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A 6 kDa protein homologous to the N-terminus of the HMG1 protein promoting stimulation of murine erythroleukemia cell differentiation

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Abstract **Murine erythroleukemia (MEL) cells, in addition to an mRNA coding for a 30 kDa high mobility group (HMG)-1 protein, contain an mRNA coding for a 6 kDa HMG1 protein having the following structural properties: (1) its primary structure has 90% homology with the N-terminal sequence of the 30 kDa HMG1 protein; (2) it contains a consensus region of the HMG1 protein family; (3) it is deprived of the cluster of acidic amino acids that characterizes the C-terminal region of the 30 kDa HMG1 protein. This novel small M_r HMG1 protein has been expressed in prokaryotic cells and tested to establish similarities and differences in activity compared to the homologous higher M_r HMG1 protein. It has been found that the low M_r HMG1 form is not released from MEL cells following induction to erythroid differentiation, but is still effective, although with much less efficiency, when added to the external medium, in promoting acceleration in the rate of MEL cell differentiation as well as in activation of α-protein kinase C. Altogether these results provide evidence for the presence in MEL cells of a multigene family that encodes at least two different HMG1-type sequences most presumably involved, at distinct cellular sites, in different functions although commonly related to the promotion of cell differentiation. Additional information can be considered concerning the relationship between the characteristic N-terminal sequence of HMG1 protein and the extracellular activity on MEL cell differentiation.**

Key words: Murine erythroleukemia cell differentiation; HMG1 protein type; Protein kinase C stimulation

1. Introduction

MEL cells are known to contain a HMG1 protein [1], belonging to the high mobility group protein family, due to its fast electrophoretic migration at acidic pH [2]. HMG1 protein has recently been shown to be a crucial factor in the promotion of MEL cell differentiation induced by hexamethylenebisacetamide (HMBA) [3]. The protein is expressed in undifferentiated growing MEL cells and remains located in the intracellular space [4]. Upon addition of the inducer, and consistently before the onset of cell differentiation, the protein factor is released into the extracellular compartment from where it delivers its stimulatory activity on erythroid differentiation [5], a conclusion strongly supported by the observation that the effect of HMG1 protein can be observed upon its addition to the cell medium [5]. These findings have been interpreted as indicative of an additional function of HMG1 type protein, which has been also shown to be a potent activator of α-protein kinase C (PKC) isozyme [6]. In the course of studies designed to establish the cDNA sequence of MEL cell HMG1 protein, we have identified, in addition to the mRNA coding for the 30 kDa HMG1 [7], a second highly homologous transcript, with a stop codon at base position 234, thus coding for a protein composed of 54 amino acids. This protein is characterized by a primary structure having more than 90% homology with the N-terminal region of the 30 kDa HMG1 protein and contains the HMG1/2 consensus sequence [8], a property on the basis of which it has been defined by us as 'low M_r HMG1 protein'.

In spite of the significant differences in molecular structure also the low M_r HMG1 protein has a stimulatory effect on MEL cell differentiation although only a third as efficient as that promoted by the 30 kDa HMG1 protein, suggesting that the N-terminal region of this class of proteins is involved in promoting MEL cells differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

N23 and C44 MEL cell clones [9] were cultured in α-MEM containing 10% fetal calf serum as previously described [10]. N23 MEL cells (10^5 cells/ml) were induced to differentiate by addition of 5 mM HMBA. After 72 h the amount of differentiated cells was measured by staining with benzidine [11]. When indicated the induction mixture also contained amounts of different HMG-type protein.

2.2. Polymerase chain reaction (PCR)

Total RNA was obtained from C44 MEL cells by extraction with guanidium thiocyanate [12] and 5 μg was reverse transcribed using a Moloney murine leukemia virus reverse transcriptase and an oligo$(dT)_{15}$ as a primer. The oligonucleotide primers used for the amplification reaction were based on the sequence of cDNA for mouse HMG1 [7]. The sense primer was 5′-TAGGATCCCTGTGCCTCGCGGAGGAAAATC (nucleotides 43–64) and contained a *Bam*HI restriction site at the 5′ end; the antisense primer was 5′-GCGAATTCGCGCTAGAATCAACTTATTCATC (nucleotides 712–734) and contained an *Eco*RI restriction site at the 5′ end. PCR was carried out using AmpliTaq DNA polymerase (Perkin Elmer), according to the manufacturer's instructions. Thirty cycles of denaturation (60 s at 94°C), annealing (60 s at 60°C) and extension (60 s at 72°C) were carried out on a Perkin Elmer Thermal Cycler.

2.3. Sequencing of cDNA

The products of PCR, obtained as described above, consisted of a fragment of approximately 700 bp which was cloned into a pUC18 plasmid vector (Boehringer Mannheim). *E. coli* TOP 10 F′ cells were then transfected by electroporation (Bio-Rad Gene Pulser) and recombinant plasmids were sequenced in both directions with the Sequenase 2.0 kit (U.S. Biochemical Corp.). A cDNA sequence identical with that named by us 'low M_r HMG1' was also found starting from total mRNA isolated from the N23 MEL cell clone.

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Abbreviations: MEL, murine erythroleukemia; HMG1, high mobility group 1; HMBA, hexamethylenebisacetamide; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; α-PKC, α-protein kinase C

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2.4. Expression of low M_r HMG1 protein

The cDNA fragment coding for the low M_r HMG1 was ligated into *Eco*RI/*Bam*HI cut pGEX2T expression vector (Pharmacia LKB Biotechnology). TOP 10 F′ cells were transfected as above and recombinant plasmids were sequenced to establish the in-frame fusion with the coding region of the GST cDNA. As controls, cells were transfected using intact pGEX2T vector to express recombinant glutathione *S*-transferase (GST). Expression and purification of recombinant proteins was then carried out as indicated by the manufacturer.

2.5. Purification of HMG1 native and recombinant proteins

The soluble fraction of C44 MEL cells was used as source of native 30 kDa HMG1 protein. The protein was purified to homogeneity following the procedure previously described [3]. Recombinant low M_r HMG1 protein was obtained by digestion of the GST-low M_r HMG1 fusion protein with thrombin [13]. The low M_r HMG1 protein was then purified by affinity chromatography on a heparin-immobiliszd column as previously described [3].

2.6. Purification and assay of α-PKC activity

α-PKC was isolated from MEL cells [9] and assayed as previously specified [14]. α-PKC stimulating activity of different protein preparations was evaluated as specified elsewhere [3].

3. Results

3.1. Characterization of a low M_r HMG1 protein

In the course of experiments designed to amplify HMG1 cDNA obtained from MEL cell mRNA, a second cDNA molecule, with 92% homology in nucleotide sequence, was identified. The more conserved region appears to be the 5′ end, in which only few substitutions are observed (Fig. 1). The protein coding region of this newly identified cDNA begins at position 73 and stops at position 234, thus corresponding to a protein containing 54 amino acid residues with a primary structure having almost 90% homology with the N-terminal region of the mouse HMG1 protein (Fig. 1). Thus this resulting low M_r HMG1 protein appears to contain approximately two thirds of the first HMG box DNA binding motif [15], and, at the C-terminus, the HMG1/2 consensus sequence [8].

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Low Mr HMG1     AACTAAACATGGGCAAAGGAGATCCTAAGAAGCCGAGAGGCAAA
                          M  G  K  G  D  P  K  K  P  R  G  K
MEL cell HMG1   AACTAAACATGGGCAAAGGAGATCCTAAGAAGCCGAGAGGCAAA108
                          M  G  K  G  D  P  K  K  P  R  G  K

Low Mr HMG1     ATGGCCTCATATGCATTTTTTGTGCAAACCTGCCCGGAGGAGCTC
                 M  A  S  Y  A  F  F  V  Q  T  C  P  E  E  L
MEL cell HMG1   ATGTCCTCATATGCATTCTTTGTGCAAACTTGCCGGGAGGAGCAC153
                 M  S  S  Y  A  F  F  V  Q  T  C  R  E  E  H

Low Mr HMG1     AAGAAGAAGCACCCGGATGCTTCTGTCAACTTCTCAGAGTTCTCC
                 K  K  K  H  P  D  A  S  V  N  F  S  E  F  S
MEL cell HMG1   AAGAAGAAGCACCCGGATGCTTCTGTCAACTTCTCAGAGTTCTCC198
                 K  K  K  H  P  D  A  S  V  N  F  S  E  F  S

Low Mr HMG1     AAGAAGTGCTCAGAGAGGTGGAAGACCATTTCTGCTTAAGAAAAG
                 K  K  C  S  E  R  W  K  T  I  S  A
MEL cell HMG1   AAGAAGTGCTCAGAGAGGTGGAAGACCATGTCTGCTAAAGAAAAG243
                 K  K  C  S  E  R  W  K  T  M  S  A  K  E  K

Low Mr HMG1     GGGAAATTTGAAGATATGGGAAAGGCTGACAAGTCTCGTTATGAA
MEL cell HMG1   GGGAAATTTGAAGATATGGCAAAGGCTGACAAGGCTCGTTATGAA298

Low Mr HMG1     AGA-AAATGAAAACCTACATTCTCCACAAATGGGGAGACCAAAAA
MEL cell HMG1   AGAGAAATGAAAACCTACATCCCCCCCAAA-GGGGAGACCAAAAA332

Low Mr HMG1     GAAGTTCAAGGACCCCAATGCACCCAAAAGGCCT-------CCTT
MEL cell HMG1   GAAGTTCAAGGACCCCAATGCACCCAAGAGCCCTCCTTCGGCCTT377

Low Mr HMG1     CTTCTTGTTCTGTTCTGAGTACCGCCCCAAAATCA
MEL cell HMG1   CTTCTTGTTCTGTTCTGAGTACCGCCCCAAAATCA412
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Fig. 1. Alignment of cDNA and amino acid sequences of MEL cell HMG1 and low M_r HMG1. The two nucleotide sequences, listed in the 5′ to 3′ direction, were obtained from cDNA prepared starting from C44 or N23 MEL cell mRNA. The translation start codon in both cDNA molecules and the stop codon in the low M_r HMG1 cDNA are underlined. Other nucleotide or amino acid differences are also underlined in the low M_r HMG1 sequence. Only the first 57 amino acids of the HMG1 protein are reported for comparison with the low M_r HMG1 protein.

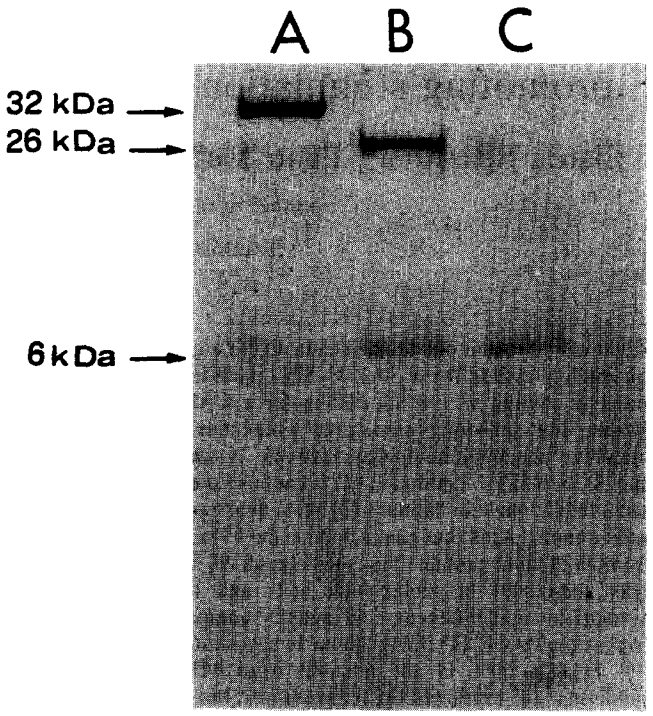

Fig. 2. Isolation of the low M_r HMG1 protein expressed in *E. coli*. Low M_r HMG1 protein was expressed and isolated from transfected *E. coli* cells as a fusion protein with GST (see Section 2). The fusion protein was then cleaved by thrombin and the 6 kDa (low M_r HMG1) protein was purified by chromatography on a heparin-immobilized column (see section 2). Samples of purified fusion protein (A), thrombin digested fusion protein (B), and isolated low M_r HMG1 protein (C) were subjected to SDS-PAGE [17] on a 12% polyacrylamide slab gel. The arrows indicate the mass of each protein band as calculated from the migration of standard proteins.

3.2. Effect of low M_r HMG1 protein on MEL cell differentiation and on α-PKC activity

In order to establish if this low M_r HMG1 protein has functional properties identical with those of the 30 kDa HMG1 protein, we first expressed this polypeptide in *E. coli* cells as a fusion protein with GST. Following purification, the fusion protein was cleaved with thrombin and the low M_r HMG1 protein isolated by affinity chromatography. The resulting protein preparation contains a single band in SDS-PAGE with a migration corresponding to a molecular mass of approximately 6 kDa (Fig. 2).

Both the fusion protein and the purified low M_r HMG1 protein were then tested for their effect on MEL cell differentiation. As shown in Fig. 3, addition of increasing amounts of GST-low M_r HMG1 fusion protein to the extracellular medium of low responding MEL cells, induced with HMBA, produces a progressive increase in the accumulation of differentiated cells. Maximal effects are observed at approximately 1 nM protein concentration. A similar effect is

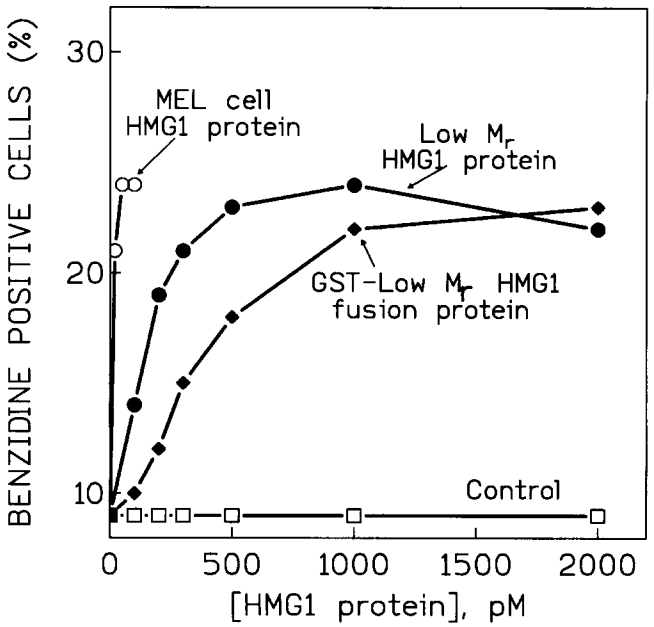

Fig. 3. Effect of low M_r HMG1 protein on the rate of differentiation of MEL cells. N23 MEL cells were induced with 5 mM HMBA in the presence of the indicated amounts of purified HMG1-type proteins or, as control, in the presence of recombinant GST. After 72 h of cell culture the percentage of benzidine positive cells was determined.

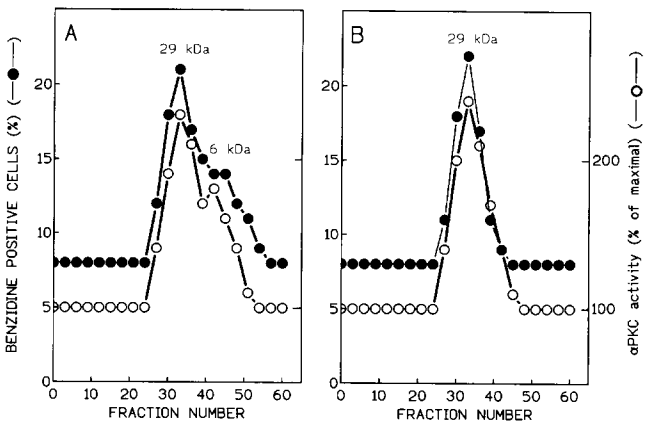

Fig. 5. Localization of the low M_r HMG1 protein during MEL cell differentiation. C44 MEL cells (2×10^8 cells) were suspended in 10 ml of serum-free culture medium containing 5 mM HMBA. Following 4 h of culture the extracellular medium and the cell soluble fraction, prepared as reported in [3], were chromatographed onto a heparin-immobilized column [3]. The peaks of eluted proteins were loaded onto a Superose 12 column (1×35 cm) equilibrated in 50 mM sodium phosphate buffer, pH 7.0. The flow rate was 0.3 ml/min and fractions of 0.3 ml were collected. Aliquots of the eluted fractions (10 μl) were assayed for their effect on α-PKC activity. Alternatively, 0.1 μl aliquots of the same fractions were used to assay their effect on MEL cell differentiation. (A) Cell-soluble fraction. (B) Extracellular medium.

also exerted by the isolated low M_r HMG1 protein, with a 2–3 times greater efficiency. As controls, cells were induced in the presence of corresponding concentrations of recombinant GST; this was totally ineffective. Thus also the low M_r HMG1 protein is capable of stimulating MEL cell differentiation although with an efficiency approximately 10-fold lower than that expressed by the complete HMG1 protein (see Fig. 3).

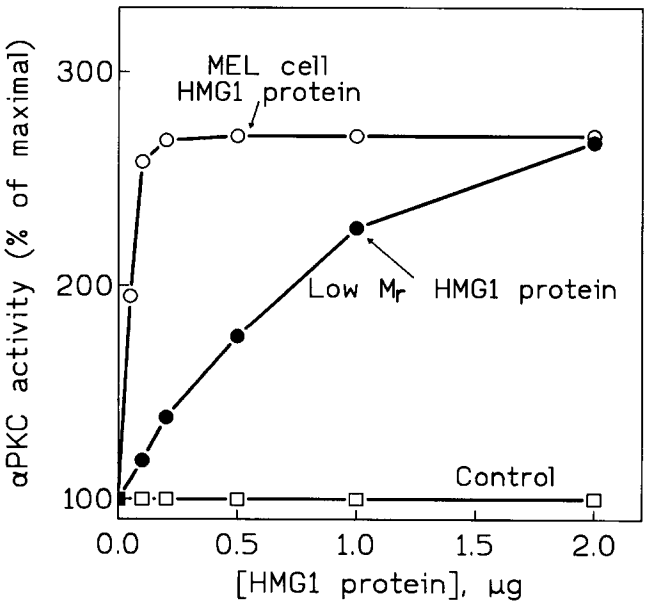

Fig. 4. Efficiency of low M_r HMG1 protein in stimulation of α-PKC activity. α-PKC was assayed in a complete assay mixture in the presence of the indicated amounts of purified HMG1-type proteins or, as control, in the presence of recombinant GST.

These data indicate that the single HMG box fragment present in this low M_r HMG1 protein contains all the information necessary for the stimulatory effect on MEL cell differentiation. This assumption has been verified by the finding that concomitant addition of sub-optimal concentrations of the two proteins, 30 kDa HMG1 and 6 kDa HMG1, to HMBA-stimulated N23 MEL cells promotes maximal stimulation in the rate of differentiation (data not shown).

The low M_r HMG1 protein causes a progressive increase in the phosphorylating activity of purified MEL cell α-PKC (Fig. 4) with a maximal effect at approximately 2 μM protein concentration; its activating effect is, however, 3–4-fold less efficient than that induced by the HMG1 protein.

3.3. Localization of low M_r HMG1 protein in MEL cells

We have previously shown that, upon exposure of MEL cells to HMBA, HMG1 protein is released into the external medium and seems to be active in this extracellular localization. We have investigated if the low M_r HMG1 protein undergoes a similar release in response to the chemical inducer. The results reported in Fig. 5 indicate that the low M_r HMG1 protein is almost completely retained in the cell (Fig. 5A) and does not appear in the extracellular medium (Fig. 5B), following 4 h of incubation of MEL cells with HMBA, a time at which more than 60% of HMG1 protein is released.

Although this result can be interpreted on the basis of the profound differences in the molecular structure of the two proteins, and related to the expression of a different function in a different cell localization, it must also be considered that these proteins contain an identical specific domain and that both, following addition to MEL cell medium, promote an increased rate of differentiation.

4. Discussion

In previous papers, we have demonstrated that HMG1 protein is released by chemically induced MEL cells and that, in this extracellular localization, the protein is effective in promoting an acceleration of the differentiation process [5]. HMG1 protein has an unusual molecular structure, composed of two basic domains, indicated as DNA interacting regions, and a third one containing almost exclusively aspartic and glutamic acid residues [7]. To identify the protein region active in the stimulation of MEL cell differentiation, we have expressed in prokaryotic cells a cDNA coding for a protein showing a high degree of homology to the first 54 amino acid residues of the HMG1 protein. This cDNA was prepared from total mRNA isolated from MEL cells. The corresponding protein contains the consensus region common to the HMG1/2 protein family [8] and approximately two thirds of the amino acid residues forming the first HMG box [16] present in the HMG1 protein. The purified recombinant protein is shown to be active, when added to the external medium, in stimulating MEL cell differentiation as well as in activating α-PKC catalytic activity. These two properties are typical of the HMG1 protein [3], although the low M_r HMG1 protein is less efficient. These data suggest that the first HMG box of the HMG1 protein contains not only a site acting as a PKC activity enhancer, but also a sequence that carries sufficient molecular information for the stimulation of MEL cell differentiation. This conclusion is further supported by the additive enhancing effects on MEL cell differentiation found following concomitant addition of both low M_r and 30 kDa HMG1 proteins.

Finally, in spite of these similarities, the low M_r HMG1 protein is not released into the extracellular medium during the early stages of MEL cell differentiation. It can only be suggested that in MEL cells two related HMG1-type proteins, belonging to a multigene protein family [18], due to their molecular composition are localized at different cell sites, although both are involved in the multistep highly coordinated process of cell differentiation.

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