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Purification and Characterization of a Novel Protamine Kinase in HL60 Cells

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A protamine kinase from HL60 cells was purified to near homogeneity by DEAE-Sephacel, protamine-agarose, Hydroxylapatite, and S-200 chromatography. It was purified by 75.8-fold through four chromatographic steps, and 0.67% of total activity was recovered. The purified enzyme had an apparent molecular mass of 120 kDa and was activated by Mg^{2+} or Mn^{2+}, but inhibited by Ca^{2+}. Neither phospholipid nor phorbol ester significantly affected the enzyme activity. Staurosporine was the most potent inhibitor of the enzyme among the protein kinase inhibitors tested, K_{252a}, H_7, heparin, and staurosporine. The purified protamine kinase exhibited a maximum velocity of 5,000 pmol/min/mg and K_m of 1.3 mM for protamine sulfate as a substrate. Myelin basic protein and protamine sulfate served as the best substrates for the protamine kinase among those tested. The activity of the protamine kinase remained unchanged upon treatment with PMA, retinoic acid, dimethyl sulfoxide, or 1,25 dihydroxy vitamin D_3 for 15 min, while treatment with a differentiating agent, 1,25 dihydroxy vitamin D_3, for one week increased its activity. These results suggest that protamine kinase in HL60 cells is involved in the late stage of the macrophage-monocytic differentiation pathway and may play a role in maintenance of the differentiation after HL60 cells are committed.

Key words: differentiation, HL60 cells, kinase, protamine, protein kinase C.

Protamine kinases preferentially phosphorylate protamine in the absence of Ca^{2+}, phospholipid, or cyclic nucleotides, and are widely distributed in yeast, fungi, fish, and mammals (*1–5*). Although protamine kinases have been characterized in numerous species, they display distinct biochemical properties, which indicate the diversity of this enzyme. For instance, protamine kinase in rainbow trout exhibits Mg^{2+}, thiol, and cAMP dependency and phosphorylates protamine more efficiently than histone in the presence of 0.3 M NaCl. In contrast, protamine kinase isolated from rat brain is independent of cAMP and different from the catalytic subunit of cAMP-dependent protein kinase. Protamine kinase from rat brain exhibits its maximum activity at 20 mM Mg^{2+}. Protamine kinase from bovine kidney mitochondria with an apparent M_r of 45 kDa and optimal Mg^{2+} concentration at 1.5 mM is inhibited by 50% by 0.5 mM Ca^{2+} (*6*). Protamine kinase from HL60 cells requires Mg^{2+} and is dependent on thiol for its activity (*4*). This enzyme exists predominantly in the cytosol and is strongly inhibited by fluoride and *N*-ethylmaleimide. Protamine kinases from HL60 cells and rat hepatocytes are stimulated by treatment with retinoic acid and dimethylformamide (*4*) and insulin (*7*), respectively, which suggests their involvement in cellular regulation in response to external stimuli. However, the physiological roles of protamine kinases have not been elucidated in detail.

HL60 cells can be induced to differentiate into monocytes, granulocytes, and macrophages by a variety of agents including dimethyl sulfoxide (DMSO), phorbol esters, retinoic acid (RA), and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (*8*). HL60 cells differentiate into granulocytes upon exposure to DMSO or retinoic acid (*8, 9*), whereas they differentiate into cells exhibiting distinct monocyte/macrophage characteristics when treated with 1,25-$(OH)_2D_3$ (*10*). From studies on the role of protamine kinase in HL60 cell differentiation, it was suggested that the enzyme is involved in the late stage of the differentiation pathway induced by dihydroxy vitamin D_3, but not by PMA, DMSO, or retinoic acid. Here we report the purification and characterization of a novel protamine kinase from HL60 cells.

EXPERIMENTAL PROCEDURES

Materials—1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was from Seikagaku Kogoyo (St. Petersburg, FL). Fetal calf serum (FCS) was from JRH Biosciences (Lenexa, KS). [γ-^{32}P]ATP (specific activity 3,000 Ci/μmol) and [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu, specific activity 20 Ci/mmol) were purchased from DuPont–New England Nuclear (Waltham, MA). Nitrocellulose sheets and #34 glass filter papers were from Schleicher & Schuell (Keene, NH). Enhanced Chemical Luminescence (ECL)

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Abbreviations: DMSO, dimethyl sulfoxide; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ERK, extracellular signal regulated kinase; H_7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; [^{3}H]PDBu, [^{3}H]phorbol 12,13-dibutyrate; HAP, hydroxylapatite; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethyl sulfonyl fluoride; RA, retinoic acid.

Western blot detection solution, goat-antirabbit-horseradish peroxidase–linked antibody and Hyperfilm-ECL were obtained from Amersham International (Amersham, UK). HL60 cells were from American Type Culture Collection (Rockville, MD). Large-scale culture of HL60 cells was performed by Endotronis (Minneapolis, MN). All reagents were of the highest grade of purity.

Cell culture-HL60 cells were grown in RPMI-1640 medium (10.2 g/liter) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 24 mM $NaHCO_3$ in a humidified incubator under 5% CO_2 at 37°C. Cells were plated at an initial density of 2.5×10^4/ml.

Protamine Kinase Assay—Protamine kinase activity was determined in 100 μl of assay mixture containing 50 mM Pipes (pH 6.5), 10 mM $MgCl_2$, 0.4 mM EGTA, and 200 μg/ml protamine sulfate as substrate. Background level was determined in the absence of $MgCl_2$. The reaction was initiated by the addition of 10 mM ATP and [γ-^{32}P]ATP (1 μCi/tube), allowed to proceed at 37°C for 5 min, and stopped by the addition of cold 230 mM phosphoric acid. The mixture was filtered onto P 81 membranes, which were washed four times with 75 mM phosphoric acid. The incorporated ^{32}P was measured by Cerenkov scintillation counting. Protamine kinase activity (pmol/min/mg) is represented as the activity stimulated by the presence of a divalent cation, Mg^{2+} or Mn^{2+}, minus background.

Protein Kinase C Assay—Protein kinase C (PKC) was assayed in 100 μl of reaction mixture containing 50 mM Pipes (pH 6.5), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 0.25 mM EGTA, 50 μg/ml phosphatidylserine, 25 μg/ml 1,2-dioleoyl-*rac*-glycerol, and 200 μg/ml histone III-S as substrate (*11*). Briefly, the assay was initiated by the addition of 10 mM ATP and [γ-^{32}P]ATP (1 μCi/tube), allowed to proceed at 37°C for 5 min, and stopped by the addition of cold 230 mM phosphoric acid. The mixture was filtered through P 81 membranes, which were washed four times with 75 mM phosphoric acid. The incorporated ^{32}P was counted by Cerenkov scintillation counting. PKC activity (pmol/min/mg) is represented as the difference between the activity in the presence and absence of phosphatidylserine and 1,2-dioleoyl-*rac*-glycerol.

Preparation of Cell Extracts—1×10^{10} HL60 cells were suspended in 100 ml of sonication buffer consisting of 20 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF, 10 mM aprotinin, 10 μg/ml leupeptin, and 5 mM NaCl. After disruption of cells by sonication on ice, homogenates were treated with 0.1% (v/v) Nonidet P-40 and 10 mM EGTA for 1 h at 4°C with gentle inversion followed by centrifugation at 10,000 ×*g* for 30 min. The pellet was discarded and the supernatant was used for the purification of protamine kinase by sequential column chromatographies, as follows.

DEAE-Sephacel Chromatography—The supernatant was filtered through glass wool and applied to 300 ml of 50% DEAE-Sephacel slurry which had been equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 100 μM PMSF). After binding to DEAE-Sephacel with moderate agitation for 1 h, proteins were transferred to a 5.5 cm × 25 cm column. The column was washed with 1,000 ml of Buffer A containing 20 mM NaCl and developed with a 500-ml linear gradient from 20 to 600 mM NaCl in Buffer A. The flow rate was 50 ml/h, and 6.25-ml fractions were collected. A 30-μl aliquot of every third fraction was assayed for protein kinase activities.

Protamine Agarose Affinity Chromatography—Protamine agarose was equilibrated with Buffer B (20 mM Tris-HCl, pH 7.5, 350 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, and 100 μM PMSF). The pooled DEAE-Sephacel fractions with protamine kinase activity were bound to 12 ml of protamine agarose in Buffer B with gentle agitation for 1 h. The slurry was transferred to a 1.5 cm × 10 cm column, which was developed with an 80-ml linear gradient from 0.35 M to 3.5 M NaCl in buffer B at a flow rate of 50 ml/h. Every third fraction (1 ml) was assayed for protamine kinase activity. The fractions with active enzyme were pooled and concentrated to 4 ml with an Amicon Centricon 10 filter. Glycerol (10% v/v) was added to the concentrated enzyme.

Hydroxylapatite Chromatography—Fractions with protamine kinase activity from protamine agarose chromatography were pooled and dialyzed against Buffer C (20 mM KPO_4, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, and 100 μM PMSF) overnight. The dialyzed enzyme was bound to 10 ml of hydroxylapatite by gentle agitation for 1 h. The enzyme was eluted with two-step linear gradients from 10 mM KPO_4 to 120 mM KPO_4 and from 110 mM KPO_4 to 300 mM KPO_4 at a flow rate of 50 ml/h. Every third fraction (1 ml) was assayed for protamine kinase activity. Conductivity was determined for active pooled fractions.

S-200 Chromatography—The concentrated enzyme of HAP 1 from Hydroxylapatite chromatography was loaded onto an S-200 gel filtration column (1.5 cm × 70 cm) which had been equilibrated with Buffer D (20 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 50 mM 2-mercaptoethanol, and 100 μM PMSF). Protamine kinase activity was recovered by developing the column with buffer D at a flow rate of 50 ml/h. Every third fraction (1 ml) was assayed for protamine kinase. Apparent molecular weight was determined by calibration of the column with native marker proteins eluted under the same conditions.

SDS–Polyacrylamide Gel Electrophoresis—Samples were precipitated with 6% trichloroacetic acid and deoxycholic acid (0.68 mg/ml) for 10 min on ice. After centrifugation at 12,000 ×*g* for 15 min, supernatants were removed and protein precipitates were dissolved by the addition of sample buffer [375 mM Tris-HCl, pH 8.8, 40 mg/ml SDS, 50 mM 2-mercaptoethanol, and 0.0004% (w/v) bromophenol blue]. Samples were boiled for 10 min and subjected to 10% SDS–polyacrylamide gel electrophoresis (*12*). Protein was visualized by either Coomassie Blue or silver staining. Protein concentration was determined by the method of Bradford (*13*).

Phorbol Ester Binding Assay—[^{3}H]PDBu binding was measured in 200 μl of reaction mixture containing 27.5 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mg/ml bovine serum albumin, 0.1 mg/ml phosphatidylserine, and 1 nM [^{3}H]PDBu (0.5 μCi) as described (*14*). Non-specific binding was determined by the addition of an excess of unlabeled PDBu in parallel incubations. Reaction was initiated by the addition of 50 μl of proteins, allowed to proceed for 45 min at room temperature, and stopped by placing the mixture on ice. The reaction mixture was filtered through glass filters, which were washed twice with 1 ml of

20 mM Tris-HCl (pH 7.6) and 1 mg/ml bovine serum albumin solution. Scintiverse E (2 ml) was added to vials containing filters, and the amount of bound [^{3}H]PDBu was determined by liquid scintillation counting. Specific binding was calculated by subtraction of non-specific binding from total binding.

Changes of Protamine Kinase Activity in HL60 Cells—Cells were harvested by centrifugation at 5,000 ×*g* for 10 min after the growth as described in "*Cell Culture*." To examine the role of protamine kinase in the early stage of differentiation, cells were resuspended with glucose (5 mg/ml) in phosphate-buffered saline (pH 7.6) and treated with an inducer, 1 nM PMA, 1 μM retinoic acid, or 1.25% (v/v) DMSO, for 15 min. Cold phosphate-buffered saline (pH 7.6) was added to stop the reaction. Cells were harvested by centrifugation at 5,000 ×*g* for 5 min and washed twice with phosphate-buffered saline (pH 7.6). To obtain whole cellular fractions, cells were disrupted by mild sonication in an ice-water bath after resuspension with 1 ml of sonication buffer. The lysates were treated with 0.1% Nonidet P-40 and 10 mM EGTA, bound to 1 ml of 50% DEAE-Sephacel slurry, and transferred to a plugged Pasteur pipette. Protamine kinase was eluted with 1 ml of 400 mM NaCl in buffer A after washing the column with 5 ml of buffer A containing 150 mM NaCl. With this step, the classical PKC was removed from protamine kinase, since classical PKC and protamine kinase were eluted from DEAE-Sephacel at 90 and 280 mM NaCl, respectively. The activity of protamine kinase was then assayed in triplicate as described above. To examine the enzyme activity at the subcellular level, cytosolic and particulate fractions were separated as follows. After treatment with inducers, cells were disrupted by mild sonication in an ice-water bath and centrifuged at 12,000 ×*g* for 15 min. The supernatant was saved as the

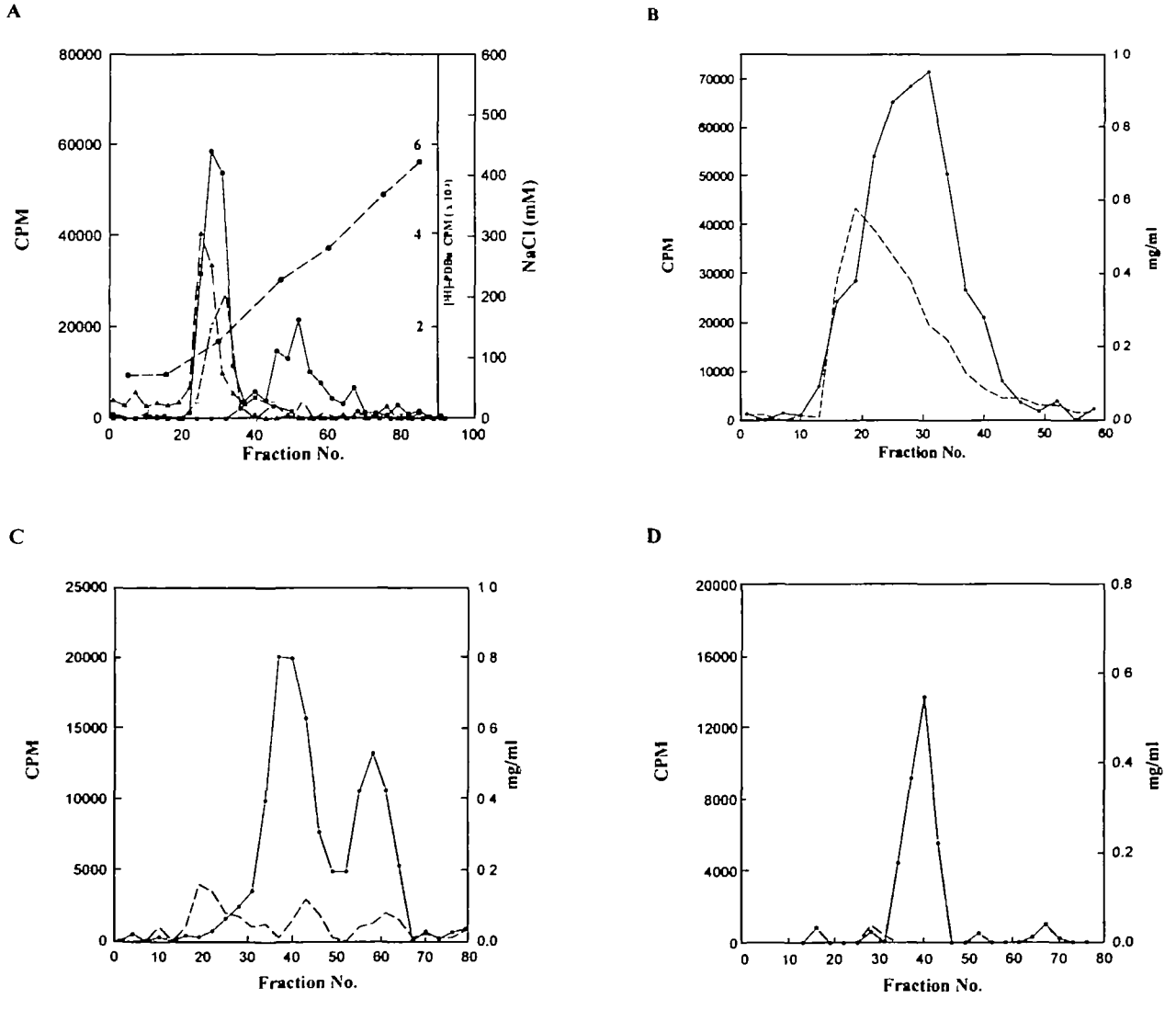

Fig. 1. **Sequential chromatographic steps for the purification of protamine kinase.** The protamine kinase was purified by four successive chromatographies (A, DEAE-Sephacel; B, protamine agarose; C, hydroxylapatite; and D, S-200). Protamine kinase activity is shown by closed circles (A–D). In A, PKC activity is expressed by closed triangles, casein kinase I by closed squares, PDBu binding activity by open squares, and NaCl gradient by open circles. The elution profiles of total proteins are shown in short-dashed line (B–D). Protein kinases were assayed as described in "MATERIALS AND METHODS."

cytosolic fraction, and the pellet was resuspended with 0.1% Nonidet P-40 and 10 mM EGTA in the sonication buffer. The particulate fraction was extracted with gentle agitation for 1 h, and the insoluble material removed by centrifugation at 12,000 ×*g* for 15 min. The cytosolic and particulate fractions were bound to 1 ml of 50% DEAE-Sephacel slurry and transferred into plugged Pasteur pipettes. To examine the role of protamine kinase in the late stage of differentiation, cells were treated with the same inducers as mentioned above for 1 week. The protamine kinase was eluted as described above and its activity assayed in triplicate.

RESULTS

Purification of protamine kinase was accomplished by successive chromatographies on DEAE-Sephacel, protamine agarose, hydroxylapatite, and S-200 matrices. The first step in the purification was DEAE-Sephacel chromatography (Fig. 1A). This step separates protamine kinase from classical PKC isoforms and casein kinase. Activity of PKC was

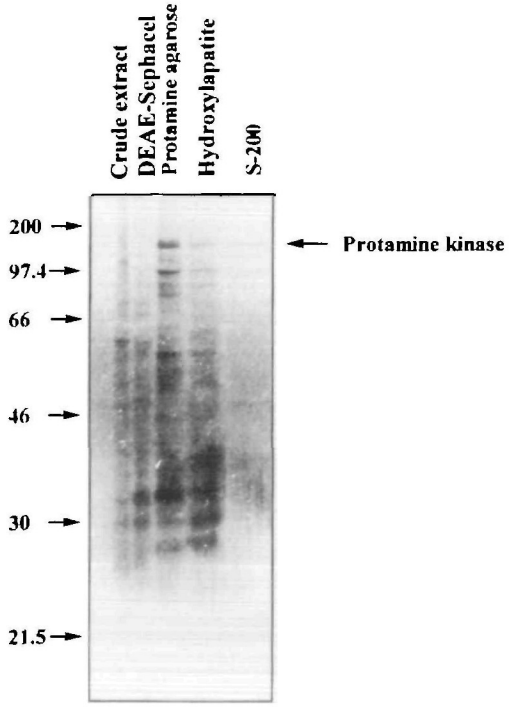

Fig. 2. **Coomassie blue staining of pooled fractions.** Proteins (3 μg/lane) from crude extract, pools of DEAE-Sephacel, protamine agarose, hydroxylapatite (HAP), and S-200 columns were separated by electrophoresis on 10% SDS–polyacrylamide gel, and stained with Coomassie Blue. Numbers on the left represent molecular mass of protein molecular markers in kDa.

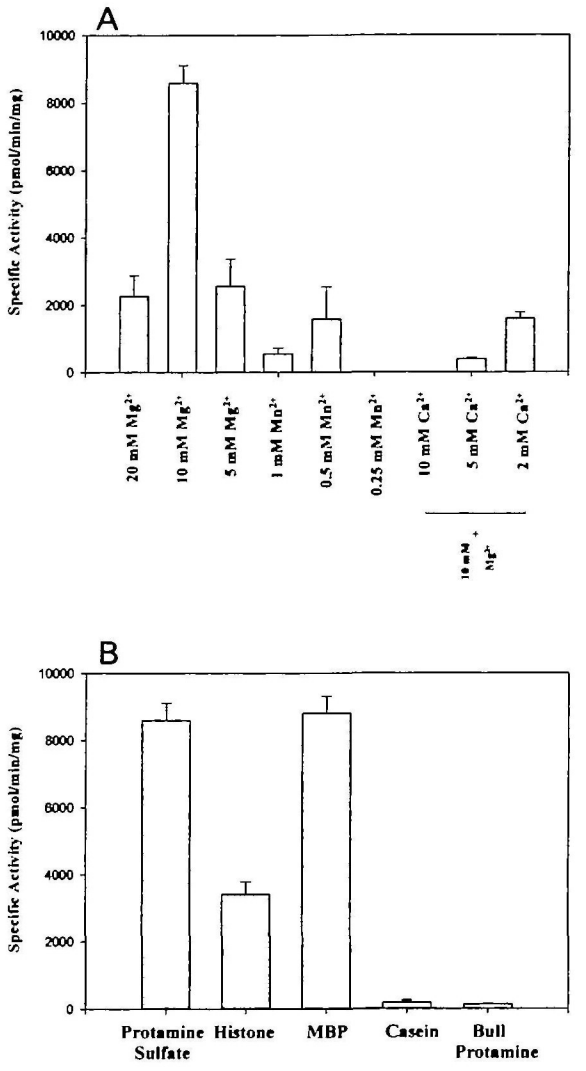

Fig. 3. **Effects of cations on protamine kinase activity and substrate specificity.** (A) The activity of purified protamine kinase (50 ng) was assayed in 100 μl of reaction mixture with various concentrations of Mg^{2+}, Mn^{2+}, and Ca^{2+}. These values are the mean ± SD of an assay conducted in triplicate. They represent four separate experiments with similar responses. The data are expressed as pmol phosphate incorporate/min/mg protein. (B) Protamine sulfate, histone III-S (histone), myelin basic protein (MBP), casein, and bull protamine, each at a concentration of 0.2 μg/ml, were incubated with purified protamine, kinase. The data are expressed as pmol phosphate incorporated/min/mg protein.

TABLE 1. **Purification of protamine kinase.**

Step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (pmol/mg/min)	Purification (fold)	Recovery (%)
Crude extract	120	20.3	2436	45		
DEAE-Sephacel	300	1.89	566	115.7	1	100
Protamine agarose	18	0.29	5.29	1795	15.5	14.5
Hydroxylapatite	13	0.06	0.78	1730	15	2.1
S-200	10	0.005	0.05	8767	75.8	0.7

The data are from one of seven similar purifications. Because protamine sulfate can be phosphorylated by several protein kinases induding protein kinase C, recovery and purification are calculated relative to the DEAE-Sephacel eluate rather than crude extract.

measured to confirm that the eluted protamine kinase activity contained little or no PKC activity. The pooled enzyme had a significant amount of protamine kinase activity, with little or no classical PKC activity. The pool was bound to protamine agarose matrix and eluted with a linear gradient of 750 mM to 3.5 M NaCl (Fig. 1B). This pool with protamine kinase activity was further purified by hydroxylapatite chromatography, which was developed in two steps with KPO_4 gradients (Fig. 1C). Two peaks of protamine kinase activity eluted at 153 mM KPO_4 (HAP I) and 238 mM KPO_4 (HAP II), respectively (the first peak was designated as HAP I and the second one as HAP II). Coomassie blue staining of proteins from the hydroxylapatite column showed that the kinase peak predominantly contains a 120-kDa protein predominantly stained (Fig. 2). The peak fractions from HAP I were concentrated to 3.5 ml and further purified on an S-200 gel filtration column. The peak activity of protamine kinase eluted in the 40th fraction (Fig. 1D), corresponding to a molecular mass of 120 kDa. An aliquot of every third fraction of the S-200 column was analyzed by 10% SDS-PAGE and stained with Coomassie blue (Fig. 2). As shown in Fig. 2, a near homogeneous protein with a specific activity of 8,700 pmol/min/mg was detected after S-200 chromatography. Through four successive chromatographic steps, protamine kinase was purified by 75.8-fold, and about 0.67% of total activity was recovered (Table I).

The effects of divalent cations on protamine kinase activity were assessed to characterize the biochemical properties of the enzyme. As shown in Fig. 3A, maximal activity of protamine kinase was observed at 10 mM $MgCl_2$ and 0.5 mM $MnCl_2$, respectively. For the activation of protamine kinase, however, $MnCl_2$ was only about 20% as effective as $MgCl_2$. Next, Ca^{2+} was added to the reaction mixture along

TABLE II. **Phorbol ester binding to PKC and protamine kinase.**

Enzyme	CPM
PKC	88.8 ± 5.4
Protamine kinase	6.8 ± 0.8

Partially purified PKC and protamine kinase from DEAE-Sephacel chromatography were each incubated with [^{3}H]-PDBu and unlabelled PDBu as described. Binding was calculated by subtraction of non-specific binding from total binding. These results are the mean ± SD of an assay conducted in triplicate (n = 3). The data are expressed as [^{3}H]-PDBu CPM bound/μg protein.

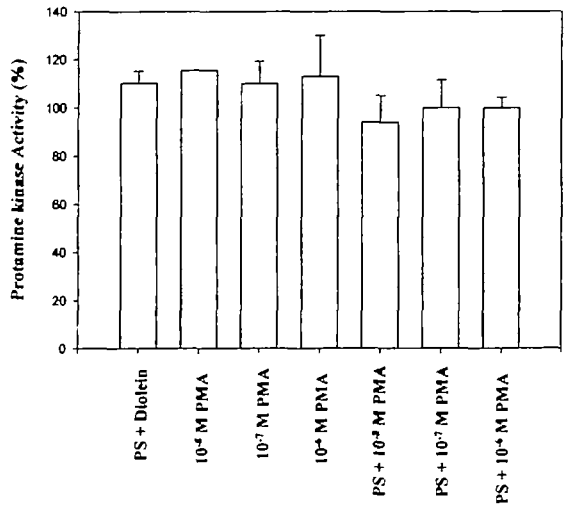

Fig. 4. **Effects of PMA and/or phospholipid on protamine kinase activity.** Purified protamine kinase (50 ng) was assayed in 100 μl of reaction mixture with PMA and/or phospholipid [0.1 mg/ml of phosphatidylserine (PS) and 0.05 mg/ml of diolein]. The values are the mean ± SD of an assay conducted in triplicate. They represent five separate experiments with similar responses. Activity is expressed as a percentage of the activity in the absence of PMA and phospholipid.

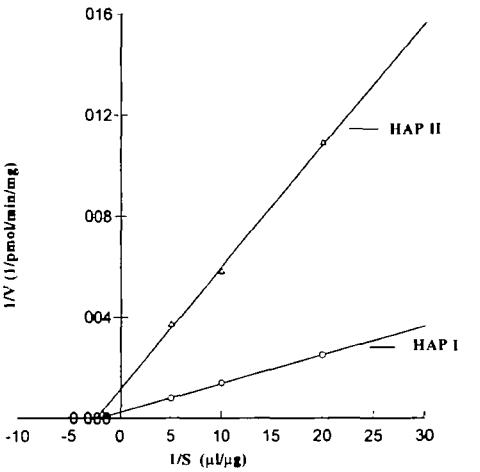

Fig. 5. **Lineweaver-Burk plot of protamine kinase, HAP I, and HAP II.** HAP I (circles) and HAP II (triangles) in amounts of 500 ng were incubated at various concentrations of protamine sulfate. Kinetic parameters were determined by linear regression analysis.

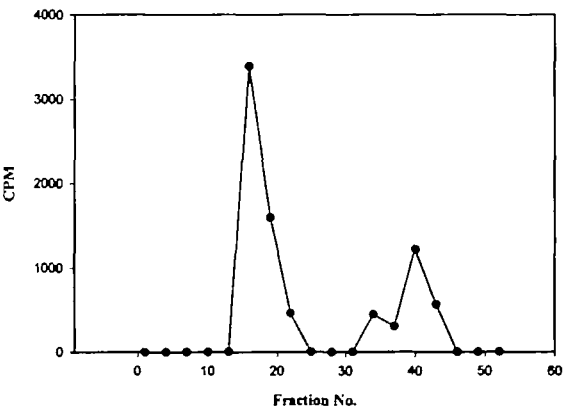

Fig. 6. **Dephosphorylation of HAP II by acid phosphatase.** HAP II (200 μg) was incubated for 5 min at 37°C in a reaction mixture containing 10 mM KPO_4, pH 7.5, 4 units of potato acid phosphatase, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM aprotinin, 1 μM leupeptin, 50 mM 2-mercaptoethanol, and 100 μM PMSF. The phosphorylated HAP II enzyme was bound to 2.5 ml of hydroxylapatite slurry and developed with two-step gradients of 25 ml as described in "MATERIALS AND METHODS." Every third fraction was assayed for protamine kinase activity.

with 10 mM Mg^{2+}, since Ca^{2+} is required for the activation of some protein kinases such as PKC and Ca^{2+}/calmodulin-dependent protein kinase. Unlike PKC, $CaCl_2$ inhibited the protamine kinase. The apparent IC_{50} of Ca^{2+} was 10.4 and 6.8 mM in Mg^{2+}- and Mn^{2+}-stimulated reaction, respectively (data not shown). This result clearly distinguishes the present protamine kinase from that reported by Damuni *et al.* (*5*), of which Ca^{2+} did not affect the kinase activity. Protamine sulfate and myelin basic protein (MBP) appeared to be the best substrates for the protamine kinase, while histone III-S was a good substrate, as evidenced by significant phosphorylation (Fig. 3B).

The effect of phorbol 12-myristate 13-acetate (PMA), a tumor promoter, on the activity of protamine kinase was also examined (Fig. 4). Treatment wih 10^{-8} to 10^{-6} M did not significantly effect the activity PMA *in vitro*. Combined treatment with phosphatidylserine and diolein did not seem to increase the protamine kinase activity. When PMA and phosphatidylserine were added simultaneously to the reaction mixture, they did not have any additive or synergistic effect on the kinase activity, suggesting that protamine kinase does not have binding sites for PMA or phospholipid.

To examine whether phorbol ester binds to protamine kinase, the specific binding of [^{3}H]PDBu to the purified protamine kinase and PKC was determined. Table II shows that phorbol ester did not bind significantly the protamine kinase, which is consistent with the result shown in Fig. 4.

To analyze biochemical properties, we determined the kinetic parameters of our protamine kinase from HL60 cells. Lineweaver-Burk plot curves of 1/HAP I (or HAP II) initial velocity *vs.* 1/protamine sulfate concentration showed that HAP I displayed a greater maximum velocity (V_{max} = 5,000 pmol/min/mg) than HAP II (V_{max} = 770 pmol/min/mg) (Fig. 5). The K_ms of HAP I and HAP II for protamine sulfate were 1.3 and 0.9 mM, respectively. Thus, the less phosphorylated form (HAP I) has a greater maximal velocity (V_{max}) than the highly phosphorylated form (HAP II), but both forms possess similar affinity (K_m) for protamine sulfate: the phosphorylation state of protamine kinase affects the maximal kinase activity without affecting its affinity.

To examine whether HAP I and HAP II are same protein in different states of phosphorylation, HAP II was incubated with acid phosphatase. The dephosphorylation of HAP II with acid phosphatase generated two peaks from hydroxylapatite chromatography, representing HAP I and HAP II peaks (Fig. 6).

As seen in Fig. 7, protamine kinase was completely inhibited by 0.1–10 μM staurosporine. K_{252a} and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H_7) also strongly inhibited the activity of protamine kinase. However, heparin, an inhibitor of casein kinase II, did not significantly inhibited protamine kinase activity at 5 μM or higher concentration. The results reveal that staurosporine acts as the most potent inhibitor of protamine kinase activity.

In the case of PKC, protamine was reported to induce the autophosphorylation of PKC (*15*). Protamine kinase did not undergo autophosphorylation in the absence of protamine sulfate, but did so in the presence of protamine sulfate (Fig. 8). This result suggested that protamine sulfate might be required for the autophosphorylation of protamine kinase as well as the stimulation of kinase activity. Therefore, protamine sulfate could serve as an inducer of autophosphorylation as well as a substrate of protamine kinase.

Because HL60 cells contain a significant amount of protamine kinase activity, it was of interest to investigate whether protamine kinase is involved in the differentiation process. Protamine kinase was predominantly cytosolic, because 75% of total activity was present in the cytosol (75%) and 25% in the particulate fraction (data not shown). To examine the *in vivo* effect of PMA on the activity of protamine kinase, HL60 cells were treated with PMA (10^{-9} M), RA (10^{-6} M), DMSO (1.25%), and 1,25-$(OH)_2D_3$ (10^{-6} M) for 15 min (Fig. 9A). The activity of protamine kinase in both cytosol and particulate fractions appeared to remain

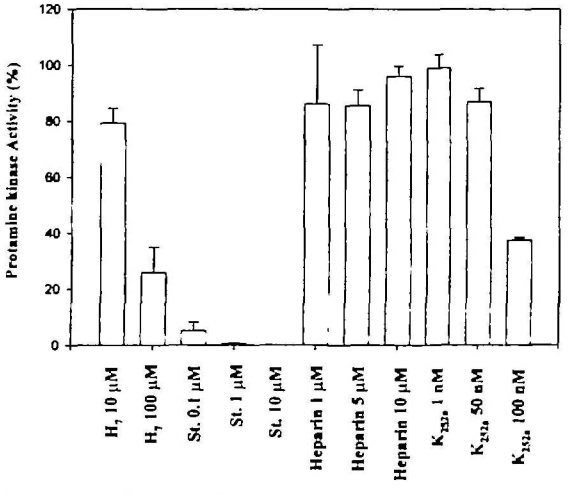

Fig. 7. **The effects of inhibitors on protamine kinase activity.** Purified protamine kinase (50 ng) was assayed in 100 μl of reaction mixture with various concentrations of kinase inhibitors [H_7, staurosporine (St) heparin and K_{252a}] as indicated. The results are the mean ± SD of an assay conducted in triplicate. They represent five separate experiments with similar responses. Activity is expressed as a percentage of the activity in the absence of inhibitors.

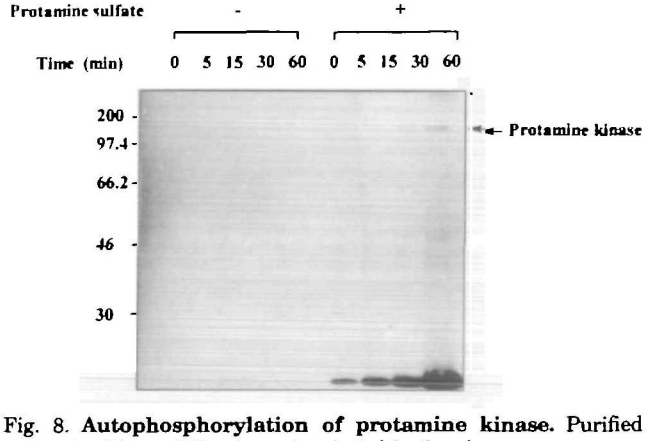

Fig. 8. **Autophosphorylation of protamine kinase.** Purified protamine kinase (50 ng) was incubated in the absence or presence of exogenous substrate for indicated times. The reaction was initiated by the addition of [γ-^{32}P]ATP, stopped by addition of 50 μl of sample buffer, and the mixture was subjected to SDS-PAGE. The gel was dried and exposed to X-ray film for autoradiography. Numbers on the left represent molecular mass of protein molecular markers in kDa.

unchanged in PMA-treated cells and PMA/cAMP-treated cells. Retinoic acid and DMSO, inducers of the differentiation of HL60 cells to neutrophil-like cells, had no effects on the activity or the translocation of protamine kinase. An inducer of HL60 cell differentiation to monocyte-macrophage phenotype cells, 1,25-$(OH)_2D_3$, also did not modulate the activity of protamine kinase in either cytosol or particulate fractions. Therefore, protamine kinase seemed not to be involved in the early events of differentiation caused by any of the inducers.

The modulation of protamine kinase was examined after cells were committed to differentiation. HL60 cells were treated with PMA (10^{-9} M), RA (10^{-6} M), DMSO (1.25%), and 1,25-$(OH)_2D_3$ (10^{-6} M) for one week, and total activity of protamine kinase was determined (Fig. 9B). In PMA-treated cells, the protamine kinase activity appeared to diminish slightly. The protamine kinase activity remained unchanged in DMSO-treated cells, while there was a slight decrease in enzyme activity in RA-treated cells, similar to those treated with PMA. Interestingly, the activity of protamine kinase was significantly increased in 1,25-$(OH)_2D_3$–treated cells, indicating that the differentiation to monocyte-macrophage phenotype cells might be induced through the protamine kinase pathway. There was no direct correlation between the extent of differentiation to macrophage-like cells or mature neutrophil-like cells and the modulation of protamine kinase. However, the differentiation with 1,25-$(OH)_2D_3$ resulted in an elevation of protamine kinase activity, suggesting that the differentiation pathway to macrophage or neutrophil might be different from the pathway to monocyte-macrophage.

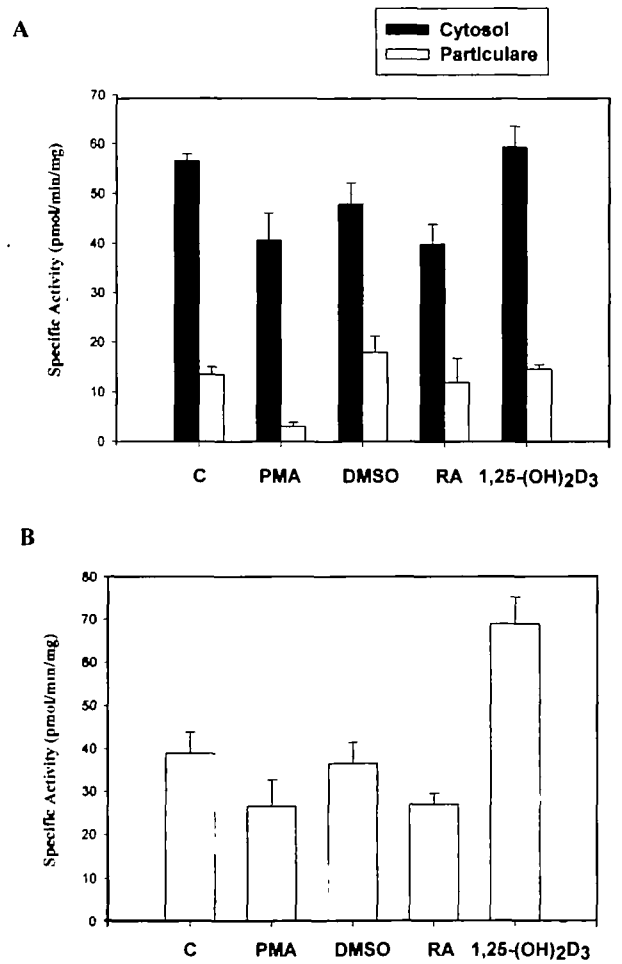

Fig. 9. **The effects of inducers on protamine kinase activity during the early and late stages of HL60 differentiation.** (A) HL60 cells were harvested, resuspended in phosphate-buffered saline with glucose (1 mg/mg) and bovine serum albumin (1 mg/ml), then stimulated with PMA (10^{-9} M), RA (10^{-6} M), DMSO (1.25% v/v), or 1,25-$(OH)_2D_3$ (10^{-6} M) for 15 min. After separation of the cytosol and particulate fractions, protamine kinase activity was determined as described in "MATERIALS AND METHODS". (B) HL60 cells were stimulated with PMA (10^{-9} M), RA (10^{-6} M), DMSO (1.25% v/v), and [1,25-$(OH)_2D_3$ (10^{-6} M)] for 1 week. Control represents the activity in the absence of inducers. These results represent the mean ± SE of four separate assays (n = 4) with triplicate determinations. The values are expressed as pmol phosphate incorporated/min/mg protein.

DISCUSSION

Because the crude extract contained protein kinases that could phosphorylate protamine sulfate, the purification yield and recovery of total enzyme activity were calculated based on the total activity present in the enzyme fraction eluted from DEAE-Sephacel. Only 14.5% recovery of activity from the protamine agarose column indicated that there were many unidentified protein kinases in pooled fractions that phosphorylated protamine sulfate after DEAE-Sephacel chromatography. Even though hydroxylapatite chromatography did not significantly increase the purification yield, it resolved protamine kinase activity into two major peaks. The two peaks displayed the same molecular weight as judged by SDS-PAGE and gel filtration column (S-200) chromatography. In addition, the generation of two peaks from hydroxylapatite chromatography by dephosphorylation of HAP II (Fig. 6) implies that protamine kinase exists in two forms: dephosphorylated and phosphorylated.

Durham *et al.* (*4*) reported a partially purified protamine kinase from HL60 cells which had an apparent molecular mass of 110 kDa and was activated by Mg^{2+} with an optimum concentration at 5 mM, but not by Mn^{2+}. The calcium ion in the range of 1 μM to 500 μM was inhibitory towards the activity of protamine kinase in the presence of Mg^{2+} in their system. However, the biochemical characterization of the protamine kinase such as systematic step for purification and the physiological role of protamine kinase in differentiation or proliferation remains to be elucidated. In contrast to the protamine kinase reported by Durham *et al.* (*4*), the protamine kinase described here has a larger molecular mass and is activated by Mn^{2+} and Mg^{2+} with optimum concentrations of 0.5 and 10 mM, respectively. The molecular mass of protamine kinase was estimated to 120 kDa, judged from gel filtration column chromatography and SDS–polyacrylamide gel electrophoresis, suggesting that protamine kinase is a monomer. In addition, the activity of protamine kinase is inhibited by Ca^{2+} in the range of 2 to 10 mM, demonstrating that the protamine kinase from our laboratory appears to be different from the protamine kinase reported by Durham *et al.* (*4*).

It has been speculated that protamine kinase could be a non-classical PKC isozyme, because both kinases elute at similar salt concentration from DEAE-Sephacel chromatography and share similar properties of being independent of

Ca^{2+} and phospholipid (*16, 17*). Protamine kinase is also reported to resemble PRK1 in its size and biochemical properties (*18*). Unlike PRK1 and PKC, protamine kinase is not activated by phospholipid or inhibited by Ca^{2+}. In addition, protamine kinase phosphorylates histone type III-S significantly (Fig. 3B), while PRK1 does not phosphorylate it at all. Therefore, our data indicate that protamine kinase is different from the PRK and PKC family.

Treatment of HL60 cells with 1,25-$(OH)_2D_3$ results in a marked elevation in PKC activity at 4 to 7 days, while phospholipid- and Ca^{2+}-independent protein kinase activity remains unchanged (*19*). There is a marked modulation of cyclic AMP–dependent protein kinase and protein kinase C during the differentiation of HL60 cells, indicating that modulation of these protein kinases and phosphorylation of their substrates are prerequisite to the differentiation process. In PC12 cells, extracellular signal regulated protein kinase (ERK) responds differently to proliferative and differentiative stimuli. Several groups have reported two different responses: short-term activation of ERK, which is down-regulated within a few hours following treatment of cells with proliferation factors; and prolonged activation of ERK on treatment with differentiation factors (*20–22*). The prolonged activation appears to be necessary to maintain the differentiated state and cell survival. The biologically active form of vitamin D, 1,25-$(OH)_2D_3$, has been shown to be a natural and potent inducer of HL60 cells to monocyte-macrophage phenotype (*23*). This steroid hormone acts by binding to a vitamin D_3 receptor, which activates or represses target genes to regulate cell growth or differentiation (*24*). However, the identities of target genes have remained elusive. So far, a few proteins such as c-Fos (*25*), $p21^{Cip1/Waf1}$ (*26*), b-zip-HLH protein Mad-1 (*27*), and homeobox protein HoxA10 (*28*) are known as candidate target genes. These genes are involved in cell growth control and differentiation. It is also known that 1,25-$(OH)_2D_3$ inhibits the growth of HL60 cells, which reduced to less than 50% of control cells by day 7, and cells became loosely adherent (*19*). Our study on the role of protamine kinase during the HL60 differentiation reveals several interesting points. Some protein kinases such as PKC are known to translocate to the plasma membrane or nucleus from the cytosol in response to external stimuli (*29, 30*). Our protamine kinase did not translocate with the treatment of inducers and its activity remained unchanged during the early stage of differentiation. These findings suggest that the protamine kinase is not associated with the early events of differentiation process. However, the fact that 1,25-$(OH)_2D_3$ treatment caused an increase in its activity after commitment to differentiation suggests that the protamine kinase is associated only with the monocytic differentiation, not with macrophage or neutrophilic differentiation. This study, therefore, indicates that the differentiation pathway to macrophages or neutrophiles may be different from that to monocytes in terms of activation of protamine kinase. The protamine kinase described here could be an important enzyme delivering the membrane signal to the nucleus, because of its location in the cytosol (without translocation by inducers) and activation during the later stage of differentiation of HL60 cells.

In summary, a novel protamine kinase that is distinct from non-classical PKC and PRK was purified to near homogeneity from HL60 cells and biochemically characterized. The protamine kinase appears to be involved during the late stage of HL60 differentiation following treatment with 1,25-$(OH)_2D_3$. The mode of activation and *in vivo* substrate of protamine kinase remain to be elucidated.

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