Phorbol Ester Stimulates the Activity of a Protein Tyrosine Phosphatase Containing SH₂ Domains (PTP1C) in HL-60 Leukemia Cells by Increasing Gene Expression*

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The affinity-purified antibody to a protein tyrosine phosphatase (PTP) containing two src homology 2 domains (PTP1C) was generated. The antibody recognized two types of PTP1C (PTP1C-α and -β) of which the molecular sizes were 68 (α) and 62 kDa (β), respectively, and these two types were expressed differentially in various cell types. The immune complex phosphatase assay using the antibody demonstrated that 12-0-tetradecanoylphorbol-13-acetate (TPA) and a vitamin D metabolite increased the PTP activity of immunoprecipitated PTP1C to 230 and 150% of control, respectively. By contrast, neither dimethyl sulfoxide nor retinoic acid significantly affected the PTP activity of PTP1C in HL-60 cells. The time course increment by TPA of PTP1C activity was closely correlated with that of the acquisition by HL-60 cells of a macrophase-like phenotype. In addition, TPA increased the amount of PTP1C detected by immunoblotting and immunoprecipitation and raised the level of expression of PTP1C mRNA in HL-60 cells. The increase of PTP1C mRNA induced by TPA treatment was inhibited by cycloheximide, suggesting that new protein synthesis is required for the increase by TPA of PTP1C mRNA expression. Furthermore, TPA increased the rate of transcription of the PTP1C gene without affecting the stability of PTP1C mRNA. These results suggest that (i) two subtypes of PTP1C may exist and function in various cell types, and (ii) TPA stimulates the PTP activity of PTP1C by increasing the transcription rate of PTP1C gene expression. The possible role of PTP1C in the macrophage differentiation will be also discussed.

The phosphorylation of protein tyrosine residues is a crucial event in the regulation of normal cellular process such as proliferation and differentiation and is also involved in the malignant transformation of cells (1–3). The level of tyrosine phosphorylation is determined by the balance between the actions of protein tyrosine kinases and PTPs (4, 5). Recently, much attention has been devoted to the role of PTP in the regulation of protein tyrosine phosphorylation, and it has been shown that there are numbers of PTPs which consist of two types, namely the transmembrane type and the nontransmembrane type (4, 5). A PTP, PTP1C/HCP/SH-PTP1/SHIP (6–9), is a nontransmembrane type PTP that has been recently cloned. Of interest is that this PTP contains two SH₂ domains in its N-terminal region. The SH₂ domain (10) has recently been found in various nonreceptor-type protein tyrosine kinases such as p66°°°° (1, 10) and other cytoplasmic signaling proteins such as phospholipase C-γ (11), GTP-activating protein (1, 10), phosphatidylinositol 3-kinase (12), and actin-binding protein (10). These signaling proteins bind phosphorylated tyrosine residues on activated growth factor receptors via SH₂ domains, and through the association they become substrates for the receptor tyrosine kinase (1, 10). Thus, SH₂ domains of PTP1C may direct this unique PTP to tyrosine-phosphorylated protein, thereby modulating protein tyrosine kinase-related signal transduction (6). Subsequently, PTP1C has been shown to be expressed predominantly in hematopoietic cells (7–9). Thus, PTP1C may play an important role in a certain function that involves tyrosine phosphorylation and dephosphorylation in hematopoietic cells (7–9). Since PTPs have been suggested to function as a negative regulator of cellular proliferation (1, 13), PTP1C might suppress the proliferation of hematopoietic cells and induce cellular differentiation. It has been shown that promyelocytic leukemia cell line HL-60 is induced to differentiate by various compounds such as TPA (13–15) or MeSO (16, 16). Therefore, we have determined whether these compounds stimulate the PTP activity of PTP1C in HL-60 cells. In the present study, we have generate an affinity-purified polyclonal antibody to PTP1C to determine the effects of TPA and MeSO on the specific activity of immunoprecipitated PTP1C. Results indicate that TPA, but not MeSO, increased the specific PTP activity of immunoprecipitated PTP1C by increasing the expression of the PTP1C gene in differentiated HL-60 cells.

EXPERIMENTAL PROCEDURES

Materials—HL-60 (a promyelocytic leukemia cell line), MKN-45 (a gastric cancer cell line), KATO-III (a gastric cancer cell line) and HeLa (a cervical carcinoma cell line) were obtained from the Japanese Cancer Research Resources Bank. The breast carcinoma cell line ZR-75-1 was obtained from the ATCC. HL-60 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37°C under 5% CO₂. Other cells were cultured as recommended by the suppliers. Both human c-myc cDNA (2.2 kb) and mouse β-actin cDNA (1.2 kb) were obtained from the Japanese Cancer Research Resources Bank. A cDNA library of ZR-75-1 was obtained from Clontech. TPA, 1,25-(OH)₂VD₃, MeSO, retinoic acid, actinomycin D, and cycloheximide were purchased from Sigma. Raytide and p66°°°° tyrosine kinase were
from Oncogene Science. Affi-Gel 10 was from Bio-Rad. Alkaline phosphatase-conjugated goat anti-mouse IgG was from Promega. Immobilon was from Millipore. Tran32S-label (1,000 Ci/mmol) was from ICN. (α-32P)ATP (3,000 Ci/μmol), (γ-32P)ATP (6,000 Ci/μl), and (α-32P)UTP (3,000 Ci/mmol) were from Du Pont-New England Nuclear. Peroxidase was from Pharmacia LKB Biotechnology Inc. Centriprep-10 was from Amicon.

Affinity Purification of a Polyclonal Antibody—To generate a polyclonal antibody, a peptide containing 18 amino acids (CEKVKKQ RSDAKEKSKGS, residues 577-593) of PTP1C (6) was chemically synthesized and conjugated to keyhole limpet hemocyanin by Peptide Institute Inc. The peptide was initially injected with 0.75 mg of the purified peptide in complete Freund's adjuvant and boosted every 2 weeks with 0.5 mg of antigen in incomplete Freund's adjuvant. For affinity purification, 5 mg of peptide was rocked with 2 ml of Affi-Gel 10 at 4 °C overnight, and gels were washed with 30 ml of elution buffer containing 10 mM glycine (pH 7.5), 2 mM NaCl, and 0.5 mM EGTA. Rabbit serum was then rocked with gels overnight at 4 °C followed by washing with 20 ml of 50 mM Hepes (pH 7.4) containing 0.5 M NaCl and 0.5 mM EGTA. The antibody bound to the gels was then eluted with 8 ml of elution buffer, and the eluate was combined with 10 ml of 1 M Hepes (pH 8.0) containing 0.5 mM EGTA, followed by concentration with Centriprep-10.

Phosphatase Assay—For the immune complex phosphatase assay, HL-60 cells treated with TPA or other compounds were lysed in 1 ml (approximately 2 mg/ml protein) of ice-cold lysis buffer (RIPA buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, and 10% glycerol) containing 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. The lysates were centrifuged at 10,000 × g for 15 min at 4 °C, and the resultant supernatants were used in the phosphatase assay. The protein concentration was determined by a modification of the method of Bradford (17) for application to cell lysates (18).

Immunoprecipitation was performed by incubating 10-500 μg of HL-60 cell lysates with 5 μg of antibody prebound to Sepharose-4B-protein A beads for 4 h at 4 °C. The beads with immunoprecipitated proteins were washed once with a buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100, twice with 1 ml of WG buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, twice with assay buffer containing 40 mM MES (pH 5.0) and 1.6 mM dithiothreitol. The assay mixture (200 μl) containing assay buffer with 25 mM pNPP and the immune complex pellet were incubated at 30 °C for 30 min. The reaction was terminated by the addition of 200 μl of 1 N NaOH, and the absorbance at 410 nm was determined (19). The assay of the PTP activity of the immune complex was also performed using the [32P]-labeled synthetic peptide Raytide (20, 21). For the radioelaboration of Raytide, 30 μg of Raytide dissolved in 30 μl of assay buffer (50 mM Hepes (pH 7.3), 0.1 mM EDTA, and 0.015% Brij 35) was incubated with 27 μl of kinase buffer (assay buffer with 0.1% Brij 35, 2-mM ATP (500 μCi/ml, χ-32P)ATP, and 1 μl of p60185 tyrosine kinase as described previously (21). The reaction was incubated at 37 °C overnight and terminated by the addition of 0.5 ml of 20% trichloroacetic acid, 20 μl NaH2PO4, and 0.1 ml of 5% acetic acid. The samples were then boiled for 4 min, loaded onto a 10% SDS-acrylamide gel, and subjected to electrophoresis followed by autoradiography. The radioactivities in 85-kDa PTTP bands were determined using a Fujix BAS 200 Bio-image Analyzer.

Isolation of Human PTP1C Clones—To isolate the gene encoding PTTP1C, a 720-base pair DNA fragment of PTP1C gene was amplified by reverse transcriptase polymerase chain reaction using total RNA extracted from the gastric cancer cell line MKN-45 as described previously (22). The 5′- and 3′-ends of the primers used were 5′-GAAGATCCCGCGCCAGAGAAACAAGGCAGAAC (nucleotides 1060-1083) and 5′-ATAAGATCTCCAGTGGCAGTGGCCCAGT-AGATG (nucleotides 1774-1806), respectively. The nucleotide numbers used here correspond to the numbering used by Shen et al. (6). The primers contained flanking sequences for BamHI and EcoRI sites to facilitate subsequent cloning. This polymerase chain reaction fragment was labeled with [α-32P]dCTP (3,000 Ci/mmol) by the random primer method (23) and used to screen a cDNA bacteriophage library constructed from the breast cancer cell line ZR-75-1. Two independent clones, 1.9 and 2.0 kb in length, were obtained, ligated, and subcloned into the EcoRI site of pBluescript II for subsequent sequencing in both directions by the chain termination method as described previously (23).

Northern Blot Analysis—For the analysis by Northern blotting, total RNA (20 μg) was extracted from HL-60 cells and was subjected to electrophoresis on a 1.2% agarose/formaldehyde gel and transferred to nylon filters as described previously (24). A PTTP1C cDNA probe was prepared from plasmid pBluescript II-PTTP1C, constructed as described above, by purifying a 2.4-kilobase pair human PTTP1C cDNA fragment. The blot was hybridized with the [32P]-labeled PTTP1C cDNA probe in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5 mM EDTA overnight at 42 °C. The blots were then washed twice at room temperature in 2 × SSC and 0.1% SDS, followed by washing twice for 30 min at 50 °C in 0.1 × SSC and 0.1% SDS. The filter was then rehybridized with [32P]-labeled human c-myc or with [32P]-labeled mouse β-actin probe.

Assay for Nuclear Transcription—Transcription of nuclear mRNA was compared in cells cultured for 24 or 48 h in the medium without TPA and cells induced to differentiate with 10 nM TPA. The assay was performed as described previously (25, 26). Briefly, 3–5 × 106 nuclei were prepared by using the lysis buffer containing 30% sucrose, 0.5 M NaCl, 2.5 M urea, 20 μg/ml RNase A, and 1 μg/ml RNase T1 at 37 °C for 30 min, followed by reverse transcriptase polymerase chain reaction using total RNA and the PTTP1C gene cDNA probe in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5 mM EDTA overnight at 42 °C. The blots were then washed twice at room temperature in 2 × SSC and 0.1% SDS, followed by washing twice for 30 min at 50 °C in 0.1 × SSC and 0.1% SDS. The filter was then rehybridized with [32P]-labeled human c-myc or with [32P]-labeled mouse β-actin probe.

Immunoblotting and Immunoprecipitation—For immunoblotting experiments, cellular lysates were prepared as described above. A 10-50 μg sample of each lysate was subjected to electrophoresis on a 10% SDS-polyacrylamide gel which was then electroblotted to Immobilon P. The blots were blocked for 60 min with 3% BSA in TBST (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20). Blots were incubated with the anti-PTTP1C antibody (1:10,000 dilution) in TBST for 2 h followed by two washes (15 min each) in TBST and visualized with alkaline phosphatase-conjugated goat anti-mouse IgG (1:5000) in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (21).

For [32P] labeling experiments, HL-60 cells were labeled overnight in methionine-free, cysteine-free RPMI 1640 supplemented with 10% dialyzed serum using Tran32S-label at 100 μCi/ml. Labeled cells were lysed in RIPA buffer as described above and centrifuged. The lysates were incubated with 5 μl of the purified antibody prebound to Sepharose-protein A beads for 4 h at 4 °C. The immunoprecipitated proteins were washed twice in buffer A (20 mM NaH2PO4, (pH 8.6), 0.5% Triton X-100, 0.1% SDS, 1 M NaCl, 0.1% BSA), twice in buffer B (20 mM NaH2PO4, (pH 8.6), 0.5% Triton X-100, 0.1% SDS, 0.5 M NaCl and 0.5 mM EGTA) and twice in WG buffer before SDS sample buffer was added. The samples were then boiled for 4 min, loaded onto a 10% SDS-acrylamide gel, and subjected to electrophoresis followed by autoradiography. The radioactivities in 85-kDa PTTP bands were determined using a Fujix BAS 2000 Bio-image Analyzer.
of the C terminus of PTPlC as described under "Experimental Procedures." This antibody immunoprecipitated a 66-kDa protein from HL-60 cells metabolically labeled with [35S]methionine (Fig. 1A, lane 2), whereas the corresponding serum from the same rabbit did not (Fig. 1A, lane 1). In addition, when the immunoprecipitation was performed in the presence of excess amounts of the peptide used to raise the antibody, a 66-kDa protein was specifically eliminated (Fig. 1A, lane 3). The molecular size (66 kDa) of the radiolabeled protein specifically recognized by the antibody closely corresponded to the molecular size predicted from the amino acid sequence of cloned PTPlC (6-9). As shown in Fig. 1B, lane 1, Western blotting using the anti-peptide antibody showed a single immunoreactive protein band in the HL-60 cell lysate. In the HeLa cell lysate (Fig. 1B, lane 2) this antibody also recognized a single protein whose relative molecular size was smaller than that of the protein detected in HL-60 cells and was estimated to be 62 kDa. Thus, we tentatively named these two different PTPlC proteins PTPlC-α (HL-60 cell type) and PTPlC-β (HeLa cell type), respectively. Furthermore, in two gastric cancer cell lines MKN-45 and KATO-III, both PTPlC-α and -β were simultaneously expressed (Fig. 1B, lanes 3 and 4). In the breast cancer cell line ZR-75-1, from which the PTPlC cDNA was originally isolated (6), PTPlC-α was predominantly expressed (Fig. 1B, lane 5).

Immunoprecipitation was carried out with increasing amounts of HL-60 cell lysate, and the PTP activity of the precipitate was then assayed using pNPP as a PTP substrate. PTP activity was recovered in the immune complex, and the assay was linear in the range of 10 μg up to 500 μg of HL-60 cell lysate (Fig. 2A). When radiolabeled Raytide was used as a PTP substrate, PTP activity was also observed in the immunoprecipitate prepared from HL-60 cell lysate (Fig. 2B). Furthermore, the PTP activity in the immune complex was completely inhibited by 1 mM vanadate, a inhibitor of PTP (Fig. 2, A and B). These results indicate that the purified polyclonal antibody specifically recognizes PTPlC and allows us to measure the PTP activity of PTPlC immunoprecipitated from HL-60 cells.

**Effects of TPA on PTPlC Activity in HL-60 Cells.**—We next determined the changes in the PTP activity of PTPlC when HL-60 cells were treated with various compounds that induce HL-60 cell differentiation. When HL-60 cells were treated with 10 nM TPA for 72 h, TPA increased PTPlC activity in HL-60 cells to 230 ± 18% of control (n = 6) (Fig. 3). In addition, a vitamin D₃ metabolite, 1,25-(OH)₂D₃, also increased PTPlC activity to 150 ± 8% of control (n = 3) after a 72-h exposure. In contrast, when HL-60 cells were treated with 1.2% Me₂SO or 100 nM retinoic acid, no significant effect on PTPlC activity was detected, even after a 120-h exposure (Fig. 3). After a 120-h exposure of HL-60 cells to 1.2% Me₂SO, cells attained the characteristics of granulocytes, as measured by their capacity to reduce nitro blue tetrazolium to insoluble formazan granules (67 ± 8% of cells positive, n = 3). TPA increased the PTPlC activity in a time-dependent manner; the detectable increase in PTPlC activity was observed after a 24-h exposure of HL-60 cells to TPA, and the maximal increase of PTPlC activity stimulated by TPA was observed after a 72-h exposure (Fig. 4A). The time course of the stimulation by TPA of PTPlC activity was closely related to the acquisition of nonspecific esterase activity, a marker of the monocyte-macrophage phenotype (25) (Fig. 4A). Furthermore, TPA stimulated PTPlC activity in a concentration-dependent fashion with a detectable increase observed at 1 nM TPA and a maximal stimulation at 10 nM TPA (Fig. 4B).

**Effects of TPA on the Protein Level of PTPlC in HL-60 Cells.**—To explore the mechanism by which TPA increases PTPlC activity in macrophage-like differentiated HL-60 cells, we next examined TPA-induced changes in the PTPlC protein level as detected by immunoblotting. As shown in Fig. 5A, both 10 nM TPA and 100 nM 1,25-(OH)₂D₃, significantly increased the intensity of the PTPlC band, whereas neither Me₂SO nor retinoic acid had any effect. When HL-60 cells were treated with 10 nM TPA for 6-72 h and the lysates were immunoblotted, the intensity of the PTPlC band was increased in a time-dependent fashion (Fig. 5B). Furthermore, when HL-60 cells were treated with or without 10 nM TPA for 48 h followed by incubation with [35S]labeled amino acids for another 16 h, TPA increased the radioactivity of the PTPlC 66-kDa band to 255 ± 24% of control (n = 3) (Fig. 5C). Thus, these results suggest that TPA may increase the level of PTPlC protein, thereby elevating the PTP activity of PTPlC in macrophage-like differentiated HL-60 cells. It was constantly observed that a 25-kDa protein coimmunoprecipitated with PTPlC (Fig. 5C, lanes 1 and 2), suggesting that this protein may specifically form a complex with PTPlC in HL-60 cells.

**Effects of TPA on PTPlC mRNA Expression in HL-60 Cells.**—Since TPA increased the level of PTPlC protein, it seemed possible that TPA might stimulate the synthesis of PTPlC protein by increasing PTPlC gene expression in differentiated HL-60 cells. To determine the level of PTPlC gene expression we isolated PTPlC cDNA from a breast cancer cell (ZR-75-1) cDNA library. To isolate the PTPlC gene, we generated a 750-base pair DNA fragment corresponding to the nucleotide sequence of the PTPlC domain of PTPlC by reverse transcriptase polymerase chain reaction and used it as a probe for library screening. The sequence of the cloned PTPlC cDNA was identical to the nucleotide sequence published by Shen et al. (6) except that our PTPlC cDNA contained 32 additional nucleotides (GGAGAAGACGAGGTTTCCCTCAAGAGGAAGT) in the 5′-noncoding region. This suggests that two different PTPlC cDNAs may utilize different 5′ exons.

When HL-60 cells were treated with 10 nM TPA as a function of time, TPA significantly increased a 2.4-kb transcript of PTPlC after a 24-h exposure and maximally increased mRNA expression at 48 h (Fig. 6A). Measurement of radioactivities in 2.4-kb bands showed that TPA increased...
PTPlC mRNA expression of the PTPlC gene overlapped closely with that of an increase in the PTPlC activity in TPA-treated HL-60 cells.

The increase of PTPlC mRNA in response to TPA may require new protein synthesis. Since TPA increased the levels of PTPlC mRNA only after a 24-h exposure, we next determined the effect of cycloheximide on TPA-induced PTPlC gene expression. Although the increased level of PTPlC mRNA was observed after treatment of HL-60 cells with TPA for 48 h, simultaneous treatment with cycloheximide inhibited the increase in the level of PTPlC mRNA induced by TPA (Fig. 6B). This result suggests that the increase of PTPlC mRNA induced by TPA may require new protein synthesis.

The increase of PTPlC mRNA in response to TPA may result from either alteration of transcription rate or degradation rate of mRNA. To examine these possibilities we next performed nuclear run-on transcription assays. The results of these experiments were normalized to the rate of transcription of the β-actin gene. As shown in Fig. 7A, a significant increase in transcription of PTPlC gene was observed in HL-60 cells treated with 10 nM TPA for 24 or 48 h. The rate of PTPlC gene transcription was increased 1.9 ± 0.3- and 3.5 ± 0.5-fold (n = 3) in nuclei from cells treated with 10 nM TPA for 24 and 48 h, respectively. This increment corresponds well with the increase in PTPlC mRNA measured by Northern blotting. The stability of PTPlC mRNA in cells treated with or without TPA was also tested by inhibiting mRNA transcription with actinomycin D. After HL-60 cells were treated with or without 10 nM TPA for 48 h, cells were incubated with actinomycin D, and subsequently total RNA was ex-

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**Fig. 2.** Assay of the PTP activity of immunoprecipitated PTPlC from HL-60 cells. Varying amounts of HL-60 cell lysates were immunoprecipitated with 5 μg of affinity-purified polyclonal antibody to PTPlC. *Panel A,* immunoprecipitates were then incubated with 25 mM pNPP for 30 min, and their absorbance at 410 nm was determined as described under "Experimental Procedures." The value of the absorbance observed in the presence of preimmune serum instead of antibody was subtracted from each value. *Panel B,* immunoprecipitates were also incubated with 32P-labeled Raytide for 30 min as described under "Experimental Procedures." After centrifugation, the radioactivity of the supernatant was determined. The value of radioactivity observed in the presence of preimmune serum instead of antibody was subtracted from each value. The assay was performed in the presence (closed circles) or absence (open circles) of 1 mM vanadate. All data points were the mean of duplicate determinations, and the result presented is a representative of three separate experiments.

**Fig. 3.** Changes in the PTP activity of PTPlC during the differentiation of HL-60 cells. HL-60 cells were treated with 10 nM TPA or 100 nM 1,25-(OH)2VD3 for 72 h or with 1.2% MeSO or 100 nM retinoic acid for 120 h. After the cells were lysed, 100 μg of each lysate was immunoprecipitated and then assayed for PTP activity using pNPP as a phosphatase substrate as described under "Experimental Procedures." The value of the absorbance observed in the presence of preimmune serum instead of antibody was subtracted from each value, and the net increase was expressed as a percentage of the control value. Each value is the mean of duplicate determinations, and the result shown is a representative of three separate experiments. VD3, 1,25-(OH)2VD3; DMSO, dimethyl sulfoxide; RA, retinoic acid.

**Fig. 4.** Time- and concentration-dependent increases of PTPlC activity stimulated by TPA in differentiated HL-60 cells. *Panel A,* HL-60 cells were treated with 10 nM TPA for the indicated times. Cells were lysed, and immunoprecipitated PTPlC was assayed using pNPP as described under "Experimental Procedures." The nonspecific esterase activity of the HL-60 cells was also determined at the indicated times. *Open column,* PTP activity; *dotted line,* the percentage of HL-60 cells expressing nonspecific esterase activity. *Panel B,* HL-60 cells were treated with increasing concentrations of 10 nM TPA for 72 h. The PTP activity of PTPlC immunoprecipitated from the cell lysates was then assayed. Each value is expressed as a percentage of control and the mean of duplicate determinations. The result shown is a representative of three separate experiments.
FIG. 5. Comparison of the levels of PTP1C proteins in differentiated HL-60 cells. Panel A, HL-60 cells were treated with either 10 nM TPA (lane 2), 100 nM 1,25-(OH)2VD3 (lane 3), 1.25% MeSO and 100 nM retinoic acid (lane 4), or 100 nM retinoic acid (lane 5). Cells were treated with TPA and 1,25-(OH)2VD3 for 72 h or with MeSO and retinoic acid for 120 h. After treatments, cells were harvested, and 20 μg of cell lysate prepared from each was blotted with the purified antibody against PTP1C as described under "Experimental Procedures." Lane 1, control cells without any compounds. Panel B, HL-60 cells were treated with 10 nM TPA for 6-72 h. Cell lysates were prepared, and each 15 μg of cell lysate was blotted. Lane a, time 0; lane b, 6 h; lane c, 12 h; lane d, 24 h; lane e, 48 h; lane f, 72 h. Panel C, HL-60 cells were treated with or without 10 nM TPA for 48 h. The cells were then incubated with 10 nM TPA and 100 μCi/ml Tran32P-label for 16 h before harvesting. The cell lysates (250 μg) were immunoprecipitated as described under "Experimental Procedures." Lane 1, control; lane 2, 10 nM TPA. Immunoprecipitation was also performed in the presence of 5 μg of the peptide used for immunization (lane 3). A 25-kDa protein coimmunoprecipitated with PTP1C is indicated by the arrow. The results represent three independent experiments.

FIG. 6. Regulation of PTP1C mRNA expression in TPA-treated HL-60 cells. Panel A, HL-60 cells were treated with 10 nM TPA for 0-72 h, and total RNA was extracted. Twenty μg of total RNA was transferred to a nylon filter and then probed with radiolabeled PTP1C cDNA (upper), c-myc cDNA (middle), or β-actin (lower). Lane 1, time 0; lane 2, 12 h; lane 3, 24 h; lane 4, 48 h; lane 5, 72 h. Panel B, cells were treated with (lanes 2 and 3) or without (lane 1) 10 nM TPA for 48 h, and total RNA was extracted. Ten μg/ml cycloheximide was also added 20 h before harvesting the cells (lane 3). Twenty μg of total RNA from each was transferred to a nylon filter and then probed with radiolabeled PTP1C cDNA. The results shown represent three separate experiments.

FIG. 7. Effects of TPA on relative transcription rate of the PTP1C gene (panel A) and stability of PTP1C mRNA (panel B). Panel A, nuclei were prepared from HL-60 cells treated with or without 10 nM TPA for 24 and 48 h and incubated with [32P]UTP. The labeled RNA was extracted and hybridized to plasmid DNAs (5 μg/lane) containing PTP1C cDNA or β-actin cDNA insert or without any insert (pBluescript). Lane a, control; lane b, 24-h TPA treatment; lane c, 48-h TPA treatment. The radioactivity in each band was determined by a Fujix BAS 2000 Bioimage Analyzer, and transcription rates are expressed as the percentage of β-actin transcription rate. Panel B, after HL-60 cells were treated with 10 nM TPA for 48 h, 5 μg/ml actinomycin D was added to the medium, and total RNA was extracted from cells at indicated times. Northern blotting was carried out as described under "Experimental Procedures." Lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 5 h; lane 5, 8 h. The radioactivity in each band was determined by a Fujix BAS 2000 Bioimage Analyzer, and the half-life of PTP1C mRNA was estimated. The results represent three independent experiments.

antibody specifically recognized PTP1C as a 66-kDa protein in the HL-60 cell lysate. Furthermore, in the HeLa cell lysate this antibody recognized another type of PTP1C (PTP1C-β) of which the estimated molecular size was 62 kDa. In the breast cancer cell line ZR-75-1, PTP1C-α was predominant, whereas both types of PTP1C were expressed in two gastric cancer cell lines. Furthermore, both types of PTP1C were also expressed in two pancreatic cancer cell lines (Panc-1 and MIA PaCa-2) and three colon cancer cell lines (SW480, SW837, and WiDr).2 Thus, these results indicate that different types of PTP1C proteins were expressed differentially and may function in various cell types. The molecular size of PTP1C-α (66 kDa) closely corresponds to the molecular size estimated by PTP1C cDNA sequence published previously (6-9). In addition, PTP1C-α is predominantly expressed in ZR-75-1 cell and cDNA of PTP1C has been originally isolated from a cDNA library of ZR-75-1 cell (6). Thus, PTP1C-α might be encoded by the PTP1C gene originally described (6). On the other hand, alternative splicing of PTP1C-α gene may generate both α and β forms, or the α and β forms may be encoded by distinct PTP1C genes. The isolation of a cDNA clone of PTP1C-β from a HeLa cell cDNA library is currently being carried out in our laboratory.

The affinity-purified antibody was used to immunoprecipitate PTP1C and allowed us to measure the changes in the PTP activity of PTP1C during the differentiation of HL-60 cells. By immune complex phosphatase assay, we have demonstrated that TPA increases the PTP activity of PTP1C in macrophage-like differentiated HL-60 cells. This stimulatory effect of TPA on PTP1C activity was found to be time- and TPA concentration-dependent. The concentrations of TPA required for the induction of an increase in PTP1C activity correspond well with those required to induce the macrophage-like differentiation of HL-60 cells. Furthermore, the time course of the TPA-induced differentiation of HL-60 cells and that of the stimulation of PTP1C activity by TPA closely overlapped. A derivative of vitamin D, 1,25-(OH)2D3, which induces the monocyte-like differentiation of HL-60 cells (13), also increased the PTP activity of PTP1C. By contrast, neither MeSO nor retinoic acid, both of which induce the myelocyte-like differentiation of HL-60 cells (13), affected

DISCUSSION

In the present study, we generated an affinity-purified polyclonal antibody to a C-terminal peptide of PTP1C. Both immunoprecipitation and immunoblotting showed that the
the PTP1C activity in HL-60 cells. It has been suggested that PTPs function as a counterpart of protein tyrosine kinases; protein tyrosine kinases generally stimulate cellular proliferation, whereas PTPs negatively regulate cellular proliferation. The cellular differentiation that is part of the maturation process involves the programmed shutdown of the proliferation capacity of the cell (13). In addition, PTP1C has been demonstrated to be highly expressed in macrophage-derived cell lines (7). Taken together, the present results suggest the possibility that PTP1C might be involved in the process of the macrophage differentiation. To examine this possibility further it is required to investigate whether transfection of PTP1C cDNA or microinjection of PTP1C protein to HL-60 cells induces macrophage-like differentiation of HL-60 cells or not; these experiments are being performed at present in our laboratory. Although MeSO did not increase PTP1C activity in HL-60 cells, it has been demonstrated that both TPA and MeSO increase the total PTP activity in differentiated HL-60 cells (29). Therefore, another PTP, but not PTP1C, might be involved in the myelocyte-like differentiation of this leukemia cell line. In fact, it has recently been demonstrated that MeSO induces the gene expression of CD45, a transmembrane-type PTP, during the myelocyte-like differentiation of HL-60 cells (30).

In the present study, the mechanism underlying the increase of PTP1C activity induced by TPA has been also investigated. TPA increased both the synthesis of PTP1C and PTP1C gene expression in HL-60 cells. Thus, the results suggest that the TPA-induced increase of PTP1C activity may be in part, caused by the stimulation of the expression of PTP1C protein by increasing the expression of the PTP1C gene. TPA and 1,25(OH)2D3 have been demonstrated to induce the expression of several genes including c-fms (31) and c-fos (32) in HL-60 cells. By contrast, these compounds depress c-myc expression during the differentiation of HL-60 cells (13, 15). The depression by TPA of c-myc expression occurs rapidly after a 6-h exposure of the cells to TPA, and become maximal following 48–72 h of induction. In the case that an increase of PTP1C mRNA detected by Northern blotting. Thus, this 25-kDa protein may be the putative target protein of PTP1C, and further characterization and purification of this protein are necessary to establish its identity.

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