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Identity in Molecular Structure between "Differentiation Enhancing Factor" of Murine Erithroleukemia Cells and the 30 kD Heparin-Binding Protein of Developing Rat Brain

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A 29 kD protein previously isolated from murine erythroleukemia (MEL) cells and shown to enhance the rate of differentiation of these cells has now been demonstrated to possess an amino acid sequence identical to that reported for the 30 kD heparin-binding protein from developing rat brain, named amphoterin after its highly dipolar structure. The identity between the two proteins has been established on the basis of a strong heparin binding affinity and a complete homology in the amino acid sequences of N-terminal region as well as of several tryptic peptides. Furthermore, the cDNA encoding this protein has been isolated from MEL cell mRNA, by means of reverse transcriptase-polymerase chain reaction, and its sequence was found to correspond to that of amphoterin. The MEL cell differentiation enhancing factor, previously abbreviated as DEF, is again confirmed to reduce the latent period preceding the appearance of hexamethylenebisacetamide induced cell commitment and to stimulate the catalytic activity of α-protein kinase C. Thus, here we demonstrate that a protein expressed in MEL cells, whose sequence is identical to that previously reported for amphoterin, plays an essential role in promoting cell differentiation, thereby indicating a new relevant function of amphoterin.

A number of reports from our and other laboratories have pointed out that protein kinase C (PKC) isozymes play a fundamental role in the early phases of the differentiation process of MEL cells (1-5). We have previously demonstrated that MEL cells contain a protein factor (DEF) which stimulates the activity of the α-PKC isoform and, once added to MEL cell culture, enhances the rate of differentiation triggered by HMBA (6). In an attempt to identify the potential mechanism of action of this protein we have found that it can replace diacylglycerol, one of the natural activating cofactors of PKC (7). The data so far obtained suggest that this protein factor is synthesized in MEL cells, retained in cytoplasmic vesicles, and, upon stimulation with HMBA, released into the external cell environment, where it undergoes binding to cell membranes and delivers its specific signal (8). The differentiation enhancing activity of this protein factor has been demonstrated not to be restricted to MEL cells, but extended to other cell types, such as HL60 promyelocytic cells,

Abbreviations: MEL, murine erythroleukemia; HMG1, high mobility group 1; PKC, protein kinase C; DEF, differentiation enhancing factor; HMBA, hexamethylenebisacetamide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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promoting a ten fold decrease in the requirement for phorbol esters or retinoic acid as inducers of differentiation of this human cell line (9). Many attempts to identify the primary structure of this protein factor have previously failed. The introduction of a new affinity–chromatography step in the purification procedure, by using a column with immobilized–heparin, resulted into an increase in the yield of the purified protein, sufficient for structural and functional analyses. In the present study, this protein has been confirmed to possess functional properties identical to those previously established to be characteristic of DEF molecule (6,8). Furthermore, the amino acid sequence of tryptic peptides obtained from this protein and the complete coding sequence of its cDNA, cloned from MEL cells mRNA, revealed the structural identity between DEF and mouse amphoterin, a member of the High Mobility Group 1 family (HMG1) (10). Taken together, these findings suggest the existence of a new class of protein factor(s) involved in differentiation of transformed cells.

MATERIALS AND METHODS

Cell culture and differentiation. MEL cell clones with low (N23) or high (C44) rate of differentiation were obtained and cultured as previously described (1, 11). MEL cells (10^5 cells/ml) were induced with 5 mM hexamethylenebisacetamide (HMBA). At the indicated times the amount of differentiated cells was evaluated by staining with benzidine (12).

Purification of the α–PKC stimulating protein factor. C44 MEL cells ($2x10^{10}$ cells), were collected during log phase growth (approximately $2–2.5x10^6$ cells/ml) and washed twice with 20 mM sodium phosphate buffer containing 0.14 M NaCl, pH 7.4. Cells were finally resuspended in 200 ml of 50 mM sodium phosphate buffer, pH 6.8, containing 0.25 M sucrose, 1 mM EDTA, 100 μM leupeptin and 2 mM phenylmethylsulphonylfluoride (buffer A) and lysed by sonication (9 strokes of 10 sec each at 0°C). The suspension was centrifuged at 40,000xg for 10 min at 5°C, the clear supernatant was collected and heated at 85°C for 3 min. The precipitated material was discarded by centrifugation at 40,000xg for 10 min and the clear supernatant was collected and loaded onto a 5 ml heparin–immobilized column (Econo–Pac column, Bio–Rad) previously equilibrated with buffer A without sucrose. The column was washed with 4 column volumes of the same buffer and the adsorbed proteins eluted with a 60 ml linear gradient of 0 to 1 M NaCl. Fractions of 1 ml were collected and 10 μl used for the assay of α–PKC stimulating protein activity. The fractions containing the activity were collected, dialyzed against 20 mM sodium phosphate buffer, pH 7.0 and submitted to ion exchange chromatography by using a 1 ml CM column (Econo–Pac CM column, Bio Rad). After washing, the adsorbed material was eluted with a 30 ml linear gradient of 0 to 1 M NaCl. Fractions of 0.5 ml were collected and aliquots (10 μl) were used for the assay of α–PKC stimulating protein activity. The fractions containing the activity were collected, concentrated to 1 ml by ultrafiltration, brought to 0.1 ml by Speed Vac concentration and loaded onto a TSK gel G–2000 column equilibrated in 50 mM sodium phosphate buffer pH 7.0. Fractions of 0.4 ml were collected and 10 μl used to assay α–PKC stimulating protein activity. The fractions containing α–PKC stimulating protein were collected and concentrated to 0.2 ml. The purity of the preparation was evaluated by SDS–PAGE (13).

Determination of the amino acid sequence of the α–PKC stimulating protein. The purified protein (5 μg) was submitted to automated Edman degradation using a gas phase sequencer on line with a PTH–amino acid analyzer (Beckman LF 3000). Alternatively, 5 μg of purified protein were digested with 0.25 μg of trypsin (sequencing grade, Boehringer Mannheim) for 20 hours at 20°C. The resulting peptides were separated by reversed–phase HPLC and sequenced as described above.

Sequencing of MEL cell cDNA for α–PKC stimulating protein factor. Total RNA was isolated from C44 MEL cells by extraction with guanidium thiocyanate (14), and 5 μg were reverse transcribed by using oligo $(dT)_{15}$ as a primer. The oligonucleotide primers for the amplification reaction were based on the sequence of cDNA for mouse HMG1 protein (15). Sense primer was: 5' TGGGATCCGGCAAAGGAGATCCTAAGAAG (nucleotides 75 to 96) and contained a BamHI restriction site at the 5' end; antisense primer was: 5'GCGAATTCGCGCTAGAATAACTTATTCA

(nucleotides 714 to 734) and contained an EcoRI restriction site at the 5' end. Polymerase chain reaction was carried out by using UlTma DNA polymerase (Perkin-Elmer), according to the manufacturer instructions. After 25 cycles of amplification (1 min at 94°C, 1 min at 64°C, and 1 min at 72°C) the product was analyzed on a 4% NuSieve agarose gel (FMC Corp. Bio Products), and consisted of a single fragment of 675 bp, which was cloned into pGEM-7Zf+ vector (Promega). The nucleotide sequence of the insert was determined by the dideoxynucleotide chain termination method (16) using a Sequenase 2.0 kit (U.S. Biochemical Corp.).

Purification and assay of α-PKC. α-PKC was isolated from MEL cells following the procedure previously described (1). The catalytic activity of α-PKC was measured in the presence of Ca^{2+}, diacylglycerol and phosphatidylserine as in Ref.17.

Assay of α-PKC stimulating protein activity. The activity of the α-PKC stimulating protein factor was determined by measuring the increase in V_{max} of α-PKC, evaluated in the presence of optimal cofactors concentrations, following the addition of appropriate amounts of the protein factor to the complete α-PKC assay mixture. One unit of α-PKC stimulating protein activity is defined as the amount that causes a two fold increase in V_{max} of α-PKC , in the specified conditions.

Isolation of amphoterin from rat brain. Rat brain tissue (7.8 g) was first disgregated in 160 ml of ice cold buffer A with 3 strokes in a Potter-Elvehjem homogenizer and then submitted to sonication followed by identical treatments and chromatographyc steps as described above for the purification of MEL cell protein.

RESULTS

Purification of the α-PKC stimulating protein factor from MEL cells. The major problem encountered during isolation of DEF from MEL cells was the long time required for each chromatographic step and for the assay of its biological activity on MEL cell differentiation. To overcome these difficulties, we have set up a new purification procedure, including an affinity-chromatography step, along with a more rapid assay of the purification efficiency and of the overall yield in each step. The latter goal was achieved by measuring the α-PKC stimulating activity of

Table I

Purification of α-PKC stimulating protein factor from MEL cell soluble fraction

Step	Protein (mg)[a]	α-PKC stimulating protein activity (units)[b]	Specific activity (units/mg)	Yield
Crude extract following heating	22.0	3500	159	1
Affinity chromatography	2.9	2190	755	0.63
Ion exchange chromatography	0.32	1215	3800	0.35
Gel chromatography	0.022	944	42000	0.27

[a]Protein concentration was determined with the procedure described by Bradford (28).

[b]α-PKC stimulating protein activity was assayed as described in Materials and Methods. The units were calculated from the area of the α-PKC stimulating protein activity peak eluted in each chromatographic step.

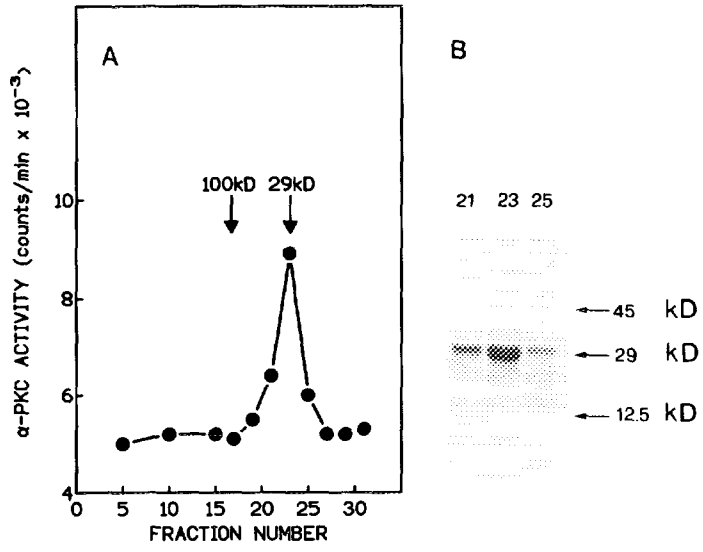

Fig. 1. *Determination of the molecular mass of α-PKC stimulating protein purified from MEL cells.* (A) Following ion-exchange chromatography, the partially purified α-PKC stimulating protein obtained from MEL cells was submitted to gel filtration as specified in Materials and Methods. The eluted fractions were assayed for the presence of α-PKC stimulating protein activity as described in Materials and Methods. The arrows indicate the elution volumes of standard proteins having the indicated molecular masses. (B) The eluted fractions (100 μl) were analyzed by SDS-PAGE (12% gel) in the presence of 0.25 M 2-mercaptoethanol. The corresponding fraction numbers and the migrations of standard proteins are indicated.

DEF at each purification step. As shown in Table I, chromatography on heparin-immobilized column followed by an ion exchange chromatographyc step yielded a partially purified protein that was further submitted to a final purification consisting of a gel-filtration step. As shown in Fig. 1, the overall procedure yielded a protein retaining all α-PKC stimulating activity (Fig. 1A) and migrating as a single band in SDS-PAGE (Fig. 1B) . The molecular mass of the protein has been calculated to be approximately 29 kD in denaturing (SDS-PAGE) and non denaturing conditions (gel-filtration) (see Fig. 1), indicating the monomeric structure of the protein.

	α-PKC stimulating protein factor	Amphoterin
N-Terminal region:	GKGDPKKPRGKMSSY	GKGDPKKPRGKMSSY
Tryptic peptides:		
Peptide 1	RPPSAFFLFXSEYRPK	RPPSAFFLFCSEYRPK
Peptide 2	DIAAYR	DIAAYR
Peptide 3	MSSYAFFVQT	MSSYAFFVQT
Peptide 4	DPNAPK	DPNAPK
Peptide 5	GKPDAAK	GKPDAAK
Peptide 6	EK	EK

Fig. 2. *Comparison between partial amino acid sequences of α-PKC stimulating protein factor isolated from MEL cells and amphoterin.* The amino acid sequences of N-terminal region and of several tryptic peptides were obtained from purified α-PKC stimulating protein factor, as described in Materials and Methods, and compared with the corresponding sequences of rat brain amphoterin (10).

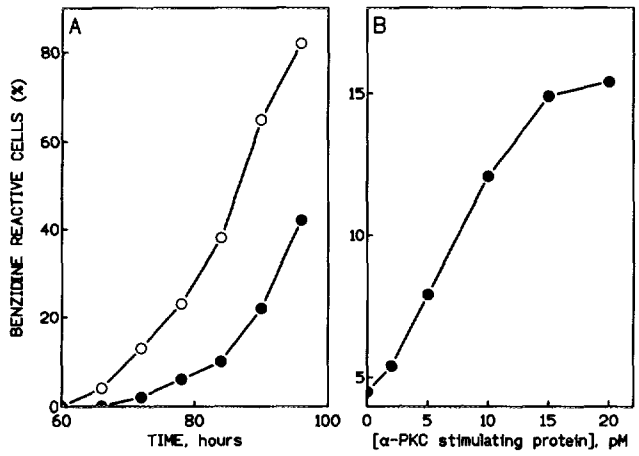

Fig. 3. *Effect of α-PKC stimulating protein on MEL cell differentiation.* (A) N23 MEL cell differentiation was induced in the absence (●) or presence (○) of purified α-PKC stimulating protein (20 pM final concentration) in the conditions reported under Materials and Methods. At the indicated times, aliquots of the cell cultures were collected and the proportion of benzidine reactive cells was determined as described in Materials and Methods. (B) N23 MEL cells were induced in the presence of the indicated concentrations of purified α-PKC stimulating protein. After 72 hours of culture, the proportion of benzidine reactive cells was determined.

Determination of the amino acid sequence of α-PKC stimulating protein factor. The purified protein, obtained as described above, was submitted directly to Edman degradation to establish its N-terminal sequence or, alternatively, digested with trypsin followed by isolation of the resulting peptides and sequencing. As shown in Fig. 2, the sequences found in both cases were identical to those reported for rat brain amphoterin (10). The presence of a cDNA open reading frame encoding the complete amino acid sequence of amphoterin was further confirmed by cDNA cloning, using reverse transcriptase-polymerase chain reaction, from MEL cell RNA (data not shown).

Effect of the isolated protein on MEL cell differentiation. To establish if the protein isolated from MEL cells, according to the new purification procedure, and monitored on the basis of α-PKC stimulating activity, corresponded to the differentiation enhancing factor previously isolated from these cells, its effect on HMBA induced MEL cell differentiation, was investigated. As shown in Fig. 3A, the addition of this protein resulted into a 3 fold increase in the rate of appearance of differentiated cells. The effect was dose dependent (Fig. 3B) and reached saturation at a concentration of 20 pM, thereby indicating the complete correspondence of the biological effects displayed by the protein factor originally identified in MEL cells and amphoterin.

Effect of rat brain amphoterin on α-PKC activity and MEL cell differentiation. To verify if amphoterin purified from other cell sources displayed DEF-like activities, this protein was purified from rat brain following the procedure utilized for the isolation of the MEL cell α-PKC stimulating factor and the effects on both MEL cell differentiation and α-PKC activation were evaluated. As shown in Fig. 4A, in these conditions a single protein was obtained, showing an electrophoretic mobility identical to that of the protein isolated from MEL cells. When added to a MEL cell culture, together with HMBA, this protein (Fig.4B) induced an increase in the rate of appearance of

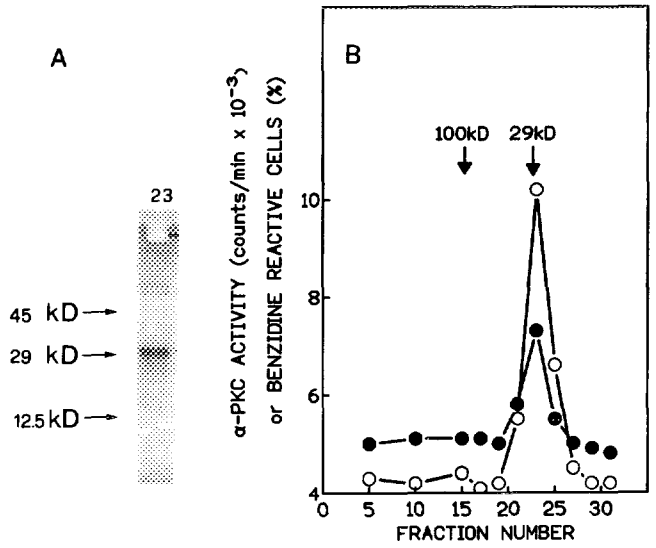

Fig. 4. *Stimulation of α-PKC activity and MEL cell differentiation by rat brain amphoterin.* (A) Partially purified rat brain amphoterin, obtained from the ion-exchange chromatographic step, was submitted to gel-filtration, as described in Materials and Methods. Aliquots (10 µl) of the eluted fractions were assayed for their effect on α-PKC activity (●). Alternatively, 0.1 µl of the same fraction as added to complete induction mixtures of N23 MEL cells and, after 72 hours of culture, the proportion of benzidine reactive cells was determined (○). (B) SDS-PAGE was carried out using 100 µl of the eluted fractions, as in the legend to Fig. 1. The corresponding fraction number and the migrations of standard proteins are indicated.

benzidine reactive cells similar to the effect induced by DEF. Furthermore this purified amphoterin preparation resultad equally efficient in promoting activation of α-PKC (Fig.4B).

DISCUSSION

A series of previous observations have indicated the prominent role of PKC isozymes as modulators of the rate of MEL cell differentiation (1,3, 18-20). The levels of δ-PKC isozyme resulted to be inversely correlated to HMBA sensitivity of various MEL cell clones (3), whereas the specific cell depletion of this kinase isoform was accompanied by a significant increase in the rate of differentiation (submitted for publication). On the contrary, the level of the only c-PKC isoform present in MEL cells, α-PKC (20), was demonstrated to be directly correlated to the sensitivity of these cells to the inducer (1). We have also identified in MEL cells a protein factor, which we have named DEF, that is released in the extracellular medium by HMBA stimulated cells and is capable to promote a large increase in the rate of MEL cell differentiation when administered in association with HMBA (6, 8). The purified form of this protein factor resulted to be a good stimulator of the α-PKC isozyme isolated from MEL cells, as well as from other tissues (7). The activity of DEF was not restricted to MEL cells, as indicated by its enhancing activity on human HL60 promyelocytic cell differentiation (9).

Following changes in the purification procedure, we have now isolated from the cytosolic fraction of MEL cells a 29 kD protein having a partial protein sequence identical to that of rat and mouse

amphoterin (10, 15). In previous studies on developing rat brain, Parkkinen et al. (21) reported the occurrence of multiple proteins co-purifying with amphoterin and displaying highly related primary structures. However, distinct sequence features where located both at the N-termini and in several tryptic peptides from each HMG1-type protein. None of those amphoterin-related polypeptides appears to co-purify with amphoterin isolated from MEL cells, since analyses of both N-terminal and tryptic peptide sequences revealed a single amino acid sequence corresponding to that of amphoterin. This protein expresses both the activities previously found to be characteristic of DEF isolated from MEL cells: it stimulates α-PKC activity, and, once added to the cell culture medium together with the inducer, promotes a large increase in the rate of cell differentiation. The identification of DEF as amphoterin is further supported by the observation that this protein, isolated from rat brain, expresses the same biological and biochemical activities. Many functions have been attributed to amphoterin, particularly related to its DNA binding capacity (22–27). However, the subcellular localization of this protein is still controversial (21) and an extracellular role on enhancement of neurite outgrowth has been reported (10). The results presented in this paper ascribe a new function to amphoterin as a stimulatory factor for the differentiation process of MEL cells and probably of other cells. Further experiments are in progress to define the mechanisms by which amphoterin elicits its effects on both α-PKC activity and cell differentiation.

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