INDUCTION OF CYCLIC AMP-DEPENDENT PHOSPHORYLATION IN CYTOSOL OF DIFFERENTIATING MOUSE ERYTHROLEUKEMIA CELL LINE 179

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1. Introduction

Differentiation of the murine erythroleukemia cell line established in [1] can be initiated by treatment with a variety of agents [2–4]. Terminally differentiated cells undergo morphological changes, cease to proliferate [1], accumulate globin and other proteins associated with the mature erythroid phenotype [5], and demonstrate alterations in protein synthesis [6] and phosphorylation of nuclear and membrane proteins [7–10]. Since both calcium and cyclic AMP have been implicated in the regulation of cell proliferation and differentiation [9,11,12] and both of these agents can exert an influence on the state of phosphorylation of cellular proteins, we have examined their effects on the in vitro phosphorylation of proteins in a soluble fraction from MEL cells at various times during the process of differentiation.

2. Materials and methods

2.1. *Cell culture and induction*

The tetraploid MEL cell line 179, kindly provided by Dr Albert Deisseroth, was grown in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum. Induction of the cells was performed by inoculating at 5×10^4 cells/ml, and following 2–3 generations, DMSO, DMF (Sigma), or ActD was added to a final concentration of 1.25% (v/v), 120 mM and 4 nM, respectively. The proportion of benzidine-positive (i.e., hemoglobin-containing) cells was monitored at various times using a benzidine peroxide stain [15].

Abbreviations: MEL, mouse erythroleukemia; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ActD, actinomycin D; M_r, relative molecular mass; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-*N*'-2-ethanesulfonic acid

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2.2. *Preparation of cytosol*

Cells were harvested by centrifugation at 5×10^3 *g* . min and washed 3 times with 20 mM Hepes (pH 7.3), 120 mM sucrose, 5 mM $MgSO_4$. All manipulations were carried out at 4°C. Cell pellets were resuspended in 2 vol. 5 mM Hepes (pH 7.3), 5 mM $MgSO_4$, 17 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml 1-tosylamide-2-phenylethylchloromethyl ketone (Sigma) and gently homogenized using a motor-driven Dounce homogenizer. Greater than 80% lysis was achieved as determined by trypan blue exclusion. The homogenate was buffered to 40 mM Hepes (pH 7.3) prior to centrifugation for 3×10^5 *g* . min. Protein concentration of the resulting supernatant was determined by the dye binding method in [13] and all supernatants were diluted to equivalent protein concentrations (5 mg/ml) with Hepes buffer.

2.3. *Protein phosphorylation assay and electrophoresis*

All points were assayed at 30°C for 2 min in a final volume of 50 μl containing 40 mM Hepes (pH 7.3), 10 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid, 2 mM $MgSO_4$, 20 μM [γ-^{32}P]ATP spec. act. 0.2 Ci/mol (1 Ci = 3.7×10^{10} Bq) and 90 μg supernatant protein. Conditions were adjusted to 100 μM free Ca^{2+} and/or 50 μM cyclic AMP where indicated. The reaction was terminated by addition of an equal volume 0.1 M Tris–HCl (pH 6.8), 2% mercaptoethanol, 2% glycerol and heat

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for 2 min at 100°C. SDS–PAGE was done as in [14] using a gradient slab gel (6–17% acrylamide). The protein bands were stained with Coomassie Blue R-250 (Bio-Rad Labs.), dried on filter paper and radioactive proteins visualized by autoradiography using Kodak X-Omat film.

3. Results

3.1. *Effect of DMF on protein phosphorylation patterns*

As early as 48 h, DMF induced a decrease in phosphorylation of a protein of M_r 120 000 that was unaffected by either Ca^{2+} or cyclic AMP (fig.1). This is accompanied by an increased phosphorylation in the low-M_r region of the gel over the same time period.

3.2. *Effect of calcium on protein phosphorylation*

Supernatant fractions from control and induced cells were incubated in the absence or presence of 100 μM free Ca^{2+} which stimulated the phosphorylation of a protein with M_r 100 000 (fig.1) in both control and differentiated cells. This Ca^{2+} effect appears to be independent of the state of differentiation of the cells.

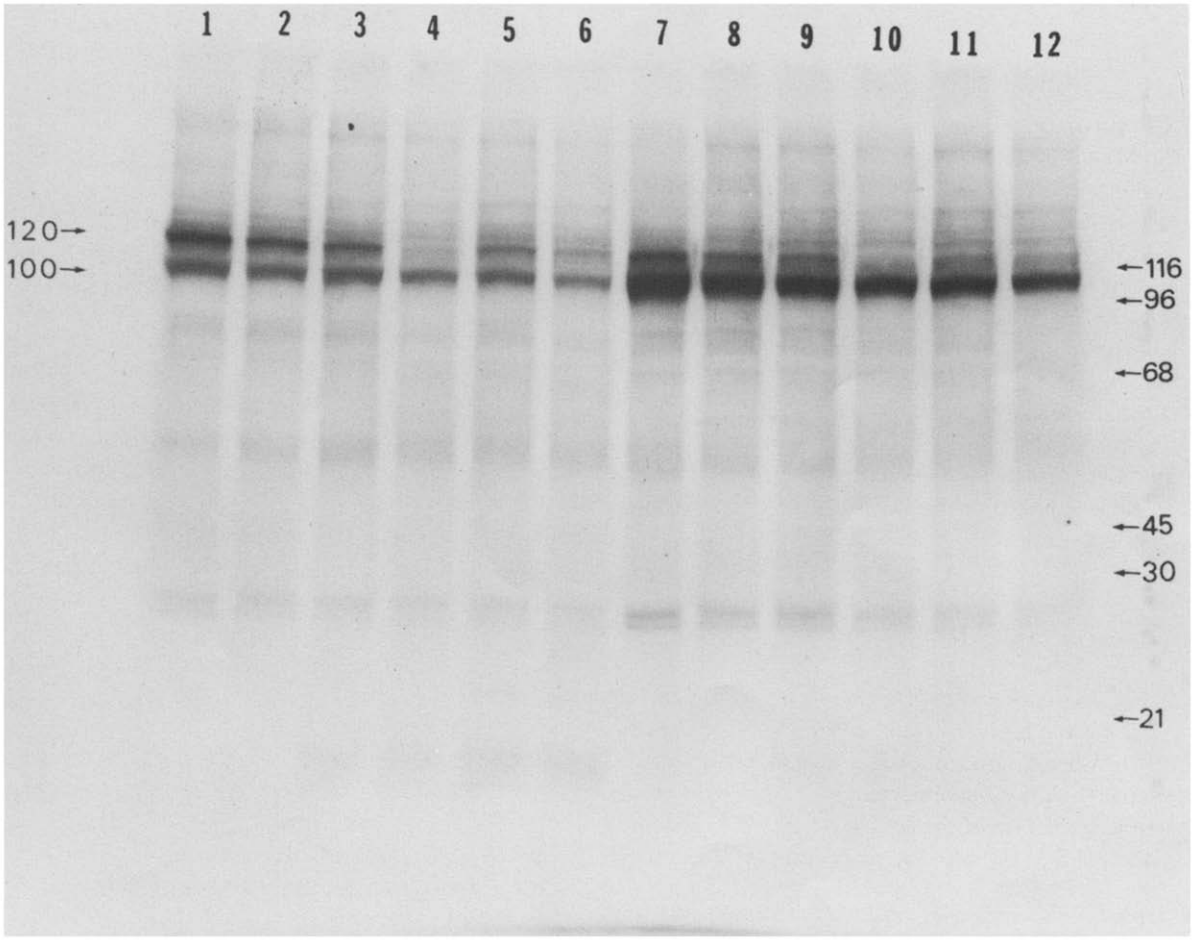

Fig.1. Autoradiogram of Ca^{2+} effect on supernatant protein phosphorylation from time course of MEL cells induced to differentiate with DMF. Cells were cultured and assayed as in section 2. Lanes 1–6 represent supernatant from cells exposed to inducer for 0, 1, 2, 3, 4 and 5 days, respectively, which was assayed in the absence of exogenous Ca^{2+} and cyclic AMP. Lanes 7–12 also represent supernatant from cells exposed to inducer for 0, 1, 2, 3, 4 and 5 days, respectively, but assayed in the presence of 100 μM free Ca^{2+}. Arrows on the left indicate the proteins with M_r 120 000 and 100 000 described in the text. M_r-Standards indicated on the right include: β-galactosidase, phosphorylase *b*, bovine albumin, ovalbumin, catalase and trypsin inhibitor with M_r 116 000, 96 000, 68 000, 45 000, 30 000 and 21 000, respectively.

3.3. *Effect of cyclic AMP on phosphorylation*

Cyclic AMP increased the phosphorylation of a protein at M_r 58 000 in cells induced with DMF over that seen with control cells. A prominent cyclic AMP-dependent, Ca^{2+}-independent phosphoprotein appears at M_r 70 000 after the first 24 h exposure to DMF (fig.2). To determine whether these changes were unique to the differentiated cell and not a function of the inducer used, cells were treated with either DMSO or ActD or 120 h and the supernatant phosphoproteins compared with the DMF results. Greater than 80% of the cells differentiated regardless of inducer employed. Two features of differentiation are clearly evident (fig.3):

(i) The loss of phosphorylation at M_r 120 000 is dependent on the agent used to induce differentiation. The addition of either DMF or DMSO results in the loss in phosphorylation at this M_r but ActD does not;

(ii) The appearance of a cyclic AMP-dependent Ca^{2+}-independent phosphorylation at M_r 70 000 is apparent regardless of inducer used.

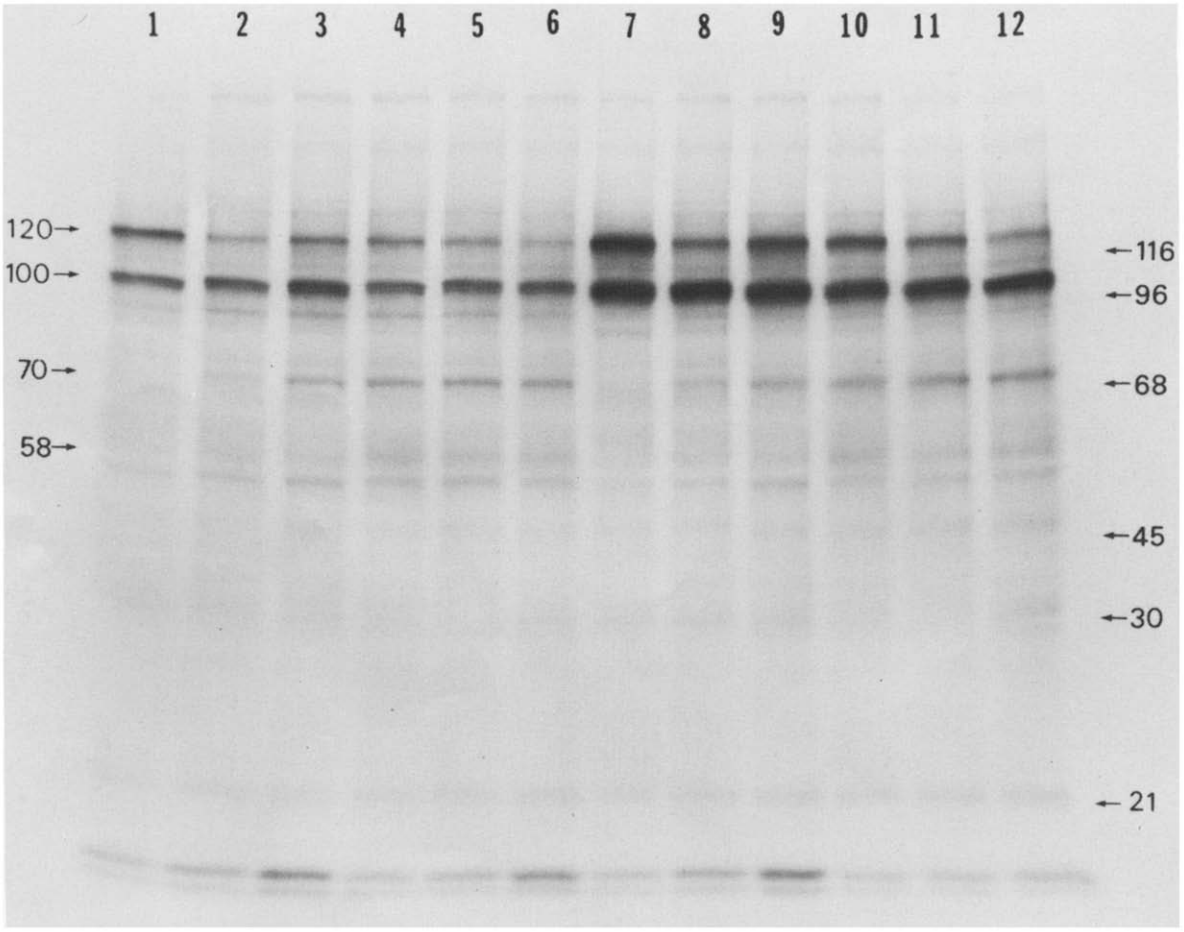

Fig.2. Autoradiogram of the Ca^{2+}, cyclic AMP effect of supernatant protein phosphorylation from time course of MEL cells induced to differentiate with DMF. Cells were cultured and assayed as in section 2. Lanes 1–6 represent supernatant from cells exposed to inducer for 0, 1, 2, 3, 4 and 5 days, respectively, which was assayed in the absence of exogenous Ca^{2+}, but in the presence of 50 μM cyclic AMP. Lanes 7–12 represent supernatant from cells exposed to inducer for 0, 1, 2, 3, 4 and 5 days, respectively, and assayed in the presence of 100 μM free Ca^{2+} and 50 μM cyclic AMP. Arrows on the left indicate phosphorylation at M_r 120 000 and 100 000 described for fig.1 and cyclic AMP-dependent phosphorylations at M_r 70 000 and 58 000 described in the text. M_r-Standards are indicated on the right.

4. Discussion

The addition of various agents with marked chemical diversity results in the differentiation of the MEL cell with the acquisition of the mature erythroid phenotype. We have demonstrated marked alterations in the phosphorylations of cytosolic proteins during the induced differentiation. We have shown a marked decrease in the phosphorylation of a protein with M_r 120 000, 48 h following the addition of DMF. This decrease in protein phosphorylation appears to be dependent on the inducer utilized and independent of either cyclic AMP or calcium. Synthesis and phosphorylation of cytosol and membrane proteins during the induced differentiation of MEL cells was examined in [10]. A marked dephosphorylation of plasma membrane proteins shortly after treatment with several inducers was noted.

We have also demonstrated the presence of a specific cyclic AMP-dependent protein phosphorylation at M_r 70 000 within 24 h following the addition of the inducer and prior to the onset of the differenti-

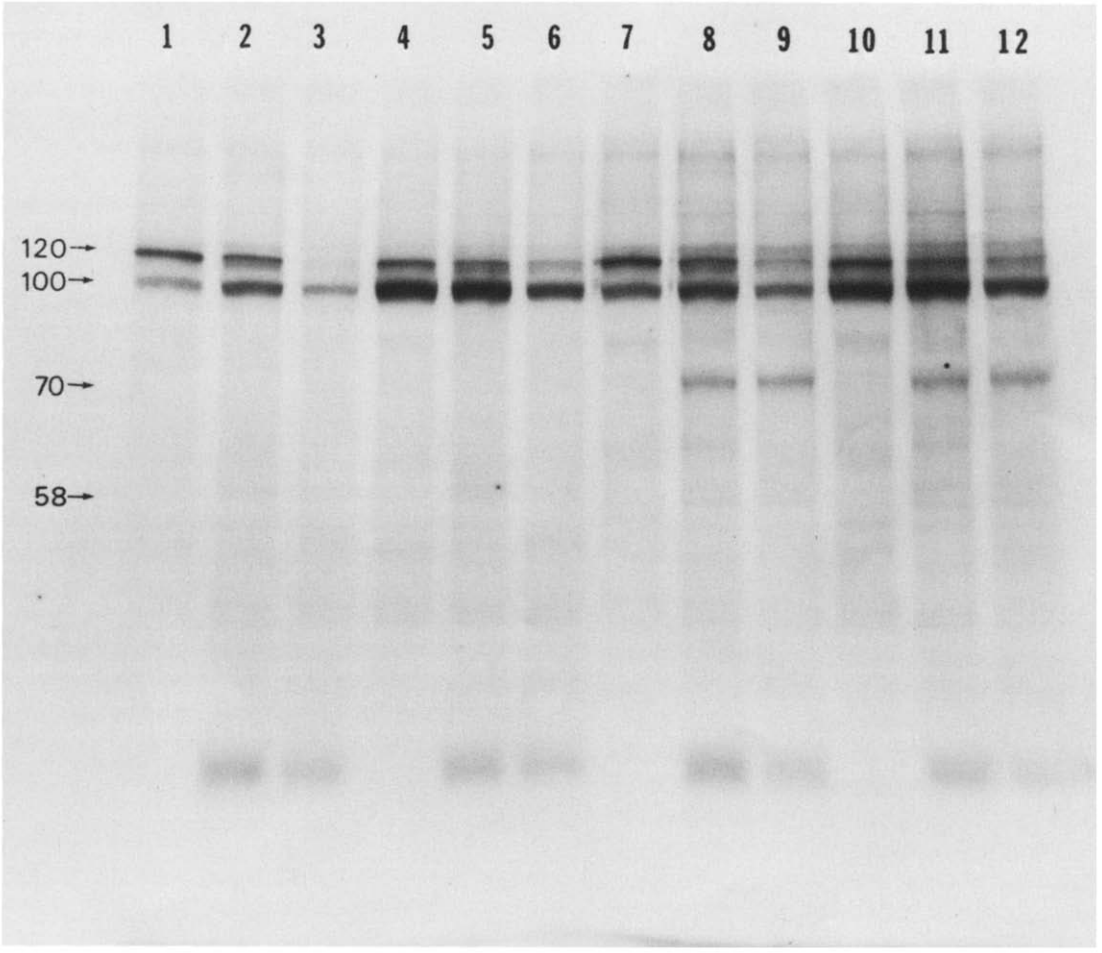

Fig.3. Autoradiogram of Ca^{2+} and cyclic AMP effect on supernatant protein phosphorylation from MEL cells induced to differentiate with DMSO and ActD. Cells were cultured with no additions or for 5 days in the presence of ActD or DMSO. Supernatant was prepared and assayed as previously described. Lanes 1–3 represent supernatant from control, ActD- and DMSO-treated cells, respectively, assayed in the absence of exogenous Ca^{2+} and cyclic AMP. Lanes 4–6 represent supernatant from control, ActD- and DMSO-treated cells, respectively, assayed in the presence of 100 μM free Ca^{2+}. Lanes 7–9 represent supernatant from control, ActD- and DMSO-treated cells, respectively, assayed in the absence of Ca^{2+} and in the presence of 50 μM cyclic AMP. Lanes 10–12 represent supernatant from control, ActD- and DMSO-treated cells, respectively, assayed in the presence of 100 μM free Ca^{2+} and 50 μM cyclic AMP. M_r-Values of proteins referred to in the text are indicated on the left.

ated state. This cyclic AMP-dependent protein phosphorylation is independent of the inducer used, and occurs when inducers with different initial mechanisms of action are utilized [4].

Using MEL-synchronized cells, cells cultured with inducers manifested a transient 5–6-fold increase in cyclic AMP during mid-S phase ~4 h following the addition of the inducer [17]. Strains of the mouse erythroleukemia cell which were resistant to DMSO-induced differentiation did not demonstrate elevation of cyclic AMP upon the addition of DMSO. In addition, these authors demonstrated that several cyclic nucleotide phosphodiesterase inhibitors also elevate cyclic AMP content, prolong the G_1, and induce murine erythroleukemia cell differentiation [18]. Cyclic AMP growth inhibition has been shown to occur in the G_1/early S period of the cell cycle [16], which is significant because commitment of the MEL cell to erythroid differentiation appears to also occur in the late G_1/early S phase with prolongation of the G_1 [11,12]. These observations, along with the fact that cyclic AMP almost universally mediates its effects via stimulation of protein phosphorylation, implicates the early cyclic AMP-dependent protein phosphorylation events that we have demonstrated as crucial in the differentiation process.

Acknowledgements

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