Tyrosine Kinase and Phosphotyrosine Phosphatase Activity in Human Promyelocytic Leukemia Cells and Human Polymorphonuclear Leukocytes

By Andrew S. Kraft and Roger L. Berkow

Although an increase in protein phosphorylation on tyrosine was first noted as a result of cell transformation or the application of growth factors to cells, recent reports have shown high levels of tyrosine kinases in nondividing tissues. For that reason, we have investigated whether normal human polymorphonuclear leukocytes (PMN) contain tyrosine kinase and phosphatase activity. Using a copolymer of glutamine:tyrosine as a substrate for the phosphotransferase reaction, we have demonstrated that PMN contain a cytosolic tyrosine kinase activity that elutes as a single peak from Sephacryl S-200 chromatography and has a molecular weight of 70 kilodaltons. Human promyelocytic leukemia cells (HL-60), contain a similar activity (as demonstrated by column chromatography), with only 25% of the activity found in PMN. This cytosolic tyrosine kinase can phosphorylate angiotensin II and a fragment of the src protein containing tyrosine 416, which suggests a similar substrate specificity to other tyrosine-phosphorylating protein kinases. In addition, we have demonstrated that PMN have double the amount of phosphotyrosine phosphatase (PTPase) activity of that found in HL-60 cells. This enzyme has a $K_m$ of 0.932 mmol/L and a $V_{max}$ of 0.355 $\mu$mol inorganic phosphate released/mg protein/min, which is similar to other cellular PTPase. Activation of PMN with f-Met-Leu-Phe and phorbol esters causes a slight but statistically significant drop in PMN PTPase activity. These results suggest that terminally differentiated myeloid cells have high tyrosine kinase and phosphatase activity, which may play a role in stimulus response coupling in the mature PMN.

PHOSPHORYLATION and dephosphorylation of specific proteins have been implicated in the initiation of myelomonocytic differentiation and polymorphonuclear leukocyte (PMN) activation. Recent findings demonstrating that the receptors for many growth factors are tyrosine-phosphorylating protein kinases has led to the concept that phosphorylation and dephosphorylation involving tyrosine residues may be of importance in regulating cellular events. This is further supported by the observation that the protein products of transforming viral oncopgenes are tyrosine kinases. Many of these transforming genes have a normal cellular homologue. The finding of phosphotyrosine-specific phosphatases in a wide variety of cells and the observation that inhibitors of these phosphatases must be included when isolating oncogene-associated tyrosine kinases further suggest that phosphorylation and dephosphorylation of tyrosine may regulate cellular events.

Recent reports have demonstrated that normal hematopoietic cells contain oncogene-coded tyrosine kinases. Platelets have been shown to contain c-src, the cellular homologue of the Rous sarcoma virus-transforming protein. In addition, c-src has been shown to increase in HL-60 cells induced to mature to granulocytes or macrophages. Furthermore, the platelet-derived growth factor (PDGF), whose receptor is known to have tyrosine kinase activity, has been shown to activate PMN to undergo chemotaxis, to produce superoxide, and to release granule contents. These data suggest that tyrosine phosphorylation and dephosphorylation may play a specific role in terminally differentiated myeloid cells.

We demonstrate that both HL-60 cells and normal human PMN contain a 70-kilodalton (kDa) cytosolic tyrosine kinase activity and that neutrophils have fourfold greater activity than is found in the HL-60 cells. In addition, PMN contain a membrane-associated phosphotyrosine phosphatase activity that has twofold greater activity than is seen in the HL-60 cells. Activation of PMN with either f-Met-Leu-Phe (fMLP) or phorbol esters induces a significant drop in tyrosine phosphatase activity. Our data suggest that tyrosine kinase and phosphatase activity increases during myelocytic differentiation and that these enzymes may play a role in stimulus response coupling in the mature PMN.

MATERIALS AND METHODS

Materials. Copolymer-containing tyrosine:glutamic acid (20:80) and angiotensin II were purchased from Sigma Chemical Co, St Louis; Filtron-X scintillation fluor was purchased from National Diagnostics, Manville, NJ; all trans-retinoic acid was purchased from Eastman Kodak, Rochester, NY; and $[^{32}P]$ adenosine triphosphate (ATP) was purchased from Amersham Corp, Arlington Heights, IL. Phorbol myristate acetate (PMA) was supplied by PL Biochemical Corp, Milwaukee, and the peptide RR-src (Src) was supplied by Peninsula Labs, Belmont, CA.

Phosphotyrosine, phosphoserine, phosphothreonine, EDTA, sodium orthovanadate, HEPES, p-nitrophenylphosphate, and fMLP were obtained from Sigma. All other chemicals were reagent grade.

Cell culture. HL-60 cells were provided by Dr J. Fontana (West Virginia University, Morgantown) and were early passage; KG-1 cells were supplied by Dr P. Koeffler (UCLA) and K-562 by Dr L. Bortoli (University of Alabama at Birmingham). All cell lines were grown at 37°C in RPMI 1640 media (GIBCO, Grand Island, NY) containing 10% fetal calf serum, antibiotics (50 U/mL of penicillin and 50 $\mu$g/mL of streptomycin), and 4 mmol/L glucose in a 5% CO2 humidified atmosphere. PMN were isolated from normal human donors by counterflow centrifugal elutriation according to our previously described method.

After isolation, PMN were suspended in either 0.1 mol/L sodium...
acetic buffer, pH 6.0, with 1 mmol/L EDTA and 0.2% (vol/vol) Triton X-100 (buffer A) or a buffer containing 145 mmol/L NaCl, 4.9 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgSO₄, 16 mmol/L HEPES, and 1 mg/mL glucose, pH 7.4 (buffer B).

**Protein kinase assays.** Cells were washed three times with Dulbecco's phosphate-buffered saline. They were dounce homogenized 30 strokes in a buffer containing 20 mmol/L Tris-HCl, pH 7.5, 2 mL of 2% (vol/vol) ethylene glycol tetraacetic acid (EGTA), 0.33 mol/L sucrose, and 2 mmol/L phenylmethylsulfonil fluoride. They were then centrifuged at 100,000 g for one hour and the supernatant used as a source of cytosolic enzyme. Tyrosine kinase activity was measured by using copolymer-containing tyrosine-glutamic acid (20:80) as a phosphotransferase acceptor. The reaction was initiated by the addition of 50 µL of enzyme preparation to a 250-µL reaction mixture containing 20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L Mg acetate, 2.5 mmol/L Mn chloride, 100 µmol/L [γ-³²P] ATP (60 cpm/pmol), and 250 µg copolymer. After incubation for five minutes at 30°C, the reaction was terminated by the addition of 2 mL of 20% trichloroacetic acid, and the precipitate was collected by filtering over a Millipore (Freehold, NJ) filter (0.45 µm). Filters are washed with 10 mL of 5% trichloroacetic acid, dissolved in Filtron-X scintillation fluid, and counted for radioactivity. Enzyme activity was linear up to 15 minutes. Triplicate determinations agreed to within ±10%. To determine specific tyrosine phosphotransferase activity, the amount of ³²P incorporation in the absence of copolymer was subtracted from the amount of ³²P incorporated with copolymer present.

**Phosphorylation of peptide substrates.** The phosphorylation reaction was done, as described previously, in a 20-µL volume containing 20 µg of RR-src or angiotensin II, 20 mmol/L Tris-HCl, pH 7.5, 100 µmol/L vandate, 10 mmol/L MgCl₂, 2.5 mmol/L MnCl₂, 10 µmol/L ATP, and 10 µL of partially purified tyrosine kinase. The reaction was started by the addition of 2 µL of [γ-³²P] ATP and incubated at 30°C for ten minutes. The reaction was terminated by the addition of 20 µL of 60% acetic acid and spotted onto Whatman P-81 paper. The filter squares were then washed in 30% acetic acid for 45 minutes followed by a wash in acetic acid for five minutes. The filter squares were counted in Aquasol-2 (New England Nuclear, Boston).

**Enzyme assays.** Alkaline phosphatase activity was determined as follows according to the method of Leis and Kaplan.21 PMN at 2 x 10⁶/mL in buffer A were sonicated to disrupt the cells and centrifuged at 400 g to remove unbroken cells and cell nuclei. The supernatant was then used for the assay (400-g supernatant). To a total volume of 0.5 mL is added 75 µL of 400-g supernatant containing approximately 75 µg of protein and 10 mmol/L phosphotyrosine in buffer A. The reaction was allowed to proceed for 15 minutes at 37°C and was stopped by the addition of 150 µL of 100% (wt/vol) trichloroacetic acid. The tube was then cooled on ice for 60 minutes. The precipitate was removed by centrifugation at 10,000 g and the supernatant assayed for inorganic phosphate (Pi) according to the method of Fiske and Subbarow.

Protein was determined by the method of Lowry et al or the method of Bradford.

**RESULTS**

**Tyrosine kinase activity of HL-60 cells and circulating PMN.** Using a copolymer containing tyrosine and glutamic acid as a phosphotransferase acceptor, we partially purified a tyrosine kinase from HL-60 cells. This enzyme can be measured in crude homogenates of HL-60 cells and was further purified by elution from a diethylaminoethyl (DEAE)-cellulose column with 0.05 mol/L NaCl (Fig 1A). After dialysis, this tyrosine kinase activity was further purified by phosphocellulose chromatography (Fig 1B). This enzyme, which eluted as a single peak from both of these columns, was active in the presence of manganese or magnesium ions, with the former stimulating greater phosphorylation of copolymer (Fig 2). In comparison, the addition of varying concentrations of calcium, cobalt, or zinc had no effect on phosphotransferase activity. Also, the addition of

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**Fig 1.** Partial purification of tyrosine kinase from HL-60 cells. (A) DEAE-cellulose chromatography. The 100,000-g supernatant prepared from 10⁰ HL-60 cells was applied to a DEAE-cellulose column (0.9 x 20 cm) equilibrated with buffer C (20 mmol/L Tris-HCl, pH 7.5, 0.58 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonil fluoride). After sample addition, the column was washed extensively with buffer C and then eluted with a linear gradient of 70 mL of buffer C plus 70 mL of buffer A containing 0.2 mol/L NaCl. Fractions of 2 mL were collected. Aliquots (50 µL) were assayed for tyrosine kinase activity (○). Protein kinase activity is expressed as counts per minute of ³²P incorporated per five minutes per 50-µL aliquot. NaCl concentration is denoted by (■). Absorbance at 280 nm is denoted by (□). (B) Phosphocellulose chromatography. Fractions containing tyrosine kinase activity eluted from DEAE-cellulose were dialyzed against three changes of buffer C. They were then loaded onto a phosphocellulose column (0.9 x 11 cm) and washed extensively with buffer C. The column was then eluted with a 80-ml 0 to 0.4 mol/L salt gradient in buffer A. Two-milliliter fractions were collected and assayed as before. The NaCl concentration is denoted by (■). Absorbance at 280 nm is denoted by (□).
stopped by the addition of 60% acetic acid. The data presented are the reaction was carried out as described in Materials and Methods and protein src jmol/L), or insulin (0.2 mmol/L) did not stimulate this tyrosine serine (80 μg/mL), epidermal growth factor (0.3 to 3 μmol/L) or toward macrophages with PMA (10 nmol/L). For comparison we assessed the activity of this enzyme in HL-60 cells.

Because HL-60 cells are a promyelocytic leukemia cell line blocked at an early stage in differentiation, we were interested in whether tyrosine kinase activity would be altered as the cell matures. To examine this possibility, we assayed tyrosine kinase activity in HL-60 cells induced to differentiate toward granulocytes with retinoic acid (1.0 mmol/L) or toward macrophages with PMA (10 nmol/L). For comparison we assessed the activity of this enzyme in mature granulocytes isolated from human peripheral blood as well as in the KG-1 cell line, a myeloid cell line less mature than HL-60 cells, and the K562 cell line, which has properties of both myeloid and erythroid progenitors.

The tyrosine kinase activity was measured on equivalent amounts of protein from the 100,000-g supernates of these cell lines. Table 2 shows that human PMNs have approximately fourfold greater activity than was found in HL-60 cells. Differentiation of the HL-60 cells to granulocyte-like cells caused small increases in enzyme activity, whereas differentiation with PMA to macrophage-like cells caused a slight decrease in activity. Both KG-1 and K562 cell lines had less cytosolic tyrosine kinase activity than that found in the HL-60 cells.

Since HL-60 cells and mature PMN contain cytosolic tyrosine kinase activity, we examined whether this activity might be determined by similar if not identical proteins. The tyrosine kinase activity in both PMN and HL-60 cytosols elutes from a DEAE-cellulose column at 0.05 mol/L NaCl. In addition, partially purified tyrosine kinase activity from the cytosol of PMN has a similar dependence on manganese (Fig 2). By using a Sephacryl S-200 molecular sieve column both the HL-60 and PMN cytosolic tyrosine kinases were found to have a molecular weight of approximately 70 kDa (Fig 3).

Phosphotyrosine phosphatase activity of circulating PMN and HL-60 cells. Because PMN demonstrated a high cytosolic tyrosine kinase activity, we hypothesized that these cells should contain phosphotyrosine phosphatase activity. We therefore determined phosphotyrosine phosphatase activity in a 400-g supernatant of human PMN obtained as described in Materials and Methods. Analysis of this enzyme activity revealed 0.399 ± 0.014 μmol Pi released from the phosphotyrosine substrate per milligram of cell protein per minute (mean ± SE, n = 17). This activity was linear with respect to both the amount of cellular protein assayed, from 10 to 150 μg and with respect to the time of incubation of the protein with substrate, 15 to 30 minutes. Under these same conditions and with 10 mmol/L phosphoserine and phosphothreonine as the substrates, the PMN 400-g supernatant demonstrated 0.012 ± 0.001 and 0.045 ± 0.001 μmol Pi released from the substrate/mg/min (mean ± SE, n = 3), respectively. This indicated that by using phosphoamino acids the phosphotyrosine phosphatase activity was greater in the 400-g supernatant than phosphatases for phosphoserine or phosphothreonine. The kinetics of phosphotyrosine phosphatase activity in the PMN 400-g supernatant was then determined by Lineweaver-Burke analysis. A double reciprocal plot (Fig 4) demonstrated that this enzyme

Phosphorylation of Peptides by Partially Purified Tyrosine Kinase From HL-60 Cells

<table>
<thead>
<tr>
<th>Additions</th>
<th>Tyrosine Kinase Activity (cpm/20 μg Protein/10 min)</th>
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<tbody>
<tr>
<td>RR-src</td>
<td>6,206</td>
</tr>
<tr>
<td>Antiotensin II</td>
<td>2,777</td>
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<tr>
<td>None</td>
<td>1,139</td>
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Tyrosine kinase is purified by DEAE-cellulose chromatography and added to a reaction mixture containing RR-src or angiotensin II. The reaction was carried out as described in Materials and Methods and stopped by the addition of 60% acetic acid. The data presented are the means of duplicate experiments that vary ± 15%.

Table 2. Relative Tyrosine Kinase Activity in PMNs and Leukemic Cell Lines When Compared With HL-60 Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Relative Tyrosine Kinase Activity (%)</th>
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<tbody>
<tr>
<td>HL-60 treated with retinoic acid</td>
<td>133 ± 21</td>
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<tr>
<td>(1.0 μmol/L) x 5 d</td>
<td></td>
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<tr>
<td>HL-60 treated with PMA</td>
<td>68 ± 16</td>
</tr>
<tr>
<td>(10 nmol/L) x 5 d</td>
<td></td>
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<tr>
<td>KG-1</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>K562</td>
<td>34 ± 14</td>
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<tr>
<td>PMN</td>
<td>460 ± 56</td>
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Equivalent amounts of protein from a 100,000-g supernatant were assayed for tyrosine kinase activity as described in Materials and Methods. Control HL-60 supernatants incorporated approximately 100 cpm/μg protein per 5 min into copolymer. Results are the means of triplicate determinations ± SD.
described. When the 400-g supernatant was centrifuged at 100,000 g at 4°C for 60 minutes, the 100,000-g pellet contained phosphotyrosine phosphatase activity equivalent to 0.315 ± 0.013 μmol of substrate hydrolyzed/mg protein/min. This represented 87% of the total cellular activity. The 100,000-g supernatant contained 0.046 ± 0.007 μmol/mg/min and represented 13% of the total activity (mean ± SE, n = 5).

We next determined the optimum pH of the phosphotyrosine phosphatase in the PMN 400-g supernatant. Figure 5 shows that this enzyme demonstrated a broad range of activity, with optimum hydrolysis of phosphotyrosine occurring at pH 7.0.

In membrane-associated fractions, PMN are known to contain a large amount of alkaline phosphatase activity. This enzyme activity is dependent on magnesium ions and is inhibited by chelators of divalent cations.45 It was important therefore to distinguish our phosphotyrosine phosphatase activity from PMN alkaline phosphatase. Alkaline phosphatase activity assessed at pH 10.0 using p-nitrophenyl phosphate as the substrate demonstrated a requirement for magnesium ions and a distinct dose-dependent inhibition of activity by 0 to 10 mmol/L EDTA (36% of control at 2 mmol/L EDTA in the presence of 1 mmol/L MgCl2). Figure 6 demonstrates that EDTA over a concentration range of 0 to 10 mmol/L had no effect on the hydrolysis of phosphotyrosine by the PMN 400-g supernatant. Similarly, CaCl2 and MgCl2 (0 to 10 mmol/L) had no effect on phosphotyrosine phosphatase activity (data not shown). In comparison a dose-dependent inhibition of hydrolysis of phosphotyrosine occurred when sodium orthovanadate, ZnCl2, or MnCl2 was added to the PMN 400-g supernatant before addition to the substrate. This pattern of inhibition is similar to that seen for phosphotyrosyl protein phosphatases from other tissue sources.13,29

To determine whether phosphotyrosine phosphatase activ-
with the stated concentration of sodium orthovanadate, ZnCl₂, or ±ated undifferentiated HL-60 cells for phosphotyrosine phosphatase activity. PMN phosphotyrosine per milligram of cell protein. This was 52.8% of the amount of phosphotyrosine phosphatase activity found in the PMN.

To help understand the role of the phosphotyrosine phosphatase in the PMN we examined the activity of this enzyme in PMN suspended in buffer B and stimulated with varied concentrations of PMA, fMLP, or fMLP plus 5 µg/mL of cytochalasin B (CB). Figure 7 demonstrates that within five minutes of incubation with PMA or fMLP plus cytochalasin B, a dose-dependent reduction in phosphotyrosine phosphatase activity was seen. At the highest concentration of PMA or fMLP tested, phosphotyrosine phosphatase activity of 71.6% ± 6.6% and 75.8% ± 8.5% of control value, respectively, was found (mean ± SD, n = 5). In contrast to the PMN granule constituents lysozyme, myeloperoxidase, and vitamin B₁₂-binding protein, which are released into the incubation media upon PMN activity with PMA or fMLP, no phosphotyrosine phosphatase activity was released upon PMN stimulation by these agents (data not shown).

DISCUSSION

In this communication, we have presented data demonstrating that myeloid leukemia cell lines and mature circulating neutrophils contain both cytosolic tyrosine protein kinase activity and membrane-associated phosphotyrosine phosphatase activity. The cytosolic tyrosine kinase activity from both the PMN and HL-60 cells has a similar molecular weight of 70 kDa. The elution profile from DEAE-cellulose and sensitivity to Mg²⁺ and Mn²⁺ are also similar, which suggests that these may be related if not identical enzymes. Further characterization will be needed, however, to establish their identity. Although PMN have approximately fourfold more cytosolic tyrosine kinase activity than the uninduced HL-60 cells, treatment of the HL-60 cells with retinoic acid to induce a granulocyte phenotype resulted in only a slight elevation of activity. Since we assayed the effects of PMA and retinoic acid five days after treatment, it is possible that we did not observe earlier changes in tyrosine kinase activity induced by these agents. Of interest is the ability of the 70-kDa cytosolic tyrosine to phosphorylate a peptide-containing tyrosine 416 of the src protein. Activation of other tyrosine kinases, for example, the PDGF receptor, has been shown to modulate membrane-associated src tyrosine kinase activity in fibroblasts. Our result might imply a possible role for the 70-kDa cytosolic tyrosine kinases in modulation of membrane-bound tyrosine-phosphorylating protein kinases. Furthermore, both platelets and rat liver contain a cytosolic tyrosine kinase activity with a similar molecular weight to that found in the present study, thereby suggesting that this activity is not specific to myeloid cells or myeloid differentiation.

Numerous substrates have been used to evaluate phosphotyrosine phosphatase activity including phosphorylated histone, angiotensin, fragments of the src oncogene, casein, albumin, and myosin light chains. Although the physiological substrates of this enzyme are not known, others have demonstrated that acidic protein substrates are dephosphorylated by phosphotyrosine phosphatase whereas alkaline substrates are dephosphorylated by membrane-associated alkaline phosphatase. We have used phospho-
tyrosine as the substrate in the present study. Although this is not a peptide or protein substrate, our data and that of others suggest that this phosphorylated amino acid may be used to measure specific phosphotyrosine phosphatase activity.

The data presented demonstrate that PMN and cells contain phosphotyrosine phosphatase activity that is localized to the cell particulate fraction. This enzyme has activity over a broad pH range (Fig 5), which indicates that the PMN may contain several species of phosphotyrosine phosphatase, as demonstrated in rat spleen and bovine brain. Both the mean (0.932 mmol/L) of this enzyme and its inhibition by zinc, vanadate, and manganese ions are similar to that described for phosphotyrosine phosphatases isolated from fibroblasts, placenta, A431 cells, astrocytoma cells, and Drosophila. Unlike PMN alkaline phosphatase, PMN phosphotyrosine phosphatase is not dependent on magnesium ions, nor is it inhibited by EDTA, thereby suggesting that these phosphatases are not the same enzyme. However, purification of both enzymes will be necessary to demonstrate that these are totally separate activities. In addition, we have found that PMN contain approximately twofold greater phosphotyrosine phosphatase activity than that in uninduced HL-60 cells. Our results are in accord with recently presented data demonstrating an increase in membrane-associated phosphotyrosine phosphatase activity induced in HL-60 cells by retinoic acid.

We have evaluated the effect of fMLP and PMA on PMN phosphotyrosine phosphatase activity. Treatment of PMN for five minutes with fMLP plus CB or PMA causes a slight drop in phosphotyrosine phosphatase activity (P < .05). Because no phosphotyrosine phosphatase activity is found in the incubation media after PMA activation, these data suggest that PMN phosphotyrosine phosphatase is not secreted and is probably not a granule constituent. Furthermore, in preliminary experiments (data not shown), we have demonstrated that activation of PMN with PMA or fMLP leads to increased phosphorylation of protein bands that demonstrate resistance to alkali treatment, thereby implying phosphorylation on tyrosine residues. These data may indicate that ligand binding to PMN leads to phosphorylation of proteins on tyrosine residues by the activation of a tyrosine kinase and the inhibition of phosphotyrosine phosphatase activity.

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