Extracellular release of the 'differentiation enhancing factor', a HMG1 protein type, is an early step in murine erythroleukemia cell differentiation

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Abstract Differentiation enhancing factor (DEF) is a 29 kDa protein expressed in murine erythroleukemia (MEL) cells and active in promoting a significant increase in the rate of hexamethylenebisacetamide induced differentiation of these cells. The factor was recently shown to possess an amino acid sequence identical to that reported for one of the HMG1 proteins, designated as 'amphoterin' on the basis of its highly dipolar sequence. In the present study, we have expressed DEF cDNA in an *E. coli* strain and found that the recombinant protein has functional properties identical to those observed with native DEF. Furthermore, we demonstrate that, following MEL cell stimulation with the chemical inducer, DEF is secreted in large amounts in the extracellular medium. In fact, the N-terminal sequence and the partial amino acid sequence of tryptic peptides from the secreted protein correspond to those of DEF isolated from the soluble fraction of resting MEL cells. These results are indicative for an extracellular localization as the site of action of DEF and suggest a novel function for proteins belonging to the HMG1 family. Finally, the early decay of DEF mRNA, in chemical induced MEL cells, support the hypothesis that the involvement of the enhancing factor occurs and is completed in the early phases of cell differentiation.

Key words: Murine erythroleukemia cell differentiation; HMG1 proteins; Expression in *E. coli* cell

1. Introduction

MEL cells are induced to terminal erythroid differentiation by hexamethylenebisacetamide (HMBA) through a multistep process [1,2], which involves also down-regulation of specific protein kinase C (PKC) isoforms shown to be functionally related to the differentiation process [3–6]. The rate of appearance of differentiated cells has previously been shown to undergo a significant acceleration following addition to the cell culture medium of a protein factor named 'differentiation enhancing factor' (DEF), which is synthesized in resting cells and retained in the intracellular compartment [7]. Altogether, these observations suggested an extracellular function for DEF, and that this localization could be achieved only through a secretory process, indicating DEF secretion as one of the earlier events in cell differentiation. Moreover, the powerful in vitro activating effect of DEF on α-PKC isotype was considered of potential interest for the understanding of the biological activity of the factor [8]. Recently, it has been demonstrated that this protein factor possesses an amino acid sequence identical to that reported for a heparin binding protein isolated from developing rat brain [9]. Due to its highly dipolar sequence, characteristic of the family of HMG1 polypeptides, this protein has been designated as 'amphoterin' [10]. The identity between MEL cell DEF and 'amphoterin' was confirmed by analysis of the sequence of the cDNA encoding DEF protein [10]. In order to provide additional data that could unequivocally attribute the differentiation enhancing activity to the protein factor isolated from MEL cells, we have expressed DEF in *E. coli* cells and evaluated the biological activity of the recombinant protein. In addition, we have isolated and characterized the protein carrying DEF activity present in the extracellular medium of MEL cells following stimulation with HMBA. The results obtained indicated that both recombinant DEF and native DEF purified from the extracellular medium expressed a biological activity corresponding to that of the protein factor present in the soluble fraction of resting MEL cells. Evidence for the identity in molecular structure between the secreted and the intracellular DEF protein is also provided. Taken together the present and previous results [9] strongly indicate a novel function of proteins belonging to the HMG1 family [11].

2. Materials and methods

2.1. Cell culture and differentiation

MEL cell clones N23 and C44 were cultured in α-MEM supplemented with 10% fetal calf serum, as previously described [12,13]. N23 cell clone was used for the assay of DEF activity, due to its low rate of differentiation. C44 cell clone was utilized as the source of intracellular and extracellular DEF. N23 MEL cells (10^5 cells/ml) were induced to differentiate by addition of 5 mM hexamethylenebisacetamide (HMBA) and, at the indicated times the amount of differentiated cells was evaluated by staining with benzidine [14].

2.2. Purification of DEF from MEL cell soluble fraction

C44 MEL cells were collected, washed and lysed as previously reported [9]. The cell soluble fraction was prepared and the protein factor isolated following the purification procedure previously described [9].

2.3. Purification of DEF from MEL cell extracellular medium

44 MEL cells (2×10^{10} cells) were collected and washed as above and resuspended in 500 ml of serum-free culture medium containing 5 mM HMBA. After 4 h of incubation at 37°C, the cell suspension was centrifuged at $800 \times g$ for 10 min; thereafter the cells and the extracellular medium were separately collected and processed as follows: cells were washed and lysed as reported above and the cell soluble fraction was submitted to chromatography on heparin-immobilized column as previously described [9]; the extracellular medium was concentrated by ultrafiltration to 10 ml, heated at 85°C for 3 min and the precipitated material discarded by centrifugation. The clear supernatant was diluted to 50 ml with 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA, 100 μM leupeptin and 2 mM phenylmethylsulfonilfluoride and the DEF protein purified following all the chromatographic steps described above for the purification of DEF from the cell soluble fraction.

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Abbreviations: MEL, murine erythroleukemia; HMG1, high mobility group 1; GST, glutathione *S*-transferase; DEF, differentiation enhancing factor; HMBA, hexamethylenebisacetamide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2.4. Assay of DEF activity

N23 MEL cells (10^5 cells/ml) were incubated in standard culture conditions with 5 mM HMBA in the absence or presence of DEF samples. After 72 h the amount of differentiated cells was evaluated by staining with benzidine [14]. One unit of DEF activity is defined as the amount which doubles the proportion of benzidine reactive cells in the specified assay conditions.

2.5. Expression of DEF as fusion protein in E. coli

Total RNA was isolated from C44 MEL cells by extraction with guanidium thiocyanate [15], and 5 μg were reversed transcribed by using oligo$(dT)_{15}$ as a primer. The oligonucleotides primers for the amplification reaction and the polymerase chain reaction conditions have been previously described [9]. The insert was ligated into *Eco*RI/*Bam*HI cut pGEX2T expression vector (Pharmacia LKB Biotechnology) and *E. coli* TOP 10 F′ cells were transformed using electroporation (BioRad Gene Pulser). Recombinant plasmids, were then sequenced by the dideoxynucleotide chain termination method [16]. As a control, bacterial cells were transformed by using intact pGEX2T vector and used to express glutathione *S*-transferase (GST). Expression and purification of GST and GST-DEF were carried out as indicated by the manufacturer. Where indicated, GST-DEF was cleaved with thrombin [17] followed by isolation of recombinant DEF protein by affinity chromatography on heparin-immobilized column [9].

2.6. Electrophoretic methods

SDS-PAGE was carried out according to Laemmli [18]. For Northern blotting 20 μg of total RNA from C44 MEL cells were submitted to electrophoresis on 1.2% agarose gel [15] and transferred to a nylon filter (Hybond-N$^+$, Amersham Int.). cDNA clones, from nucleotide 75 to 734 of mouse HMG1 protein [19] and from nucleotide 217 to 540 of mouse β-actin cDNA [20], were labelled using [^{32}P]dCTP (Rediprime kit, Amersham Int.) and used to probe the filter. The radioactive bands were excised from the filter and counted in a scintillation β-counter.

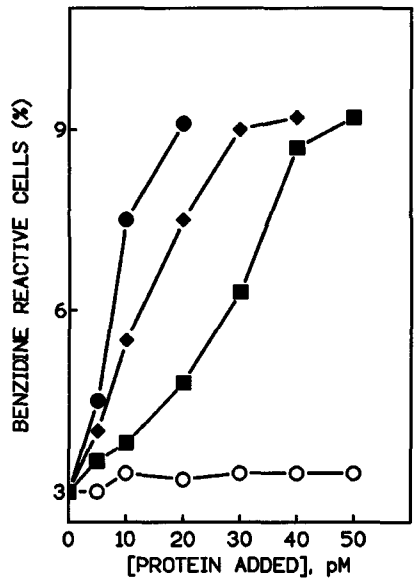

Fig. 1. Efficiency of native and recombinant DEF on MEL cell differentiation. N23 MEL cell differentiation was induced as reported in section 2 in the absence or presence of the indicated amounts of recombinant or native DEF, or GST as follows: (●), DEF from MEL cell soluble fraction; (◆), recombinant DEF isolated after digestion of the fusion protein with thrombin; (■), recombinant GST-DEF fusion protein; (○), recombinant GST. After 72 h of culture the proportion of benzidine reactive cells was determined as described in section 2.

3. Results

3.1. Expression of DEF protein

Starting from MEL cell total RNA, DEF cDNA was prepared and used to express DEF in bacterial cells as a fusion protein with the *Schistosoma japonicum* GST. The fusion protein was then purified by affinity chromatography (see section 2). The addition of increasing amounts of fusion protein to the culture medium of N23 MEL cells, together with the inducer HMBA, promoted a dose-dependent acceleration in the rate of accumulation of differentiated cells, indicating that the protein sinthesized by bacterial cells displayed a differentiation enhancing activity identical to that shown by native DEF protein obtained from MEL cell soluble fraction (Fig. 1). The maximal stimulatory effect was obtained at fusion protein concentrations of approximately 40–50 pM, indicating a specific activity of this protein approximately two-fold lower as compared to pure native DEF.

In order to obtain the 29 kDa protein fragment, the fusion protein was cleaved by digestion with thrombin. The efficiency in the stimulation of MEL cell differentiation was not only tested for the cleaved 29 kDa protein but also for GST expressed by the same bacterial cells, as an internal control. The 29 kDa protein fragment was maximally effective at concentrations of 20–30 pM, almost identical to those observed with native DEF (Fig. 1). GST revealed no significant effect even at the highest concentrations utilized.

3.2. Isolation of DEF from MEL cell culture medium

Previous results indicated that DEF had to be secreted into the extracellular compartment in order to express its biological activity. This observation emerged from data indicating that the protein, although present in the soluble fraction of uninduced cells, expressed its differentiation enhancing activity when added to MEL cell culture medium. Thus it was reasonable to postulate that the protein was released from the intracellular compartment during stimulation with the chemical inducer. To test directly this hypothesis, cells were suspended in fresh serum-free culture medium, in order to avoid contamination by exogenous proteins during the isolation procedure, and then induced with HMBA. At time intervals, the cell-free medium was collected and analyzed for its content in DEF activity. As shown in Fig. 2A, DEF activity was progressively released from the cells, reaching a maximum between the third and the fourth hour after the addition of HMBA. No appreciable amounts of DEF activity was detected in the medium in which cells had been incubated in the absence of HMBA.

In order to identify the nature of this protein factor and evaluate the extent of the secretion process, MEL cells, suspended in serum-free medium, were incubated for 4 h with HMBA, then both cells and medium were separately collected, submitted to affinity chromatography on heparin-immobilized column, and analyzed for their content in the protein expressing DEF activity. As shown in Fig. 2B, a large amount of this protein was recovered from the extracellular medium as a single peak of DEF activity. A lower protein peak, eluting at the same salt concentration and containing the same activity was recovered from the cell soluble fraction (Fig. 2C). The amount of activity present in the extracellular medium resulted to be approximately 1.5–1.6-fold greater than that recovered from the cell soluble fraction, indicating that approximately 60–65% of the total DEF protein had been released during cell stimulation. The possibility that this high recovery in the extracellular me-

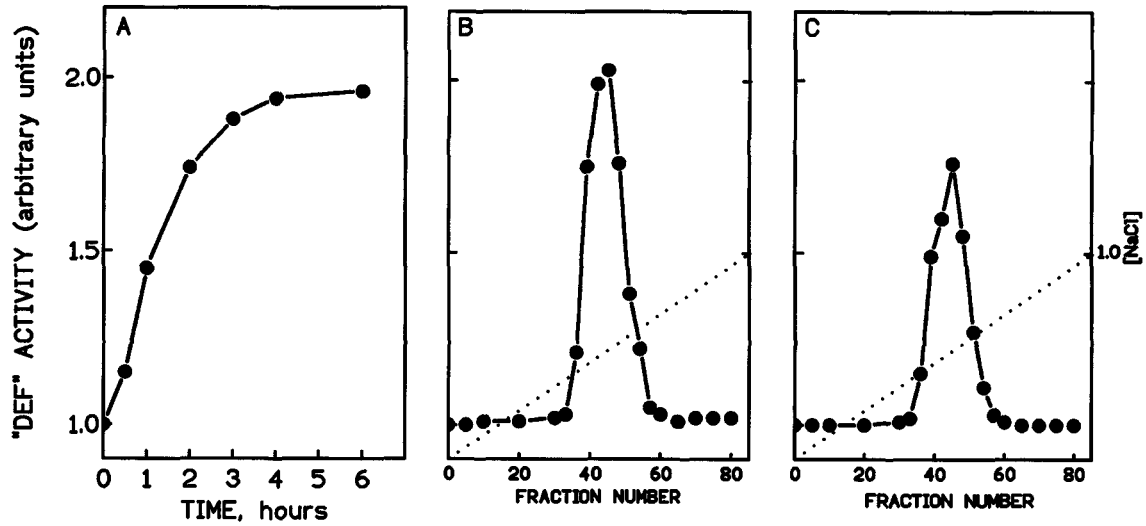

Fig. 2. Release of differentiation enhancing activity in the culture medium of stimulated MEL cells. C44 MEL cells (4×10^7 cells) were resuspended in 1 ml of serum-free culture medium containing 5 mM HMBA and incubated at 37°C. (A) At the indicated times, aliquots (0.1 ml) of cell suspension were collected, cells were pelletted by centrifugation and 1 μl of cell-free extracellular medium was used for the assay of DEF activity as described in section 2. (B and C) After 4 h of stimulation, cells and extracellular medium were separately collected by centrifugation and the cell soluble fraction prepared as specified under section 2. Both cell soluble fraction (B) and extracellular medium (C) were submitted to chromatography on a heparin-immobilized column as described in section 2. Aliquots (1 μl) of the eluted fractions were used for the assay of DEF activity.

dium could be due to cell death was ruled out on the basis of the large difference between the amount of DEF protein released and the extent of cell death, which involved only 6–8% of total cells, as measured during the entire experiment.

The peak of DEF protein, obtained from the extracellular medium of MEL cells, was further purified, following the procedure described in section 2, and finally analyzed using a size-exclusion chromatography (Fig. 3). The elution volume of

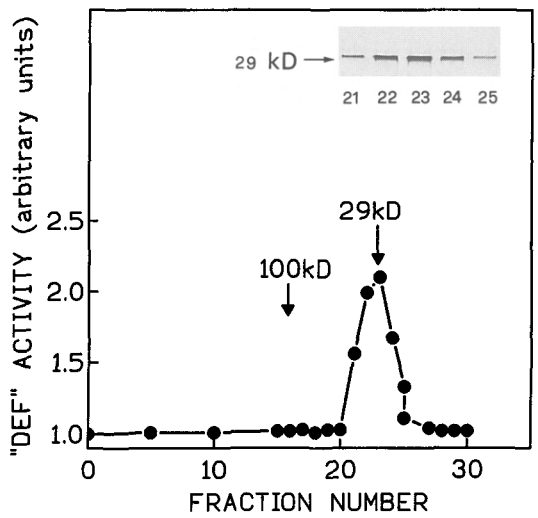

Fig. 3. Determination of the molecular mass of DEF purified from the extracellular medium of MEL cells. DEF was purified from the extracellular medium of C44 MEL cells, as reported in section 2, and loaded onto a TSK gel G-2000 column equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Fractions of 0.4 ml were collected and aliquots of 1 μl assayed for DEF activity as described under section 2. The arrows indicate the elution volumes of standard proteins having the indicated molecular masses. In the inset: SDS-PAGE of the indicated fractions was performed, as specified in section 2. The arrow indicates the migration of a 29 kDa standard protein.

the peak indicated that the molecular mass of the protein corresponded to 29 kDa; moreover the protein under this peak, submitted to SDS-PAGE, was found to migrate as a single band, with a mobility corresponding again to the same molecular mass (see inset in Fig. 3).

3.3. Molecular and functional comparison between DEF protein isolated from cell soluble fraction and from extracellular medium

The two preparations of DEF protein, respectively isolated from the soluble fraction and from the extracellular medium of MEL cells, were tested for their efficiency in promoting increase in the accumulation of benzidine reactive cells, following exposure of MEL cells to HMBA. As shown in Fig. 4, both proteins showed identical efficiencies. Moreover, the N-terminal sequences and the sequences of three tryptic peptides were analyzed and resulted to be identical in the two proteins (data not shown). It has been reported that HMG1 preparations from rat brain contain at least three homologous polypeptides [21]. The absence of an Ala residue at the positions 1 and 14 and of a Lys residue at the position 9, which have been described to be present in co-purifying proteins [21], excluded the presence of such HMG1 isoforms in the intracellular as well as in the extracellular DEF protein purified from MEL cells. These results demonstrate that following induction with HMBA, DEF is rapidly released from the intracellular compartment of MEL cells and progressively accumulated in the culture medium, thus suggesting an extracellular localization as the site of expression of the DEF function.

3.4. Decay of DEF mRNA during cell differentiation

To gain insight into the function of DEF and specifically to tentatively verify if its stimulatory effect could be related to the completion of the entire differentiation process, we have evaluated the level of DEF mRNA during the early times of MEL

cells differentiation. As shown in Fig. 5, the level of DEF mRNA decreases, following the addition of HMBA, and after 4 h is 3-fold reduced with respect to the original value. This result could be preliminarly interpreted as an indication for the requirement for DEF limited to the early steps of cell differentiation.

4. Discussion

We have previously reported that MEL cells contain a 29 kDa protein, which we named DEF, that, upon addition to the culture medium, increases 3–4-fold the rate of chemically induced differentiation of these cells [7]. Structural studies have indicated that the amino acid sequence of this protein is identical to that of a mouse HMG1 protein [19], designated as 'amphoterin' due to characteristic distribution of charged amino acids [10]. A number of biological functions have been suggested for HMG1 protein, mostly derived from its proposed nuclear localization [11]. It has been reported that HMG1 protein binds to DNA, thereby modifying, through a calcium dependent mechanism, its structural organization [22]. However, the peculiar highly charged nature of HMG1 protein may also suggest an association with other cell components such as the internal surface of plasma membranes, an hypothesis that at present has not yet been tested. Of particular interest is the observation that in N18 neuroblastoma cells the functional localization of this protein seems to occur at the level of the external surface of plasma membranes or of the extracellular matrix, where it is involved in neurite outgrowth [10]. To provide an unequivocal identification and characterization of this protein factor from MEL cells, in the present study we have explored the functional properties of the recombinant protein obtained by the expression in *E. coli* of the DEF cDNA obtained from MEL cells. The fusion protein was shown to be equally efficient, as the native protein, in stimulating α-PKC activity (data not shown) and in accelerating the rate of HMBA induced MEL cell differentiation. Likewise amphoterin, DEF seems to be functionally dependent from an extracellular localization as suggested by the observation that its activity is expressed following addition to the MEL cell culture medium. In the present paper, we provide additional evidence to this hypothesis by the direct demonstration that DEF activity, detectable in the culture medium, following stimulation of MEL cells with HMBA, is the result of a secretory process. In fact, it has been found that the extracellular accumulation of DEF is paralleled by a corresponding decrease in its intracellular level. Furthermore, amino acid sequencing of secreted DEF revealed a complete identity of this protein with DEF prepared from the soluble fraction of MEL cell, and with brain amphoterin [10]. In conclusion, the present data not only suggest a novel biological function for HMG1 type proteins but emphasize, with respect to MEL cell erythroid differentiation, the significant role of a differentiation enhancing factor (DEF) that is expressed and retained in the intracellular compartment and is rapidly secreted to become active when these cells are stimulated by the inducer. Further experiments are in progress to explore the site of action of DEF in MEL cells with special emphasis to PKC activation and decay, as suggested by the well documented stimulatory effect of this factor on α-PKC activity [8,9].

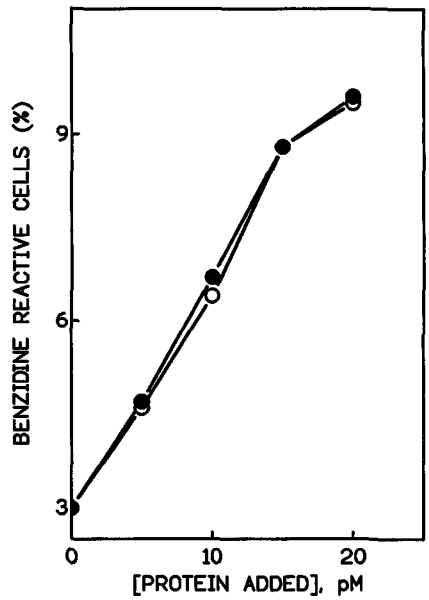

Fig. 4. Efficiency of DEF obtained from intracellular and extracellular MEL cell sources. DEF was purified from the MEL cell soluble fraction of unstimulated cells (●) or from the serum-free medium obtained after 4 h of MEL cells stimulation with 5 mM HMBA (○), as specified in section 2. The indicated amounts of purified protein were used to assay DEF activity as reported in section 2.

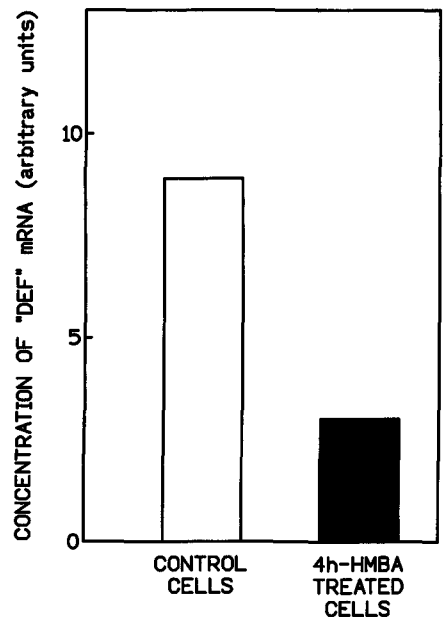

Fig. 5. DEF mRNA in resting and HMBA stimulated MEL cells. C44 MEL cells (7×10^5 cells/ml) were incubated for 4 h at 37°C in complete culture medium in the absence or in the presence of 5 mM HMBA. Total mRNA was then isolated and submitted to Northern blot analysis as reported in section 2 using ^{32}P-labelled cDNA probes for HMG1 and β-actin. Radioactivity associated with the β-actin band was evaluated to normalize the amount of mRNA used for both samples.

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