Differentiation-induced Changes in Protein-Tyrosine Phosphatase Activity and Commensurate Expression of CD45 in Human Leukemia Cell Lines


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ABSTRACT

Pharmacologic differentiation of the promyelocytic leukemia HL60 is associated with an increase in cellular tyrosine phosphatase activity. We asked (a) if this increase might, at least in part, be due to changes in a transmembranous protein-tyrosine phosphatase, CD45; and (b) if CD45 changes similarly in other differentiating leukemias. Differentiation of HL60, several chronic myelogenous leukemias, a monocytic leukemia (THP-1), and a monoblastoid leukemia (U-937) could be induced by phorbol ester, 1,25-dihydroxy vitamin D₃, dimethyl sulfoxide, or cyclic AMP analogues. This differentiation was associated with a marked increase in (a) total cellular tyrosine phosphatase activity (2-4-fold as measured by the ability to dephosphorylate a tyrosine-phosphorylated peptide); (b) CD45-specific tyrosine phosphatase activity (2-4-fold); (c) CD45 cell surface expression by flow cytometry (2-5-fold); (d) synthesis of both exon B-dependent M₉, 205,000 and exon ABC M₉, 185,000 CD45 proteins, as revealed by immunoprecipitation with antisera specific for CD45 isoforms. Both isoforms have enhanced electrophoretic mobility when isolated from the differentiated cells. This enhanced mobility did not appear to be due to decreased stoichiometry of CD45 phosphorylation on serine/threonine residues. Interestingly, 12-O-tetradecanoylphorbol-13-acetate transiently reduced CD45 protein-tyrosine phosphatase activity in the chronic myelogenous leukemia cell RWLeu4 without altering the CD45 amount (as measured by cell surface immunofluorescence).

Modulation of CD45 tyrosine phosphatase activity (and protein levels) may play a role in differentiation or in maintaining cells in a nonproliferative state or may represent a phenotypic marker of differentiation.

INTRODUCTION

Studies of many transforming retroviruses and normal mammalian growth factor receptors have demonstrated that proliferation of both normal and neoplastic cells is dependent on the phosphorylation of certain proteins on tyrosine residues (for a review, see Ref. 1). For example, the binding of polypeptide growth factors such as epidermal growth factor, platelet-derived growth factor, fibroblast growth factors, insulin-like growth factor I, insulin, and colony-stimulating factor I to their respective receptors causes activation of the intrinsic tyrosine kinases of their receptors, typically resulting in the tyrosine phosphorylation of several important cellular proteins, including the raf serine kinase, phospholipase C γ, a M₉, 85,000 subunit of the 3'-phosphatidylinositol kinase, the ras-GTPase activating protein, and a M₉, 62,000 protein associated with the ras-GTPase activating protein (1, 2). Mutated receptors which either lack tyrosine kinase activity or fail to associate with and phosphorylate certain of these cellular proteins fail to transduce growth factor signals (1-3). Similarly, many neoplastic cells rely on abnormally expressed tyrosine kinases, growth-factor receptors, and growth factors to demonstrate their transformed phenotype.

The extent to which a protein is tyrosine phosphorylated is the result of a dynamic equilibrium between two opposing reactions: phosphorylation by protein-tyrosine kinases and dephosphorylation by tyrosine phosphatases (4-7). Altering this equilibrium can have profound effects on cells. For example, vanadate, a potent inhibitor of tyrosine phosphatases, can cause normal cells to take on a transformed phenotype (8). In contrast, tyrosine phosphatase activity increases markedly during pharmacologically induced terminal differentiation of HL60 promyelocytic leukemia cells (9-10). The potential role played by the changes in tyrosine phosphatase activity during differentiation is particularly interesting.

The leukocyte common antigen CD45 has recently been demonstrated to be a protein-tyrosine phosphatase (6, 7, 11-13). CD45 is a family of structurally related, high-molecular-weight, membrane-spanning molecules found in all hematopoietic cells except mature red cells, platelets, and their immediate progenitors. Here we sought to determine if the increase in tyrosine phosphatase activity during the differentiation of HL60 might be due, at least in part, to changes in CD45 expression and activity, and to extend our observations to other leukemia cells.

MATERIALS AND METHODS

Reagents. 1,25-Dihydroxy vitamin D₃ was kindly provided by R. Uskokovic (Hoffman-LaRoche, Inc., Nutley, NJ). Stock solutions were prepared at 0.2 mM in ethanol, protected from direct light, and stored at −20°C. PMA (Sigma Chemical Co., St. Louis, MO) was dissolved in acetone at 10 µg/ml (17 µM), protected from direct light, and stored at −20°C. DMSO (cell culture grade), Nonidet-P40, phenylmethylsulfonyl fluoride, bovine serum albumin (radioimmunoassay grade), leupeptin, aprotinin, and pepstatin were all obtained from Sigma. Rat p43v ahl and the p43v α' tyrosine kinase were obtained from Oncogene Science (Unionville, NY). Affinity matrices (wheat germ agglutinin bound to Sepharose and protein A bound to Sepharose) were obtained from Pharmacia (Piscataway, NJ). RPMI-1640 was purchased from Gibco (Grand Island, NY).

Monoclonal Antibodies. Monoclonal antibodies to CD45 included 13.4, GAP 8.3, CMRF.11(806), UCHL 1(826), and PD-7/26/l6(816).

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6 The abbreviations used are: VD₃, 1,25-dihydroxyvitamin D₃; PMA, 12-O-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; HEPEs, N-(2-hydroxyethyl)piperazine-N'-2(ethanesulfonic acid); PTPase, protein-tyrosine phosphatase; CML, chronic myelogenous leukemia; FCS, fetal calf serum; PBS, phosphate-buffered saline; PKC, protein kinase C.
Monoclonal antibodies 13.4 and GAP 8.3 (raised from the corresponding hybridomas obtained from the American Type Culture Collection) are specific for all members of the CD45 family (14). Antibodies 806, 816, and 826 were obtained from the Third Leukocyte Typing workshop (15). Antibody 806 is dependent on the presence of the CD45 A exon, antibody 816 is dependent on the presence of the B exon, and antibody 826 requires the absence of the A, B, and C exons and therefore recognizes the smallest, Mₐ, 180,000 CD45 isoform. Monoclonal antibody OKT8, which is specific for CD8, normal mouse IgG, or a mouse IgG1k anti-dinitrophenyl monoclonal antibody (gift of H. Eisen, Massachusetts Institute of Technology) served as negative controls for flow cytometry and immunoprecipitations. Antibody to β₂-microglobulin served as a positive control for changes in cell size (16). Monoclonal antibodies to the macrophage-specific antigens, CD11b (Mo1) and CD14 (Mo2), were obtained from Coulter Corporation (Hialeah, FL), and monoclonal antibodies to the macrophage activation antigen, Mo3, were from R Todd (Ann Arbor, MI). Monoclonal antibody to the granulocyte antigen, CD15, was obtained from Dako (Carpinteria, CA).

Cells Lines and Cell Culture. HL-60 human promyelocytic leukemia cells (17) (obtained from the American Type Culture Collection, Rockville, MD); human chronic myelogenous leukemia cell lines BV173 (obtained from J. Ritz) (18), EM2 and EM3 (19) (obtained from A. Keating), RWLeu4 (16, 20, 21), and K562 (22) (obtained from the American Type Culture Collection); and the monocytic leukemia-cell lines U-937 and THP-1 (obtained from H. Lazarus) were maintained in logithmic growth in RPMI 1640 supplemented with 10% FCS, non-essential amino acids, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% air/5% CO₂ atmosphere.

Induction of Differentiation. Cells growing logarithmically were sedimented for 5 min at 400 × g and resuspended in culture media containing various concentrations of maturation agents (typically 17 nM PMA, 50 nM VDS, or 1.2% DMSO) at 2 × 10⁶ cells/ml. The cells were then typically cultured in 6-well tissue culture plates (Costar, Cambridge, MA) containing 5 ml of media/well for 0 to 5 days. Differentiation along the monocytic pathway was monitored by characteristic changes in cellular and nuclear morphology (Wright-Giemsa staining), development of adherence to plastic substratum, the appearance of typical reactive macrophage antigen, CD15, was obtained from Dako (Carpinteria, CA). Changes in cellular and nuclear morphology and the appearance of cytoplasmic granules (May Grunwald and Wright-Giemsa staining) and the level of the granulocyte antigen CD15 (26-27).

Flow Cytometry. Cells were collected, sedimented at 400 × g, washed with PBS (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4, containing 1% FCS), resuspended at 1 × 10⁶ in 200 μl of PBS containing 2% FCS, and chilled on ice for 5 min. Then, 5–10 μl of primary antibody (e.g., anti-CD45 or anti-CD11b) were added and incubated on ice for 30–45 min. The cells were washed once in PBS at 4°C, and if the first antibody added was not fluorescently labeled, the cells were then resuspended in 100 μl of PBS containing 5 μl of fluorescein isothiocyanate goat anti-mouse IgG (Tago, Inc., Burlingame, CA) and incubated on ice for 30 min. The cells were washed 3 times with 4 ml of PBS and fixed with 1% formaldehyde in 1 ml of PBS. If the first antibody was fluorescently labeled, the cells were fixed in 1% formaldehyde solution after the washes with PBS. The samples were stored at 4°C and analyzed within 3 days by flow cytometry using an EPICS model C flow cytometer (Coulter).

Cell Fractionation. Adherent cells were removed from tissue culture plates after a 15-min incubation in 0.14 M NaCl, 0.01 M sodium phosphate, 0.1% EDTA, pH 7.4, on ice by dislodging with gentle trituration using a Pasteur pipet and centrifuged at 400 × g for 5 min at 4°C. For the assay of PTPase activity associated with the particulate fraction of cells, cell pellets were resuspended in buffer (10 mM HEPES, 5 mM EDTA, (pH 7.0), 15 mM 2-mercaptoethanol, 0.14 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin, aprotinin, and pepstatin) at 1 × 10⁶ cells/ml. The cells were disrupted by 10 strokes in a Dounce homogenizer (B pestle) or by sonication (three to six 5-s bursts at power setting 5 with a microprobe-equipped sonicator; model W140D; Ultrasonic, Plainview, NY) and centrifuged at 8000 × g for 30 min at 4°C to sediment particulate material. The pellet was resuspended in 25 mM HEPES, 1 mM EDTA (pH 7.0), 50 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, and 1% Triton X-100 and centrifuged for 10 min at 4°C. The resulting detergent-soluble portion of the pellet was aliquoted and stored at -70°C. Alternatively, for PTPase assays of immunoprecipitated CD45, cells were harvested as described above and then extracted at 2 × 10⁶ cells/ml with lysis buffer (1% Triton X-100, 0.15 M sodium chloride, 20 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.04% bovine serum albumin, 0.04% FicolI, and 0.04% polyvinylpyrrolidone, pH 8.0) for 10 min on ice. The extract was clarified by centrifugation (400 × g for 5 min) and reacted with 25 μl of protein-A Sepharose beads which had been precoated with rabbit antibody to mouse IgG (Gateway) and the GAP 8.3 monoclonal antibody to CD45. After rotating overnight at 4°C, the CD45-immunosorbent complex was washed three times with 1 ml of the lysis buffer and three times with PBS. The immunosorbent complex was then directly assayed for its ability to hydrolyze 32P-Tyr-Raytide as described below.

PTPase Assays. Tyrosine-phosphorylated Raytide was prepared using the tyrosine-containing peptide, Raytide, [γ-32P]ATP, and the p43-everted kinase, as directed by packaging instructions (Onco- gene Science). Typically, 10–15% of the 32P radioactivity was incorporated into the peptide; by phosphoamino acid analysis, all of the label was in the tyrosine (data not shown). Nonincorporated [32P]ATP was removed by multiple precipitations of the labeled peptide using 10% trichloroacetic acid and, finally, acetone. The labeled peptide was then redissolved in 0.2 ml Tris, pH 8.0, and stored at -20°C until used. The phosphatase reaction was performed essentially as described by Streuli et al. (28). Briefly, 5 μl of reaction buffer (250 mM HEPES, 50 mM EDTA, 100 mM dithiothreitol, pH 7.3, 1.5 μl of water, and 25 μl of the sample to be assayed (e.g., CD45-immunosorbent complex described above) were combined with 5 μl of 32P-Raytide (20,000 cpm) and incubated at 30°C for 20 min (reaction was still linear at 30 min at low PTPase concentrations). Reactions were terminated by adding 750 μl of 4% Norit A charcoal in 0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM monosodium phosphate, and unhydrolyzed 32P-Raytide was sedimented at 8000 × g for 10 min at 4°C. A 400-μl aliquot of the supernatant was taken for measurement of hydrolyzed 32PPO₄ by scintillation spectrometry. Dilutions of PTPase were utilized that gave between 5% and 20% hydrolysis of the 32P-Raytide. The release of 4000 cpm corresponded to the hydrolysis of 2.4 pmol of P0₄⁻.

Data are expressed as the percentage of substrate hydrolyzed; where indicated, this has been normalized to the activity extractable from the same cell lines but which had not been exposed to maturation agents, to facilitate comparison of different cell lines.

Immunoprecipitation of Metabolically Labeled CD45. For metabolic labeling of CD45 with [32S]methionine, cells were washed twice in methionine-free media, resuspended at 10⁶ cells/ml in the same media containing 25 μCi i-35S methionine (ICN, Irvine, CA), and incubated at 37°C for 4 h (or as indicated) in a humidified 5% air/5% CO₂ atmosphere. For metabolic labeling of CD45 with [33P], cells were washed twice in phosphate-free media, resuspended at 10⁶ cells/ml in the same media containing 1 μCi 32P/μl (ICN), and incubated at 37°C for 3 h as for methionine labeling. Proteins were then extracted from the cells with detergent (1% Triton X-100, 10 mM Tris, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, pH 7.6, at 4°C), and cellular debris was removed by centrifugation (8000 × g for 15 min at 4°C). To partially purify the glycosylated, mature CD45 proteins, clarified protein extract was rotated for 1 h at 4°C with 25 μl of wheat germ agglutinin linked to Sepharose beads, after which the beads were washed three times with 1 ml of the extraction buffer, and glycoproteins were specifically eluted.
using 40 µl of 0.3 M  N-acetyl glucosamine prepared in extraction buffer. CD45 proteins in this eluate were reacted overnight at 4°C with the indicated anti-CD45 monoclonal antibody (e.g., 13.4, GAP 8.3, etc.) and then coprecipitated using protein A-Sepharose immunosorbent which had been precoated overnight with rabbit antibody to mouse immunoglobulin G. The CD45-immunosorbent complex was washed twice with 1-ml aliquots of the extraction buffer (lacking bovine serum albumin) and finally eluted by boiling for 5 min in Laemmli sample buffer. CD45 proteins were resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (5-7.5% acrylamide) and visualized by fluorography (for 35S) or by autoradiography using preexposed Kodak XAR-5 film and Lightning Plus intensifying screens, exposing at ~70°C for 1-2 days. Densitometric analysis was performed on the autoradiograms using a GS300 scanning densitometer and GS370 Macintosh software (Hoefer, San Francisco, CA). The CD45 peak was integrated by Gaussian curve fitting of the densitometric difference between the anti-CD45 lane and the anti-dinitrophenyl nonspecific control lane.

Cell Proliferation. Cells were seeded into wells of 24-well tissue culture plates at 1 × 10⁴ cells/well (~20% confluence) in 1 ml culture media containing the indicated concentrations of maturational agents. After 24, 48, and 72 h, cells were harvested by treatment with trypsin-EDTA and enumerated using a Coulter counter (model Channelyzer, ZM).

RESULTS

Pharmacological differentiation of the promyelocytic leukemia HL60 is associated with an increase in cellular tyrosine phosphatase activity (9, 10, 29). We were interested in whether similar changes would be observed during pharmacologically induced differentiation of other leukemia cell lines, in particular cell lines derived from chronic myelogenous leukemias. One recently derived CML cell line, RWLeu4, appears to be arrested at a developmental stage very similar to that of HL60. The RWLeu4 cells clearly lie on the myelomonocytic pathway; they lack Fc receptors, have a basophilic cytoplasm with prominent cytoplasmic granules, have a high nuclear:cytoplasmic ratio, bear β₂-microglobulin and CD11b antigen on their surface, lack immunoglobulin, and display histocytochemically both peroxidase and esterase activity (16, 20, 21). Furthermore, like HL60 (30-34), these cells can be induced to differentiate along the monocytic pathway into macrophages by VD₃ (15) and by PMA (Figs. 1-3). Exposure of RWLeu4 cells to 10 nM PMA caused them to adhere to the plastic tissue culture substratum and to develop lobulated nuclei and irregular cellular morphology with pseudopodia (Fig. 1, A and B). These morphological changes were accompanied by striking increases in the myeloid/mature monocytic-specific antigen CD11b (Fig. 2A) but a decrease in the granulocyte-specific antigen CD15.

Also like HL60 (35, 36), RWLeu4 can be differentiated along the myeloid pathway into granulocytes by polar solvents such as DMSO (Figs. 1 and 2). DMSO causes RWLeu4 cells to become adherent and to exhibit morphological changes similar to but less extreme than those described for PMA-induced differentiation (Fig. 1C). Furthermore, unlike PMA, DMSO caused no significant decrease in the granulocyte-specific antigen CD15 (Fig. 2B).

Both PMA and DMSO showed a marked, dose-dependent inhibition of RWLeu4 proliferation (Fig. 3), with 50% inhibition doses of <1 nM and 1.2%, respectively. RWLeu4 viability was >95% after 3 days' exposure to 10 nM PMA and >60% after 3 days' exposure to 1.5% DMSO, as measured by trypan blue exclusion.

PTPase Activity during RWLeu4 Differentiation. We sought first to determine the effect of PMA-induced differentiation on PTPase activity. PTPase activity was assayed using tyrosine-phosphorylated Raytide, an excellent substrate, compared to several other artificial substrates, for the CD45 families of tyrosine phosphatases (28, 37, 38). Preliminary experiments indicated that most PTPase activity was associated with the particulate fraction of the HL-60 and RWLeu4 cells, irrespective of their exposure to maturational agent, and therefore all subsequent data are reported on that fraction. Raytide PTPase activity in HL60 cells increased by 90% (P < 0.01) after 1 day.
CD45 AND PTPase DURING LEUKEMIC DIFFERENTIATION

Fig. 2. PMA and DMSO cause the appearance of differentiation antigens on RWLeu4 cells. RWLeu4 cells were exposed to 16 nM PMA or 1.2% DMSO as in Fig. 1 for the indicated times, harvested by chilling and gentle trituration with a pipet and analyzed for the presence and relative amount of antigenic markers of monocytic and granulocytic differentiation, using flow cytometry (see “Materials and Methods”). Data are expressed as percentage positive cells, where each data point is the average ± SE, typically of 4–6 replicates from two or more experiments. , CD11b; , CD14; , Mo3; , CD15.

Fig. 3. PMA and DMSO inhibit proliferation of RWLeu4 CML cells. RWLeu4 cells were seeded into culture dishes as in Fig. 1 and cultured in the presence of various concentrations of PMA (A) or DMSO (B). At the indicated times cell numbers were determined with a Coulter cell counter. Cell numbers expressed are the means of at least six separate determinations in three experiments. Bars, SE, typically <5% of the mean. Inhibition of cell growth by PMA was statistically significant (P < 0.01 by Student’s t test) at all times and concentrations of PMA, and inhibition by DMSO was statistically significant at DMSO concentrations >1%.

Effects of PMA on CD45 Expression. CD45 is a major, transmembranous PTPase found on nearly all cells in the hematopoetic lineage. Therefore we asked whether CD45 expression changed in response to PMA-induced differentiation and whether any changes correlated with the changes in cellular PTPase observed above. Various leukemia cell lines were exposed to PMA for 6, 24, 48, and 72 h, harvested, and examined for CD45 expression by flow cytometry (Table 1 and data not shown). As expected, nearly all lines showed ≥95% of their cells to be positive for CD45 expression. However, there were marked differences in the amount of CD45 expressed per cell, with untreated RWLeu4 cells displaying several times more CD45 than HL60 cells or K562 cells. Exposure of RWLeu4 and HL60 cells to PMA caused an increase in cell surface expression of CD45, first detectable in HL60 cells after 6 h (no change detected after a 30-min PMA exposure) and maximal after 24 h in RWLeu4 and 48 h in HL60 (Table 1). Interestingly, CD45 increased to nearly the same final amount on HL60 and on RWLeu4, even though this represents a 200% increase for HL60 and only a 60% increase for RWLeu4 (Table 1). Of the eight cell lines examined, only K562 failed to show significantly increased CD45 in response to PMA (Table 1). K562 is unique in its position on the erythroid lineage and is induced by PMA along a differentiation pathway to megakaryocytes (39).

To test whether the increase in CD45 was specific for maturation induced by PMA, we examined the effects of three other maturational agents: DMSO, 1,25-dihydroxyvitamin D3, and 8-chloro-cAMP. As mentioned above, DMSO differentiates HL60 and RWLeu4 along a granulocytic pathway, 1,25-dihydroxyvitamin D3 along a monocytic/macrophage pathway, and 8-chloro-cAMP along a monocytic pathway (40). Each of these agents induced marked increases in the cell surface expression of CD45, with DMSO and VD3 inducing CD45 expression levels in RWLeu4 surpassing those induced by PMA (Table 2).

Effects of PMA on CD45 Synthesis and Electrophoretic Mobility. The increased level of cell surface CD45 could have resulted from increased CD45 synthesis, decreased degradation, or transfer of already synthesized CD45 from an intracellular compartment to the cell surface. To begin to address these possibilities, we examined the effects of PMA on CD45 synthesis in RWLeu4. RWLeu4 cells were metabolically labeled with [35S]methionine, and mature, glycosylated CD45 was partially purified from cell extracts by lectin-affinity chromatography and specific immunoprecipitation. Fluorography of the immunoprecipitated CD45 revealed a broad band, frequently resolved as a pronounced doublet, corresponding in migration to Mc ~ 210,000–230,000 proteins (Fig. 5, Lanes 0 h). Exposure to PMA caused a dramatic increase in CD45 synthesis in RWLeu4: PMA-induced CD45 synthesis levels in RWLeu4 surpassing those induced by PMA (Table 2).

Fig. 4. Protein-tyrosine phosphatase activity during PMA-induced differentiation of HL60 (C) and RWLeu4 (A) leukemia cells. Cells were seeded in tissue culture dishes and exposed to 16 nM PMA or carrier solvent for 0 min, 30 min, 2 h, 24 h, 48 h, or 72 h. Cell extracts were prepared and assayed for tyrosine phosphatase activity as described in “Materials and Methods,” using 32P-Tyr-Raytide as substrate. Phosphatase activity is expressed relative to the activity in an equal number of untreated cells. Data points (±SE) are the average of 4–5 experiments. In each experiment, 4–10 replicate serial dilutions of cell extract were used to determine the enzymatic activity.
Table 1 Changes in cell surface CD45 during PMA-induced differentiation

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<td>HL60</td>
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<td>RWLeu4</td>
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* Cells were exposed for the indicated times to 16 nm PMA (except BV173, which required 160 nm PMA to induce differentiation) and then examined for cell surface CD45 by flow cytometry as detailed in "Materials and Methods." Numbers, CD45 mean fluorescence intensity compared to untreated cells. Each measurement has been repeated typically 8 to 10 times.

** CD45 fluorescence was significantly greater (P < 0.01) than in cells cultured in media or exposed to carrier solvent alone.

* ND, not determined.

The electrophoretic mobility of CD45 increased slightly, but very consistently, after 24 h of PMA exposure in RWLeu4 (Fig. 5) and in all other cells examined, including HL60, U937, EM2, and EM3, and in response to 1,25-dihydroxyvitamin D3 (data not shown). The increased mobility persisted in the differentiated cells (Fig. 5 and data not shown). Because CD45 exists in several splicing isoforms (41-43), and because isoform usage has been reported to change during differentiation (28, 44, 45), we asked whether the increased mobility was due to changes in splicing isoforms. To address this question, RWLeu4 cells were exposed to PMA for various times, metabolically labeled, extracted with detergent, and subjected to lectin-affinity chromatography as before. Immunoprecipitation of CD45 with isoform-specific antibodies revealed that the CD45 doublet seen with the non-isoform-specific CD45 antibody (13.4; see Figs. 5 and 6) consisted of a Mr ~215,000 exon B-dependent isoform and a Mr ~190,000 isoform lacking the A, B, and C exons (Fig. 6). After exposure to PMA, synthesis of both isoforms increased severalfold, but they each now migrated slightly faster, with apparent Mr of ~205,000 and ~180,000. Exon A-containing CD45 isoforms were not detected.

Another possible explanation for the increased electrophoretic mobility was a PMA-induced hypophosphorylation: less phosphorylated species migrate faster than more highly phosphorylated species (46). Therefore, we examined the effects of PMA on the extent of CD45 phosphorylation. RWLeu4 cells were exposed to PMA for various times as before, metabolically labeled, extracted with detergent, and subjected to wheat-germ affinity chromatography and specific immunoprecipitation using a monoclonal antibody broadly reactive with CD45 isoforms (monoclonal 13.4; see "Materials and Methods"). The partially purified CD45 proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% acrylamide, resolving doublet seen with the non-isoform-specific CD45 antibody revealed that the CD45 expressed slightly faster, with apparent Mr ~205,000 and ~180,000. Exon A-containing CD45 isoforms were not detected.

Effects of PMA on CD45 PTPase Activity. Mechanisms regulating CD45 PTPase activity are not well understood but may involve posttranslational modifications such as changes in serine or tyrosine phosphorylation or the addition of a lipid moiety (47-49). The PMA-induced increase in CD45 protein (Table 1) might have been responsible for at least a part of the increase in PTPase activity seen after PMA exposure (Fig. 4), but this would have been possible only if the CD45 from cells exposed to PMA is fully active. To test this, CD45 was isolated from untreated and PMA-treated RWLeu4 cells and then tested for its ability to catalyze the release of 32P from 32P-Tyr-Raytide. Interestingly, PMA acutely and transiently inhibited CD45 PTPase activity (50% inhibited, P < 0.01, after a 30-min exposure) (Fig. 8A). By 2 h of exposure, cellular CD45 activity had begun to climb, and activity peaked near 24 h at approximately 3-4 times its initial activity. One can obtain a crude measure of CD45 relative specific activity by dividing CD45 PTPase activity (Fig. 8A) by the relative amount of CD45 determined by flow cytometry (Table 1 and data not shown). Examining this measure of specific activity as a function of PMA exposure (Fig. 8B), CD45 specific activity decreased.
generally observed during the pharmacological maturation of the promyelocytic leukemia cell line HL60. In this regard, Frank et al. (9, 10) have reported severalfold increases in PTPase activity after 1 day of PMA exposure and remained at approximately that specific activity thereafter.

DISCUSSION

Mounting evidence has pointed to pivotal roles for tyrosine phosphorylation of cellular proteins in growth factor signal transduction, immune recognition (T- and B-cell responses to antigen), and progression through the cell cycle. The steady-state level of tyrosine phosphorylation of any particular protein is the result of a dynamic balance between tyrosine phosphorylation and tyrosine dephosphorylation. Although much is known about the dozens of tyrosine kinases and the roles they play in cellular processes, comparatively little is known about the counterbalancing PTPases. One PTPase, CD45, is found in large amounts on virtually all cells of hematopoetic lineages (except cells late in the erythroid lineage). CD45 appears to play a critical role in the antigen activation of T-cells by removing a negative regulatory phosphate from tyrosine at position 505 of the c subunit of the T-cell receptor; CD45 plays in cellular processes, comparatively little is known about the counterbalancing PTPases. One PTPase, CD45, is found in large amounts on virtually all cells of hematopoetic lineages (except cells late in the erythroid lineage). CD45 appears to play a critical role in the antigen activation of T-cells by removing a negative regulatory phosphate from tyrosine at position 505 of lck, a src family tyrosine kinase which, when activated, appears to dephosphorylate the c subunit of the T-cell receptor; CD45 appears to play a similar role in the antigen activation of B-cells (50–57). The function of CD45 in other cells is not known, however.

One interesting question is whether PTPases play a role in normal or in pharmacologically induced differentiation. In this regard, Frank et al. (9, 10) have reported severalfold increases in PTPase activity during pharmacologically induced differentiation of the promyelocytic leukemia cell line HL60.

Here we asked whether changes in PTPase activity were more generally observed during the pharmacological maturation of leukemia cell lines and whether changes in PTPase activity might be due, at least in part, to changes in the number of CD45 molecules per cell or in the specific activity of CD45 PTPase in these cells. We chose to focus most of our studies on a CML cell line, RWLeu4, which is rather similar to HL60 in that it is positioned along the myeloid lineage (Refs. 16, 20, 21, and Figs. 1–3), and along a granulocytic pathway by exposure to DMSO (Figs. 1–3). However, differentiation induced by PMA is not completely equivalent to differentiation induced by VD3. Whereas both PMA and VD3 caused similar morphological changes (Fig. 1), increases in the early macrophage-specific antigen CD11b (Fig. 3) and inhibition of proliferation (Fig. 2), VD3 but not PMA increased the late macrophage differentiation antigen CD14, and PMA but not VD3 increased the macrophage activation antigen Mo3 (Fig. 3). By contrast, both PMA and VD3 increased CD11b and CD14 on HL60. Another difference between RWLeu4 and HL60 is seen with the myeloid marker CD15. CD15 is highly expressed in RWLeu4, reduced during PMA and VD3 monocyctic differentiation, but maintained at high levels during DMSO-induced granulocytic differentiation (Fig. 3). In HL60, CD15 is expressed at low levels and induced by DMSO but not by PMA or VD3.

Clearly, PMA-induced differentiation of RWLeu4, as well as of HL60, is associated with a general increase in PTPase activity (Fig. 4). Using a substrate (32P-P-Tyr-Raytide) that the CD45 PTPase family hydrolyzes well, the maximal increase in PTPase activity was observed after HL60 cells had been exposed to PMA for only 1 day. This would suggest that the major increase in PTPase seen after 1 day of PMA exposure was due to CD45 (or a CD45 family member). However, this is not completely consistent with the flow cytometric evaluation of CD45 following PMA treatment of HL60 (Table 1 and data not shown). Although nearly all HL60 cells expressed CD45, they did so at a relatively low level per cell; exposure to PMA caused PMA (hrs) 0 24 48

Fig. 6. Effects of PMA on synthesis of CD45 isoforms. RWLeu4 cells were exposed to 16 nM PMA for the indicated times, metabolically labeled with [35S]-methionine, and extracted with detergent as in Fig. 5. CD45 was partially purified from the detergent extracts of equal numbers of cells by affinity chromatography on wheat germ agglutinin and then immunoprecipitated with monoclonal antibody 1.1.4 (Un), which reacts with all CD45 isoforms; monoclonal antibody 806 (A), which reacts with CD45 isoforms containing exon A; monoclonal antibody 816 (B), which reacts with CD45 isoforms containing exon B; or monoclonal antibody 826 (N), which reacts with a CD45 isoform lacking the A, B, and C exons. Fluorography was for 20 h.

markedly and transiently within 30 min of PMA exposure ($P < 0.01$) but then displayed a trend toward increased activity after 1 day of exposure and remained at approximately that specific activity thereafter.
a marked increase in CD45 expression, but this increase did not peak until at least 2 days of PMA exposure: CD45 increased by only 60% after 1 day, 180% after 2 days, and 210% after 3 days of exposure to PMA (Table 1). This suggests that in HL60, PMA maximally induces within 1 day some PTPase other than CD45 having a preference for hydrolyzing 32P-Tyr-Raytide. Alternatively, the specific enzymatic activity of CD45 may be greatest after 1 day of PMA exposure.

Frank and Sartorelli (9, 10) reported previously that PMA causes a 2-fold increase in HL60 PTPase activity after 1 day’s exposure and 8-fold increase after 2 or 3 days’ exposure. They used a copolymer of glutamine, alanine, and tyrosine, phosphorylated on tyrosine residues, as a substrate for the cellular PTPases. Because similar polyamionic copolymers of glutamine and tyrosine actually inhibit some PTPases (particularly PTPase 1A, 1B, and 2P5) (38, 58), it is difficult to compare the kinetics and magnitude of their PTPase changes with our results obtained with 32P-Raytide as a PTPase substrate.

The overall time course of changes in RWLeu4 PTPase was similar to that seen in HL60 cells. However, in RWLeu4, in contrast to HL60, PMA caused a commensurate and coincident increase in total particulate Raytide PTPase (Fig. 4) and in CD45 Raytide PTPase per cell (Fig. 4a). This suggests that CD45 constitutes a large fraction of RWLeu4 PTPase activity. Consistent with this interpretation, CD45 was present at much higher levels on untreated RWLeu4 cells than on untreated HL60 cells (data not shown).

In RWLeu4 (but not reproducibly in HL60 or in U937; data not shown), PMA caused a transient, marked inhibition of Raytide PTPase. Interestingly, the time course of this inhibition corresponded to the time course of PMA-induced decrease in CD45 total PTPase activity (Fig. 8b) and specific PTPase activity (Fig. 8A). The mechanism whereby CD45 PTPase activity is inhibited is unclear. We did not detect gross changes in the phosphorylation of CD45 that might be responsible for the decrease in CD45 PTPase activity, although subtle changes in the phosphorylation of individual sites might well have occurred. (Alternatively, CD45 function may be inhibited by some other posttranslational modification or by complexing with an inhibitory protein.) CD45 may well be the target of regulatory protein kinases other than PKC: CD45 is highly phosphorylated in RWLeu4 cells under conditions where PKC is >90% down-regulated (Fig. 7; data not shown).

In some human T-cells PKC increases the serine phosphorylation of CD45, and this correlates with a transient decrease in CD45 PTPase activity in human T-cells (59, 60). In contrast, however, direct phosphorylation of CD45 by PKC in vitro does not alter CD45 PTPase activity (37). Furthermore, Ostergaard and Trowbridge (61) have recently reported that ionomycin, but not PMA, reduces the serine phosphorylation of a specific site on CD45 in mouse thymocytes. Coincident with this decrease, they observed a decrease in CD45 PTPase activity.

The biological significance of the rapid decrease in CD45 specific activity is unclear. Inasmuch as we have detected this in response to PMA-induced differentiation of RWLeu4 but not HL60 or U937, this transient decrease in CD45 PTPase activity may not play a general role in PMA-induced differentiation. This would also be consistent with the severalfold variation in CD45 levels expressed by different myelomonocytic leukemic cell lines (Table 1; data not shown) that differentiate in response to PMA. Nevertheless, rapid increases in CD45-mediated dephosphorylation of tyrosine 505 of the lck protein are believed to be important in T-cell activation (55). T-cell activation by antigen results in the activation of PKC (see Ref. 62): we could speculate that one result of this might be to feedback-down-regulate CD45 activity, tightly regulating responses to antigen.

In addition to the general increase in the amount of CD45 that occurs during PMA-induced differentiation of most leukemia cell lines, we also observed a clear increase in the electrophoretic mobility of CD45 (Figs. 5–7). Because of reports of changes in CD45 isotype usage during T-cell differentiation (44, 45), we initially speculated that analogous changes were occurring in RWLeu4 and other leukemia cell lines. This clearly was not the case, however (Fig. 6): one isoform we detected contained the B exon but lacked the A exon, while the other isoform lacked the A, B, and C exons. PMA caused no change in the usage of these exons.

It is also well known that decreases in the stoichiometry of phosphorylation can increase electrophoretic mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (46). We had already determined that PMA down-regulated PKC in RWLeu4 cells (data not shown). We speculated that, in the absence of PKC, CD45 might be serine phosphorylated to a much lower extent. Two findings argue against this possibility.
First, allowing cells to recover to normal levels of PKC (by washing away phorbol ester and then culturing cells an additional 24 h in its absence) does not result in return of CD45 to its slower electrophoretic mobility (data not shown). Second, PMA exposure causes commensurate increases in CD45 (by flow cytometry; Table 1) and in CD45 phosphorylation (by densitometric analysis of Fig. 7). We are left, then, with four possible explanations. PMA may change the usage of some other CD45 exon or may cause a posttranslational modification in polypeptide length (proteolysis), carbohydrate content, or lipid moieties. In this regard, PKC is known to activate a protease which cleaves the colony-stimulating factor 1 receptor (63).

Our data also suggest that CD45 specific activity (Fig. 8B) increases concurrently with increases in CD45 mobility (Figs. 5–7). Our observation of a trend to increased CD45 specific activity is limited in that we determined cellular CD45 using flow cytometry. Our antibodies do not immunoblot, and concurrent changes in the rate of CD45 synthesis and protein half-life complicated attempts to compare CD45 in undifferentiated and differentiated cells by isotopic labeling. This raises the possibility that the faster-migrating CD45 may be a more enzymatically active form of CD45. Alternatively, the apparent increased specific activity of CD45 may reflect the down-regulation of PKC. This could be easily tested by washing away the phorbol ester and allowing PKC to return to normal levels. Although this tactic does not alter CD45 migration (Fig. 7), we do not know whether this would return CD45 apparent specific activity to levels found normally in RWLeu4 cells.

The generality of increases in CD45 during the pharmacologically induced differentiation of leukemia cells was seen in the effects of PMA on several leukemia cell lines (Table 1) and in the effects of other maturation agents on RWLeu4 and HL60 (Table 2). This raises the question of whether the increased expression of CD45 (and CD45 PTPase activity) plays a role in the differentiation process itself or, rather, plays a role in the function of the differentiated cell. Our data do not distinguish between these possibilities. Although one CML cell line, K562, showed no changes in CD45 during PMA-induced differentiation, PMA causes K562 to differentiate along a megakaryocytic pathway, and mature megakaryocytes would not be expected to have high levels of CD45, inasmuch as platelets, produced by megakaryocytes, lack CD45 (64). Thus the failure of CD45 to increase during the differentiation of these cells is not surprising. However, other PTPases may substitute for increased CD45 during differentiation of these cells. Consistent with this possibility, Butler et al. (65) have recently reported increases in a M, 40,000 cytosolic PTPase during PMA-induced differentiation of K562.

Our results with HL60 cells are also generally consistent with a recent report of increases in CD45 expression and CD45 PTPase activity during PMA-induced monocytic and DMSO-induced granulocytic differentiation (66). Our results appear to differ from theirs regarding U937 cells, however. We detect a 100% increase in the amount of CD45 on U937 cells during PMA-induced differentiation (Table 1), whereas Taetle et al. (66) mention that PMA does not increase CD45 immunofluorescence on U937 cells.

Coordinate expression of CD45 and various antigenic markers of monocytic and myeloid differentiation has been examined in human bone marrow by flow cytometry (64). In normal monocytic differentiation, CD45 is expressed at high levels on monocyctic precursors and increases further during monocyctic differentiation. In contrast, CD45 is present at low levels on myeloid precursors and remains low and constant during granulocytic differentiation. Thus while our data (in contrast to Taetle’s) are consistent with normal monocyctic differentiation (monocytic differentiation induced by PMA and VD3 caused increased expression of CD45 in RWLeu4, HL60, BV173, EM2, U937, and THP-1), our (and Taetle’s HL60) observations on DMSO-induced granulocytic differentiation (CD45 increases) do not appear consistent with normal myeloid differentiation.

What might be the function of the large amounts of CD45 in the differentiated monocyctic cells? Given that CD45 appears to play a role in transducing antigen recognition signals in both T-cells and B-cells, it is tempting to speculate about a role for CD45 in signal transduction in mature monocytes/macrophages, perhaps transducing signals from Fc or other cell surface receptors.

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Differentiation-induced Changes in Protein-Tyrosine Phosphatase Activity and Commensurate Expression of CD45 in Human Leukemia Cell Lines

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