Biochemical Characterization of Tyrosine Kinase and Phosphotyrosine Phosphatase Activities of HL-60 Leukemia Cells

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ABSTRACT

The cellular phosphotyrosine content of the HL-60 promyelocytic leukemia markedly decreased during the induced granulocytic and monocytic maturation of these cells. This occurs in the face of major increases in tyrosine kinase and protein phosphotyrosine phosphatase activities (D. A. Frank and A. C. Sartorelli, Biochem. Biophys. Res. Commun., 140: 440-447, 1986). In the present work, these two activities were characterized in the particulate fraction of HL-60 cells, since both enzymes are membrane bound. The tyrosine kinase activity utilized ATP as a phosphate donor, although GTP and other nucleotides were competitive with ATP. The enzyme was temperature sensitive, had a pH optimum of 6.5, and required Mg2+ or Mn2+ for activity, with additional stimulation of activity being produced by Zn2+. Agents such as epidermal growth factor and insulin, which stimulate other tyrosine kinase enzymes, were without effect on the tyrosine kinase activity of HL-60 cells. Enzyme activity was stimulated, however, by non-ionic detergents and was inhibited by quercetin. The protein phosphotyrosine phosphatase activity was paralleled by that of p-nitrophenyl phosphatase, was inhibited by VO2+, Zn2+ and F-, and was maximally active at a pH of 7 to 8. The characteristics of the tyrosine kinase and the protein phosphotyrosine phosphatase activities were distinct from those of other known proteins of these classes.

Tyrosine kinase activity was predominantly located on the plasma membrane, while the protein phosphotyrosine phosphatase activity was concentrated on internal membranes. The activities of both enzymes present on the plasma membrane appeared to exist on the cytoplasmic face of this membrane.

Further characterization of the activities of these enzyme systems and their contribution to the regulation of tyrosine phosphorylation would appear to be important to an understanding of the control of cellular proliferation and differentiation.

INTRODUCTION

The regulation of the maturation of hematopoietic cells in the bone marrow is subject to complex physiological regulatory processes. Thus, a single stem cell is capable of giving rise to erythrocytes, leukocytes, and platelets, each of which matures in a number of discrete steps, and each of which responds to diverse stimulatory and inhibitory factors (1). A number of model systems are available to use in studies designed to understand the mechanisms involved in the cellular regulation of hematopoietic differentiation. The HL-60 promyelocytic leukemia is one such system which has been used to study the processes of both granulocytic and monocytic maturation (2-5).

A number of oncogenes have been found to code for tyrosine kinases, and in several systems, elevated levels of tyrosine phosphorylation have been shown to be associated with increased cellular proliferation (6, 7). Since terminal differentiation represents in part the organized shutdown of proliferative processes, we have speculated that alterations in tyrosine phosphorylation might be of major importance to these events. In keeping with this concept, we have shown that the induced differentiation of HL-60 leukemia cells along both the granulocytic and the monocytic pathways is accompanied by a pronounced decrease in the cellular content of P-tyr. We (8-10) and others (11-15) have shown that this change in P-tyr residues is accompanied by major increases in tyrosine kinase and protein phosphotyrosine phosphatase activities, with the latter enzymic activity responsible for cleaving phosphotyrosyl bonds being increased significantly more than that of tyrosine kinase activity. We have further shown (10) that these changes are specific for the differentiation process per se, rather than being due to growth cessation alone, and that similar changes occur with the granulocytic maturation of the WEHI-3B D+ mouse myelomonocytic leukemia. Since these processes appear to be important to the differentiation process, the present report provides information on the initial characterization of tyrosine kinase and protein phosphotyrosine phosphatase activities of HL-60 leukemia cells.

MATERIALS AND METHODS

Materials. Radioactive isotopes were purchased from Amersham Corp., Arlington Heights, IL. Tissue culture media and serum were obtained from Gibco, Grand Island, NY. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise noted.

Culture Conditions. HL-60 promyelocytic leukemia cells were a gift from Dr. R. C. Gallo, National Cancer Institute. Cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated (56°C for 30 min) fetal bovine serum at 37°C in a humidified 95% air/5% CO2 atmosphere. Cells were seeded at a level of 2 x 10^6 cells/ml, and were allowed to attain a maximum density of 1.5 x 10^8 cells/ml before being passed into fresh medium. All studies used cells that were between passages 32 and 60.

A431 cells were obtained from the American Type Culture Collection (Rockville, MD). These cells were seeded at 0.5 to 1 x 10^6 cells/100 cm^2 and were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a humidified 90% air/10% CO2 incubator at 37°C. Cells were routinely removed from flasks by exposure to 0.05% trypsin and subcultured before reaching confluence.

Cell Fractionation. Four to 5 x 10^6 cells were collected, washed twice in PBS, resuspended at a level of 10^6 cells/ml in buffer A (5 mM HEPES, pH 7.4; 1 mM MgCl2; and 1 mM EDTA) and disrupted with a Branson sonicator (Danbury, CT) using two 10-s bursts at a setting of 20. Nuclei and unbroken cells were removed by centrifugation at 1,000 x g for 10 min and the supernatant was centrifuged at 30,000 x g for 30 min. The resulting supernatant was designated the soluble fraction.

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3 The abbreviations used are: P-tyr, phosphotyrosine; EGF, epidermal growth factor; GAT, glutamic acid:alanine:tyrosine, 6:3:1; GAT, glutamic acid:alanine:tyrosine, 4:1; NEM, N-ethyl maleimide; PBS, phosphate-buffered saline (140 mM NaCl, 12.7 mM KCl, 6.7 mM Na2HPO4, H2O, 1.5 mM KH2PO4); PNPP, p-nitrophenyl phosphate; PNPase, p-nitrophosphophosphatase; TCA, trichloroacetic acid; TLCK, N-p-tosyl-l-lysine chloromethyl ketone; TPA, 12-O-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.
fraction. The pellet was resuspended in 0.3 ml of buffer B (25 mM HEPES, pH 7.4; 5 mM 2-mercaptoethanol; and 0.1% (v/v) Nonidet P-40), shaken vigorously, and centrifuged for 5 min at 12,000 x g in a microcentrifuge. The resulting supernatant was designated the particulate fraction. Protein was determined by the method of Bradford (16), using bovine serum albumin as a standard.

Plasma Membrane Isolation. The cationic silica method of Chaney and Jacobson (17) was used to isolate plasma membranes with only their cytoplasmic face available for molecular interactions. Three to 5 x 10^8 cells were washed twice with ice-cold 70 mM NaCl, 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5 (wash buffer). Four ml of colloidal silica donated by Dr. B. S. Jacobson (University of Massachusetts, Amherst, MA) was diluted to 6–8% (w/v) silica with attachment buffer (140 mM sorbitol; 20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5). The cells, in a volume of 4 ml of attachment buffer, were then slowly added to the silica with gentle mixing. Immediately after mixing, the suspension was diluted to 40 ml with wash buffer. The silica-coated cells were recovered by centrifugation (650 x g for 2.5 min), and resuspended in 5.0 ml of wash buffer. The cells were then slowly added to 5.0 ml of a 2-mg/ml solution of polyacryl acid (M, 90,000; Aldrich Chemical Co., Milwaukee, WI) in wash buffer (pH 6.5). After mixing, the suspension was again diluted to 15 ml in wash buffer and centrifuged as above. This procedure was repeated twice, forming a pellicle three layers thick. The cells were lysed by three cycles of rapid freezing and thawing, and the membrane fraction was recovered by centrifugation (800 x g for 5 min).

Cellular Membrane Separation. Plasma membranes were separated from internal cellular membranes by the density isolation method of Hertel et al. (18). Cells were washed twice in PBS and then incubated with 0.25 mg/ml of concanavalin A in PBS for 20 min at 4°C. Cells were lysed by hypotonic shock in 1 mM Tris, 2 mM EDTA, pH 7.4, for 20 min at 4°C. Lysates were layered on top of a step gradient consisting of 2 ml of 60%, 35 ml of 38%, and 3.5 ml of 15% sucrose (w/v in 20 mM HEPES-HCl, pH 7.4) and centrifuged for 30 min at 100,000 x g. The HIMTris-HCl, pH 7.4) and centrifuged for 30 min at 100,000 x g. The resulting supernatant was designated the particulate fraction. Protein was determined by the method of Bradford (16), using bovine serum albumin as a standard.

REGULATION OF TYROSYNE PHOSPHORYLATION

RESULTS

While others have reported both cytosolic and particulate tyrosine kinase activity in HL-60 cells (15), under the conditions used in these experiments, essentially all of the tyrosine kinase activity of HL-60 leukemia cells was found in the particulate fraction (8–10); experiments were performed to characterize the tyrosine kinase activity present in this fraction. Tyrosine kinase activity was temperature dependent, being minimal but detectable at 0°C with activity increasing through 37°C (Fig. 1). γ-ATP serves as a phosphate donor for the tyrosine kinase reaction in a concentration-dependent manner; phosphorylation was linear with ATP concentration to a level of about 50 µM, at which point it reached a plateau (Fig. 2). The apparent Km for ATP was approximately 22 µM at 23°C. The possibility of incorporation of 32P by mechanisms other than transfer of the γ-phosphate of ATP was considered. Two potential alternate mechanisms include high affinity binding of ATP to the

Fig. 1. Temperature dependence of the tyrosine kinase activity of HL-60 cells. Tyrosine kinase activity was determined at 0°C (O), 23°C ( ), and 37°C ( ), using 20 µg of particulate fraction protein, GT as substrate (1 mg/ml), and 25 µM ATP. Points, mean of 3 separate experiments, each done in duplicate; bars, SE.
such that a 1 mg/ml solution of GT has a tyrosine concentration of 0.61 mM. GT has an average molecular weight of 28,000, an average chain length of 190; it contains 8% tyrosine, and is 20-fold greater for GT on a weight basis. GAT has an average molecular weight of 25,000 with a degree of polymerization of 160, and a tyrosine content of 17%, such that a 1 mg/ml solution of GT has a tyrosine concentration of 0.97 mM. Thus, expressed in terms of tyrosine concentration, the initial velocities were similar for the two polymers. Expressed in terms of tyrosine concentration, the apparent $K_{m}$ for the two substrates does vary, being 0.49 mM for GAT and 0.73 mM for GT. At relatively high substrate concentrations a decrease in activity occurred with both polymers. In the absence of substrate, $^{32}$P incorporation was approximately 10% of that of the GT-containing reaction.

Table 1: Effect of nucleotides on tyrosine kinase activity of HL-60 leukemia cells

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>% of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>5</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
</tr>
<tr>
<td>AMP</td>
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</tr>
<tr>
<td>Adenosine</td>
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<tr>
<td>GDP</td>
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</tr>
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<td>GMP</td>
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<td>dGTP</td>
<td>23</td>
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<tr>
<td>Deoxyguanosine</td>
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<tr>
<td>UTP</td>
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</tr>
<tr>
<td>CTP</td>
<td>57</td>
</tr>
<tr>
<td>CDP</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 1 Effect of nucleotides on tyrosine kinase activity of HL-60 leukemia cells

A 100-fold excess of unlabeled nucleotide (1 mM) was added to the standard tyrosine kinase assay and activity was measured. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

The effects of cyclic AMP on tyrosine kinase activity were assessed; at concentrations up to 10$^{-4}$ M no effect was discernible.
activity was measured. Values represent the mean of two experiments each done to obtain the enzymic activity or in the tyrosine kinase assay itself, and enzyme assays were performed at 23°C, using 15 ng of particulate fraction protein, 1 mg/ml of GAT, and Mg\(^{2+}\) (Δ), or Mn\(^{2+}\) (Ο) concentrations as shown. Points, mean of 3 separate experiments, each done in duplicate; bars, SE.

### Table 2 Effect of detergents on tyrosine kinase activity of HL-60 leukemia cells

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Concentration (mM)</th>
<th>% of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effect on extraction</td>
<td>Effect on assay</td>
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<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nonidet P-40</td>
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<td>248</td>
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<tr>
<td></td>
<td>0.5</td>
<td>148</td>
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<tr>
<td></td>
<td>1.0</td>
<td>148</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>165</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>0.1</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>87</td>
</tr>
</tbody>
</table>

able, in agreement with findings that tyrosine kinases are cyclic AMP independent. The effects of stimulators of protein kinase C activity were also evaluated. The phorbol ester, TPA, showed no effect up to 10\(^{-3}\) M. Ca\(^{2+}\) had no effect up to 10\(^{-2}\) M; at 10\(^{-3}\) M Ca\(^{2+}\), however, kinase activity was inhibited 40%, and at 10\(^{-1}\) M Ca\(^{2+}\) enzyme activity was totally abolished.

Given that a number of growth factor receptors possess tyrosine kinase activity, it was of interest to determine whether the activity detected in HL-60 leukemia cells was that of a receptor. Two possibilities were examined: the insulin receptor and the EGF receptor. Neither insulin (up to 10\(^{-6}\) M) nor EGF (up to 10\(^{-6}\) M) had any effect (stimulatory or inhibitory) on the tyrosine kinase activity of HL-60 cells.

It has been suggested that tyrosine kinases also may phosphorylate glycolipids, particularly phosphoinositol. To determine whether the HL-60 tyrosine kinase had the ability to phosphorylate phosphoinositol, the glycolipid was added to the tyrosine kinase reaction mixture to determine whether tyrosine phosphorylation would be inhibited. At concentrations of phosphoinositol up to 200 μg/ml, no inhibition occurred, implying that phosphoinositol was not a substrate for the enzyme.

The trypsin and papain inhibitor, TLCK, was also examined for its action on tyrosine kinase activity. A concentration-dependent inhibition was seen, with up to a 68% decrease in enzyme activity being obtained at a level of TLCK of 10 mM (Table 2). Since high concentrations of polymeric substrates inhibited enzyme activity, it was possible that the inhibition was due to the relatively large amounts of negatively charged groups (i.e., the γ-carboxyl moieties of the glutamic acid) present in the substrate molecule. To examine this possibility, heparin was evaluated as a potential inhibitor, since it is a negatively charged polymer of comparable molecular weight that cannot be phosphorylated. Heparin caused a relatively slight inhibition, with a maximal inhibition of 29% occurring at a concentration of 10 μg/ml.

It has been suggested that the α-vanadate ion (VO\(^{4+}\)) can directly stimulate a number of tyrosine kinases (22), including the insulin receptor (23). Therefore, the effect of this ion on HL-60 leukemia tyrosine kinase activity was evaluated. At concentrations of VO\(^{4+}\) up to 10\(^{-3}\) M, no effect was discerned.

The effect of a number of detergents on the tyrosine kinase activity of HL-60 cells was also examined. Tyrosine kinase activity could be recovered from the particulate fraction of HL-60 cells in the absence of any detergent (Table 2), although enzyme extraction with either 0.1% (v/v) Triton X-100 or 0.1% (v/v) Nonidet P-40 led to about a 2.5-fold enhancement of recoverable activity. The same concentration of sodium deoxycholate had no effect. In the reaction mixture itself, 0.5% (v/v) of either Triton X-100 or Nonidet P-40 led to maximal 1.6- to 1.9-fold increases in enzyme activity, respectively.

The effects of NEM, which inactivates sulfhydryl groups, on tyrosine kinase activity was also assessed; activity was inhibited by NEM in a concentration-dependent (Fig. 5, top) and time-dependent (Fig. 5, bottom) manner. The effects of a number of other potential inhibitors of tyrosine kinase activity were also evaluated (Table 3). Up to 5 mM free phosphoserine or phosphothreonine had no effect on enzyme activity. P-Tyr had no effect up to a concentration of 0.1 mM; at 1.0 mM, however,
94% of the enzyme activity was abolished. At 1.0 mM, free tyrosine inhibited enzyme activity by 76%. Disodium phenylphosphate, which could be considered an analogue of P-tyr, caused maximal inhibition of 26% at a level of 10⁻³ M. Amiloride, an inhibitor of Na⁺/H⁺ exchange, has also been found to inhibit the EGF receptor tyrosine kinase (24). In assays with the HL-60 tyrosine kinase, however, amiloride had no effect at concentrations of up to 1 mM. Quercetin, a bioflavonoid compound, which has among its activities the capacity to inhibit tyrosine kinases from rat lung (25), Rous sarcoma virus (26), and a mouse mammary tumor (27), inhibited the HL-60 tyrosine kinase activity by 36% at 10⁻³ M, and by 96% at 10⁻³ M. Levamisole, an immunomodulator with some reported clinical success against human tumors (28), was without effect on the tyrosine kinase activity of HL-60 cells at concentrations up to 10 mM.

Heating of the HL-60 cell particulate fraction protein at 56°C for 10 min completely abolished tyrosine kinase activity. The exposure of the particulate fraction to pronase for 10 min at 37°C also destroyed all of the enzymatic activity; this effect did not appear to be due to the degradation of the substrate, since the addition of pronase after completion of the phosphorylation reaction had no effect on the recovery of the phosphorylated peptide. The 10-min incubation at 37°C also was not a significant factor, since incubation with RNase under these conditions caused no significant diminution in enzyme activity.

A number of tyrosine kinases have been shown to have altered substrate specificities and sensitivity to inhibitors following treatment of the cells with agents (29-34). To ascertain whether this phenomenon was operative with the HL-60 enzyme preparation, particulate fractions were preincubated with ATP for up to 10 min. Preincubation for 30 s led to a 42% increase in tyrosine kinase activity; this rose to 64% at 1 min, and remained at this level for up to 10 min thereafter.

Like tyrosine kinase activity, >90% of the protein phosphotyrosine phosphatase activity was found in the particulate fraction. The phosphatase activity was linear with particulate protein concentration to about 25 μg/assay, and the enzymatic activity was able to liberate 100% of the ³²P present in the substrate. The initial velocity was also linear with substrate concentration to 4 μg/assay, and then plateaued. In addition, activity was temperature dependent, being minimal at 0°C, and increasing through 37°C; the reaction was also initially linear with time before reaching a maximum, and in the absence of enzyme, the substrate was extremely stable (Fig. 6).

It has been shown in other systems that the ability to hydrolyze PNPPase may be a property solely of protein phosphotyrosine phosphatases (21). The PNPPase activity of HL-60 leukemia cells was almost completely recovered (>90%) in the particulate fraction. Like protein phosphotyrosine phosphatase, PNPPase activity was linear with protein concentration; the Kₘ for PNPP was approximately 1.9 mM. PNPPase activity also showed a time course and a temperature dependence similar to that of the protein phosphotyrosine phosphatase, and increased in parallel with this phosphatase during granulocytic and monocytic differentiation induced by DMSO and TPA, respectively.

The response of protein phosphotyrosine phosphatase and PNPPase to a number of ions and small molecules closely paralleled each other. Thus, neither EDTA up to 0.5 mM, MnCl₂ up to 1 mM, nor MgCl₂ up to 1 mM had a significant effect on either activity (Table 4). ZnCl₂, however, caused nearly complete inhibition of both activities at 10 mM.

NaF is a known inhibitor of many serine/threonine phosphatases. Furthermore, one of the first described distinctive features of protein phosphotyrosine phosphatases was the unusual response to the fluoride ion. The enzyme described by Nelson and Branton (35) and the phosphotyrosine phosphatase activity of the alkaline phosphatases described by Swarup et al. (36)
were both resistant to inhibition by F⁻. The activity examined in A431 membranes by Brautigan et al. (37) was actually stimulated slightly by fluoride, an effect the authors suggested was due to the complexing and removal of Zn²⁺ by this anion. The protein phosphotyrosine phosphatase activity of HL-60 cells was decreased somewhat by fluoride, with 65% inhibition found at 50 mM (Table 4). This feature apparently distinguishes it from other such enzymes that have been described. The most potent inhibitor of the protein phosphotyrosine phosphatase and PNPPase activities that was assayed was orthovanadate, which at 1 mM inhibited both activities by >90%.

The ionic detergent sodium dodecyl sulfate caused 60 to 70% inhibition at a concentration of 0.001% (w/v) and complete inhibition of enzyme activity at higher concentrations, while neither quer cetin up to 10⁻⁴ M, levanosile up to 10⁻² M, ATP up to 1 mM, nor inorganic pyrophosphate up to 10 mM altered these activities. The addition of 10 mM PNPP to the protein phosphotyrosine phosphatase assay decreased activity by 14%.

The pH optimum for both enzymic activities differed slightly, being pH 8 for protein phosphotyrosine phosphatase and pH 7 for PNPPase (Fig. 7). Protein phosphotyrosine phosphatase had activity at a pH value as low as 4 and as high as 9, while the activity range for PNPPase was pH 3 to 8. To determine whether commercially available alkaline or acid phosphatases possessed phosphotyrosine phosphatase activity, three such proteins were tested in this assay. Two alkaline phosphatases, one from pig kidney and one from calf intestine, hydrolyzed PNPP in the pH range of 9 to 14, with a maximum at pH 11, but failed to cleave phosphotyrosine at any pH value. Prostatic acid phosphatase possessed PNPPase activity, with maximum activity at pH 4 and an activity range of from pH 3 to 8. The acid phosphatase cleaved the phosphotyrosine-containing substrate slightly at pH values of from 5 to 7, although the specific activity of the commercial preparation was less than one-tenth that of the protein phosphotyrosine phosphatase from HL-60 cells.

Since the particulate fraction contained all of the HL-60 cellular membranes, an effort was made to further define the location of these activities. Using the technique of Hertel et al. (18), plasma membranes were separated from internal cellular membranes by density gradient centrifugation. This methodology relies on the ability of concanavalin A to cross-link surface membrane proteins, such that when cells are lysed by hypotonic treatment, the plasma membrane forms sheets while the internal membranes remain as vesicles. A step sucrose gradient was then used to obtain quantitative separation and recovery of each fraction (18). Using this technique, 5 times more tyrosine kinase activity was found on the plasma membrane than on the internal membranes; conversely, nearly 4 times more protein phosphotyrosine phosphatase activity was located on the internal membranes (Table 5).

Additional experiments were directed at further defining the subcellular location of these enzymes. Given that they are predominantly membrane associated, an experiment was conducted to determine which side of the membrane they resided upon. The technique of Chaney and Jacobson (17) was used to recover pure plasma membranes with only the cytoplasmic face available for enzymatic reactions. This method relies on the formation of a thick pellicle around the external face of the plasma membrane. Cationic silica was used to coat the outside membrane with a positively charged layer; this was then followed by treatment with polyacrylic acid to form an anionic layer. This technique was then repeated to form a thick pellicle.

Lysis of the cells by alternate cycles of freezing and thawing, followed by centrifugation, then allowed recovery of the plasma membranes with only the cytoplasmic side exposed. Cells were then treated with 0.1% (w/v) trypsin for 5 min at 37°C either before pellicle formation or after the plasma membranes were recovered. As seen in Table 6, trypsinization after membrane isolation completely abolished both tyrosine kinase and protein phosphotyrosine phosphatase activities. This finding indicated that at least a portion of these enzymic activities resides on the cytoplasmic face of the membrane.

Although tyrosine kinase and protein phosphotyrosine phosphatase activities increase in differentiated HL-60 cells, it is unclear whether this reflects increased synthesis of the same or new enzymes, decreased degradation of enzymes, modification of enzymes already present in the cells, or removal of inhibitors of these proteins. To address the question of the possible presence of soluble inhibitors, experiments were performed in which particulate fractions from undifferentiated HL-60, and from those induced to differentiate along the granulocytic pathway by DMSO and the monocytic pathway by TPA were mixed. All possible combinations showed additive activity, indicating

![Graph](image-url)

Fig. 7. pH dependence of protein phosphotyrosine phosphatase and PNPPase activity of HL-60 cells. Each assay was performed at the indicated pH in HEPES buffer. Points, mean of 2 experiments, each done in duplicate; the range was less than 5% of the mean.
that a reduced level of inhibitors in differentiated cells was an unlikely mechanism for the increased enzyme activities observed.

To examine further the relationship between the enzymes present before and after differentiation, the activities were compared for a number of biochemical properties. As shown in Table 7, the properties of cells induced to differentiate to granulocytic or monocytic forms were nearly identical to those of the untreated cells.

The serum from patients with a number of malignancies has been shown to contain elevated levels of tyrosine kinase activity, perhaps having been shed from tumor cells (38). Given this observation, experiments were conducted to determine whether either tyrosine kinase or protein phosphotyrosine phosphatase activities could be recovered from HL-60-conditioned medium. Medium was concentrated by diafiltration and tested for these enzymic activities. Neither fresh medium [RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum] nor medium conditioned by untreated HL-60 cells, or those treated with DMSO or TPA contained detectable kinase or phosphatase activity.

DISCUSSION

In the period since the kinases that phosphorylate tyrosine were identified, major effort has been expended to elucidate the role of this modification in cellular physiology. Evidence has accrued suggesting that tyrosine phosphorylation is important in the regulation of cellular proliferation (6, 7) and differentiation (8–12). Previous work from this laboratory has shown that the terminal differentiation of the HL-60 promyelocytic leukemia and the WEHI-3B D+ myelomonocytic leukemia is associated with a marked decrease in cellular P-tyr residues (8–10). These changes occur even though cellular tyrosine kinase activity is increasing, and presumably are the result of an even greater increase in cellular protein phosphotyrosine phosphatase activity. We have hypothesized that these changes are central to the differentiation process.

To extend our understanding of these processes, we have carried out an initial characterization of these two enzyme activities by using cellular particulate fractions. Partial purification of these activities by our laboratory and by Kraft and Berkaw (39) have shown a single peak of activity for each of these enzymatic activities, suggesting that a single enzyme is involved in each case. Recent purification of tyrosine protein kinase activity by Yu and Glazer (15) has, however, demonstrated two activities which appear to be p60src and p95src.

The tyrosine kinase activity of HL-60 cells is temperature dependent, and it utilizes the terminal phosphate group of ATP, although ATP apparently can be replaced by a number of other nucleotides. The kinase can phosphorylate several tyrosine-containing substrates, it has a pH optimum of 6.5, a requirement for Mn2+ or Mg2+, and is further activated by Zn2+. The tyrosine kinase is not activated by a number of growth factors and ligands which are known to stimulate other kinases. Furthermore, the HL-60 tyrosine kinase is activated by nontoxic detergents, and is inhibited by TLCK, NEM, and quercetin.

The protein phosphotyrosine phosphatase activity is also associated with the particulate fraction, and its activity is closely mirrored by that of PNPPase activity, as reported in other systems (21). Protein phosphotyrosine phosphatase activity is inhibited by VO2+, Zn2+, and to a lesser degree by F−; it is most active at pH 7 to 8.

These features distinguish these enzymic activities from other such enzymes previously described (21, 37, 40–44), although it is clear that properties could be influenced by the unpurified state of the enzyme extracts. Given the apparent importance of tyrosine phosphorylation in the physiology of cellular proliferation and differentiation, further evaluation of the tyrosine kinase and protein phosphotyrosine phosphatase activities will be required to fully understand these processes. The present work suggests that although the HL-60 cell tyrosine kinase and protein phosphotyrosine phosphatase are both membrane bound, the kinase activity is located predominantly on the plasma membrane, while the phosphatase activity is present largely on internal membranes. Both activities present on the plasma membrane seem to be positioned on the cytoplasmic face of the membrane. The significance of this partitioning is unclear, but is consistent with the finding that both enzyme activities increase with differentiation, the phosphotyrosine phosphatase activity increasing to the greatest extent, while the proportion of cellular P-tyr is decreasing. The initial biochemical characterization of these enzymic activities has suggested that the tyrosine kinase and protein phosphotyrosine phosphatase activities are identical in parental HL-60 cells and in those differentiated along the granulocytic and monocytic pathways by DMSO and TPA, respectively.

Although the precise details of the regulation of cellular P-tyr content remain obscure, it would seem that further study of the tyrosine kinase and protein phosphotyrosine phosphatase activities in model systems such as the HL-60 leukemia will aid in the elucidation of the factors involved in the regulation of proliferation and differentiation. An understanding of these mechanisms in normal and neoplastic cells, and the development of agents to specifically alter these enzymatic processes, may provide therapeutic benefits as well.

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Unpublished results.


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