products, Cambridge, MA) and protein A/G-plus agarose beads on a rotating shaker for 1 hour at 4°C. The beads were washed five times with IP buffer, boiled in SDS sample buffer, and the proteins were analyzed with SDS-7% PAGE followed by autoradiography.

In Vitro Kinase Assay

HeLa cells (5×10^6 cells grown for 24 hours) that had been treated with 500 ng/ml of TSA for the indicated times were lyzed in IP buffer. CDKs were immunoprecipitated from the clarified cell lysates (500 μg protein) using an anti-human Cdk2 antibody (Santa Cruz Biotechnologies, Inc.) and an anti-human Cdk4 antibody (Santa Cruz Biotechnologies, Inc.). The immune complexes were washed thoroughly three times with IP buffer and

twice with kinase buffer (20 mM MOPS, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, pH 7.2). Kinase assays were performed with a cdc2 kinase assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instruction. An Rb peptide (Santa Cruz Biotechnologies, Inc.) was used as the substrate for Cdk4.

Extraction of Histones and Acid-Urea-Triton Gel Electrophoresis

The level of histone acetylation was analyzed by slab gel electrophoresis using an acid-urea-Triton (AUT) gel as described previously[2]. The gels were stained with Coomassie Brilliant Blue.

Western Blot Analysis

Cell lysates from HeLa cells treated with various concentrations of TSA for the indicated times were prepared by lyzing with brief sonication in IP buffer. Lyzed cells were centrifuged for 10 minutes at 4°C, and the supernatants were frozen until analysis. An equal amount of protein (30 μg protein/lane) was loaded and electrophoresed on SDS-polyacrylamide gels, and proteins separated were transferred onto an Immobilon-P membrane (Millipore Co, Bedford, MA). After the membrane had been treated with an anti-human Cyclin A (Santa Cruz Biotechnologies, Inc.), an anti-human Cyclin E (Upstate Biotechnology, Lake Placid, NY), an anti-human $p21^{WAF1/Cip1}$ (Transduction Laboratories Inc.), an anti-human $p27^{Kip1}$ (Transduction Laboratories Inc.), an anti-human Cdk2 (Santa Cruz Biotechnologies, Inc.), or an anti-human Cdc25A (Santa Cruz Biotechnologies, Inc.) antibody, the immune complexes were detected with an ECL Western blotting kit (Amersham Pharmacia Biotech.).

Gel Filtration Chromatography

Cell lysates (2~5 mg protein) prepared by freeze-thawing in IP buffer were fractionated on a Superose 12 gel filtration column using a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden). The fractions (0.5 ml) eluted with 50 mM Tris-HCl (pH 7.4), 200 mM KCl as the eluent were collected and the proteins were concentrated by acetone precipitation prior to SDS-PAGE and Western blotting. The void volume of the column was determined with blue dextran 2000 (Pharmacia Fine Chemicals). The column was standardized by employing a protein mixture of a gel filtration calibrating kit (Pharmacia Fine Chemicals) containing aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The standards were run before and after the analyses for verifying the efficiency of separation.

Results

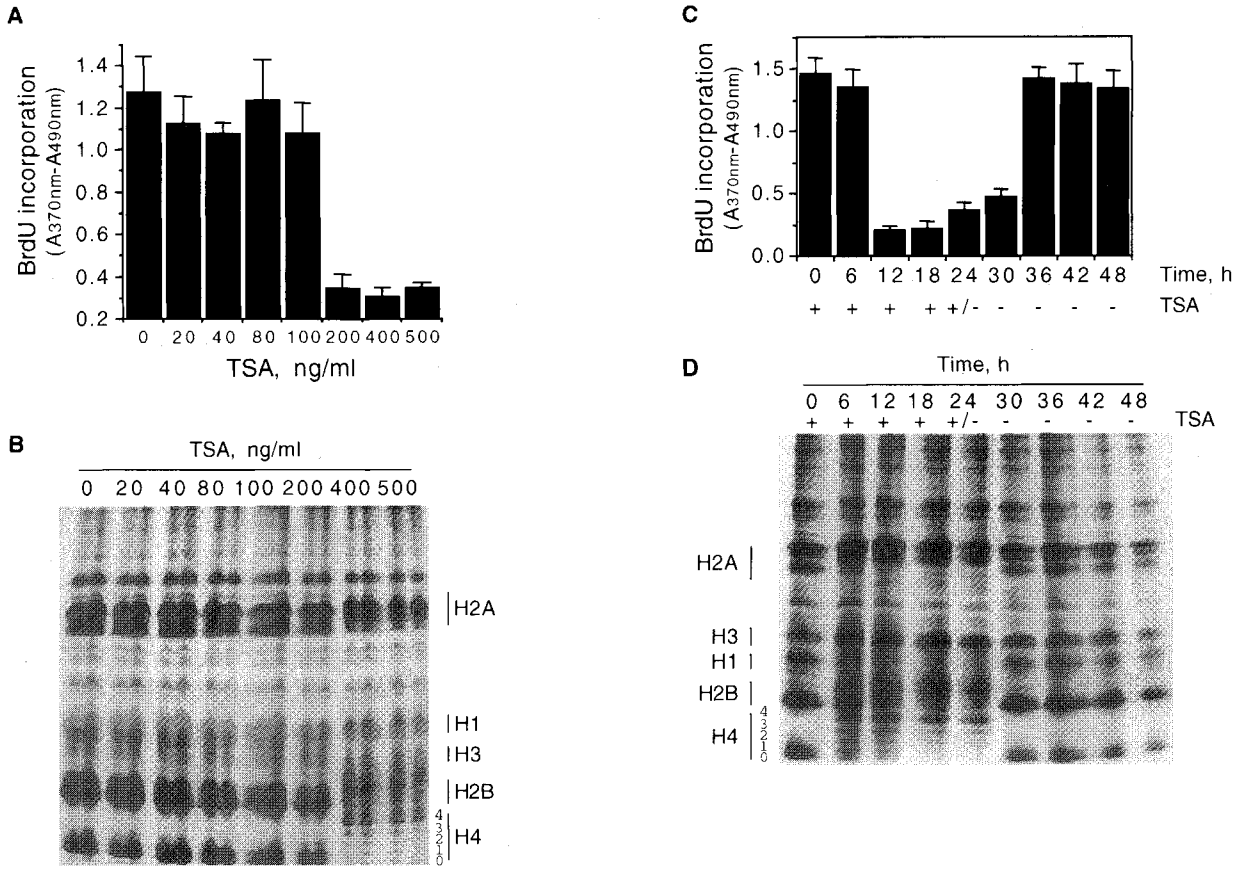
Reversible G1 Block by TSA in HeLa Cells

TSA blocks the cell cycle mainly in the G1 phase in HeLa cells[5]. We analyzed the relationship between TSA-induced cell cycle arrest and histone acetylation by measuring DNA synthesis using 5-bromodeoxyuridine (BrdU) incorporation and histone acetylation using acid-urea-Triton (AUT) gel electrophoresis. When HeLa cells were treated with various concentrations of TSA for 24 hours, BrdU incorporation was inhibited (Fig. 2A) and highly acetylated histone were accumulated (Fig. 2B) at the TSA concentrations of higher than 200 and 400 ng/ml, respectively, suggesting that the overall acetylation of bulk histones is not required for the cell cycle arrest in HeLa cells. The correlation between the cell cycle arrest and changes in histone acetylation were further analyzed by following the time courses. As shown in Fig. 2C, the growth of HeLa cells was inhibited 12 hours after TSA addition, whereas their cell cycle was recovered 12 hours after the removal of TSA from the culture. There is a time lag of 12 hours between the arrest and TSA challenge as well as the resumption of the cell cycle and TSA removal. On the other hand, histones were acetylated immediately after the addition of TSA and deacetylated after removal of the drug (Fig. 2D). These data suggest that the histone hyperacetylation by itself does not directly prevent cell cycle progression but affects expression of some proteins essential for cell cycle regulation, thereby inhibiting cell cycle progression.

Effect on pRb Phosphorylation and G1 CDK Activity

Although pRb, a key player in the regulation of G1 to S transition[11], is functionally inactivated by the human papillomavirus E7 oncoprotein in HeLa cells, it is still a good substrate for G1 CDKs. We therefore examined an effect of TSA on the activity of G1 CDKs by analyzing the phosphorylation level of pRb. pRb was immunoprecipitated from [^{35}S]-labeled cell lysates using a monoclonal antibody that recognizes both the hypo- and hyperphosphorylated forms of pRb. The immunoprecipitated pRb from the lysate of control cells consisted of both a slow-migrating hyperphosphorylated (ppRb) and a fast-migrating hypophosphorylated (pRb) form. On the other hand, the hyperphosphorylated forms were almost absent from the

Fig. 2. Relationship between TSA-induced G1 arrest and histone hyperacetylation.



(A) Dose response of TSA-induced G1 arrest. HeLa cells (seeded at a density of 1×10^4/well and grown for 24 hours in 96-well plates) were treated with TSA at various concentrations for 24 hours and then DNA synthesis was measured using a BrdU incorporation assay. Data are shown as means of quadruplicate assays (bars, SD).

(B) Dose response of TSA-induced histone hyperacetylation. The acetylation level of histone molecules in the cells treated with TSA at various concentrations for 24 hours was monitored by AUT gel electrophoresis.

(C) Time-course of TSA-induced G1 arrest. HeLa cells were added with 500 ng/ml of TSA at time 0, cultured for the indicated times (+), then released from the block 24 hours after the TSA challenge (+/−), and further cultured for an additional 24 hours without TSA (−). TSA-induced G1 arrest was monitored by BrdU incorporation assay.

(D) Time-course of TSA-induced histone hyperacetylation. The acetylation level of histone molecules in the cells treated with (+) or without (−) TSA was monitored by AUT gel electrophoresis.

cells treated with TSA for 24 hours (Fig. 3A). The result indicates that the *in vivo* activity of G1 CDKs to phosphorylate pRb is reduced by treatment with TSA.

Cdk2 and Cdk4 are major Rb kinases in HeLa cells. Although D-type cyclins and Cdk4 are supposed not to be required in Rb-negative tumor cells in general[12], it has recently been reported that Cdk4 is important in cell cycle progression in HeLa cells[13]. Therefore, we determined whether Cdk2 and Cdk4 activity was influenced by the TSA treatment by using *in vitro* kinase assays. As shown in Fig 3B, the histone H1 kinase activity in the immunoprecipitate containing Cdk2 was not significantly affected until 6 hours after TSA addition, but was reduced at 12 hours. At 24 hours, the activity was less than one fifth of the initial level. The inhibition was continued for at least 6 hours even after the drug removal, but it was completely abolished 12 hours after removal of TSA. The time course of the Cdk2 activity correlated well with the cell cycle arrest and restart. On the other hand, the Rb kinase activity of Cdk4 was unaffected by TSA (Fig. 3B). The suppression of Cdk2 activity was not due to the reduced expression, since the Cdk2 protein level was constant,

irrespective of the presence and absence of TSA, as determined by Western blotting of the Cdk2 immunoprecipitates used for the kinase assay (Fig. 3C). Cdk2 is also post-translationally regulated by phosphorylation and dephosphorylation. However, no obvious band shift implying changes in the phosphorylation level of Cdk2 was observed (Fig. 3C). Furthermore, Western blot analysis demonstrated that the protein level of Cdc25A phosphatase, a Cdk2 activator, was also constant.

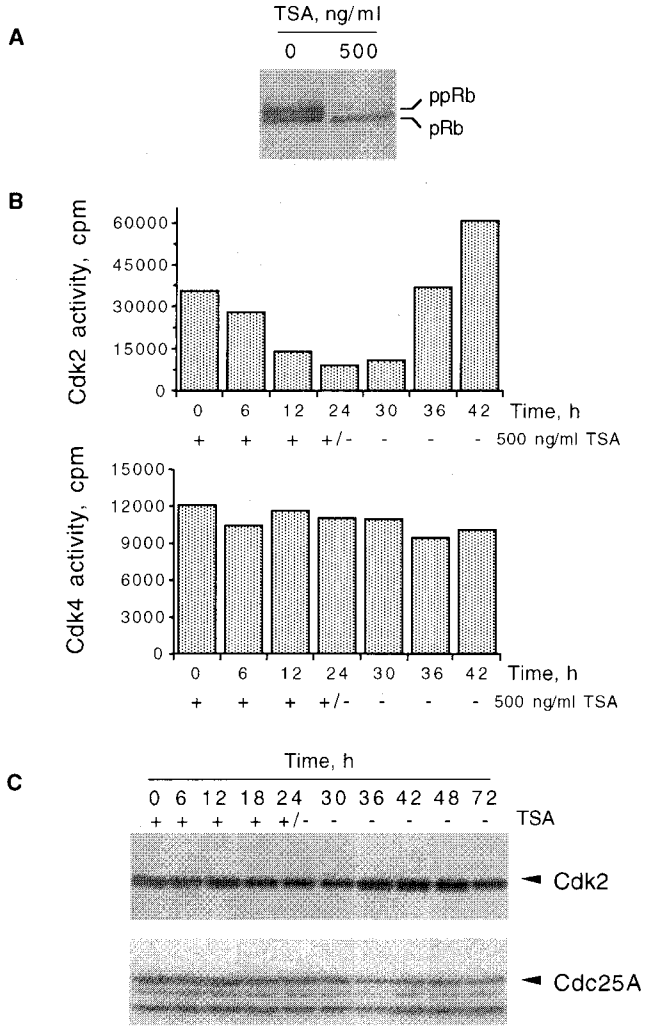
TSA Alters Expression of Cyclin A and Cyclin E

To elucidate the mechanism by which TSA suppresses Cdk2 activity, we analyzed the amounts of the positive regulatory subunits for Cdk2 in the cells. When whole cell lysates from HeLa cells treated with TSA at various concentrations were examined by Western blotting, the expression of cyclin A, a component of Cdk2 complex required for G1/S transition, was markedly suppressed by TSA at the concentrations higher than 200 ng/ml (Fig. 4A) where the cell cycle progression is inhibited (Fig. 2A), whereas cyclin E, another partner of Cdk2, was up-regulated in a dose-dependent manner. Time course experiments showed that the amount of cyclin A decreased 12 hours after TSA addition but increased again 12 hours after TSA removal (Fig. 4B). The dose- and time-dependent changes in the amount of cyclin A correlated with those of BrdU incorporation and Cdk2 activity. In contrast, the amount of cyclin E began to increase within 6 hours after TSA addition and the level was retained even 18 hours after drug removal. The amount of cyclin E induced by TSA was much larger than that in control exponentially growing cells, suggesting that it is sufficiently high for Cdk2 to be active. These results suggest that the decrease in the amount of cyclin A alone cannot fully account for down-regulation of Cdk2 activity.

TSA Up-regulates p21 Expression

To test whether the changes in the amount of CKIs are responsible for the decreased Cdk2 activity, we examined the effect of TSA on expression of p21 and p27, both of which inhibit the Cdk2 kinase complex. The amount of p21 was markedly elevated in the cells treated with more than 200 ng/ml TSA, whereas that of p27 was not significantly altered (Fig. 4A). Similar analysis with extracts from the cells collected at various times during TSA treatment showed a dramatic increase in the amount of p21 when treated with 500 ng/ml of TSA for 12 hours or longer (Fig. 4B), at which time Cdk2 activity was markedly reduced

Fig. 3. Inhibition of pRb phosphorylation and Cdk2 activity by TSA.

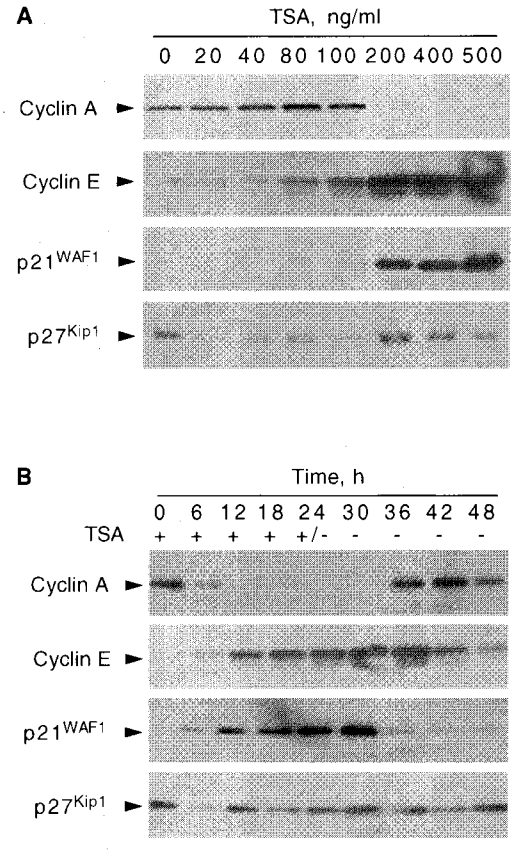


(A) Induction of pRb hypophosphorylation by TSA. HeLa cells treated with TSA for 24 hours were [^{35}S]-labeled, and pRb immunoprecipitated was analyzed by SDS-PAGE. The slow-migrating hyperphosphorylated form and the fast-migrating hypophosphorylated form are indicated by ppRb and pRb, respectively.

(B) Effects of TSA on Cdk2 and Cdk4. HeLa cells were added with 500 ng/ml of TSA at time 0, cultured for the indicated times (+), then released from the block 24 hours after the TSA challenge (+/−), and further cultured without TSA (−). Each CDK was immunoprecipitated and analyzed by the *in vitro* kinase assay as described in Material and Methods.

(C) Effects of TSA on the expression of Cdk2 and Cdc25A. The protein levels of Cdk2 and Cdc25A in the above samples used for the kinase assay were determined by Western blotting.

Fig. 4. Effect of TSA on the expression of cell cycle proteins.



(A) Dose response. HeLa cells (1×10^6 cells) were treated with TSA at the indicated concentrations for 24 hours. Changes in the amounts of the endogenous cell cycle proteins were analyzed by Western blotting.

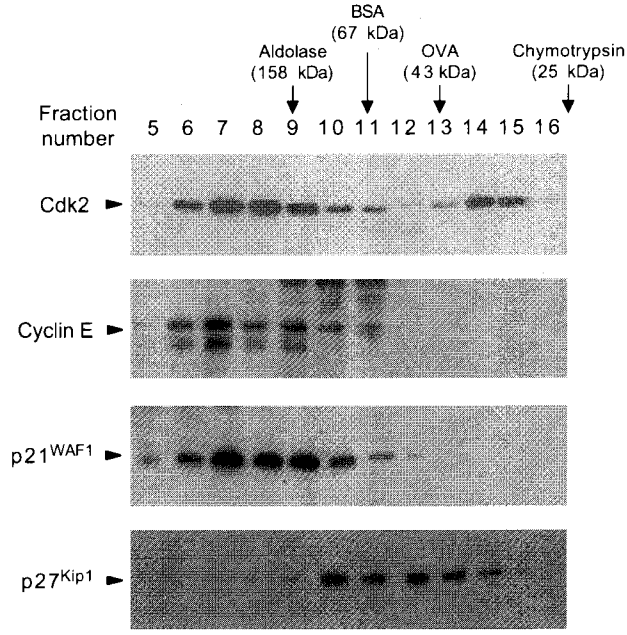
(B) Time-course. HeLa cells were added with 500 ng/ml of TSA at time 0, cultured for the indicated times (+), then released from the block 24 hours after the TSA challenge (+/−), and further cultured for additional 24 hours without TSA (−). Changes in the amounts of the endogenous cell cycle proteins were analyzed by Western blotting.

(Fig. 3B). The level of p21 decreased 12 hours after the drug removal. These patterns of change in p21 in response to TSA correlate oppositely with those in Cdk2 activity and cell cycle progression. This observation raised a possibility that the increase in the amount of p21 is responsible for the decrease in the Cdk2 kinase activity.

Association of p21 with Cdk2

Each component in the CDK complexes with different compositions and activities can be resolved by gel filtration chromatography. To investigate whether the elevated p21 is

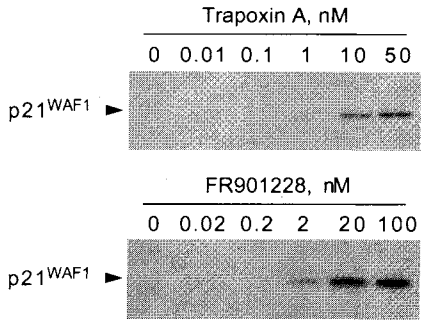
Fig. 5. Complex formation of up-regulated p21 with Cdk2 in TSA-treated HeLa cells.



HeLa cells (1×10^6 cells) were cultured for 24 hours in the presence of 500 ng/ml TSA. Cell lysates were fractionated on a Superose 12 HR gel filtration column. Fractions were analyzed by Western blotting using antibodies recognizing Cdk2, Cyclin A, Cyclin E, p21 and p27. Cyclin A was not detected. Each experiment was repeated at least three times and typical results are shown.

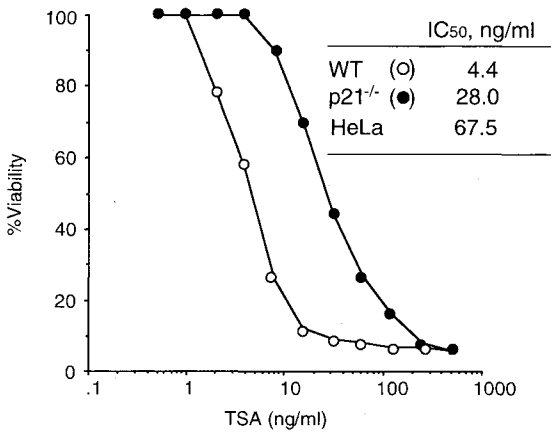
actually associated with the cyclin-Cdk2 complex, we analyzed the components in the complexes including Cdk2 by using a Superose 12 HR gel filtration column, which separates native proteins at high resolution. Figure 5 shows a typical pattern obtained from three independent experiments; each fraction obtained by chromatography was analyzed by immunoblotting for p21, p27, Cdk2, cyclin E and cyclin A. Cdk2 eluted in two major peaks. One peak eluted at an apparent molecular mass of ~40 kDa, close to the expected position of monomeric Cdk2 (Fig. 5). The other peak, representing the majority of the Cdk2, eluted as a broad peak with an apparent molecular mass of 1000 kDa~100 kDa. These results are consistent with previous observations by others[14]. Cyclin E coeluted with the peak containing the larger Cdk2 complex (Fig. 6). Cyclin A was not detected by immunoblotting over the entire fractions (data not shown), consistent with the above-described observation that the amount of cyclin A decreased in TSA-

Fig. 6. Induction of p21 by trapoxin A and FR901228.



HeLa cells (1×10^6 cells) were treated with trapoxin A and FR901228, HDAC inhibitors structurally unrelated to TSA, at the indicated concentrations for 24 hours. The cell lysates were analyzed by Western blotting.

Fig. 7. Effect of TSA on cell proliferation of p21-null colorectal carcinoma cells.



p21-null HCT116 (p21−/−) cells (●) and its parental (p21+/+) cells (○) seeded at a density of 1×10^4/well on day 0 were challenged with serial dilutions of TSA on day 1, and incubated for 3 days. Inhibition of the cell growth was determined by staining with MTT.

treated cells (Fig. 4). The fractions containing p21, which eluted as a single major broad peak, encompassed the larger Cdk2 complex probably associated with cyclin E. p21 neither eluted in the fractions of expected position of its monomeric form nor coeluted with the other peak of Cdk2. Association of p21 with Cdk2 was also verified by immunoprecipitation (data not shown). These data demonstrate that most Cdk2 proteins form a complex with not only cyclin E, but also with p21 in TSA-treated cells. The tight association of the increased p21 with the cyclin E-Cdk2 complex explains why the cyclin E-Cdk2 complex diminishes the Cdk2 kinase activity in TSA-treated HeLa cells. On the other hand, p27 did not coelute with the larger Cdk2 complex, but eluted as a single major peak with a smaller apparent molecular mass than that of the p21-Cdk2 complex (Fig. 5).

Up-regulation of p21 Caused by Other Inhibitors of HDAC

Since TSA is a specific inhibitor of HDAC, it is likely that other HDAC inhibitors causing histone hyperacetylation also induce p21 expression. This possibility was tested by using two structurally unrelated inhibitors of HDAC, trapoxin A, a fungal cyclic tetrapeptide compound which possesses a 2-amino-8-oxo-9,10-epoxydecanoyl (AOE) moiety[3], and FR901228, a recently identified bacterial bicyclic depsipeptide with strong anticancer activity[4] (Fig. 1). These inhibitors strongly induce histone hyperacetylation in the cell. As expected, these two compounds also induced p21 expression in HeLa cells (Fig. 6), suggesting that p21 induction is a general phenomenon caused by histone hyperacetylation.

Effect of TSA on Cell Proliferation of p21-Deleted Human Colorectal Carcinoma Cells

The above results suggest that the enhanced expression of p21 is responsible for TSA-induced cell cycle arrest in HeLa cells. However, it is difficult to prove conclusively its essential role in HeLa cells, since the p21-negative HeLa cells are yet unavailable. Instead, we tested whether a p21-null human colorectal carcinoma cell line HCT116 (p21−/−) is resistant to TSA or not. As shown in Fig. 7, the parental cell line HCT116 (p21+/+) was fairly sensitive to TSA compared with HeLa, but HCT116 (p21−/−) was found to be resistant to TSA. The IC_{50} was calculated to be 28 ng/ml for HCT116 (p21−/−) and 4.4 ng/ml for HCT116 (p21+/+). However, the growth of HCT116 (p21−/−) cells was still inhibited by TSA in a concentration range similar to that for HeLa cells. These results suggest that p21 is important, but not the sole determinant for the TSA-induced growth inhibition in HCT116.

Discussion

In this study, we investigated the mechanism by which TSA causes a G1 cell cycle block in HeLa cells. First, we showed that the dose and time of TSA treatment to cause accumulation of bulk histone hyperacetylation did not coincide with those for the inhibition of the cell cycle, suggesting it unlikely that histone hyperacetylation *per se* prevents the onset of DNA replication. Upon TSA treatment, the phosphorylation of pRb by cyclin-CDK complexes was markedly reduced in the cell. Because HeLa cells express the papillomavirus E6 and E7 proteins, this cell line lacks functional p53 and pRb[15]. In the absence of the pRb function, cells no longer require D-type cyclins for G1 progression[12], although cyclin D-Cdk4 has recently been shown to play an important role in the G2 cell cycle progression in HeLa cells[13]. Among CDKs, Cdk2 was found to be specifically inhibited in the TSA-treated cells. These results strongly suggest that TSA-induced histone hyperacetylation suppresses Cdk2 activity leading to hypophosphorylation of pRb and cell cycle inhibition at G1.

Cdk2 is controlled by at least three different mechanisms, phosphorylation/dephosphorylation, association with positive regulators (cyclins), and association with inhibitory proteins (CKIs). Phosphorylation regulates Cdk2 activity both positively and negatively. Stimulation of the kinase activity requires phosphorylation at Thr-160 in the T-loop by CDK-activating kinase[16] and dephosphorylation at Thr-14 and Tyr-15 by Cdc25A phosphatase[17]. TSA did not cause any detectable changes in Cdk2 phosphorylation or expression of Cdc25A. On the other hand, expression of cyclin E and cyclin A was dramatically altered by TSA. Cdk2 is normally activated by the sequential association with cyclin E and cyclin A in late G1 and the G1/S boundary, respectively. Dose response and time-course experiments showed that marked down-regulation of cyclin A coincided with the cell cycle inhibition. However, the decrease in cyclin A may not fully account for the inhibition of Cdk2 activity, since cyclin E, another positive regulator for Cdk2, was up-regulated by TSA. Among the CKIs that inhibit Cdk2, p21 was overexpressed by TSA in a dose-dependent manner. The pattern of p21 expression matched completely with the inhibition of Cdk2. Most cyclin E-Cdk2 complexes were associated with p21 but not p27 (Fig. 5). These results deny the possibility that p27 suppresses E-Cdk2 kinase activity in response to TSA, although previous reports showed that some negative growth signals can cause cell cycle arrest in G1 by increasing the association of p27 with a target CDK complex without modulating its level of expression[14]. p21 was also overexpressed in the cells treated with other HDAC inhibitors, trapoxin, FR901228 (this study) and oxamflatin[18]. These findings indicate that p21-mediated inhibition of Cdk2, but not the change in p27 expression, Cdk2 phosphorylation or cyclin expression, contributes to the cell cycle arrest imposed by drug-induced histone hyperacetylation.

Recently, several groups demonstrated a correlation between G1 arrest and changes in p21 by HDAC inhibitors[18~22]. However, the requirement of p21 induction for the cell cycle arrest caused by HDAC inhibitors is still disputable. ARCHER *et al.*[19] showed that DNA synthesis occurred in HCT116 (p21−/−) in the presence of HDAC inhibitors at low concentrations. On the other hand, VAZIRI *et al.*[21] demonstrated that the growth of p21-null mouse embryonic fibroblasts was inhibited similarly to that of the parental cells by *n*-butyrate. In this study, we compared TSA-sensitivity between p21-positive and -negative clones of HCT116 colorectal carcinoma cells. The p21-deleted cells were significantly resistant to TSA, but their growth was still inhibited by TSA at a high concentration. These results indicate that the induction of p21 is actually important in this cell line, but that some other mechanisms are also involved in the inhibition of the cell cycle. Since TSA strongly caused apoptosis in HCT116 cells (unpublished results), it seems possible that an apoptosis-inducing protein(s) of which expression is altered by TSA plays an additional role in the growth inhibition of HCT116 cells.

It has been proposed that the repression of cyclin E expression in quiescent cells is mediated by pRb[23]. The cyclin E promoter contains E2F-binding sites, and this promoter activity can be inhibited by pRb in transient transfection assays. Recently, the E2F-pRb complex was shown to recruit HDAC to repress transcription and TSA released this repression by inhibiting HDAC[7,8]. Since HeLa cells lack the functional pRb due to inactivation by the E7 viral protein, some other mechanism is probably involved in TSA-induced up-regulation of cyclin E. It is also possible that the pRb function is not completely abrogated in HeLa cells. The cyclin A promoter also contains E2F-binding sites[24], and pRb or related pocket proteins such as p130 and 107 are involved in the repression of the cyclin A promoter. Therefore, the cyclin A promoter could be activated by the HDAC inhibitors as is the cyclin E promoter. In contrast to this prediction, however, our data clearly showed that cyclin A was down-regulated by inhibiting HDAC in HeLa cells. The decrease in cyclin A expression in the butyrate-treated NIH3T3 cells was also

described by others[21], although another group reported an opposite result using TSA in the same cell line[25]. The modulation of the cyclin A promoter by TSA is probably complicated, since by which time cyclin A is transcribed the TSA-treated cells may have already overproduced p21. It was recently reported that p21 binds directly to E2F and inhibits its transcriptional activity[26].

In summary, we demonstrated that induction of p21 and inhibition of Cdk2 activity as a result of association of p21 with the cyclin E-Cdk2 complex play a critical role in TSA-induced growth inhibition in HeLa cells. Further experiments are needed to elucidate the mechanisms by which TSA induces specific induction of p21 and down-regulation of cyclin A expression, which may be important for understanding how HDAC inhibitors induce strong antitumor effects *in vivo*.

Acknowledgments

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