ABSTRACT

In order to determine if cyclic adenosine 3':5'-monophosphate-(cyclic AMP)-dependent protein kinase has a role in the expression of chemically induced differentiation of HL-60 cells, levels and subcellular distribution of this enzyme were studied during this process. Cyclic AMP binding protein and stimulated kinase activities increased moderately in cytosol and more markedly in nucleosomal and nonhistone chromatin-associated protein fractions of cells induced to differentiate with dimethyl sulfoxide or 12-O-tetradecanoylphorbol-13-acetate. Retinoic acid induced similar cytosolic changes but less marked intranuclear increases. Nuclear increases did not occur in the differentiation-resistant subline, HL-60 Blast II, treated with dimethyl sulfoxide. DEAE-cellulose chromatography, as well as photoaffinity labeling and gel electrophoresis, disclosed higher ratios of type I to type II kinase in cytosol than in intranuclear fractions. Differences of the qualitative binding protein patterns between cytosol and nucleosomal were enhanced following chemically induced differentiation. Dibutyryl cyclic AMP increased cytoplasmic and nuclear binding protein levels when given alone or in combination with retinoic acid or dimethyl sulfoxide, and it enhanced differentiation. These results suggest that intranuclear cyclic AMP-dependent protein kinase is associated with the expression of the differentiative program in HL-60 cells.

INTRODUCTION

It is thought widely that the failure of cells to differentiate normally is a central defect in cancer. In several experimental systems, including the HL-60 cell line (11), this block may be partly reversible, and study of such models may help to illuminate the mechanisms responsible. cAMP and the protein kinases it acts through have been suspected of having roles in the control of differentiation of a variety of cell types. cAMP-dependent protein kinase occurs in distinctive isozymic forms, which may exhibit differential subcellular localization. Nuclear translocation of protein kinase (3,17) has been implicated in regression of hormone-dependent mammary tumors (5) and has also been studied in virally transformed fibroblasts (22, 23). cAMP has been shown to be capable of directly influencing transcription of specific messenger RNAs of diverse cell types, including those for phosphoenolpyruvate carboxykinase, prolactin, and lactate dehydrogenase (13, 18, 21). Such effects have been linked to phosphorylation of particular chromatin constituents by the catalytic subunit of protein kinase (13, 21). Significant interactions of the regulatory subunit with chromatin which could influence transcriptional activity have also been described (22, 23).

We have studied protein kinase levels, chromatographic and electrophoretic characteristics, and subcellular distribution during chemically induced differentiation of the HL-60 human promyelocytic leukemic cell line. The major finding of this study, as described below, was a marked increase of cAMP-dependent protein kinase in soluble nuclear and chromatin protein subfractions during such differentiation. This supports the hypothesis that nuclear translocation of protein kinase may play a role in the expression of the differentiative program of such cells.

MATERIALS AND METHODS

Cells. The HL-60 cell line (11) was the generous gift of Dr. S. Collins and Dr. R. Gallo of Bethesda, MD, and the HL-60 Blast II cell line (19) was the generous gift of Dr. P. Major of Boston, MA. These were passaged in modified McCoy's medium 5A with 15% fetal calf serum using standard tissue culture techniques. Differentiation was triggered with chemicals as indicated, and cells were harvested for study by centrifugation and by scraping from plastic surfaces when adherent cells were present. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. For assessment of differentiation, cytocentrifuge-prepared slides were stained with Wright-Giemsa, for acid phosphatase, and for nitroblue tetrazolium reduction, using either the bacterial stimulant prepared by Sigma for this purpose or TPA (100 ng/ml). The cytochemical stains were performed using Sigma kits. Results were expressed as the percentage of cells positive for nitroblue tetrazolium reduction or strongly positive (greater than a baseline of weak reactivity for all HL-60 cells) for acid phosphatase.

Subcellular Fractionation. Subcellular fractionation was performed by the following scheme, which was modified in minor respects from methods described previously (20, 32). Following 3 washes with cold phosphate-buffered saline, cells were resuspended at a cellular concentration of 10^6/ml in TKM buffer and then frozen at −70°C. The samples were thawed to −20°C, and cells were disrupted with 7 strokes of a Dounce homogenizer. The nuclei were pelleted at 3300 x g for 12 min at −20°C in a Beckman J-21C centrifuge. The pellets were then washed as above twice with TKM plus 0.3% Triton X-100, twice with TKM plus 0.15% Triton X-100, and then once with TKM. Protein determinations performed upon the successive Triton X-100 washes confirmed exhaustive removal of residual proteins, and microscopic examination of the pellets disclosed an appearance of broad, intact nuclei. The nuclei were then extracted twice with 0.25 M EDTA-0.07 M NaCl, pH 7.5, at 4°C by aspiration through 22-gauge needles, followed by centrifugation at 17,000 x g for 15 min using a Beckman J-21C centrifuge. This extract was referred to as nuclearosol. The remaining microscopically amorphous chromatin was then extracted with 0.5 M NaCl, pH 7.0, by aspiration through a 22-gauge needle. This fraction was designated NHCP. The

1 Supported in part by Grant CH-224 from the American Cancer Society, Inc. This study has been presented in part at the 74th Annual Meeting of the American Association for Cancer Research, May 25 to 28, 1983, San Diego, CA (8).

2 The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; dbcAMP, N,N'-dibutyl cyclic AMP; DMSO, dimethyl sulfoxide; NHCP, nonhistone chromatin-associated proteins; RA, trans-retinoic acid; TKM, 10 mM Tris-HCl (pH 7.5)-10 mM KCl-1.5 mM MgCl_2-50% glycerol; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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JULY 1984
RESULTS

Levels of cAMP binding protein during DMSO-induced HL-60 differentiation were characterized by increases in cytosol, nucleosol, and NHCP subfractions, with the greatest relative increases occurring in the nuclear, and especially NHCP, subfractions (Chart 1; Table 1). Dependence upon both time and DMSO concentration was noted, with increases observed within 24 hr and with concentrations of DMSO as low as 1.0%. Maximal levels occurred between 3 and 6 days. The HL-60 Blast II subline, which does not differentiate in response to chemical signals (19), exhibited higher base-line levels of cAMP binding protein and similar increases in cytosol binding activity after DMSO treatment. The intranuclear increases characteristic of HL-60 were, however, absent in HL-60 Blast II. In contrast, binding activities of Blast II NHCP decreased markedly. Despite the resistance of HL-60 Blast II to the differentiation-inducing effects of DMSO, it exhibited equal or slightly greater sensitivity to its growth-inhibitory effect, compared to the HL-60 line (Chart 2). A similar pattern of increases was noted of TPA-induced differentiation. RA-induced differentiation was accompanied by less marked intranuclear increases (Table 1).

Biochemical Determinations. Protein kinase activity was determined using reaction mixtures containing 70 μl of the enzyme source, 20 μM [γ-32P]ATP (200,000 to 400,000 cpm/tube), 3 mM MgCl2, 50 mM Tris-HCl (pH 7.5), and either 2 mg of calf thymus histone (II-AS)/ml or 5 mg of casein as substrate/ml, in a final volume of 140 μl. Incubations were run with and without 1 μM cAMP. [γ-32P]ATP was purchased from New England Nuclear, Boston, MA. Standard 10-min incubations at 31° were used for assays of fractions after chromatography. Aliquots of 50 μl were spotted onto Whatman No. 3MM filter paper discs, which were then washed in 5 changes of cold 10% trichloroacetic acid-1% sodium pyrophosphate, followed by two 95% ethanol washes. The paper discs were then dried and counted in a Beckman liquid scintillation counter. CAMP binding was determined by incubating 50-μl aliquots with 50 μl of 50 mM sodium acetate (pH 4.0) and 10 μl of 0.01 μM [3H]cAMP (42,000 cpm/tube) (from New England Nuclear) for 90 min at 4°, harvesting onto Millipore GS 0.22-μm filters with 20 mM potassium phosphate (pH 6.0), and counting by liquid scintillation (7). Each assay result presented is based upon replicate determinations, which had average variances of 4.2% for cytosol and nucleosol samples and 6.4% for NHCP samples. Protein concentrations were determined using Bio-Rad reagent with bovine serum albumin as a standard. Because of competing phosphatase activity, which precluded accurate kinase determinations upon crude homogenates in this series of experiments, kinase activities were only measured following DEAE-cellulose chromatography. This was accomplished as described previously (9) using columns with 0.5 ml of bed volume, with 20 ml of 0 to 0.3 M NaCl in 5 mM KH2PO4-K2HPO4, pH 7.4-10% glycerol-0.1% 2-mercaptoethanol gradients for cytosol samples and 10-ml gradients for nuclear samples. Fraction sizes were 1.0 ml for the cytosols and 0.5 ml for the nuclear fractions. Corresponding pairs of samples were run in parallel under identical conditions to allow for direct comparison of results. Units were defined as pmol bound for binding activities and pmol of 32P transferred per min for kinase activities. cAMP-dependent histone kinase activities are presented as the net activity with 1 μM cAMP less activity without cAMP. Photoaffinity labeling was performed using 2 μM β-azido-[32P]cAMP (from ICN, Irvine, CA) as described previously (10, 30). Binding protein bands were detected by autoradiography following sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 10% polyacrylamide gels.
Further analysis of DEAE-cellulose chromatography disclosed the increases of cAMP binding protein to be paralleled by increases in cAMP-dependent histone kinase activity; no evidence of free binding proteins was noted (Chart 3). The modest increases in the cytosol were comprised of components eluting early (0 to 0.05 mM NaCl) from DEAE-cellulose (type I kinase). In contrast, the bulk of activity in the nucleosol, with or without DMSO treatment, eluted later from DEAE-cellulose (0.10 to 0.15 mM NaCl) and was thus identified as the type II cAMP-dependent protein kinase. The NHCP subfraction contained lower amounts of protein, of lower specific activity, making these determinations technically more difficult, even when large numbers of cells were used. The pattern was nonetheless clearly similar to that which was noted in the nucleosol. Neither differentiation nor the accompanying cAMP binding protein increases of RA- or DMSO-treated cells were blocked by the prostaglandin synthetase inhibitor indomethacin (data not shown). In contrast to the cAMP-dependent histone kinase activity, cAMP-independent casein kinase activity of each subcellular fraction decreased following DMSO treatment (Chart 3). Similar results were noted using photoaffinity labeling with 8-azido[32P]cAMP, gel electrophoresis, and autoradiography (Fig. 1). Quantitative increases in binding protein were apparent in both cytosol and nucleosol following DMSO or TPA treatment. Untreated cells showed a greater proportion of higher molecular weight, type II kinase, designated Band b, in nucleosol than cytosol. Expression of Band b in the nucleosol was particularly enhanced by DMSO treatment. TPA treatment was accompanied by increased Band b expression, as well as increased expression of a type II variant band previously designated d in the cytosol (10). As was noted with DEAE-cellulose chromatography (Chart 3), expression of type I kinase in the cytosol was preferentially enhanced by DMSO (Fig. 1).

As has been described previously (4, 27), exogenous cAMP supplied as dbcAMP induced slight differentiation of HL-60 cells. This was detectable mainly by cytochemical staining (Table 2) and was paralleled by slight increases in binding protein levels and histone kinase activity and a slight shift in elution characteristics from DEAE-cellulose (Chart 4). dbcAMP enhanced both differentiation and cAMP binding protein changes of cells treated with low concentrations of RA and DMSO (Table 2). Butyrate may be released intracellularly from dbcAMP and may also induce HL-60 differentiation when applied at an optimal concentration of 0.5 mM. The effects observed of 0.1 mM dbcAMP could not, however, be reproduced by 0.1 mM butyrate.

DISCUSSION

The major finding of this study was the appearance of relatively large increases of cAMP-dependent protein kinase in subfractions of HL-60 cells induced to differentiate. The changes noted in the cytosol of DMSO-treated cells (increased cAMP-dependent histone kinase type I relative to type II and decreased cAMP-independent casein kinase) were towards a pattern more characteristic of mature granulocytes than of primitive myeloblastic leukemic cells (9, 10, 29). TPA-treated cells exhibited a distinctive pattern of change of cytosolic binding proteins, which may reflect the alternate differentiative pathway these cells follow. Such cytosolic changes are reasonably interpreted as a consequence of differentiation, rather than as a step facilitating the process of differentiation. Increases in cAMP-dependent kinase and binding activity and endogenous protein phosphorylation have also been reported in cytosol of differentiating Friend murine erythroleukemia cells (7, 33).

The nuclear changes were unlikely to be nonspecific effects of DMSO unrelated to differentiation (such as growth arrest or increased permeability of the nuclear membrane), since they did not occur in the differentiation defective HL-60 Blast II line. Observations that cAMP can influence HL-60 (4, 27) and mouse myeloid leukemia (14) differentiation and can directly affect gene transcription (13, 18, 21) make it furthermore plausible to suggest that these intranuclear increases are pertinent to the mechanism of chemically induced differentiation. High nuclear levels of cAMP-dependent protein kinase are certainly not the sole trigger for differentiation, since similar high base-line intranuclear levels were noted in HL-60 Blast II in the absence of differentiation. Whether or how these higher levels of the Blast II line are related to the early blockage in differentiation of these cells is unclear.

The presence of both binding and phosphorylating activity within the nuclei implies that the mechanism of translocation is not the same as that of the catalytic subunit upon holoenzyme dissociation by high cAMP levels (3, 28) but may be similar to the phosphorylated ternary complex proposed to arise in regressing mammary tumors (5). Translocated protein kinase could affect chromatin structure and thus influence transcriptional activity in several possible ways. An increase in histone H2A phosphorylation of differentiating Friend cells has been reported (24). Increased phosphorylation of another basic nuclear protein has been correlated with induction of prolactin mRNA transcription in pituitary cells by cAMP (21). Direct effects of the regulatory subunit on chromatin have also been suggested (22). Delineating the consequences of protein kinase translocation in HL-60 cells may thus prove to be a fruitful area for future study.

The changes in binding protein activity and in cellular differentiation were not inhibitable by indomethacin, an inhibitor of prostaglandin synthesis. This is in accordance with previous
Chart 3. Effects of DMSO on enzymatic and binding protein patterns following DEAE-cellulose chromatography. HL-60 cells were cultured with (•) and without (O) 1.3% DMSO for 5 days, harvested, fractionated, and chromatographed as described in "Materials and Methods." Results shown are cAMP binding activities (A, D, and G), net cAMP-dependent histone kinase activities (B, E, and H), and cAMP-independent casein kinase activities (C, F, and I) of cytosols (A to C), nucleosols (D to F), and NHCP fractions (G to I). Amounts of protein applied to columns were: 4 mg of cytosol; 1.79 mg of nucleosol; and 0.43 mg of NHCP. Paired samples were chromatographed in parallel and under identical conditions so that results could be contrasted by superimposition as shown here. U, units; M, M.

Table 2
Effects of dbcAMP alone and in combination with DMSO and RA

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Acute phos-</th>
<th>Blasts/</th>
<th>Myelo-</th>
<th>Meta-</th>
<th>Bands</th>
<th>Cytosol</th>
<th>Nucleo-</th>
<th>NHCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBT (+)^*</td>
<td>0.5</td>
<td>3.5</td>
<td>96</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1.23</td>
<td>0.59</td>
</tr>
<tr>
<td>dbcAMP, 0.1 mM</td>
<td>7.0</td>
<td>8.5</td>
<td>89</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>2.08</td>
<td>1.30</td>
</tr>
<tr>
<td>DMSO, 1.0%</td>
<td>6.5</td>
<td>12.0</td>
<td>87</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>3.11</td>
<td>1.29</td>
</tr>
<tr>
<td>DMSO, 1.3%</td>
<td>7.5</td>
<td>12.0</td>
<td>87</td>
<td>24</td>
<td>7</td>
<td>2</td>
<td>4.11</td>
<td>2.04</td>
</tr>
<tr>
<td>DMSO, 1.0% + dbcAMP, 0.1 mM</td>
<td>13.0</td>
<td>11.0</td>
<td>90</td>
<td>17</td>
<td>3</td>
<td>0</td>
<td>5.14</td>
<td>1.93</td>
</tr>
<tr>
<td>RA, 0.1 μM</td>
<td>3.5</td>
<td>6.5</td>
<td>93</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>2.18</td>
<td>0.85</td>
</tr>
<tr>
<td>RA, 1.0 μM</td>
<td>8.0</td>
<td>12.5</td>
<td>77</td>
<td>14</td>
<td>7</td>
<td>2</td>
<td>3.72</td>
<td>1.17</td>
</tr>
<tr>
<td>RA, 0.1 μM + dbcAMP, 0.1 mM</td>
<td>17.5</td>
<td>32.5</td>
<td>63</td>
<td>34</td>
<td>3</td>
<td>0</td>
<td>4.83</td>
<td>1.28</td>
</tr>
</tbody>
</table>

* NBT, nitroblue tetrazolium, was performed using bacterial stimulant as described in "Materials and Methods.

Acid phosphatase cytochemical determinations were scored as the percentage of cells showing greater than minimal background reaction as described in "Materials and Methods."
were processed in parallel, and fractions were assayed for cAMP binding activity. 60 cells were cultured for 5 days with (•) and without (O) 0.1 μM dbcAMP. Cytosols from these cultures were used for subsequent biochemical analyses.

**Chart 4. Effects of dbcAMP upon cytosol cAMP-dependent protein kinase.** HL-60 cells were cultured for 5 days with (•) and without (O) 0.1 μM dbcAMP. Cytosols were processed in parallel, and fractions were assayed for cAMP binding activity (A) and cAMP-dependent histone kinase activity (B). Results shown are normalized to 1 mg of protein application to each column. U, units; M, μM.

At least five to eight experimental cultures were processed in parallel, and fractions were assayed for cAMP binding activity (A) and cAMP-dependent histone kinase activity (B). Results shown are normalized to 1 mg of protein application to each column. U, units; M, μM.

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**REFERENCES**


L. Elias


Fig. 1. Photoaffinity labeling of cyclic AMP binding proteins of induced and uninduced HL-60 subcellular fractions. Lanes 1 and 4, uninduced 1.3% DMSO treatment for 5 days; Lanes 2 and 4, 16 nM TPA treatment for 2 days; Lanes 3 and 6, cytosols; Lanes 4 to 6, nucleosols. Activities of NHCP fractions have been too low for optimal demonstration with this technique. Lanes are shown without excess cyclic AMP (−) and with 20 μM cyclic AMP (+) in the photoaffinity reaction mixture as a competitive inhibitor. The position of the "C-methylated molecular weight markers bovine serum albumin, ovalbumin, and α-chymotrypsinogen is shown on the right, as well as the positions of cyclic AMP binding protein variants a, b, and c (10). Binding protein bands of molecular weight lower than a are noted in increased quantities in the induced cells and are considered to represent proteolytic fragments of the higher-molecular-weight bands.
Subcellular Distribution of Cyclic Adenosine 3′:5′-Monophosphate-dependent Protein Kinase during the Chemically Induced Differentiation of HL-60 Cells

Laurence Elias and Thomas Stewart


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