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DIFFERENTIATION OF MURINE ERYTHROLEUKEMIA CELLS BY HEXAMETHYLENEBISACETAMIDE INVOLVES SECRETION AND BINDING TO MEMBRANES OF A DIFFERENTIATION ENHANCING FACTOR

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A protein factor previously shown to enhance terminal differentiation of transformed erythroid cells is synthesized by murine erythroleukemia cells and secreted in the early stages of differentiation induced by hexamethylenebisacetamide (HMBA). Secretion also occurs, constitutively, in the absence of inducer, from a murine erythroleukemia cell variant characterized by an accelerated response to HMBA. The protein factor binds to intact cells following addition of HMBA and enhances translocation of protein kinase C to the nuclear fraction. These results strongly support an important role for this natural protein factor in cell differentiation. © 1991 Academic Press, Inc.

MEL cell differentiation induced by HMBA is a multistep process involving an early 10-12 hr latent period before the beginning of irreversible commitment to terminal differentiation (1-3). We have produced a variant (V3.17[44]) resistant to low concentrations of vincristine (VC) (4), which shows an accelerated response to HMBA (virtual absence of the latent period) and is sensitive to lower concentrations of HMBA than the parental clone (N23) (4, 5). In studies, performed to characterize at a biochemical level the differences between slowly inducing N23 cells and the rapidly responding VC-resistant cells V3.17[44], we have shown that both clones contain a protein differentiation enhancing factor (DEF). The concentration of DEF is significantly lower and limited to the cytoplasmic compartment of N23 cells, whereas V3.17[44] cells both contain and secrete the factor into the extracellular medium (6). DEF is a protein with a

ABBREVIATIONS
MEL, murine erythroleukemia; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; FCS, fetal calf serum; PMSF, phenylmethylsulphonylfluoride.

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mass of approximately 30 kD that promotes, when added to a culture of N23 cells, acceleration in the rate of terminal differentiation including a partial reduction in the latent period that precedes commitment (6). In vitro, purified DEF stimulates PKC activity at low (μM) calcium concentrations, in the absence of diacylglycerol, and promotes preferential binding of one kinase isozyme to natural or artificial membrane vesicles (7).

In the present paper, we present additional studies on the properties of DEF, based on a comparative analysis of its intracellular location and properties in MEL cell variants. The effects of HMBA on the kinetics of secretion of DEF and the binding of DEF to cell membranes have been examined, as well as the effect of DEF on the translocation of PKC.

MATERIALS AND METHODS

Cell culture. N23 MEL cells and the VC-resistant clone V3.17[44] (4) were cultured in α minimum essential medium containing 10% FCS (3). Protein labelling by [^{35}S]Met was performed on $5x10^6$ cells in 10 ml with 0.5 mCi of [^{35}S]Met (>1000 Ci/mmol Amersham). After 24 hours the cells and the culture medium were collected separately by centrifugation.

Isolation of the nuclear fraction. MEL cells nuclei were prepared as reported by Nevins (8). Samples of $5x10^7$ MEL cell nuclei were suspended in 1 ml of 10 mM HEPES, pH 7.5 containing 0.25 M sucrose, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin and 2 mM PMSF (Sigma) and disrupted by sonication (6 pulses of 10 sec each) at 5°C. The suspension was centrifuged at 100,000 xg for 10 min, the supernatant collected (endonuclear fraction), and chromatographed on a DEAE (DE 52 Whatman) column, as previously reported (9). PKC activity was assayed as described (9) using 150 μl of the eluted fractions. One unit of PKC is the amount which catalyzes the incorporation of 1 pmol of [^{32}P]/min into histone III-S under the assay conditions.

Purification and assay of DEF. The conditioned culture medium from V3.17[44] MEL cells (5 litres at a cell density of $2x10^6$ cells/ml) was collected and DEF purified as previously described (7). An additional purification step was carried out by loading DEF (1 ml, 3,700 units/mg) onto a Sephadex G100 (Pharmacia) column (1.5x120 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.0 containing 0.14 M NaCl (phosphate buffered saline). Proteins were eluted at 0.2 ml/min and fractions of 1.5 ml were collected. DEF activity was assayed as described (6) using 1 μl of the eluted fractions. One unit of DEF activity is defined as the amount which doubles the proportion of benzidine reactive (hemoglobin-containing) N23 cells at 72 hr of culture with 5 mM HMBA (2). The specific activity of our purified DEF was 31,000 units/mg. SDS-PAGE analysis of DEF was performed on a 8% polyacrylamide slab-gel (10). To identify DEF activity the gel was treated, cut and the proteins eluted as previously described (6).

DEF labelling with Na[^{125}I]. Purified DEF (3 μg) was iodinated using chloramine T (11) and dialyzed against phosphate buffered saline. The specific radioactivity of DEF was $1.67x10^6$ cpm/μg.

RESULTS

Synthesis of DEF by MEL cell variants. We have previously shown that a 30 kD protein factor (DEF), present in MEL cells, accelerates the rate of HMBA-induced terminal erythroid differentiation (6). In N23 cells the factor is localized exclusively to the cytoplasm, whereas in V3.17[44] DEF is also secreted into the extracellular medium. To further examine the properties of DEF, we have cultured N23 and V3.17[44] cells with [^{35}S]Met and then submitted both the extracellular medium and the cellular soluble fraction, from each cell type, to phosphocellulose column chromatography. Radioactivity and DEF activity were measured on the eluted fractions. As shown in Fig. 1A, the extracellular

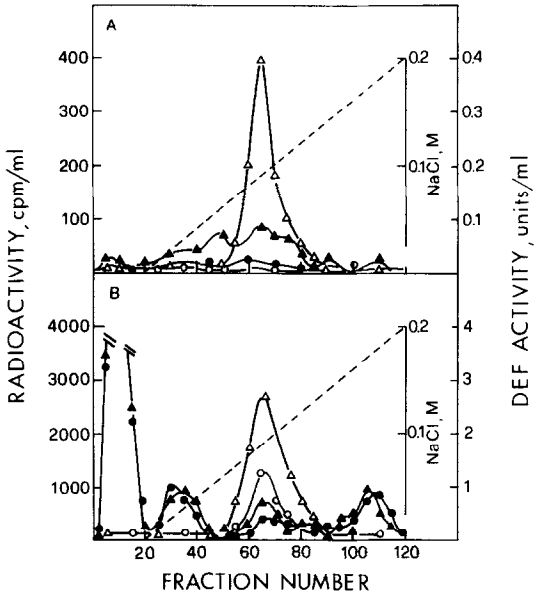

FIG. 1. Phosphocellulose chromatography of extracellular medium and cytosolic fraction from N23 and V3.17[44] MEL cells labelled with [^{35}S]Met. Both variants were labelled with [^{35}S]Met as described in Methods. After 24 hr the cell suspension was centrifuged at 600 xg for 5 min, the extracellular medium concentrated by ultrafiltration to 2 ml and dialyzed against 20 mM sodium phosphate buffer, pH 7.0 (buffer A); the cells were washed with 2 ml of phosphate buffered saline, suspended in 1 ml of this buffer containing 1 mM EDTA and lysed by 6 pulses of sonication of 10 sec each at 5°C. Lysates were centrifuged at 100,000 xg for 10 min, the particulate fraction discarded and the supernatant (cytosolic fraction) collected, heated at 90°C for 3 min and dialyzed against buffer A. The extracellular medium (A) and the heated cytosolic fraction (B) from N23 cells (circles) or V3.17[44] cells (triangles) were submitted to phosphocellulose chromatography on a column (1x4 cm) equilibrated with buffer A. Protein elution was performed with a linear gradient of NaCl from 0 to 200 mM (50+50 ml) and fractions of 1 ml were collected at a flow rate of 0.5 ml/min. Samples of 100 µl of the eluted fractions were counted in a β counter (closed symbols), while DEF activity was assayed using 10 µl of the eluted fractions (open symbols). The DEF containing fractions (from 58 to 75) were separately collected and concentrated by ultrafiltration to 1 ml.

medium of V3.17[44] cells contains DEF activity which elutes with a radioactive protein peak, no DEF activity is observed in the extracellular medium of N23 cells. The cytosolic fraction of both variants contains a radiolabelled protein which elutes with the peak of DEF activity (Fig. 1B). In agreement with previous observations, DEF is present in smaller amounts in the N23 cells compared to V3.17[44]. The radiolabelled peaks containing DEF activity, obtained from the extracellular medium or from the cytosolic fraction of N23 and V3.17[44] cells were collected and submitted to SDS-PAGE. In each case a single labelled protein band was identified by autoradiography (Fig. 2, insert) and found to contain DEF activity (Fig. 2).

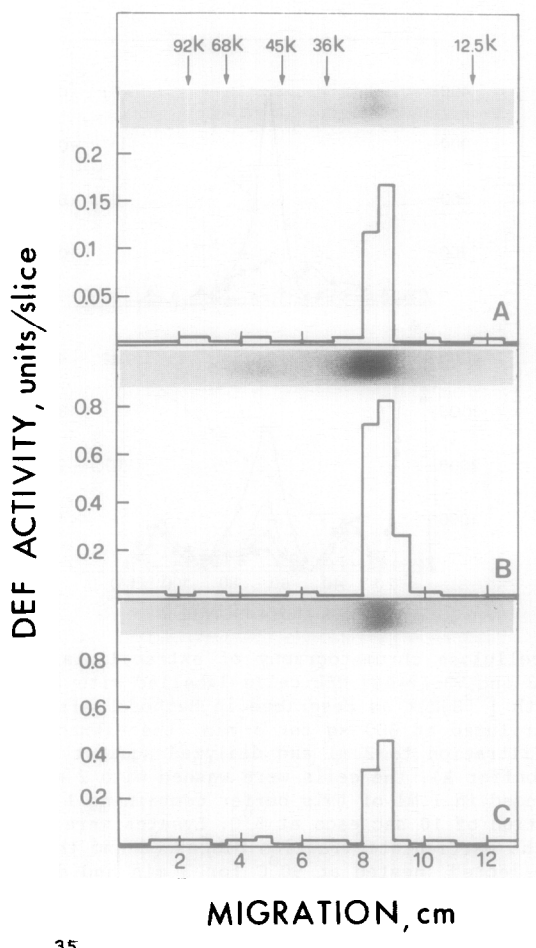

FIG. 2. SDS-PAGE of [^{35}S]Met-DEF from extracellular medium and cytosolic fraction of MEL cells. The pooled fractions containing DEF activity, obtained as reported in Fig. 1, from the extracellular medium (A) and the cytosolic fraction (B) of V3.17[44] cells and from the cytosolic fraction (C) of N23 cells were separately submitted to SDS-PAGE as described in Methods. Two lanes for each sample were run, one lane was exposed to autoradiography (insert), the other was sliced to assay DEF activity.

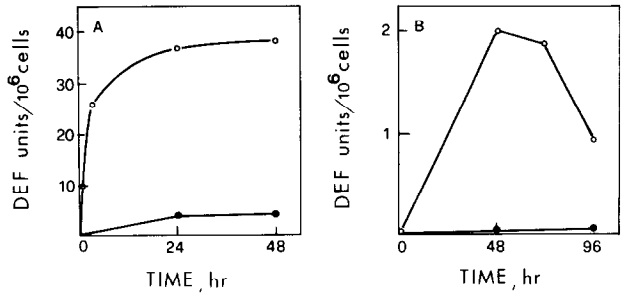

FIG. 3. Effect of HMBA on DEF secretion from MEL cells. V3.17[44] (A) and N23 (B) cells were cultured starting from a density of $2x10^5$ cells/ml, in the absence (●) or presence (○) of 5 mM HMBA. At the times indicated the cell density and the proportion of differentiated cells (benzidine-reactive cells) were evaluated. The extracellular medium was collected by centrifugation, dialyzed against buffer A and added to 0.5 ml of packed phosphocellulose previously equilibrated with the same buffer. The resin was washed three times with 5 ml of buffer A then DEF was eluted by three washes with 0.5 ml of phosphate buffered saline. The eluted fractions were pooled and DEF activity assayed using 25 ul.

Taken together, these data suggest that both MEL cell variants synthesize DEF but only V3.17[44] cells secrete the factor into the extracellular medium.

Effect of HMBA on secretion of DEF by MEL cell variants. To determine whether DEF must be secreted in order to act as differentiation enhancer, both V3.17[44] and N23 cells were exposed to HMBA and the appearance of DEF in the extracellular medium was measured over time. As shown in Fig. 3, HMBA promotes a 10-20 fold increase in the amount of DEF secreted by V3.17[44] cells and also induces secretion from N23 cells. The amount of DEF secreted by N23 cells appears to be significantly lower than by V3.17[44] cells. By the time induced differentiation was complete, secretion of DEF appears to have ceased.

Binding of DEF to MEL cell variants. In order to establish whether, to participate in cell differentiation induced by HMBA, secreted DEF must interact with the cell membrane, we have studied the binding of iodinated DEF (which retains full biological activity) to either N23 and V3.17[44] cells, in the presence and absence of the inducer. As shown in Table I, HMBA induces association of labelled DEF to membranes from either variant, suggesting that HMBA has a dual effect, inducing both secretion and binding of DEF to the cell surface. The amount of DEF bound to V3.17[44] cells is lower than that on N23 cells, probably due to competition by unlabelled DEF secreted in much larger amounts by V3.17[44] cells during exposure to HMBA. This conclusion is supported by the

TABLE I

Effect of HMBA on binding of DEF to MEL cells

Clone	Addition HMBA	Unlabelled DEF	Cell bound [^{125}I]-DEF cpm
N23	-	-	20 ± 10
N23	+	-	320 ± 50
N23	+	+	20 ± 20
V3.17[44]	-	-	20 ± 20
V3.17[44]	+	-	160 ± 20
V3.17[44]	+	+	30 ± 10

DEF was purified and labelled with [^{125}I] as reported in Methods. The cells were incubated at a density of $2x10^5$ cells/ml in 1 ml of culture medium containing [^{125}I]labelled-DEF (1 unit, 50,000 cpm) and, where indicated, 5 mM HMBA and 10 units of purified unlabelled DEF. After 15 min at 37°C the cells were collected by centrifugation, washed with 100 µl of culture medium, suspended in 10 µl of 50 mM Tris, pH 6.8, containing 10% glycerol, 2% sodium dodecyl sulfate and 2% 2-mercaptoethanol and submitted to SDS-PAGE. After autoradiographic identification the band corresponding to DEF was excised from the slab gel and counted in a γ-counter.
Each experiment was done in triplicate, the values given are the mean ± S.D.

observation that the binding of labelled DEF to MEL cells is completely abolished by the addition of excess cold DEF (see Table I).

Effect of DEF on translocation of PKC to nuclei. We have previously shown that PKC is involved in MEL cell induced differentiation (9, 12, 13). It is generally accepted that PKC mediated signal transduction involves translocation of the kinase to cell membranes, where activation and down-regulation occurs (14-20). Translocation of PKC to plasma membranes has been described following exposure of MEL cells to different stimuli (9). Furthermore, the presence of PKC activity in nuclei of stimulated cells has been suggested by Hocevar and Fields (21). We have examined the effect of HMBA on the association of PKC with the nuclear fraction of N23 and V3.17[44] cells, in the absence or presence of DEF. As shown in Fig. 4A, in N23 cells, following the addition of HMBA more PKC activity becomes associated with the nuclear fraction in the presence of DEF than in its absence. In V3.17[44] cells (Fig. 4B) HMBA also induces association of PKC activity with the nuclear fraction, but the addition of DEF (already constitutively secreted by these cells) has no additional effect.
Following translocation to the nuclear fraction, nuclear PKC activity falls and is essentially gone before the completion of commitment (Fig. 4A and B).

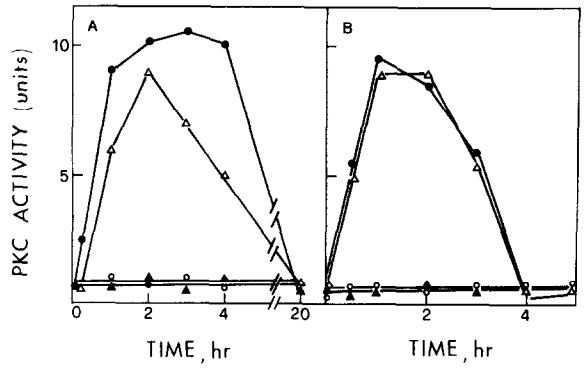

FIG. 4. Effect of DEF on PKC translocation. N23 (A) or V3.17[44] (B) MEL cells were cultured starting from a concentration of $5x10^5$ cells/ml in the absence of any addition (O), in the presence of 1 µl/ml of purified DEF (▲), 5 mM HMBA (Δ), or 1 µl of purified DEF together with 5 mM HMBA (●). At the times indicated $50x10^6$ cells were collected and the nuclei prepared as specified in Methods. The endonuclear fractions were chromatographed on DEAE columns and PKC activity was assayed using 100 µl of the eluted fractions as specified in Methods. The amount of PKC activity was calculated from the area under the eluted peaks.

DISCUSSION

One approach to understanding the events that accompany MEL cells terminal differentiation promoted by HMBA is provided by MEL cell variants such as V3.17[44] which are characterized by loss or shortening of the latent period which precedes commitment, and by a higher sensitivity to the inducer (4). Unlike the slowly differentiating N23 cells, V3.17[44] cells secrete a differentiation enhancing factor (DEF) into the medium. Both variants, however, contain DEF in their cytoplasmic fractions (6). In the present paper we provide evidence confirming that DEF is synthesized, although in different amounts, in both MEL cell variants and is constitutively secreted only by the variant characterized by a rapid response to HMBA. Exposure to HMBA initiates DEF secretion by N23 cells and increases the release of the factor from V3.17[44] cells. Secretion from both cell variants occurs during the early stages of commitment and is completed by the time the cells are fully differentiated. HMBA also appears to promote the binding of extracellular DEF to the MEL cell surface.

Data have also been obtained which provide further evidence on the involvement of PKC in MEL cell differentiation. We have demonstrated that early in the HMBA-mediated commitment process, PKC is translocated to the nuclear fraction and this is amplified by the presence of DEF. Taken together the data suggest

that DEF represents a new component of the differentiation-signalling system that promotes commitment to terminal erythroid differentiation in MEL cells.

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