Calcium-activated, phospholipid-dependent protein kinase activity and protein phosphorylation in HL60 cells induced to differentiate by retinoic acid

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Received 12 April 1985

Treatment of human promyelocytic (HL60) cells with retinoic acid for at least 48 h causes differentiation to more mature myeloid forms. Prior to commitment of cells to the myeloid pathway there is a marked increase in cytosolic calcium-activated, phospholipid-dependent protein kinase activity. This increase does not result from an intracellular redistribution of the enzyme. Concomitant with the increased enzyme activity there is enhanced phospholipid-dependent phosphorylation of proteins of 29, 49, 52, 58, 68, 69, 120, 170, 200 and 245 kDa.

Protein kinase *Leukemia* *Differentiation* *Retinoic acid*

1. INTRODUCTION

The human promyelocyte cell line HL60 can be induced to differentiate terminally into cells with many of the characteristics of mature granulocytes or macrophages by addition of the appropriate chemicals. RA induces differentiation along the myeloid pathway [1] while the phorbol ester TPA leads to transformation into macrophages [2]. It has been shown that each HL60 cell is bipotent and can differentiate along either pathway [3] and that induction by TPA is dominant. The biochemical mechanisms by which RA produces differentiation remain unclear but it causes a marked activation of cyclic AMP-dependent protein kinase both in HL60 [4] and F9 embryonal carcinoma cells [5]. In both cases, there is synergistic induction of differentiation by RA and agents which elevate intracellular cyclic AMP levels [6–8] suggesting that the primary action of RA is upon some other process. Recently, it has been shown that RA treatment also elevates the activity of a Ca^{2+},PL protein kinase in F9 cells [9] and that this enzyme is also present in HL60 [10]. The activity of a kinase phosphorylating protamine in a calcium-independent manner is elevated in RA-induced HL60 [11] and this enzyme may be identical to Ca^{2+},PL protein kinase [12]. We have therefore studied the effects of RA upon the activity of Ca^{2+},PL protein kinase in HL60 cells and upon the in vitro phosphorylation of cytosolic proteins by this enzyme.

Abbreviations: Ca^{2+}, PL protein kinase, calcium-activated phospholipid-dependent protein kinase; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; RA, retinoic acid; NSE, non-specific esterase

2. MATERIALS AND METHODS

2.1. *Cell culture*

HL60 cells were subcultured at an initial density 1×10^5 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 5% CO_2 and 100% humidity. Inducers were then added (RA, 1 μM; TPA, 64 nM) and the cells further incubated. Differentiation was assessed by measuring the ability of cells to

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00145793/85/$3.30

reduce nitroblue tetrazolium and assaying for α-naphthol AS-D acetate (non-specific) esterase (NSE) activity [3].

2.2. *Enzyme assay*

The cells were harvested by centrifugation, washed twice and the cell pellet resuspended in 2 mM Mops, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 100 μg/ml leupeptin. After disruption with a Polytron homogenizer the cell homogenate was centrifuged for 5×10^6 $g \cdot$min and the supernatant fraction taken for assay. Ca^{2+},PL protein kinase activity was determined in a reaction mixture containing 40 mM Mops, pH 7.5, 5 mM $MgSO_4$, 200 μg/ml histone f_1, 0.5 mM $CaCl_2$, 0.02 mM [γ-^{32}P]ATP (925 dpm/pmol) and 1–10 μg enzyme protein in a final volume of 120 μl. Phospholipid-dependent activity was determined by adding phosphatidylserine to a final concentration of 25 μg/ml. Protamine kinase activity was assayed as described [10]. After incubation for 5 min at 30°C duplicate 50-μl aliquots were quantitated by the disc method of Witt and Roskoski [13]. All assays were in duplicate. One unit of enzyme activity is defined as the transfer of 1 nmol ^{32}P per min. DEAE-cellulose column chromatography was carried out as in [4]. Protein was determined by the method of Bradford [14].

2.3. *Protein phosphorylation*

The phosphorylation of cytosolic proteins by endogenous Ca^{2+},PL protein kinase was performed in a reaction mixture consisting of 40 mM Mops, pH 7.5, 5 mM $MgSO_4$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 μM [γ-^{32}P]ATP (9250 dpm/pmol) and 100 μg protein in a total volume of 100 μl. Assays were carried out in the presence of 100 μM free Ca^{2+} and in the presence and absence of 25 μg/ml phosphatidylserine. Reactions were started by adding enzyme and incubation was for 2 min at 30°C. The phosphorylation was terminated by adding SDS and SDS-gradient gel electrophoresis performed according to Laemmli [15] on 6–17% gels of acrylamide:bisacrylamide (30:0.8). The gels were dried and autoradiographed using Min-R film and the autoradiograms analyzed using a Zeineh S1-504 SL laser densitometer. The density of silver grains was directly proportional to the ^{32}P radioactivity present in the phosphoproteins.

3. RESULTS AND DISCUSSION

The addition of 1 μM RA to HL60 cells causes more than 90% of the cells to differentiate along the myeloid pathway and to express NBT reduction as a marker of more mature myeloid forms [1,4]. Two aspects of this process are significant. First, maximum differentiation is observed (at 5 days) when cells are exposed to RA for 48 h but exposure for only 24 h gives a greatly reduced response (table 1). Second, the cells exposed to RA for 48 h are not irrevocably committed to differentiation along the myeloid lineage as the addition of TPA at that time will cause these cells to acquire the adherent properties and NSE activity characteristic of monocyte-macrophage differentiation. Therefore, any biochemical process expressed in the first 48 h after RA addition could be a causative influence in differentiation along the myeloid pathway.

The effect of inducers upon cytosolic Ca^{2+},PL protein kinase activity is shown in table 2. After 24 h in the presence of RA there is a small but significant elevation of enzyme activity and by 48 h activity has more than doubled. Activity continues to rise up to 96 h by which time it is elevated 4.5-fold. It has previously been shown that the addition of TPA leads to a rapid association of the Ca^{2+},PL protein kinase to membrane fractions of HL60 [16] and other cell types [17]. We have confirmed these observations in HL60. However,

Table 1

Differentiation of HL60 cells by retinoic acid and TPA

Treatment	Time (h)	NBT-positive cells (%)	NSE-positive cells (%)
None		5	3
Retinoic acid	0.5	8	N.D.
	24	24	N.D.
	48	91	5
	120	95	6
TPA	48	24	83
Retinoic acid + TPA	48 + 48	26	81

RA was present for the time indicated and NBT and NSE positive cells were analyzed 120 h after the initial addition of RA or 48 h after the addition of TPA

Table 2

Time course of the effect of retinoic acid upon cytosolic Ca^{2+} activated, PL-dependent protein kinase activity

Time after adding RA (h)	Time after adding TPA (h)	Ca^{2+},PL protein kinase activity (U/mg protein)	Protamine kinase activity (U/mg protein)
0		0.208	2.81
24		0.281	4.08
48		0.486	5.88
96		0.929	10.93
48	0.25[a]	0.267	3.83
48	1[a]	0.203	2.71
48	2[a]	0.202	2.93
48	48[a]	0.056	1.23

[a] Added after 48 h RA pretreatment

when cells are induced for 48 h with RA, and then TPA is added, there is now only a rapid loss of part of the cytosolic Ca^{2+},PL protein kinase suggesting that RA causes the appearance in the cytosol of an altered form of the enzyme which TPA will not cause to associate rapidly with particulate fractions.

As an initial approach to the study of this possibility Ca^{2+},PL protein kinase activities from control, RA-induced and RA-induced TPA-treated cells were analyzed by DEAE-cellulose chromatography (fig.1). The enzyme elutes as a single major peak at approx. 0.15 M NaCl in between the peaks of type I and II cyclic AMP dependent protein kinase. This elution profile is identical to that reported for protamine kinase activity [11], strongly suggesting that the activities measured by assaying protamine phosphorylation in the absence of Ca^{2+} and PL and histone f_1 phosphorylation in the presence of Ca^{2+} and PL are the same enzyme; a conclusion supported by the observation that the homogeneous enzyme from bovine heart has both activities [12] and the close correlation between the levels of Ca^{2+},PL protein kinase and protamine kinase activities in table 2. Although there is a single major peak of activity, this peak is not symmetrical and there is apparently another minor form, less PL-sensitive which elutes at 0.21 M NaCl. When the profiles of enzyme activity from the 3 conditions are compared there is no evidence of significant differences between them. Further-

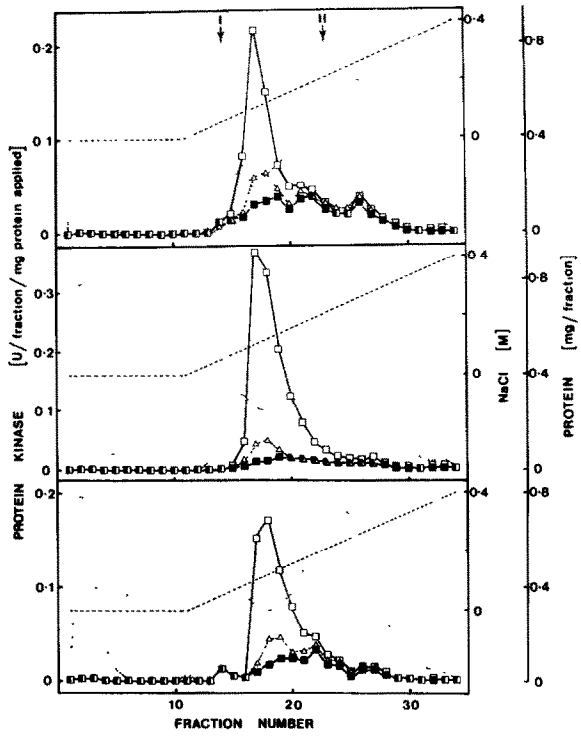

Fig.1. DEAE-cellulose chromatography of Ca^{2+},PL protein kinase. Cytosolic extracts of control (upper), 48 h RA-treated (middle) and 48 h RA-induced and 1 h TPA-treated (lower) HL60 cells were fractionated upon DEAE-cellulose and the fractions assayed: (△) with Ca^{2+}, (■) with PL, (□) with Ca^{2+} and PL. (···) Protein profile.

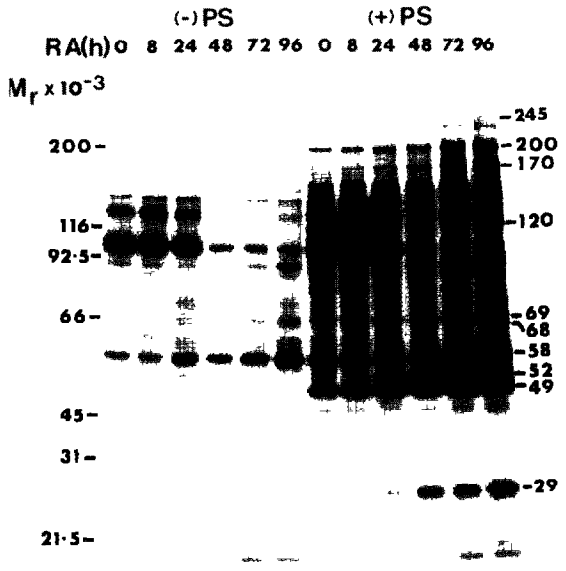

Fig.2. Effect of retinoic acid-induced differentiation upon phosphatidylserine-dependent in vitro cytosolic protein phosphorylation.

more, analysis of the properties of the enzymes from the peak fractions of each column does not reveal any significant differences in the pH profile or Ca^{2+} or PL concentration dependence (not shown). Thus although TPA inhibition suggests RA induces an altered molecular form of the enzyme we have not found any simple differences between the enzyme in control and RA-treated cells. Since Ca^{2+},PL protein kinase activity is found both in the cytosol and associated with membranes, one possibility is that RA could cause release of enzymes from the particulate fraction and TPA would cause reassociation. To assay Ca^{2+},PL protein kinase activity in particulate fractions it is necessary to detergent-solubilize the enzyme from the membranes and then reduce the detergent concentration to levels which do not activate the kinase. We have not so far succeeded in obtaining preparations which are reproducibly activated by PL. However, it is possible to extract reproducibly protamine kinase activity and we have previously shown that the particulate activity of this enzyme is raised more than 2-fold by RA treatment indicating that RA does not cause release of particulate enzyme [11].

Kuo and co-workers [10] have reported the Ca^{2+} and PL-dependent in vitro phosphorylation of proteins of 34, 38, 45 and 100 kDa in the cytosol of uninduced HL60 cells. We reproducibly find proteins of 29, 49, 52, 58, 68, 69, 120, 170, 200 and 245 kDa whose phosphorylation is either greatly enhanced or totally dependent upon the presence of phosphatidylserine and whose phosphorylation is increased upon the treatment of HL60 cells with RA (fig.2). We have quantitated the autoradiograms using a laser densitometer. Within 24 h there is a significant (>75%) increase in the incorporation of ^{32}P into proteins of 49, 52 and 58 kDa; by 48 h similar increases in 29, 68, 69, 170 and 245 kDa proteins have occurred and by 72 h the 120 and 200 kDa phosphorylations are enhanced. These changes are not the result of overall increases in protein phosphorylation as the two major phosphorylated proteins of control cells of 98 and 125 kDa begin to decrease by 24 h and are reduced by more than 80% by 48 h. Furthermore the phosphorylation of the 98 kDa protein is dependent upon the presence of calcium (not shown). The addition of 10 μM RA to the phosphorylation assay did not enhance the phosphorylation of any of the bands indicating that the effect of RA is indirect.

These results indicate that there are major increases in the Ca^{2+} and PL-dependent phosphorylation of cytosolic proteins and an increase in Ca^{2+},PL protein kinase in HL60 cells treated with RA prior to the commitment of the cells to the myeloid pathway. We do not know whether the enhanced protein phosphorylation is due to the appearance of a modified form of the Ca^{2+},PL protein kinase or to the synthesis of new protein substrates. Enhanced protein synthesis of molecules of 32, 34 and 60 kDa has been reported [18]. Further studies will determine the significance of these events to the myeloid differentiation of HL60.

ACKNOWLEDGEMENTS

The authors wish to thank M. Adams and A. Repucci for their technical assistance. The work was supported by grants from NIH CA35517 and CA37060 and the WVU Biomedical Research Fund.

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