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Protein Tyrosine Phosphatase-Dependent Activation of β-Globin and δ-Aminolevulinic Acid Synthase Genes in the Camptothecin-Induced IW32 Erythroleukemia Cell Differentiation

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SUMMARY

Camptothecin, an antitumor drug that specifically targets topoisomerase I, induced IW32 erythroleukemia cells to differentiate along the erythroid pathway, as demonstrated by the increased mRNA and protein expression of hemoglobin. Unlike other chemically induced erythroleukemia cell differentiation, no c-*myc* mRNA down-regulation was observed in the early phases of drug treatment. Among the heme-synthesizing enzyme mRNAs that were analyzed, only that of the erythroid-specific δ-aminolevulinic acid synthase (ALAS-E) was stimulated. Vanadate or benzylphosphonic acid, which inhibited protein tyrosine phosphatases (PTPase), blocked the camptothecin-induced differentiation. Maximal inhibition was attained if vanadate was added within the first 6 hr of camptothecin treatment, after which vanadate gradually lost its effectiveness. Camptothecin-induced expression of β-globin or ALAS-E transcript levels was inhibited in the presence of cycloheximide or vanadate. It was also shown that vanadate blocked differentiation of IW32 cells induced by sodium butyrate, VM-26, and p53. Increased PTPase activity could be observed 48 hr after cells were treated with camptothecin, VM-26, or sodium butyrate. Analysis of PTPase activity in the course of camptothecin treatment showed elevated levels of PTPase in the cytosol and the nucleus, with a greater increase demonstrated in the cytosol than in the nucleus. Our results suggest that by stimulating the β-globin and ALAS-E gene expression, PTPase plays a critical role in the induced differentiation of IW32 erythroleukemia cells.

The phosphorylation of protein tyrosine residues is a critical event in the regulation of normal cellular processes, including proliferation and differentiation, and is involved in the malignant transformation of cells (1–3). The extent to which a protein is tyrosine phosphorylated arises from a dynamic equilibrium between two opposing reactions: phosphorylation by protein tyrosine kinases and dephosphorylation by PTPases (4, 5). Alteration of this equilibrium can have profound effects on cells. Recently, several studies implicated the involvement of PTPases in the differentiation of a number of cell lines. For example, the DMSO-induced differentiation of MEL cells is accompanied by extensive dephosphorylation of cellular proteins at tyrosine residues, and the differentiation can be blocked by the specific PTPase inhibitor sodium vanadate (6). Furthermore, transcript levels of several PTPase isozymes have been reported to be increased (7). In the HL-60 promyelocytic leukemia cells induced to differentiation by TPA, significant elevation of PTPase activity was detected at a relatively early stage of the treatment (8). Activation of PTPase associated with various apparent molecular weights was demonstrated (9) in the neuronal differentiation of PC12 cells induced by nerve growth factor; later studies identified a novel PTPase that was regulated by nerve growth factor at the level of its mRNA expression (10).

Camptothecin is an alkaloid that has been isolated from the plant *Camptotheca acuminata* and classified as an antitumor component (11). Camptothecin targets the nuclear enzyme DNA topoisomerase I (12). Camptothecin specifically

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ABBREVIATIONS: PTPase, protein tyrosine phosphatase; ALAS-E, erythroid-specific δ-aminolevulinic acid synthase; BPA, benzylphosphonic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MEL, murine erythroleukemia; PMSF, phenylmethylsulfonyl fluoride; *p*-Npp, *para*-nitrophenyl phosphate; TBST, Tris-buffered saline/Tween 20; PBG-D, porphobilinogen deaminase; URO-D, uroporphyrinogen III decarboxylase; EGTA, ethylene glycol bis(α-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

inhibits the rejoining step of the topoisomerase I reaction by trapping the putative covalent intermediate of the enzyme/DNA complex. Because the topological state of DNA is an important determinant for DNA structure and function, topoisomerase I is thus involved in many biological processes, such as DNA replication, recombination, and transcription. It is not surprising that in addition to its cytotoxic effect, camptothecin has been reported to induce the differentiation of cells, including F9 embryonic carcinoma cells (13) and HL-60 myeloid leukemia cells (14). Camptothecin also induces MEL cell differentiation, but this occurs only in conjunction with inhibitors of protein tyrosine kinases (15).

When cells differentiate along the erythroid lineage, they accumulate massive amounts of hemoglobin. The biosynthesis of this protein requires the coordinated production of globin chains as well as heme molecules. The synthesis of globin chains primarily results from transcriptional activation of the globin genes (16), and the enhanced heme production is ensured by activation of the enzymes responsible for its biosynthesis. It has been shown that the activities of these enzymes are up-regulated during erythroid differentiation of MEL cells (17). In the erythropoietin-induced marrow cell differentiation, only the activity of porphobilinogen deaminase was activated (18). The enzyme that catalyzes the first step in heme synthesis is ALAS, which contains two isozymes. The housekeeping ALAS-N is constitutively expressed in all cell types, including erythroid cells, whereas ALAS-E expression is restricted to the erythroid cells (19). ALAS-E plays a specific role in erythroid differentiation; its mRNA level is markedly elevated during the DMSO-induced DS-19 erythroleukemia differentiation (20). Two positive *cis*-regulatory domains within the avian ALAS-E promoter have been identified, and both elements are required to confer high level, tissue-specific transcription of the gene (21). Binding motif of the erythroid-specific transcription factor GATA-1 has also been found in the regulatory domain of ALAS-E (21). All together, these findings suggest that ALAS-E expression is tightly regulated.

The IW32 MEL cells were derived from mice infected with the helper component of the Friend virus (22). These cells produce erythropoietin, the physiological regulator of red blood cell production (23), and contain erythropoietin receptors (24). We have previously shown that podophyllotoxin (25) or its derivative VM-26 (26) induced the differentiation of IW32 cells along the erythroid lineage. The underlying mechanism of VM-26- induced differentiation seems to be independent of that of the more well characterized chemically induced MEL cell differentiation, since our findings indicated that c-*myc* down-regulation, shown to be necessary and sufficient for the latter, was apparently not required for the VM-26-induced differentiation (26).

Current knowledge of the mechanisms underlying erythroleukemia differentiation have emerged largely from studies on MEL cells induced by chemicals, such as hexamethylene bisacetamide or DMSO, whose cellular targets remain elusive. In the current study, we show that the topoisomerase I-targeting anticancer drug camptothecin was capable of inducing differentiation of IW32 cells. A parallel increase in ALAS-E and globin mRNA levels was found that required protein synthesis and could be blocked by PTPase inhibitors. Vanadate also blocked the IW32 cell differentiation induced by p53, VM-26, and sodium butyrate. Furthermore, increase in PTPase activity was shown in cells treated with differentiation-inducing agents. Analysis by subcellular fractionation demonstrated that camptothecin treatment resulted in elevated PTPase activities in the cytosol and the nucleus. Our results support that PTPases play a major role in the differentiation of IW32 erythroleukemia cells.

Materials and Methods

Cell culture and benzidine staining. The IW32 cells were a generous gift from Dr. B. Varet (Hospital Cochin, Paris, France). Cells were maintained in RPMI 1640 containing 10% fetal bovine serum at 37° in a humidified atmosphere of 5% CO_2 (22). Viability of cells was assessed by the trypan blue exclusion method. For hemoglobin staining, cells were reacted with 3,3′-dimethyl-oxybenzidine and viewed under a microscope as described previously (27).

DNA probes. DNA probes that were used included the 1.8-kb *Eco*RI/*Cla*I fragment of c-*myc* genomic clone pMC41–3RC for c-*myc*, the 1.7-kb *Hha*I fragment of pMB9 for β-globin, the 1.85-kb *Pst*I fragment of pMS20 for ALAS-E, the 1.45-kb *Pst*I fragment of PBG-DH4 for PBG-D, and the 1.2-kb *Pst*I fragment of pUD3 for URO-D.

Preparation of nuclear extracts. Cells (1×10^6) were harvested by centrifugation and resuspended in 0.5 ml of lysis buffer (10 mM Tris·HCl, 40 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.9) containing freshly added protease inhibitors (0.25 mM PMSF and 2.5 μg each of antipain, leupeptin, chymostatin, and pepstatin A). Then, 0.5 ml of lysis buffer containing 1% Nonidet P-40 was added. The cells were incubated for 5 min on ice, and the nuclei were collected through centrifugation at 500 × *g* for 5 min at 4°. The nuclei were washed with 1 ml of cold wash buffer (20 mM Tris·HCl, 20% glycerol, 140 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) and resuspended in 200 μl of nuclear extraction buffer (0.35 M NaCl, 5 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 7.5) containing the protease inhibitors mentioned above. After 30 min at 4° with periodic stirring, the mixture was centrifuged at 10,000 × *g* for 15 min. Supernatant containing the nuclear extract was used immediately or stored at −70° in the presence of 10–20% glycerol.

RNA isolation and Northern blot analysis. Total RNA was prepared as follows. Approximately 5×10^6 cells were lysed in 1 ml of TRI reagent through by repetitive pipetting. The homogenate was extracted with 0.2 ml of chloroform and centrifuged at 12,000 × *g* for 15 min at 4°. The aqueous phase containing the RNA was precipitated with isopropanol. The pellet was washed with 70% ethanol.

For Northern blot hybridization, RNA was denatured with formamide/formaldehyde and applied at 20 μg/lane to a 1.2% agarose gel containing formaldehyde. After electrophoresis, RNA was blotted to a nitrocellulose filter and hybridized with ^{32}P-labeled DNA. The radioactivity was detected by exposing the nitrocellulose paper to Kodak X-ray film. Alternatively, radioactivity associated with the band was analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell fractionation. Cells (2×10^7) were washed twice in phosphate-buffered saline and resuspended in 0.5 ml of homogenizing buffer (20 mM HEPES, pH 7.4, containing 250 mM sucrose, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 0.25 mM $MgCl_2$, 1 mM DTT) with freshly added PMSF (0.2 mM) and a 10 μg/ml concentration each of antipain, leupeptin, chymostatin, and pepstatin A. Cells were disrupted in a motor-driven Potter-Elvehjem homogenizer using 20 strokes (300 rpm). Nuclei and unbroken cells were removed after centrifugation at 2,000 × *g* for 10 min at 4°, and the supernatant was centrifuged at 60,000 × g for 60 min at 4°. The resulting supernatant was designated the cytosolic fraction. The particulate was resuspended in membrane preparation buffer (20 mM HEPES, pH 7.5, 140 mM NaCl, 1% Nonidet P-40, 20 mM DTT) containing the protease inhibitors listed above. After 1 hr at 4° with periodic mixing, the solution was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was used as membrane fraction. Protein was deter-

mined according to the method of Bradford using bovine serum albumin as a control.

For preparation of total cell extract, cells were lysed in 50 mM Tris·HCl, pH 7.4, containing 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1 mM PMSF, and a 10 μg/ml concentration each of aprotinin and leupeptin at 4° for 15 min. The homogenate was centrifuged at 10,000 × g for 15 min at 4° to remove cell debris.

PTPase assay. PTPase assay was measured using the synthetic substrate of *p*-Npp (28). The assay was carried out by adding 20 μl of 10 mM *p*-Npp to a solution (0.1 ml) containing 50 mM Tris·HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 10 mM DTT with either 10 μg of cytosolic protein, 15 μg of membrane protein, or 25 μg of nuclear extract. The reaction was carried out at 30° for 30 min and was stopped by the addition of 0.9 ml of 0.2 N NaOH. The extent of each reaction was estimated by measurement of the absorbance at 410 nm.

Western blot analysis. Monoclonal antibody (anti-CC98) against nucleolin (29) was kindly provided by Dr. Ning-Hsing Yeh (National Yang Ming University, Taipei, Taiwan). For Western blot analysis, the proteins (10 μg of cytosolic protein and 25 μg of nuclear extract) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 6% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was soaked in 3% nonfat dry milk dissolved in TBST (10 mM Tris·HCl, 150 mM NaCl, pH 8.0, containing 0.05% Tween 20) for 2 hr to decrease nonspecific binding. Membrane was washed three times for 10 min with TBST and then reacted with anti-nucleolin antibody for 2 hr. Membrane was washed for three times for 10 min with TBST followed by incubation with horseradish-peroxidase-labeled goat anti-mouse antibody (1: 2000 dilution) for 1 hr. Signals were detected with ECL reagents (Amersham International), followed by exposure to X-ray film.

Results

Camptothecin induces the erythroid differentiation of IW32 cells. We wanted to determine whether camptothecin affected the growth or differentiation of IW32 cells. Cells were treated with various concentrations of camptothecin for ≤ 4 days, and hemoglobin-containing cells were determined with benzidine staining. As indicated in Fig. 1, camptothecin dose-dependently increased the benzidine-positive cells. After exposure to 0.1 μM for 48 hr, ~50% of cells contained hemoglobin, whereas cells remained viable. Proliferation of cells was also inhibited by camptothecin. At 0.1 μM, cell growth was completely blocked; FACScan revealed that cells were arrested in the G_2/M phase of the cell cycle (data not shown). Consistent with benzidine staining, Northern blot analysis showed a significant increase in β-globin mRNA expression at 24 and 48 hr of camptothecin treatment (Fig. 2). These results indicate that camptothecin induced IW32 cells to mature along the erythroid pathway.

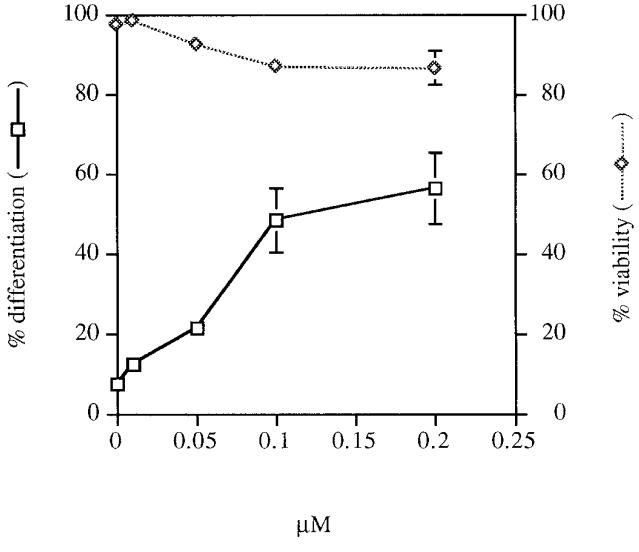

Fig. 1. Dose-dependent induction of IW32 cell differentiation by camptothecin. IW32 cells were plated at a density of 1 × 10^5 cells/ml, and increasing concentrations of camptothecin were added as indicated. After 2 days, benzidine-positive cells were counted. Viability of cells was assessed by the trypan blue exclusion method. Data represent results from three independent experiments.

It should be noted that with the concentration used to induce differentiation, there was a significant reduction in topoisomerase I activity at 2–10 hr of the drug treatment (data not shown), suggesting that topoisomerase I was the cellular target of camptothecin in IW32 cells.

c-*myc* mRNA level remains unchanged during differentiation. Evidence supports that c-*myc* down-regulation is necessary and sufficient for the differentiation of MEL cells induced by a number of chemicals (30–32). Because camptothecin inhibited nuclear enzyme topoisomerase I, it is conceivable that its mechanism of differentiation induction may be quite different from that of other differentiation promoters. We therefore examined whether down-regulation of c-*myc* gene expression was associated with camptothecin-induced differentiation. c-*myc* mRNA expression was analyzed during the course of differentiation by Northern blotting. As shown in Fig. 3, there was no apparent reduction in c-*myc* transcript levels during the 24-hr period after camptothecin treatment, suggesting that c-*myc* down-regulation was not a prerequisite for the camptothecin-induced differentiation of IW32 cells. This is in clear contrast to our previous findings of IW32 differentiation induced by podophylotoxin, sodium butyrate, or hemin; in all cases, a complete inhibition of c-*myc* mRNA expression was observed at 30 min after drug exposure (25).

Differential and coordinated induction of ALAS-E mRNA during differentiation. Hemoglobin synthesis required the coordinated production of heme. To examine the expression of the enzymes involved in the heme synthesis during differentiation, we analyzed the mRNA levels of three heme-synthesizing enzymes by Northern blotting: PBG-D, URO-D, and ALAS-E. As indicated in Fig. 4, IW32 cells

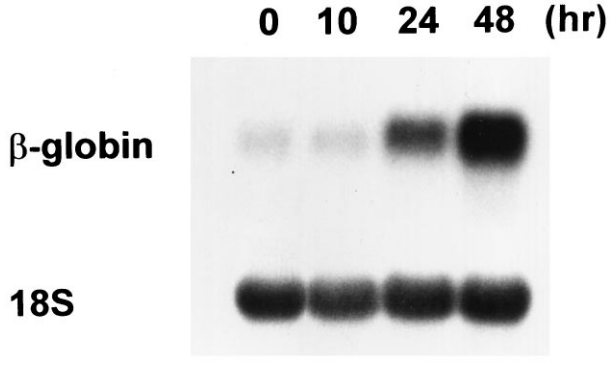

Fig. 2. Effect of camptothecin on β-globin mRNA expression. IW32 cells were treated with 0.1 μM camptothecin. Total RNA was isolated at the indicated times, and β-globin mRNA expression was analyzed by Northern blotting as described in Materials and Methods. As an internal control, the same blot was hybridized with DNA of the 18S ribosomal RNA.

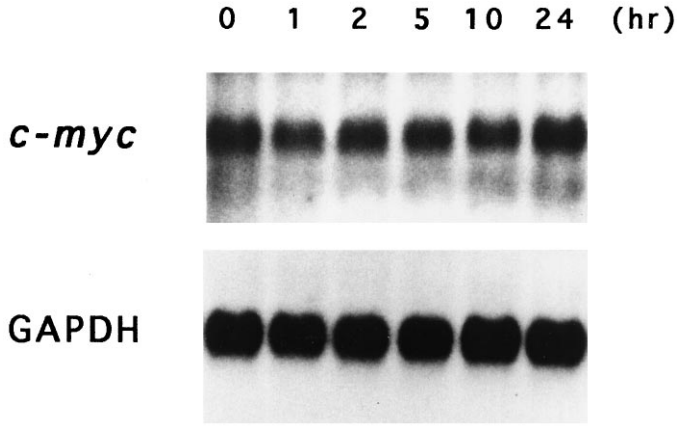

Fig. 3. Effect of camptothecin on c-*myc* mRNA expression. IW32 cells were treated with 0.1 μM camptothecin for the times indicated. Total RNA was extracted and analyzed by Northern blotting for mRNA levels of c-*myc* and GAPDH as described in Materials and Methods. Similar results were obtained from three independent experiments.

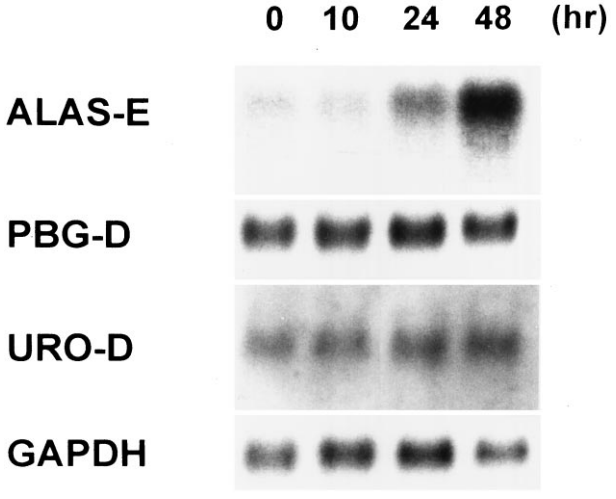

Fig. 4. Effect of camptothecin on ALAS-E, PBG-D, or URO-D mRNA expression. IW32 cells were treated with 0.1 μM camptothecin for the indicated times. Total RNA was prepared and analyzed by Northern blotting for the expression of ALAS-E, PBG-D, and URO-D. GAPDH is shown as an internal control. Similar results were obtained from three independent experiments.

constitutively expressed mRNAs of PBG-D and URO-D, and their levels remained relatively unchanged during the 48-hr period of the drug-induced differentiation. In contrast, transcript levels of ALAS-E were up-regulated in the camptothecin-treated cells. The time course of the ALAS-E mRNA induction paralleled with that of the β-globin mRNA accumulation. These results indicate that ALAS-E was up-regulated at the level of its gene expression during the camptothecin-induced IW32 cell differentiation.

Activation of globin and ALAS-E genes requires protein synthesis and is inhibited by PTPase inhibitors. Activation of β-globin and ALAS-E genes was observed at a delayed period after camptothecin administration, suggesting that protein synthesis may be required. To verify this, IW32 cells were treated with cycloheximide (10 μg/ml) for 1 hr before the addition of camptothecin. Because prolonged treatment at the dosage of cycloheximide was toxic to IW32 cells, at 12 hr after the addition of camptothecin, cells were replaced with media containing neither camptothecin nor cycloheximide. The expression of β-globin and ALAS-E mRNAs was analyzed by Northern blotting 48 hr after camptothecin addition. Under such conditions, camptothecin was still able to increase β-globin and ALAS-E mRNA accumulation (Fig. 5, *lanes 1 and 3*). However, in the presence of cycloheximide, camptothecin-stimulated expression of both genes was inhibited (Fig. 5, *lanes 2 and 4*). These findings suggest that the activation of the erythroid marker genes by camptothecin is an indirect consequence of its effect on other protein expression.

We next examined whether the signals mediating camptothecin-induced differentiation involved phosphorylation/dephosphorylation. Incubation with several kinase inhibitors, including genistein, H7, and HA1004, or depletion of protein kinase C by prolonged treatment with TPA revealed no significant effects on the camptothecin elicited-differentiation (data not shown). In contrast, however, sodium vanadate, a PTPase inhibitor, when used simultaneously with camptothecin, dose-dependently inhibited the differentiation. As indicated in Fig. 6A, the camptothecin-induced differentiation was significantly blocked by vanadate at 50 μM, a concentration shown to substantially increase the phosphotyrosine-containing proteins of IW32 cells (Fig. 6B). Similar dose-dependent inhibition of differentiation was demonstrated with another PTPase inhibitor, BPA (Fig. 6A). In both cases, complete inhibition of the camptothecin-induced differentiation was observed. In contrast, okadaic acid, a serine/threonine phosphatase inhibitor, did not show any significant inhibition on the camptothecin-induced differentiation (data not shown). Consistent with benzidine staining, Northern blot analysis demonstrated that the β-globin mRNA expression could be specifically blocked by vanadate but not by okadaic acid (Fig. 7). Furthermore, the same pattern of inhibition was observed in the camptothecin-induced ALAS-E mRNA expression. Taken together, these results confirmed

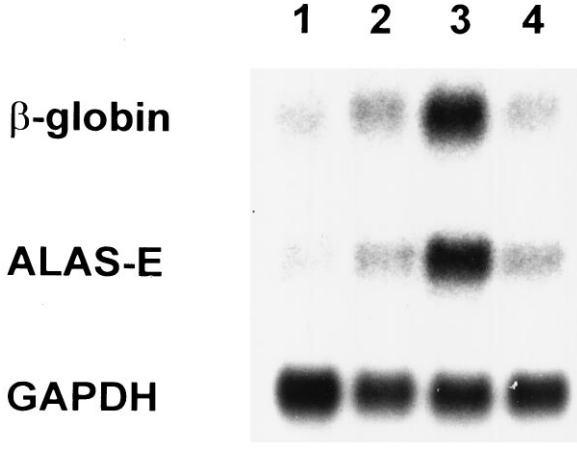

Fig. 5. Effect of protein synthesis on β-globin and ALAS-E mRNA expression induced by camptothecin. IW32 cells were pretreated with 10 μg/ml cycloheximide for 1 hr before the addition of camptothecin (0.1 μM). Twelve hours after camptothecin treatment, cells were washed and cultured in medium without camptothecin and cycloheximide. Total RNA was extracted and analyzed by Northern blotting for β-globin mRNA expression. The same blot was reprobed for ALAS-E and GAPDH transcript levels. *Lane 1*, no addition. *Lane 2*, cycloheximide. *Lane 3*, camptothecin. *Lane 4*, camptothecin plus cycloheximide. Similar results were obtained from at least three independent experiments.

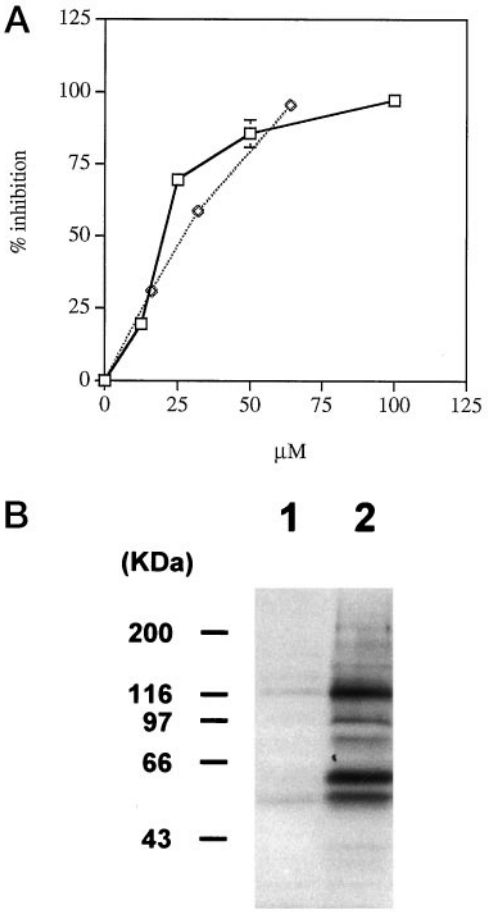

Fig. 6. A, Effect of tyrosine phosphatase inhibitors on the camptothecin-induced IW32 cell differentiation. IW32 cells were pretreated with different concentrations of sodium vanadate (□) or BPA (◇) for 30 min before the addition of camptothecin. After 48 hr, differentiation was determined by benzidine staining as described in Materials and Methods. Percent inhibition was assessed by comparing the percentage of benzidine-positive cells with that in the absence of PTPase inhibitors. B, Analysis of phosphotyrosine-containing proteins in the presence or absence of vanadate. Cells were treated (*2*) with or (*1*) without 50 μM sodium vanadate for 30 min. Total cell lysates were prepared, separated by sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose paper, and reacted with specific antibodies against phosphotyrosine. *Numbers on the left,* molecular mass markers (kDa).

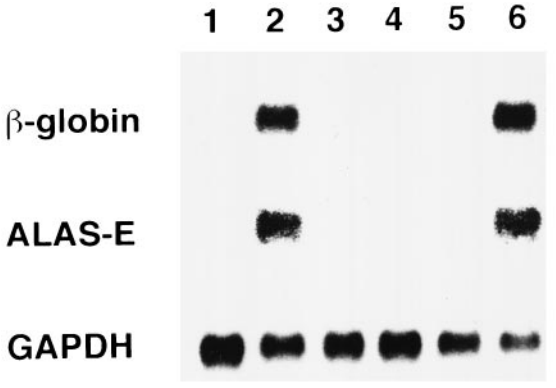

Fig. 7. Effect of vanadate or okadaic acid on β-globin and ALAS-E mRNA expression induced by camptothecin. IW32 cells were treated with sodium vanadate (50 μM) or okadaic acid (0.01 μM) for 30 min before the addition of camptothecin (0.1 μM). After 48 hr, total RNA was prepared and analyzed separately by Northern blotting for β-globin, ALAS-E, and GAPDH mRNA expression. *Lane 1*, no addition. *Lane 2*, camptothecin. *Lane 3*, vanadate. *Lane 4*, okadaic acid. *Lane 5*, camptothecin plus vanadate. *Lane 6*, camptothecin plus okadaic acid.

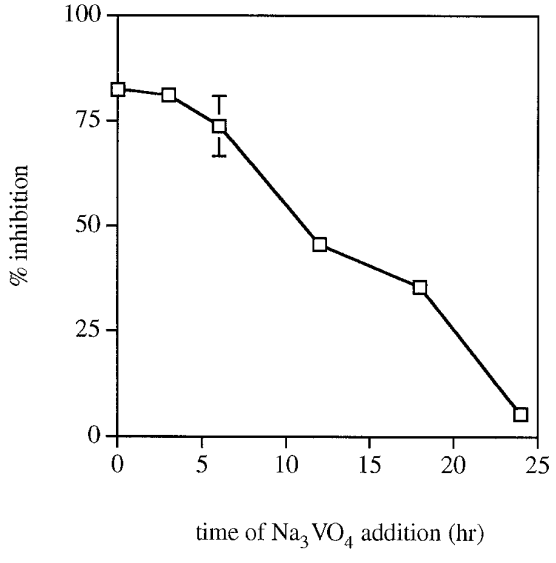

Fig. 8. Relationship between the effectiveness of vanadate inhibition and the times of its addition during camptothecin treatment. IW32 cells were treated with 0.1 μM camptothecin, and vanadate (50 μM) was added at the indicated times after camptothecin addition. Cells were harvested at 48 hr, and benzidine-positive cells were counted. Data represent mean ± standard deviation from three independent experiments, each with triplicate determinations.

that inhibitors to PTPase specifically blocked the camptothecin-induced expression of erythroid-specific marker genes.

Time window of PTPase action in the camptothecin-induced differentiation. We next examined the possible time point of PTPase action during the camptothecin-induced differentiation. Sodium vanadate was added at different times after camptothecin addition, and at 48 hr, cells were harvested and analyzed for hemoglobin production by benzidine staining. As shown in Fig. 8, vanadate remained fully active in its inhibition of differentiation if added between 0 and 6 hr after camptothecin treatment, after which it gradually lost its effectiveness. Cells became refractory to vanadate inhibition after exposure to camptothecin for >18 hr. These data suggest that PTPases act within a defined time window in mediating the camptothecin-induced differentiation.

Phosphatase as a common mediator of IW32 erythroleukemia differentiation. IW32 cells could be induced to differentiate by a number of agents, including sodium butyrate and topoisomerase II inhibitor VM-26. We have found that IW32 cells do not express p53 mRNA or protein. By stably transfecting a temperature-sensitive p53 DNA ($p53^{val135}$) into the cell, we demonstrated that temperature down-shift could promote the differentiation of the cell (data not shown). The possibility that the PTPase was involved in IW32 differentiation induced by the above-mentioned inducers was studied. Cells were induced to differentiation via VM-26, sodium butyrate, or p53 expression in the presence of 50 μM vanadate, and benzidine-positive cells were counted at 48 hr. As shown in Table 1, significant inhibition by vanadate was found in all cases studied, suggesting that PTPase played a general role in the erythroid differentiation of IW32 cells.

TABLE 1

Effect of vanadate on IW32 cell differentiation induced by VM-26, sodium butyrate, or p53 expression

IW32 cells were treated with or without sodium vanadate (50 μM) for 30 min before the addition of VM-26 (0.05 μM) or sodium butyrate (2.5 mM), and differentiation of cells was assessed with benzidine staining. Alternatively, IW32 cells stably transfected with a temperature-sensitive p53 DNA ($p53^{val135}$) were induced to express wild-type p53 by temperature down-shifting in the presence or absence of vanadate, and the benzidine-positive cells were determined. Results are given as mean ± standard deviation of three independent experiments.

Differentiation inducer	Inhibition
	%
VM-26	91.1 ± 6.2
Sodium butyrate	89.0 ± 9.4
p53	80.0 ± 1.3

We next examined whether increased PTPase activity could be detected in differentiating IW32 cells. Total cell extracts were prepared from cells exposed to camptothecin, VM-26, or sodium butyrate for 48 hr, and PTPase activity was assayed according to Dunphy *et al.* (28) using *p*-Npp as a substrate. Significant elevation in *p*-Npp-hydrolyzing activity was observed in IW32 cells induced to differentiation by all three drugs (Table 2). When the assay was performed in the presence of vanadate, it was clear that a significant portion (30–40%) of the activity measured could not be inhibited by vanadate. However, because the vanadate-insensitive activity did not vary with drug treatment, the majority of the increase in *p*-Npp-hydrolyzing activity could be attributed to the increase in vanadate-sensitive PTPase activity. As a control, we showed that okadaic acid did not have any significant inhibition on the *p*-Npp-hydrolyzing activity.

Camptothecin induces nuclear and cytosolic phosphatase activity in IW32 cells. Cells were further fractionated and analyzed for the distribution of PTPase activities in cytosols, membranes, and nuclei during the course of camptothecin treatment. The results showed that there was a time-dependent increase in cytosolic and nuclear PTPase activities in the camptothecin-treated cells (Fig. 9). A noticeable increase in cytosolic PTPase activity was found at 12 hr, and the activity remained high, even at 48 hr after camptothecin addition. Enhanced nuclear PTPase activity was not apparent until 18 hr after the drug addition. Because the cytosolic protein contents and PTPase activity far exceeded those of the nucleus, the majority (> 80%) of the induced activity was associated with the cytosol. There was a moderate but reproducible increase in membrane-associated PTPase activity at 18 hr after camptothecin treatment, although at other times, membrane-associated PTPase activity remained relatively unchanged. As a control for cell fractionation, the cytosolic and nuclear extracts were analyzed by Western blotting for levels of a major nucleolar protein nucleolin (29). As can be seen in Fig. 10, there was no detectable nuclear-to-cytosol leakage of nucleolin in the 48-hr period of camptothecin treatment or during subcellular fractionation.

Discussion

Using IW32 cells as a model system, we have shown that camptothecin induced the differentiation of the cells along the erythroid pathway. At the concentrations of camptothecin used to induce differentiation, a significant reduction in topoisomerase I activity was observed at an early stage of differentiation, suggesting that differentiation may be initiated at the nucleus through inhibition of topoisomerase I activity. It is possible that by inhibiting the activity of topoisomerase I, camptothecin alters the gene expression and promotes the differentiation program. Results showing that induction of both the globin and ALAS-E mRNA expression is abolished by cycloheximide demonstrate that activation of erythroid-specific genes by camptothecin is mediated through a newly synthesized protein, suggesting that these two tissue-specific genes are not the direct targets of topoisomerase I.

Several lines of evidence support that enzymes involved in the heme synthesis are regulated, at least in part, at the level of gene expression during erythroid maturation. Previous studies have demonstrated increased mRNA expression of PBG-D, URO-D (33), and ALAS-E (20) during the DMSO-induced MEL cell differentiation. Induction of ALAS-E mRNA has also been found in the erythropoietin-induced differentiation of J2E-1 erythroleukemia cells (34). Very low levels of ALAS-E mRNA expression were detected in the IW32 cells. Furthermore, among the heme-synthesizing enzymes that were analyzed, ALAS-E was the only enzyme that showed differential regulation at the level of gene expression by camptothecin; the transcript levels of URO-D and PBG-D remained relatively unchanged. This implies that the amount of ALAS-E expression may be limited in the heme-synthesizing pathway of differentiating IW32 cells. These results, however, do not exclude the possibility of post-tran-

TABLE 2

Increase in PTPase activity during IW32 erythroleukemia cell differentiation

IW32 cells were treated with camptothecin (0.1 μM), VM-26 (0.05 μM), or sodium butyrate (2.5 mM) for 48 hr, cells were harvested, and total cell lysates prepared as described in Materials and Methods. PTPase activity was assayed by using *p*-Npp as a substrate according to Dunphy *et al.* (28). Vanadate-sensitive PTPase activity was determined by subtracting the activity in the presence of sodium vanadate (50 μM) from that obtained in the absence of the drug, whereas okadaic acid-sensitive PTPase activity was determined by subtracting the activity in the presence of okadaic acid (0.01 μM) from that in the absence of the drug. Results are mean ± standard deviation of three experiments, each performed in triplicate.

	PTP activity						
		Camptothecin		VM-26		Sodium butyrate	
	Basal	Total	Increase over basal	Total	Increase over basal	Total	Increase over basal
	pmol of p-Npp/min/μg of protein						
Overall *p*-Npp-hydrolyzing activity	24.0 ± 1.5	35.5 ± 0.2	11.5 ± 1.1	31.7 ± 0.7	7.7 ± 1.2	31.0 ± 1.3	7.0 ± 1.4
Vanadate-sensitive phosphatase	13.5 ± 2.4	24.4 ± 1.0	10.9 ± 1.9	20.8 ± 0.5	7.3 ± 1.8	19.0 ± 1.0	5.5 ± 1.9
Okadaic acid-sensitive phosphatase	1.1 ± 1.2	2.7 ± 1.7	1.6 ± 1.5	0.8 ± 0.9	0	0.0 ± 1.1	0

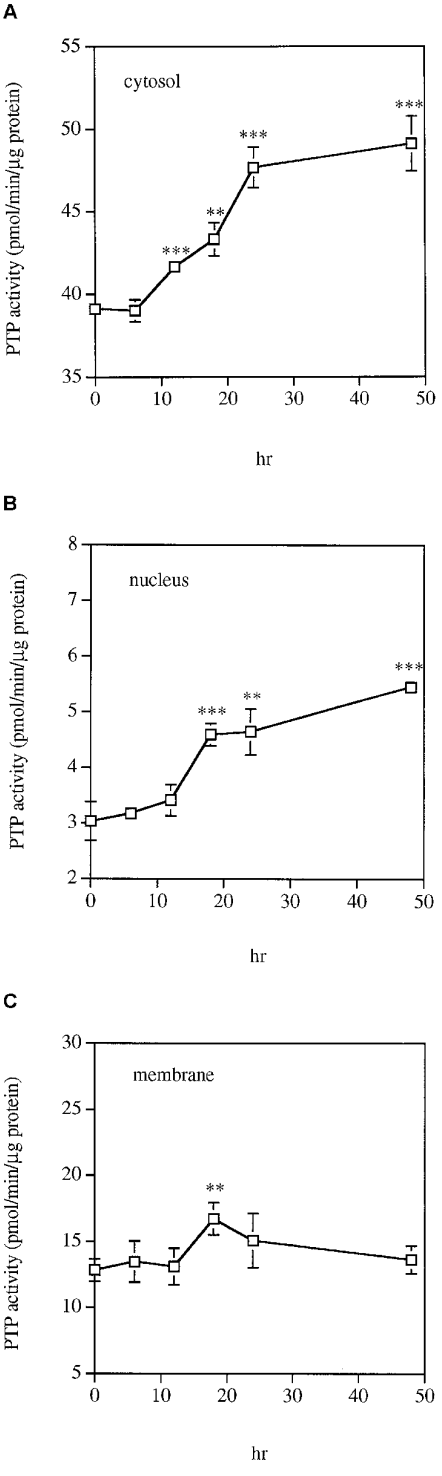

Fig. 9. Effect of camptothecin on PTPase activity. IW32 cells were treated with 0.1 μM camptothecin. At the indicated times, cells were harvested and fractionated as described in Materials and Methods. PTPase activities of (A) cytosol, (B) nucleus, and (C) membrane fractions were determined as described in Materials and Methods. Data are mean ± standard deviation from six experiments, each performed in triplicate. **, $p < 0.02$; ***, $p < 0.01$ by Student's *t* test.

scriptional control on ALAS-E or other heme-synthesizing enzymes in the camptothecin-induced IW32 cell differentiation.

Results from the current study support the hypothesis that PTPases may play a pivotal role in the induced differentiation of IW32 erythroleukemia cells. First, inhibitors of PTPases, including sodium vanadate, phenylarsine oxide, and BPA, have been shown to inhibit hemoglobin production induced by camptothecin. Second, vanadate also inhibits differentiation induced by other signals, including p53, VM-26, and sodium butyrate. Third, there is a time-dependent increase in cytosolic and nuclear PTPase activity in cells treated with camptothecin; elevated PTPase activity has also been demonstrated in differentiation induced by VM-26 and sodium butyrate.

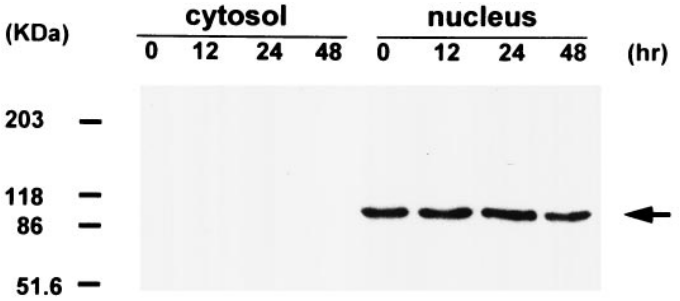

Fig. 10. Analysis of nucleolin levels in nuclear and cytosolic extracts. IW32 cells were treated with 0.1 μM camptothecin for the indicated times. Cells were harvested, and nuclear and cytosolic proteins were fractionated as described in Materials and Methods. The same amounts of proteins used for PTPase assays were analyzed by immunoblotting for nucleolin levels as described in Materials and Methods. *Arrow*, 100-kDa nucleolin.

Accumulating evidence suggests the importance of PTPase in the differentiation of several experimental cell models (35–40). In the TPA-induced HL-60 promyelocytic differentiation, enhanced activity of PTP1C has been observed, which may result from the increased transcription of the PTP1C gene (36, 38). Increased transcript levels of PTPases has also been found in DMSO-induced MEL cell differentiation (7, 40) and in retinoic acid-induced F9 embryonic carcinoma cell differentiation (39). The findings that the camptothecin-stimulated mRNA expression of β-globin and ALAS-E is inhibited by vanadate indicate that PTPase activity is necessary in mediation of the camptothecin-stimulated expression of the key differentiation markers of the erythroid cells.

It has been shown that c-*myc* down-regulation occurs at a very early stage in the DMSO-induced MEL cell differentiation. Evidence further supports that prior c-*myc* mRNA reduction is necessary and sufficient for many drug-induced MEL cell differentiations (30–32). In contrast, our results that show no significant changes in c-*myc* transcript levels during the entire period of the camptothecin-induced IW32 cell differentiation indicate that c-*myc* down-regulation does not play a role in the camptothecin action. However, despite the apparently independent mechanisms used by camptothecin and DMSO, both drug-induced differentiations could be inhibited by vanadate. This suggests the importance of PTPase in both the c-*myc*-dependent and -independent erythroleukemia differentiations. Consistent with this, we found that vanadate blocked the sodium butyrate-, p53-, and VM-26-induced IW32 differentiations. Differentiation induced by butyrate or p53 occurs in the presence whereas that by VM-26 occurs in the absence of c-*myc* down-regulation. It appears that PTPase activation may be a relatively late event that converges multiple signaling pathways in induction of IW32 erythroleukemia cell differentiation.

PTPase isozymes belongs to a large superfamily of proteins that have widespread subcellular distributions, including membrane, nucleus, and cytosol. In the camptothecin-induced IW32 cell differentiation, a significant increase in PTPase activity is found in both the cytosolic and nuclear fractions, with the cytosolic increase preceding that of the nucleus. It is not known whether the nuclear enzyme originates from the cytosol. Translocation of PTPase from one compartment to another has been shown in the TPA-induced HL-60 differentiation, in which PTP1C is translocated from cytosol to the plasma membrane (38). Alternatively, it is possible that various cytosolic and nuclear PTPases are activated during differentiation. As shown by Northern blotting in the DMSO-induced MEL cells differentiation, transcript levels of more than one PTPase isozymes are induced (7). A similar observation has been noted in the nerve growth factor-induced differentiation of PC12 cells, in which elevated PTPase activities associated with various molecular weights have been found (9).

We have shown that after 12–18 hr of camptothecin treatment, the cells become refractory to vanadate inhibition, indicating that by this time, the signals of PTPases mediating the differentiation program have been executed. It is interesting to point out that the time course of camptothecin-stimulated cytosolic PTPase activity closely correlates with the time required for vanadate addition to effectively block the differentiation. No such correlation is found in the time course increment of the nuclear PTPase activities. In this sense, the increase in cytosolic PTPase activity may be more critical in mediating the camptothecin-elicited IW32 cell differentiation.

In conclusion, we have shown that camptothecin can induce the differentiation of IW32 erythroleukemia cells. The inhibition of PTPases by vanadate blocks the coordinated expression of both the β-globin and ALAS-E genes. The observation that vanadate inhibits IW32 cell differentiation induced by multiple signals, together with the findings of elevated PTPase activities in differentiating cells, suggests PTPases play a critical role in mediating the differentiation of IW32 erythroleukemia cells.

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