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A NOVEL PROTAMINE KINASE ACTIVITY IN HUMAN PROMYELOCYTIC LEUKEMIA CELLS

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A novel protamine kinase activity is present in human promyelocytic leukemia cells (HL60). The enzyme is clearly distinct from cyclic AMP-dependent protein kinase and is not calcium activated. The enzyme requires Mg^{2+}, is sulfhydryl dependent and is strongly inhibited by fluoride. Activity is 80% cytosolic and 20% particulate but appears to be the same enzyme in the two fractions. Enzyme activity is markedly stimulated during differentiation by retinoic acid and dimethylformanide but not by a phorbol diester. However the latter treatment does lead to a redistribution of the kinase so that it becomes predominantly particulate.

INTRODUCTION

A human myeloid leukemia cell line (HL60) consisting primarily of cells resembling promyelocytes (1) can be caused to differentiate into either granulocytes or macrophages by various inducers. Retinoic acid (RA), dimethylformamide and dimethylsulfoxide induce differentiation to mature myeloid forms (2-4) while TPA and conditioned medium of human leukocytes treated with PHA lead to differentiation of HL60 cells into macrophages (5,6). Very little is known of the mechanisms of human hematopoietic cell differentiation. However wide spectrum of cellular processes appear to be regulated by protein phosphorylation mechanisms (7). Retinoic acid-induced differentiation of melanoma (8) and F9 embryonal carcinoma (9) cells appears to involve cyclic AMP-dependent protein kinase.

In the course of studies on the role of protein kinases in HL60 cell differentiation, it was observed that extracts of these cells contained high levels of a kinase preferentially phosphorylating protamine. In this paper we describe some of the properties of this kinase and the effect of HL60 cell differentiation upon the activity.

MATERIALS AND METHODS

Cell culture. HL60 cells were subcultured at an initial density of $1x10^5$ cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and incubated for 48 h at 37°C in an atmosphere of 10% CO_2 and

Abbreviations used are: RA, retinoic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMF, dimethylformamide; cAMP-DPK, cyclic AMP-dependent protein kinase.

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100% humidity. Inducers are then added (RA 1 μM; DMF 120 mM; TPA 64 nM; Sigma Chemical Co., St. Louis, MO) and the cells further incubated for 5 days with RA and DMF and 2 days with TPA.

Enzyme Assays. The cells were harvested by centrifugation, washed twice in 20 mM HEPES pH 7.2-120 mM sucrose and the cell pellet resuspended finally in 2 mM MOPS, pH 7.5-0.5 mM EDTA -0.5 mM DTT (buffer A) containing 1 mM EGTA, 0.4 mM phenylmethylsulfonylfluoride, 0.04 mM tosyl-L-lysine chloromethylketone and 10 μg/ml pepstatin A, leupeptin, antipain, aprotinin and soybean trypsin inhibitor as protease inhibitors. After allowing the cells to swell they are disrupted in a teflon/glass homogenizer, monitoring cell disruption with Trypan Blue. When >98% of the cells are disrupted the homogenate is centrifuged at 3 x 10^6 g.min to give supernatant and particulate fractions. The particulate material is extracted twice with 1·5% Triton X-100 in buffer A containing protease inhibitors and the combined Triton supernates used for protein kinase assay. Protein concentrations were determined using the method of Bradford (10). Histone kinase was assayed in a medium containing: 40 mM MOPS pH 7, 1 mM DTT, 7.5 mM $MgSO_4$, 1.33 mg/ml histone f_2b (Sigma) and protamine kinase in a medium containing 40 mM HEPES pH 8, 1 mM DTT, 5 mM $MgSO_4$ and 5.3 mg/ml protamine sulfate (Sigma). All assays contained 50 μM [γ-^{32}P]ATP (350 dpm/pmol), 1 mg/ml bovine serum albumin, and 1-20 μg enzyme protein in a final volume of 120 μl. After assaying for 5 min at 30°C, duplicate 50 μl aliquots were quantitated by the disc method of Witt & Roskoski (10). Cyclic AMP dependence was measured by assaying in the presence and absence of 10 μM cyclic AMP. All assays were in duplicate. One unit of enzyme activity was defined as the transfer of 1 nmole [^{32}P] per minute.

Column Chromatography. Ultrogel AcA34 (LKB, Gaithersburg, MD) columns, 1.5 x 45 cm, were equilibrated with 50 mM MOPS pH 7.5-1 mM DTT-25 mM NaCl. The cytosol and pellet extract were dialyzed for 2 h against the column buffer, applied to separate columns and run concurrently at 5 ml/h collecting 1·8 ml fractions. DEAE cellulose columns (DE-32, Whatman Clifton, N.J. were formed in 1 cc syringes after equilibration in buffer A. Aliquots (1ml) of cytosol and pellet extract were applied without dialysis and the columns washed with 9 ml buffer A, then a 22 ml gradient of 0 to 0.4 M NaCl in buffer A, 2 ml of 0.4 M NaCl in buffer A and 4 ml of 1 M NaCl in buffer A, running at 2 ml/h and collecting 1 ml fractions.

RESULTS

HL-60 cells contain high levels of a kinase which preferentially phosphorylates protamine. This enzyme is present in more than 12-fold the specific activity of cAMP-DPK (Table 1). The protamine kinase is predominantly cytosolic although there is a significant particulate component. Upon differentiation of the cells to mature myeloid forms with RA or DMF there is a 4-fold increase in protamine phosphorylation with no apparent shift in distribution. In contrast differentiation to macrophages with TPA does not significantly alter total cellular activity but does result in a major shift to the particulate compartment. In a number of systems protamine has been found to be a good substrate for cAMP-DPK (12). The protamine kinase is clearly distinct from the cAMP-DPK as it is not stimulated by cyclic AMP or inhibited by cAMP-DPK heat-stable inhibitor (Walsh inhibitor,13). On the other hand it is strongly inhibited by fluoride ion and reagents which react with sulfhydryl groups such as N-ethylmaleimide (Table 2).

Table 1. Protein Kinase Activities in Control and Differentiated HL60 Cell Extracts

TREATMENT	PROTEIN KINASE ACTIVITY			
	PROTAMINE		HISTONE	
	Cytosolic	Particulate	Cytosolic	Particulate
	U/mg protein			
Control	6.63±0.13 (79)	2.76±0.63 (21)	0.51±0.05 (63)	0.48±0.05 (37)
RA	26.78±3.91 (86)	6.37±1.17 (14)	1.09±0.22 (61)	0.99±0.23 (39)
DMF	21.42±4.81 (79)	9.19±2.75 (21)	1.20±0.16 (66)	1.00±0.11 (34)
TPA	4.16±0.62 (43)	5.47±1.06 (57)	0.64±0.03 (53)	0.57±0.08 (47)

Histone kinase activity determined (+) cyclic AMP. Results are average of 4 expts (±) S.E. Values in brackets are the proportion of total activity in the subcellular fraction.

The molecular forms of protamine kinase have been studied by gel permeation chromatography. A single major peak of protamine phosphorylating activity (peak III) with a molecular weight of about 110,000 is observed with the cytosolic (A) and particulate (B) fractions showing identical protamine kinase profiles (Fig. 1). Coincident with this activity is a peak of histone phosphorylation which is cyclic AMP-independent but the activity with histone f_2b is <2% of that with protamine. Both protamine and histone phosphorylation by peak III are inhibited by fluoride (not shown). The protamine kinase (peak III) is clearly separated from the cAMP-DPK (Peak II) which shows an approximately 10-fold stimulation by cyclic AMP (Fig. 2) and is not inhibited by fluoride ion (not shown). There is some protamine phosphorylating activity associated with this peak which becomes apparent when protamine kinase is plotted on the same scale as histone kinase activity (open circles in Fig. 1 are the same data points as the closed circles but with 40-fold expanded scale) but it may result from peak spreading from area III. There is also a minor peak of protamine phosphorylating activity which elutes in the void volume (peak 1).

Table 2. General Properties of the Protamine Kinase

ADDITION	CONCENTRATION	RELATIVE ACTIVITY
		%
None		100
N-Ethylmaleimide	20 mM	2.3
Cyclic AMP	10 μM	90.3
Cyclic AMP + PKI	10 μM + 200 μg/ml	93.5
NaF	10 mM	34.7
NaF + Cyclic AMP	10 mM + 10 μM	35.2

Peak III from Fig. 1 was employed as the enzyme. Protein kinase inhibitor (PKI) was partially purified from rabbit skeletal muscle (13).

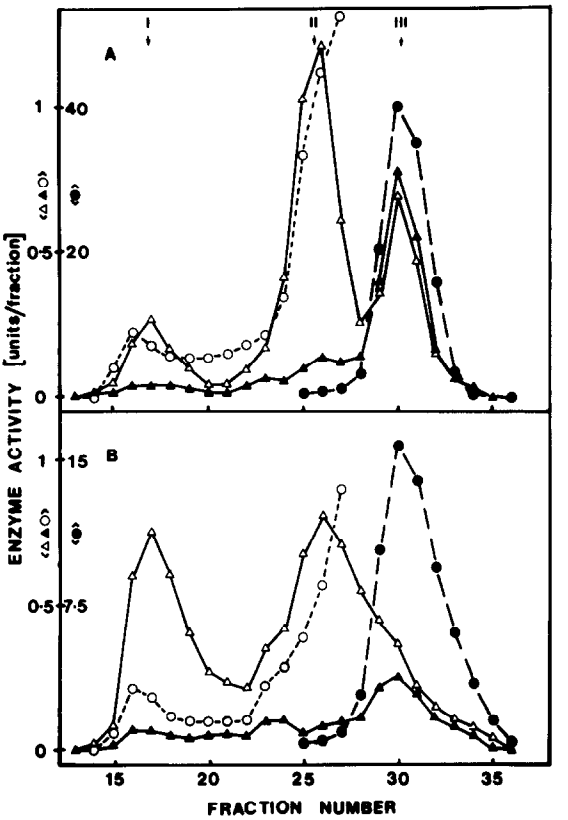

Fig. 1 Gel Permeation Chromatography of Protein Kinases in Subcellular Fractions of HL-60 cells. A, cytosol; B, Triton-extracted particulate. Protamine kinase (0,●); histone kinase with (△) and without (▲) cyclic AMP.

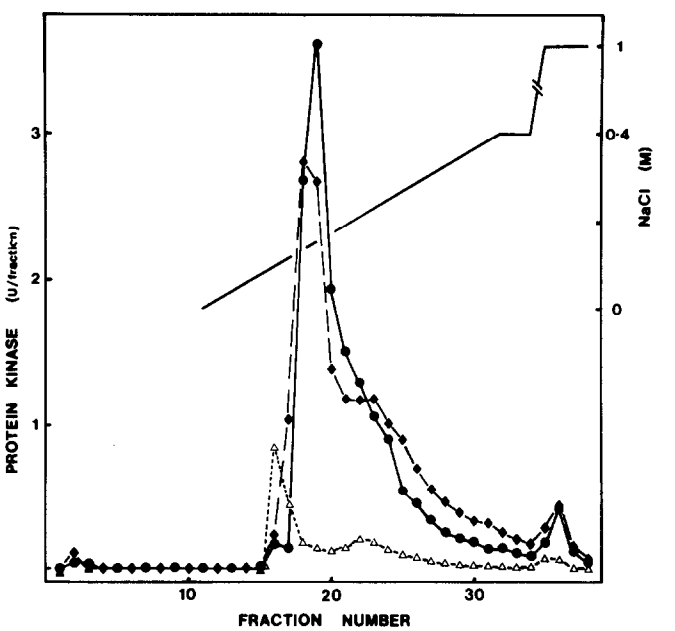

Fig. 2. DEAE Cellulose Chromatography of Protein Kinases in Subcellular Fractions of HL-60 Cells. ● cytosol, ◆ particulate, protamine kinase; △ histone (+ cyclic AMP).

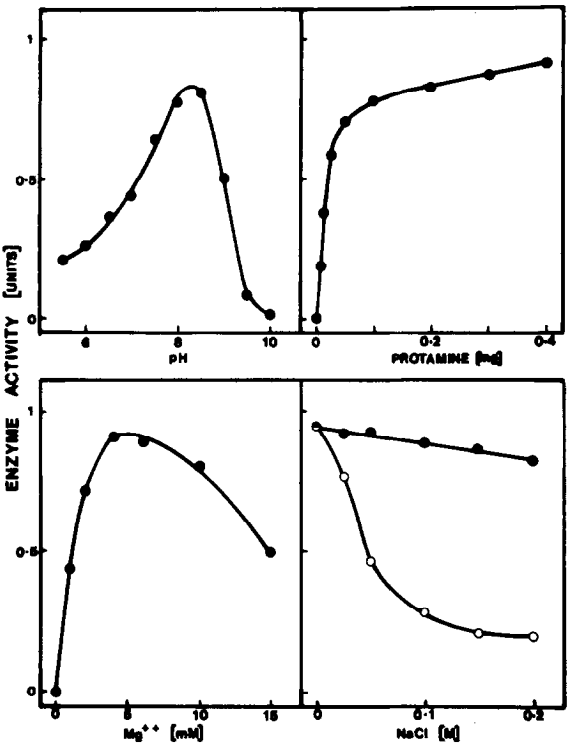

Fig. 3 Properties of Protamine Kinase. Peak III from Fig. 1A was used as enzyme source. ●, protamine; O, histone (+ cyclic AMP).

The cytsolic and particulate fractions were also chromatographed upon DEAE-cellulose. A major peak of protamine kinase elutes immediately after Type I cAMP-DPK, at an ionic strength of 0.15 M NaCl (Fig.2). However the peak is not symmetrical and there is a major trailing shoulder eluting at 0.2 to 0.3 M NaCl. Thus there may be more than one form of protamine kinase present in peak III from gel permeation. Essentially identical profiles are obtained with the cytosolic and particulate fractions. There is also a minor peak which binds strongly to DEAE and only elutes with a 1M NaCl wash.

The protamine kinase has a sharp pH optimum at about pH 8.3 (fig.3). The preferred metal ion required for activity is Mg^{2+} with a optimum at 5 mM (Fig.3). Mn^{2+} cannot effectively replace Mg^{2+}. Two types of calcium-activated protein kinases have been described one requiring calmodulin (7,14) and the other phospholipid-dependent (15). The protamine kinase does not fall into either of these categories since calcium at free levels between 1 and 500 µM is inhibitory to activity in the presence of Mg^{2+}, EGTA has no effect upon activity and neither calmodulin (40 µg/ml) nor phosphatidylinositol (25 µg/ml) has a stimulatory effect (not shown). Increasing ionic strength, in the form of NaCl has a very slight inhibitory effect upon protamine kinase whereas histone phosphorylation by cAMP-DPK is markedly inhibited (Fig.3). The

requirement for protamine is biphasic with a very sharp rise in activity as the protamine concentration is raised from 0 to 200 µg/ml followed by a shift to a slower rise in activity up to 3500 µg/ml.

The protamine kinase activity of HL60 cell extracts appears to be quite different from previously described protamine kinase activities of trout (16) and calf thymus nuclei (17) as these enzymes, in contrast to the protamine kinase described here, had a high activity with histone, utilized Mn^{2+} as metal ion, were stimulated by NaCl at low concentrations, were not inhibited by fluoride and had a pH optimum of around pH 7. Further studies are in progress to investigate its role in HL60 cellular function.

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