Expression of the Catalytic and Regulatory Subunits of Protein Phosphatase Type 2A May Be Differentially Modulated during Retinoic Acid-induced Granulocytic Differentiation of HL-60 Cells

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ABSTRACT

To elucidate the regulation of protein phosphatases types 1 (PP1) and 2A (PP2A) during all-trans retinoic acid (ATRA)-induced granulocytic differentiation of HL-60 cells, the phosphatase activity, proteins, and gene expressions of PP1 and PP2A were examined. Treatment with 1 µM ATRA caused an 85% decrease in the PP2A activity in extracts from HL-60 cells, while the PP1 activity was constant. This reduction in PP2A activity appeared to parallel phenotypic and functional changes of HL-60 cells induced by ATRA. Western blot analysis showed that the level of PP2A catalytic subunit (PP2A-C) decreased during the course of ATRA-induced differentiation, whereas expressions of A and B (M, 55,000) regulatory subunits of PP2A were relatively unaltered. Expressions of PP1 catalytic subunit isoforms (PP1α, PP1γ, and PP1β) were not significantly affected by ATRA treatment. Northern blot analysis revealed that mRNA levels of PP2A-Cβ and Aα regulatory subunits were decreased following treatment with ATRA, while levels of PP2A-Cα and B (M, 55,000) α regulatory subunit transcripts were relatively constant. Selective down regulation of PP2A-Cβ preceded the granulocytic maturation induced by ATRA. Expressions of PP2A-C isoforms and A and B regulatory subunits may be differentially modulated during ATRA-induced granulocytic differentiation of HL-60 cells.

INTRODUCTION

ATRA³ induces granulocytic differentiation in cultured leukemic HL-60 cells (1, 2) and is clinically effective as the differentiation therapy in inducing high remission rates in patients with acute promyelocytic leukemia (3, 4). The biological effects of ATRA appear to be mediated through a network of closely related nuclear retinoic acid receptors that possess discrete DNA-binding and retinoid acid-binding domains (5). Although the exact mechanism of the ATRA-induced granulocytic differentiation remains to be elucidated, protein phosphorylation/dephosphorylation is also thought to be a regulatory device eminently suited for the control of differentiation processes (6). The levels of both protein kinase C activity and the expression of its isoforms have been shown to increase during HL-60 cell differentiation induced by dimethyl sulfoxide and ATRA (7). However, little is known concerning protein phosphatases responsible for reversing the actions of protein kinase-catalyzed phosphorylation reactions in HL-60 cell differentiation.

The protein serine/threonine phosphatase catalytic subunits of mammalian cells comprise four forms which have been designated type 1 (PP1), type 2A (PP2A), type 2B (calcineurin), and type 2C (8, 9). They differ in metal ion requirements and sensitivities to two heat-stable protein inhibitors, inhibitor-1 and inhibitor-2 (8, 9). Several isoforms of PP1 catalytic subunit, PP1α, PP1γ, and PP1β have been cloned from rat cDNAs (10). PP1 isoforms comprise of multimeric structures composed of a catalytic subunit complexed to several regulatory subunits (8, 9). Formation of heteromeric complexes is thought to play an important role in regulating the activity of PP1 catalytic subunits, as yet not defined in HL-60 cells. The PP2A catalytic subunit (PP2A-C, M, 36,000) is mainly present as a holoenzyme forming a heterotrimer with A (M, 65,000) regulatory and different B regulatory subunits; the basic form is the PP2A-C/A complex, and the B subunit is associated with the dimer through A subunit (9, 11, 12). The A and B regulatory subunits were shown to modulate the phosphatase activity of the PP2A-C in vitro. cDNA clones for the two respective isoforms of PP2A-C and A subunit, PP2A-Cα and PP2A-Cβ and Aα and Aβ, respectively, have been isolated from several animal species (13–15). Based on biochemical evidence, the B subunit of PP2A holoenzyme is comprised of several distinct families of proteins of B (M, 55,000), B' (M, 54,000), B" (M, 74,000), and M, 72,000 (9). Three cDNAs for isoforms of the B (M, 55,000) subunit (α, β, γ) have been cloned from human, rabbit, rat, and yeast libraries (9, 16). A, B, and PP2A-C subunits show a high degree of sequence conservation among species (9). Although the effect of the regulatory subunits of PP2A on the substrate specificity has clearly been demonstrated in vitro (8,9), the roles of individual regulatory proteins in the regulation of metabolism, growth, differentiation, and development remain largely unknown.

We reported that OKA and calyculin-A, both potent and specific inhibitors of PP1 and PP2A, augment the granulocytic differentiation of HL-60 cells induced by ATRA but not the monocytic differentiation induced by phorbol diester (17). In addition, PP2A-C is down regulated during ATRA-induced granulocytic differentiation of HL-60 cells, whereas the PP1 catalytic subunit is unchanged (18). To determine the effects of ATRA treatment on regulation of cellular PP2A holoenzyme, we analyzed PP2A activity and expressions of the A and B regulatory subunits in addition to that of PP2A-C, following ATRA treatment.

MATERIALS AND METHODS

Cells, Culture Conditions, and Evaluation of Differentiation. Procedures for the maintenance of HL-60 cells and determination of variable cell counts have been described previously (19). Cells in the logarithmic growth phase were used for experiments. The extent of differentiation by ATRA (Sigma Chemical Co., St Louis, Mo) was assayed by the ability to produce superoxide, as monitored by the reduction of NBT (Sigma) and surface antigen analysis. The ability of NBT reduction was evaluated as described elsewhere (20). Surface antigens were assessed by cytofluorometry in a fluorescence-activated cell sorter scan (Becton Dickinson, Mountain View, CA), using monoclonal antibodies, including CD11b (OKM1; Ortho Diagnostic System Inc., Raritan, NJ), CD11c (LeuM5; Becton Dickinson), and CD54 (anti-ICAM; Cosmo Bio Co., Tokyo, Japan).

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3 The abbreviations used are: ATRA, all-trans-retinoic acid; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; PP2A-C, the catalytic subunit of PP2A; MLC, myosin light chain; OKA, okadaic acid; NBT, nitroblue tetrazolium; CDNA, complementary DNA; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Preparation of Cytosolic Fractions of HL-60 Cells. HL-60 cells (2 × 10⁶ cells each) were washed three times with ice-cold Tris-HCl-buffered (pH 7.4) saline and then disrupted in 1 ml of homogenization buffer consisting of 250 mM sucrose and buffer A (20 mM Tris-HCl, pH 7.5, 2 mM diithiothreitol, 2 mM EDTA, 2 mM ethylene glycol bis-(β-aminoethyl) ether)-N,N'-tetraacetic acid, and protease inhibitors, including 75 mg/liter phenylmethyl-sulfonylfluoride, 10 mg/liter leupeptin, 10 mg/liter N-tosyl lysine chloromethyl ketone, and 5 mg/liter N-tosyl phenylalanine chloromethyl ketone) by a glass-glass Potter-Elvehjem homogenizer at 4°C (19). The sample was then centrifuged at 1000 × g for 10 min. The resulting pellet was used as the crude nuclear fraction. The supernatant was centrifuged at 100,000 × g for 1 h to separate the cytosol and membrane fractions. The membrane and nuclear fractions were rinsed with homogenization buffer and subsequently resuspended in homogenization buffer containing 1% Nonidet P-40. Following a 1-h incubation on ice, these fractions were sonicated and further centrifuged at 100,000 × g for 1 h to obtain solubilized fractions. In preliminary experiments, we have found that Nonidet P-40 is most suitable for solubilization of phosphatase because it does not inhibit the activity of phosphatase at concentrations as high as 1%.

Assay of Protein Phosphatases. Activities of PP1 and PP2A were assayed in the absence of divalent cations using [32P]-phosphorylated isolated MLC of chicken gizzard as a substrate, because it is known to be a good substrate for both mammalian PP1 and PP2A (21). Phosphatase activity was determined by the liberation of inorganic 32P from the substrate (32P-labeled M, 20,000 MCL) at 30°C according to the method of Pato and Kerc (22). Inhibition of phosphatase activity by OKA was determined by adding OKA to the enzyme preparation 10 min prior to adding the substrate. The extent of dephosphorylation was restricted to <10%. Under these conditions, the rates of phosphorylation were linear with respect to time and enzyme dilution.

Immunoblot Analysis. Antiserum against PP1 catalytic subunit, PP2A-C, and A, B (M, 55,000), B' (M, 54,000), and M, 72,000 subunits were obtained by immunozizing rabbits with synthetic fragments of PP1α, PP1γ, and PP18 (23, 24), A (25) regulatory, B (M, 55,000), α and B (M, 55,000) (β) (22), B' (M, 54,000) (26), or M, 72,000 (27) regulatory subunits. HL-60 cells were incubated with 1 μM ATRA, and the reaction was terminated with cold 10% trichloroacetic acid-10 mM diithiothreitol-2 mM ethyleneglycol bis-(β-aminoethyl) ether)-N,N'-tetraacetic acid solution. The resulting cell pellet was washed twice with acetone-10 mM diithiothreitol and then dried. The dried acetone powder was dissolved with Laemmli sample buffer (28). The sample was subjected to 0.1% SDS-12.5% polyacrylamide gel electrophoresis followed by electrophoretic transfer to a nitrocellulose membrane (29). The membrane was reacted with the avidin-biotin peroxidase complex method (Vectastain; Vector Laboratories, Burlingame, CA). Prestained SDS-polyacrylamide gel electrophoresis standards (Bio-Rad, Richmond, CA) were used as molecular weight standards. Quantitative estimation of the level of PP1 and PP2A was carried out densitometrically with a Bio-Rad videodensitometer by scanning the immunoreactive band after immediately photographing the band. The area of an individual peak was measured above background in densitometric tracings and expressed as absorbance × mm (29).

RNA Isolation and Northern Blot Analysis. Total cellular RNA was extracted by the guanidinium isothiocyanate/cesium chloride density method (30). Total RNA (20 μg) of HL-60 cells was electrophoresed through 0.8% agarose with 18% formaldehyde and transferred to Nytran membranes (Schleicher & Schuell, Dassel, Germany). After baking at 95°C for 2 h under vacuum, the blots were hybridized at 42°C in 50% formamid, 5X Denhardt's solution, 5X SSPE (1 × SSPE is 0.15 M NaCl-10 mM NaHPO₄-0.1 mM EDTA), 0.5% SDS, 200 μg/ml of denatured salmon sperm DNA, and the 32P-labeled cDNA probe. The probes used were as follows: Pshr-EcoRI fragment of rat PP2A-Co cDNA and synthetic oligomer (3'-noncoding region) of rat PP2A-CB cDNA (31), EcoRI fragments of rat Aα and Aβ regulatory subunit cDNAs (32), HindIII fragment of rat B (M, 55,000) α and AccI fragment of rat B (M, 55,000) β regulatory subunit cDNAs (32), EcoRI fragment of rat PP1α, EcoRV-PstI fragment of rat PP1γ, and EcoRI fragment of rat PP18 (10). These probes were labeled with [α-32P]dCTP by a multiprime labeling system kit (Amersham, Buckinghamshire, England). Hybridized blots were finally washed with 0.1 × standard saline citrate (1 × is 0.15 M NaCl-15 mM trisodium citrate) and 0.5% SDS at room temperature and autoradiographed. Quantification of hybridization was determined by scanning densitometry (18).

RESULTS

ATRA-induced Granulocytic Differentiation in HL-60 Cells. Treatment of HL-60 cells with 1 μM ATRA for 4 days led to acquisition of a mature phenotype resembling that of a granulocyte (17). The mature phenotype in HL-60 cells was confirmed by flow cytometric assessments with various monoclonal antibodies and an increased NBT reduction. HL-60 cell proliferation was inhibited after treatment with 1 μM ATRA. As shown in Fig. 1, treatment of HL-60 cells with ATRA resulted in a time-dependent increase in NBT reduction and increase in activities of the cells with CD11b, CD54, and CD11c, although the kinetics of each differentiation marker acquisition were not necessarily identical. The reactivity of HL-60 cells with CD54 reached a plateau faster than that of CD11b or CD11c.

Alterations of Activity and Expression of PP1 and PP2A during ATRA-induced Differentiation. The activity of protein serine/threonine protein-phosphatase in HL-60 cells was assayed in the absence of divalent cations with 32P-phosphorylated MLC as substrate (21). The activities of cytosol, membrane, and nuclear fractions in HL-60 cells were 17.6 ± 3.1, 4.90 ± 0.65, and 5.84 ± 0.58 mmol/min/g (mean ± SE; n = 3), respectively. In untreated HL-60 cells, approximately 60-70% of the total activity of MLC phosphatase was present in the cytosol fraction, with the remaining activity located in the membrane and nuclear fractions. Incubation of HL-60 cells with 1 μM ATRA resulted in a 66% decrease in MLC phosphatase activity in the cytosol fraction, as shown in Fig. 2, but the phosphatase activity of the membrane or nuclear fraction was unaltered (data not shown), thereby suggesting that the translocation of phosphatase from the cytosol fraction to membrane or nuclear fractions did not occur. PP2A is inhibited completely by 5 mM OKA, while PP1 is hardly affected at this concentration and complete inhibition requires 500 nm (21). The varying concentrations producing 50% inhibition for different phosphatases mean that OKA can be used to distinguish between these activities in cell-free systems. Therefore, we examined effects of OKA (5 and 500 nm) on MLC phosphatase activities of HL-60 cells during ATRA-induced differentiation to estimate the relative amounts.

Fig. 1. Induction of markers of cell differentiation in HL-60 cells after treatment with ATRA. HL-60 cells were incubated with 1 μM ATRA. Percentages of NBT-positive cells (●) and surface markers using CD11b (○), CD11c (△), and CD 54 (□) were analyzed by flow cytometry, as described in "Materials and Methods." Points, means of three separate experiments expressed as percentages of positive cells.
Expressions of mRNAs of PP2A during ATRA-induced cell differentiation of HL-60 cells. To determine whether the ATRA-induced down regulation of PP2A-C in HL-60 cells was reflected at the level of RNA transcripts, Northern blot analyses using 32P-labeled cDNA probes for PP2A catalytic subunits (PP2A-Cα and PP2A-Cβ) and regulatory subunits (Aα, Aβ, B (M, 55,000) α and B (M, 55,000) β subunits) were performed on total cellular RNAs obtained from HL-60 cells following treatment with ATRA (Fig. 4). HL-60 cells expressed mRNAs of PP2A-Cα, PP2A-Cβ, and Aα and B (M, 55,000) α subunits. The 2.0-kilobase mRNA was a major transcript of HL-60 cells after hybridization to the PP2A-Cβ probe. The down regulation of PP2A-Cβ mRNA was evident within 3 h after the addition of 1 μM ATRA. The level of PP2A-Cβ mRNA was further decreased to approximately one-third of the basal level of untreated HL-60 cells within 6 h, when normalized to GAPDH mRNA expression. Rehybridization of these blots with GAPDH cDNA probe confirmed that relatively equal amounts of RNA were loaded in each lane. The PP2A-Cα probe also detected a major band of 2.0 kilobase and the expression of PP2A-Cα 2.0-kilobase transcript was relatively unaltered by treatment with ATRA. Thus, our data suggest that two isoforms of PP2A could be differentially regulated during ATRA-induced differentiation. The Aα and B (M, 55,000) α genes were also expressed in HL-60 cells, and A α and B (M, 55,000) α transcripts were approximately 3.1 and 2.6 kilobases, respectively. The levels of Aα transcript gradually decreased by treatment of HL-60 cells with ATRA, although expression of the Aα regulatory protein did not fluctuate. The levels of B (M, 55,000) α mRNA remained relatively constant after ATRA treatment. Transcripts of the Aβ and B (M, 55,000) β subunits in HL-60 cells were not detectable. The mRNA expressions of PP1α, PP1γ, and PP1β were unaltered during ATRA-induced differentiation of HL-60 cells (data not shown).

DISCUSSION

Our present results suggest that PP1 enzyme is not altered during ATRA-induced granulocytic differentiation of HL-60 cells, while PP2A activity is down regulated during ATRA-induced differentiation. Subunit composition and specific complex formation play important roles in regulating the activity and specificity of PP2A-C (9). Cellular PP2A is composed of a common PP2A-C/A complex that interacts with different families of endogenous and exogenous regulatory proteins including B (M, 55,000), B' (M, 54,000), B' (M, 74,000) and M, 72,000 proteins and tumor antigen (9). We found that the expression of PP2A-C was markedly decreased during the course of granulocytic differentiation, whereas the levels of A and B (M, 55,000) α regulatory subunits were relatively constant. Other different B regulatory subunits, including B (M, 55,000) β, B' (M, 54,000), and M, 72,000 regulatory proteins were not expressed in HL-60 cells before and after treatment with ATRA. Although the molar ratio for the PP2A-C and A and B (M, 55,000) subunits in the cells is unknown, the selective down regulation of PP2A-C may change the ratio of dimeric and trimeric PP2A holoenzymes and, therefore, may alter specific catalytic properties such as substrate specificities or response to effectors.

HL-60 cells expressed RNA transcripts of PP2A-Cα, PP2A-Cβ, and Aα and B (M, 55,000) α regulatory subunits. Treatment with ATRA led to a dramatic reduction in mRNA expression of PP2A-Cβ, whereas the level of α isoforms mRNA was unchanged after exposure to ATRA. The down regulation of PP2A-Cβ mRNA transcript clearly preceded the ATRA-induced granulocytic differentiation. The amino acid sequences of PP2A-Cα and PP2A-Cβ proteins, deduced from cDNA sequences, are 97% identical (8, 9). Although mRNAs encoding both isoforms have been detected in mammalian cells and tissues (14, 33), the steady-state PP2A-Cα mRNA is more abundant than PP2A-Cβ mRNA in various cell...
lines other than hematopoietic cells, and this differential expression is thought to be due to different promoter activities (14). PP2A-Cα and PP2A-Cβ are encoded by distinct genes whose expression appears to be differentially regulated (14, 31). Therefore, it is conceivable that the expression of PP2A-Cα and PP2A-Cβ is separately modulated in the process of ATRA-induced granulocytic differentiation. We did not demonstrate directly the differential expression of two PP2A-C isoforms at protein levels, because a specific antibody against each isoform was not available. The expression ratio of PP2A-Cα to PP2A-Cβ proteins may possibly be increased in the PP2A activity of ATRA-induced mature granulocytes.

The mRNA level of Aα regulatory subunit was also decreased following treatment with ATRA, while that of B (M, 55,000) α regulatory subunit was relatively constant. These data suggest that mRNA expressions of PP2A-C and Aα subunits are coordinately regulated in HL-60 cells following treatment with ATRA. In spite of the early decrease in mRNA expression of Aα regulatory subunit, the level of expression of the A subunit protein did not fluctuate throughout ATRA-induced differentiation. While the reason for this discrepancy remains unknown, we speculate that the A regulatory subunit may be resistant to proteolysis and, therefore, has a long life span in the cell. The ATRA-induced granulocytic differentiation of HL-60 cells appears to be mediated through the nuclear retinoic acid receptor (34). The retinoic acid receptor is a member of the thyroid hormone superfamily of transcription factors and possesses discrete retinoic acid-binding (ligand-binding) and DNA-binding domains that regulate transcription of certain target genes (5). ATRA can influence PP2A gene expression either directly, by activating transcription of the PP2A gene, or indirectly, by altering expression of genes encoding transcription factors.

In addition to the modulation of protein phosphatases, the levels of both activity of protein kinase C and expression of its isoforms (α, β, and γ) of protein kinase C was shown to increase during granulocytic differentiation of HL-60 cells induced by ATRA and dimethyl sulfoxide (7). 1,25-Dihydroxyvitamin D3-induced monocytic differentiation of HL-60 cells is also associated with an increase in both activity of protein kinase C and expression of protein kinase C-α and -β, as well as steady-state levels of mRNA of protein kinase C-α and -β (35, 36). The activation of cAMP-dependent protein kinase also appears to be involved in the regulation of HL-60 cell differentiation by TPA.
ATRA or dimethyl formamide (37, 38). Although the monocytic and granulocytic inducers share protein kinases as target molecules (6), it is also true that activation and/or modulation of protein kinases may be solely insufficient to explain the mechanisms for bringing about terminal differentiation into granulocytic or monocytic phenotypes. Thus, the modulation of protein phosphatases may play important roles in regulating the net phosphorylation of critical substrate(s) that subsequently mediate the differentiation of HL-60 cells into either phenotype. PP2A is thought to regulate multiple functions in vivo, including several metabolic pathways, protein synthesis, DNA replication, and the cell cycle (8, 9). Differentiation of HL-60 cells by chemical agents, such as ATRA, 1,25-dihydroxyvitamin D3, and phorbol diester is accompanied by withdrawal from the cell cycle (1, 2). All of these data taken together suggest that down regulation of PP2A may be associated with ATRA-induced differentiation in HL-60 cells, although the modulation of protein phosphatases is only one of several parallel events which mediate the various effects of ATRA. Further studies of HL-60 cells utilizing antisense oligodeoxynucleotides directed against PP2A-C and variant cell lines (39) arrested at discrete points of differentiation may provide insights into the mechanisms by which PP2A influences myeloid differentiation.

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REFERENCES


