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 μmol 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.

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acetate 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 mmol/L EDTA, 0.5 mmol/L ethylene glycol tetraacetic acid (EGTA), 0.33 mmol/L sucrose, and 2 mmol/L phenylmethylsulfonyl fluoride. They were then centrifuged at 100,000 g for five minutes to remove unbroken cells and cell nuclei. The supernatant was 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-²³P] ATP (60 cpm/pmol), and 250 µg copolymer. After incubation for five minutes at 30°C, the reaction was terminated with 2 µL of 20% trichloroacetic acid, and the precipitate was collected by filtering over a Millipore (Freehold, NJ) filter (0.45 µm). Filters were washed with 10 mL of 5% trichloroacetic acid, dissolved in Flotron-X scintillation fluor, 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 MnCl₂, and 10 µL of partially purified tyrosine kinase. The reaction was started by the addition of 2 µL of [γ-²³P] 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 acetone for five minutes. The filter squares were counted in Aquasol-2 (New England Nuclear, Boston).

**Enzyme assays.** Alkaline phosphatase activity was determined by the method of DeChatelet and Cooper using p-nitrophenyl phosphate as the substrate and 2-aminophenol propanol, pH 10.0, containing 1 mmol/L MgCl₂ as the buffer. Phosphoprotein phosphatase activity was determined as follows according to the method of Leis and Kaplan. PMN at 2 x 10⁷/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 µmol/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 greater stimulation by the former. The enzyme had no effect on varying concentrations of calcium, cobalt, or zinc.

**Fig 1.** Partial purification of tyrosine kinase from HL-60 cells. (A) DEAE-cellulose chromatography. The 100,000-g supernatant prepared from 10⁷ 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, 0.8 mmol/L EGTA, 2 mmol/L phenylmethylsulfonyl 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 (O--O). 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 (O---O). (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 to remove copolymer. The column was then eluted with a 80-ml 0 to 0.8 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 (O---O).
phosphatidylserine (80 µg/mL), PMA (1 µmol/L) alone or in combination with calcium (1 mmol/L) and phosphatidylserine (80 µg/mL), epidermal growth factor (0.3 to 3 µmol/L), or insulin (0.2 umol/L) did not stimulate this tyrosine kinase. Acidic and basic histones were poor substrates for this kinase, whereas a peptide-containing tyrosine 416 of the src protein (RR-src) or angiotensin II (Table 1) were both phosphorylated.

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 mol/L) or toward macrophages with PMA (10 nmol/L). For comparison we assessed the activity of this enzyme in the HL-60 cells.

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<th>Table 2. Relative Tyrosine Kinase Activity in PMNs and Leukemic Cell Lines When Compared With HL-60 Cells</th>
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<tr>
<td><strong>Cell Line</strong></td>
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<tr>
<td>HL-60 treated with retinoic acid</td>
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<tr>
<td>(1.0 µmol/L) x 5 d</td>
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<tr>
<td>HL-60 treated with PMA</td>
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<td>(10 nmol/L) x 5 d</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.

The tyrosine kinase activity was measured on equivalent amounts of protein from the 100,000-g supernates of HL-60 supernatants incorporated approximately 100 cpm/µg protein per 5 min into copolymer. Results are the means of triplicate determinations ± SD.

Fig 2. The effect of increasing divalent ion concentration on tyrosine kinase activity from HL-60 cells and PMN. The activity of tyrosine kinase from HL-60 cells partially purified as in Fig 1 was determined with increasing concentrations of manganese (O——O) or magnesium (Δ——Δ). A similar experiment was done at a later time with partially purified tyrosine kinase from PMN in which the effect of varying concentrations of manganese (O——O) was assayed.

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
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 mol 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. 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.

To determine whether phosphotyrosine phosphatase activity in the PMN 400-g supernatant was incubated at 37°C for 15 minutes with varying concentrations of phosphotyrosine as substrate in 0.1 mol/L sodium acetate buffer, pH 6.0, with 0.2% (vol/vol) Triton X-100 and 1 mmol/L EDTA. The results are a representative experiment of at least three different experiments that varied by less than 5%.

**Fig 4.** Double reciprocal plot of phosphotyrosine phosphatase activity in the PMN 400-g supernatant. Fifty to 75 μg of the PMN 400-g supernatant was incubated at 37°C for 15 minutes with varying concentrations of phosphotyrosine as substrate in 0.1 mol/L sodium acetate buffer, pH 6.0, with 0.2% (vol/vol) Triton X-100 and 1 mmol/L EDTA. The results are a representative experiment of at least three different experiments that varied by less than 5%.

**Fig 5.** Effect of pH on phosphotyrosine phosphatase activity. The activity was determined in 0.1 mol/L acetate buffer adjusted to the given pH by using 10 mmol/L phosphotyrosine as substrate under the conditions noted in Fig 4. The results are expressed as micromoles of Pi released from the substrate per milligram of protein per minute. This curve represents the means of three separate experiments performed in duplicate.
ity increases during myeloid differentiation, we next evaluated undifferentiated HL-60 cells for phosphotyrosine phosphatase activity. A 400-g supernatant prepared from HL-60 cells demonstrated 0.211 ± 0.05 amol Pi hydrolyzed from 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 various 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 B<sub>12</sub>-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 Mg<sup>2+</sup> and Mn<sup>2+</sup> 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 retinoid 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 $K_m$ (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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