Secretion and binding of HMG1 protein to the external surface of the membrane are required for murine erythroleukemia cell differentiation

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Abstract We show here that murine erythroleukemia (MEL) cells, following induction with hexamethylene bisacetamide, accumulate high mobility group (HMG)1 protein onto the external surface of the cell in a membrane-associated form detectable by immunostaining with a specific anti-HMG1 protein antibody. This association is maximal at a time corresponding to cell commitment. At longer times, immunostainable cells are progressively reduced and become almost completely undetectable along with the appearance of hemoglobin molecules. Binding to MEL cells does not affect the native molecular structure of HMG1 protein. The type of functional correlation between HMG1 protein and MEL cell differentiation is suggested by the observation that if an anti-HMG1 protein antibody is added at the same time of the inducer almost complete inhibition of cell differentiation is observed, whereas if the antibody is added within the time period in which cells undergo through irreversible commitment, inhibition progressively disappears. A correlation between MEL cell commitment and the biological effect of HMG1 protein can thus be consistently suggested.

Key words: HMG1 protein; Murine erythroleukemia cell; Cell differentiation

1. Introduction

The molecular mechanisms underlying the chemical induction of MEL cell differentiation, stimulated by polar-apolar chemical compounds [1,2], have not so far been identified. During the cell commitment period [3], among a well-established series of metabolic changes [4–7], it has been demonstrated that MEL cells rapidly release into the extracellular medium significant amounts of HMG1 protein [8]. The presence in the extracellular medium of the HMG1 protein is of crucial importance for MEL cell differentiation as indicated by the following observations: (i) the external addition of this protein promotes acceleration in the rate of differentiation of slow HMBA responding clones [8]; (ii) extracellular HMG1 protein accelerates the down-regulation of δPKC, a condition favouring MEL cell differentiation [9]; (iii) increasing or decreasing the rate of HMG1 protein release, and thereby affecting the concentration of the extracellular protein, results in an increase or decrease in the rate of MEL cell differentiation [10]. Reports from other laboratories have indicated that HMG1 protein, although considered on the basis of its molecular structure a DNA-binding protein [11], can perform specific biological functions, related to neurite outgrowth, when present in the extracellular compartment [12]. However, no precise information is available at present on the mechanism(s) of interaction of HMG1 protein with the cell membrane or on the type of signal transduced. Hori et al. [13] have recently reported that the receptor for advanced glycation end products (RAGE) is capable of binding HMG1 protein, although with an affinity of order higher than nanomolar, thus leaving open the problem concerning the existence of a specific HMG1 protein receptor. In this study we demonstrate, at first, that the release of HMG1 protein by induced MEL cells is due to a cell export mechanism which does not involve the ER-Golgi system and then we show that extracellular HMG1 protein, through the interaction with the external cell surface, expresses a new function crucially related to commitment of the cell to terminal erythroid differentiation. At present, several questions remain to be solved. It is in fact still unknown how this protein, lacking the signal peptide, can be exported from the cell, how the association with the cell membrane occurs, and finally if the protein undergoes internalization into the cell.

2. Materials and methods

2.1. Cell culture and differentiation

MEL cell clones characterized by a slow (N23 clone) or rapid (C44 clone) rate of differentiation were obtained and cultured as described [14]. The cells were free of mycoplasmal contamination as established by a routine assay performed on cell cultures using the Mycoplasma Detection Kit (Boehringer). If not otherwise indicated, cells were induced at a cell density of 10^5/ml by addition of 5 mM hexamethylene bisacetamide (HMBA) (Sigma Chemical Co.) to 1 ml of culture medium. At the indicated times, the percentage of differentiated cells was evaluated by staining with benzidine (Sigma Chemical Co.) [15].

2.2. Purification and assay of HMG1 protein

HMG1 protein was obtained from the soluble fraction of C44 MEL cells as described [16] and its activity was assayed as previously specified [8]. One unit of HMG1 protein is defined as the amount which doubles the proportion of benzidine reactive cells under the specified assay conditions. The specific activity of purified HMG1 protein was 2 units/ng.

2.3. Evaluation of HMG1 protein content in MEL cells and in the extracellular medium

MEL cells (5×10^6 cells) were incubated at 37°C in 10 ml of complete culture medium containing the additions reported in Section 3.1. After 4 h the cells and the culture medium were separately collected by centrifugation and the soluble cell fraction prepared as previously described [16]. Both the culture medium and the soluble cell fraction were submitted to affinity chromatography onto a 1 ml heparin-immobilized column (EconoPac, Bio-Rad) previously equilibrated in 50 mM sodium phosphate buffer, pH 7.0 [8]. The adsorbed proteins were

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Abbreviations: HMG, high mobility group; MEL, murine erythroleukemia; HMBA, hexamethylene bisacetamide; PKC, protein kinase C

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eluted with the same buffer containing 0.8 M NaCl. The peak of eluted proteins was collected and aliquots were used to assay HMG1 protein activity as reported above.

This step was introduced to concentrate large volumes of extracellular medium and to reduce the total contaminant protein present in the complete culture medium. To normalize the experimental conditions the same chromatographic procedure was carried out on the soluble cell fraction.

2.4. Preparation of the anti-HMG1 protein monoclonal antibody

The anti-HMG1 protein antibody (mAb 23.38) was produced and purified as described [10]. This antibody recognized HMG1 protein as a single band with a molecular mass of 29 kDa in Western blot analysis and did not cross-react with other proteins of MEL cell lysate.

2.5. Fluorescence microscopy

MEL cells (3×10^6 cells) were fixed with 4% paraformaldehyde for 30 min, washed three times with 1 ml of PBS buffer, pH 7.4 and suspended in 0.1 ml of the same buffer. Non-specific binding sites were blocked with 2% normal goat serum. The fixed cells were incubated for 18 h at 4°C with 10 μg/ml of purified anti-HMG1 protein mAb 23.38. Cells were then washed as above and suspended in 50 μl of PBS buffer, pH 7.4, containing 28 μg/ml of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories). After 1 h at 20°C in the dark, cells were washed three times as above, suspended in 150 μl of PBS buffer, pH 7.4 and analyzed with a Zeiss microscope (model IM35) equipped for epifluorescence with appropriate filters to detect specifically the fluorescein isothiocyanate. A 40× objective (Zeiss) was used in all figures shown. As control, experiments in which the anti-HMG1 protein mAb was omitted from the staining protocol were carried out in parallel; in this case no fluorescent cells were detectable.

2.6. Labelling of HMG1 protein with $Na^{125}I$

Purified HMG1 protein (10 μg) was radiolabeled employing the chloramine T method [17] using 0.5 mCi of carrier-free $Na^{125}I$ (Amersham). ^{125}I-HMG1 protein was separated from unreacted ^{125}I by gel chromatography onto a Sephadex G-25 (PD 10) column previously equilibrated in PBS buffer, pH 7.4. The specific activity of the eluted protein was about 3.7×10^7 cpm/μg and its specific activity on stimulating MEL cell differentiation was 1.8 units/ng. The radioactivity associated with HMG1 protein was 95% precipitable in 20% trichloroacetic acid and migrated as a single band of 29 kDa on SDS-PAGE carried out in the presence of 2-mercaptoethanol [18].

2.7. Evaluation of ^{125}I-HMG1 protein binding to MEL cells

MEL cells were suspended at a concentration of 10^7 cells/ml in complete culture medium containing 170 pM ^{125}I-HMG1 protein and were induced by addition of 5 mM HMBA. At the times indicated in Section 3.3, 3 ml of the cell suspension were collected, and layered on the top of a discontinuous gradient of Ficoll 400 (Pharmacia) containing 8 ml of 3% (w:v) Ficoll solution in the upper layer and 1 ml of 9% (w:v) Ficoll solution in the lower layer. After 20 min of centrifugation at $1000\times g$ the cell layer at the interphase between the 9 and 3% Ficoll steps was collected and the cells diluted at 1 ml in PBS buffer, pH 7.4 containing 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. The cells were then counted, lysed by sonication and the cytosolic and the membrane fractions were obtained as described [19]. Aliquots of cell soluble and membrane fractions, corresponding to 3×10^6 cells, were subjected to SDS-PAGE on a 12% polyacrylamide slab gel in the presence of 2% 2-mercaptoethanol.

3. Results

3.1. Characterization of the HMG1 protein secretion process from MEL cell

To define the possible mechanism of secretion of HMG1 protein, we have treated MEL cells with compounds known to affect at different levels the protein release. As shown in Table 1, untreated MEL cells release small amounts of HMG1 protein corresponding to approx. 2% of the total. This amount of extracellular HMG1 protein activity is likely due to lysis of dead cells during the incubation time and corresponds to the percentage of release of soluble marker enzymes, such as aldolase. Treatment with HMBA produces considerable release of HMG1 protein corresponding to approx. 60 and 25% of the total, for C44 and N23 cells respectively. No increase in the level of the cytosolic marker enzyme in the culture medium is observed, indicating that the release of HMG1 protein is not due to aspecific cell damage. Addition of methylamine to HMBA stimulated MEL cells promotes 35% inhibition of HMG1 protein export. Similar results (43% inhibition) are obtained by lowering the temperature of incubation of HMBA stimulated MEL cells to 16°C. Monensin, known to affect the protein traffic between the ER and Golgi complex [20], is ineffective on HMG1 protein secretion by induced MEL cells. However, treatment of MEL cells with the calcium ionophore A23187, added to the culture medium in the absence of HMBA, promotes the release of HMG1 protein in an amount comparable to that recovered after cell treatment with HMBA alone. Exposure of MEL cells to the tumor promoter phorbol 12-myristate 13-acetate results in release of HMG1 protein slightly higher than that induced by HMBA alone.

Taken together, these results indicate that the secretion of HMG1 protein is a process in which the ER or Golgi complex do not seem to be involved, and is stimulated by intracellular increase in Ca^{2+} concentration and possibly by the activation of a Ca^{2+}-dependent PKC isoform.

Table 1
Effect of drugs or treatments on the release of HMG1 protein from MEL cells

Treatment	C44 clone		N23 clone	
	HMG1 protein (% of total)	Aldolase (% of total)	HMG1 protein (% of total)	Aldolase (% of total)
None	2	1.6	2	2.0
HMBA, 5mM	61	2.8	25	4.5
HMBA, 5 mM+methylamine, 5 mM	40	2.1	15	2.8
HMBA, 5 mM+low temperature (16°C)	35	1.8	13	2.8
HMBA, 5 mM+monensin, 10 μM	59	2.5	26	3.2
A23187, 1 μM	54	2.5	21	4.0
Phorbol myristate acetate, 100 ng/ml	75	3.0	37	5.6

MEL cells (5×10^6 cells) were incubated in 10 ml of complete culture medium containing the indicated additions. Following incubation for 4 h at 37°C, the culture medium and the soluble cell fractions were obtained as specified under Section 2. To assay HMG1 protein activity, the two solutions were chromatographed onto a heparin-immobilized column and the peak of proteins eluted with 0.8 M NaCl was used. The aldolase activity was assayed on the culture medium and soluble cell fraction as described by Gracy et al. [28]. As 100% was considered the amount of activity of both HMG1 protein or aldolase present in the soluble fraction of the same quantity of untreated cells.

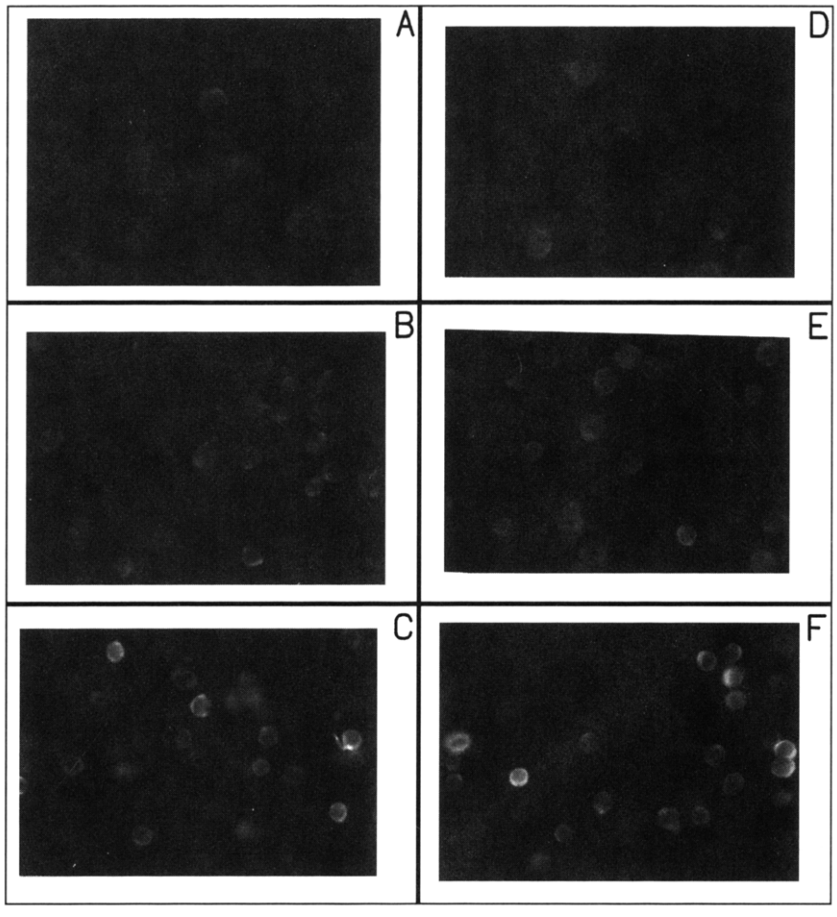

Fig. 1. Immunoreactivity of intact MEL cells with the anti-HMG1 protein mAb 23.38. Uninduced N23 (A) and C44 (D) cells or HMBA induced cells were fixed and treated with anti-HMG1 protein mAb 23.38 as primary antibody and fluorescein isothiocyanate-conjugated goat anti-mouse Ig as secondary antibody as reported under Section 2. The cells were then analyzed with a Zeiss microscope as specified in Section 2. B and C refer to the N23 clone, after 4 and 16 h of exposure to HMBA, respectively. E and F refer to the C44 clone, after 2 and 4 h of exposure to HMBA, respectively.

3.2. *Binding of HMG1 protein to intact cells*

To reveal if HMG1 protein, once released, is taken up by MEL cells, we evaluated the presence of the protein factor on the external surface of cells using immunostaining with an anti-HMG1 protein monoclonal antibody. As shown in Fig. 1, both N23 (A) and C44 (D) MEL cells are almost completely unstainable in the absence of HMBA. Following exposure to the inducer, cells become progressively immunofluorescent, indicating the presence of HMG1 protein on their surface (Fig. 1B, N23 cells after 4 h; C, N23 cells after 14 h; E, C44 cells after 2 h; F, C44 cells after 4 h). The kinetics of immunostaining are reported in Fig. 2. With C44 clone, cells become immunofluorescent very rapidly, and after 3–4 h of exposure to HMBA, approx. 70% of the cells are recognized by the anti-HMG1 protein antibody. After this time the number of immunopositive cells decreases and, at 16 h of incubation, all cells are insensitive to the antibody. MEL cell clone N23 shows different kinetics, reaching a maximum (45%) of immunostainable cells at 14–16 h of incubation; thereafter a decline in the number of positive cells is observed and at 48 h only few cells are immunofluorescent. It is important to note that the induced cells of both MEL cell clones become insensitive to HMG1 protein once they have reached the fully irreversible committed state and initiate to synthesize hemoglobin [14,21].

3.3. *Binding of ^{125}I-labelled HMG1 protein to MEL cell membranes*

The binding of HMG1 protein to the external surface of induced MEL cells was further confirmed by experiments in which radiolabelled HMG1 protein was added to the culture medium of C44 MEL cells together with the chemical inducer HMBA and, after removal of unbound ^{125}I-HMG1 protein, the membrane and soluble cell fraction were separately analyzed for the presence of ^{125}I-HMG1 protein. As shown in Fig. 3, the radiolabelled protein was found almost exclusively bound to the membrane fraction of HMBA induced cells and only 1–2% of the total radioactivity was recovered in the soluble cell fraction. The kinetics of binding, including the time required to reach maximal association (4 h), were found to be almost identical to that observed (see Fig. 2) in experiments in which the binding of HMG1 protein was evaluated on the C44 clone by immunostaining of intact cells with the anti-HMG1 protein mAb 23.38. At all times, the radioactive material analyzed by SDS-PAGE (see top of Fig. 3) was recovered as a single band with a molecular mass of 29 kDa, identical to that of the native HMG1 protein.

These results support the concept that binding of HMG1 protein occurs only in HMBA induced cells, and is not accompanied by modification of the molecular structure of the protein. Thus, the native HMG1 protein, once associated with

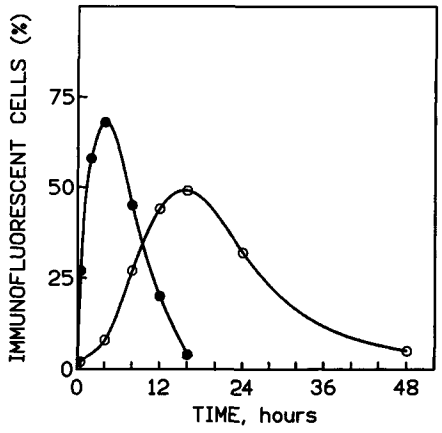

Fig. 2. Kinetics of binding of HMG1 protein to the external surface of MEL cell clones. Binding of HMG1 protein to C44 (●) and N23 (○) MEL cells was detected by immunostaining with the anti-HMG1 protein mAb 23.38, as described in the legend to Fig. 1 and in Section 2. At the indicated times, the percentage of immunofluorescent cells was evaluated by counting a total of 10^3 cells.

MEL cell membranes, can express without further modification its stimulatory activity on MEL cell differentiation.

3.4. Timing of HMG1 protein action

In order to identify the time at which HMG1 protein exerts its extracellular function, C44 cells were induced with HMBA and the anti-HMG1 protein mAb 23.38 was subsequently added at different time intervals. As shown in Fig. 4, when the antibody was added at the same time as the inducer, almost complete inhibition of the MEL cell differentiation process was observed. When the antibody was added at a time corresponding to the maximal accumulation of HMG1 protein on the external surface (4 h) or at longer times, when full cell commitment is already reached (approx. 8 h), the inhibitory efficiency of the antibody was profoundly reduced or completely abolished, respectively. This result indicates that the effect of HMG1 protein is confined to the early phase of MEL cell differentiation and correlated to the onset of cell commitment.

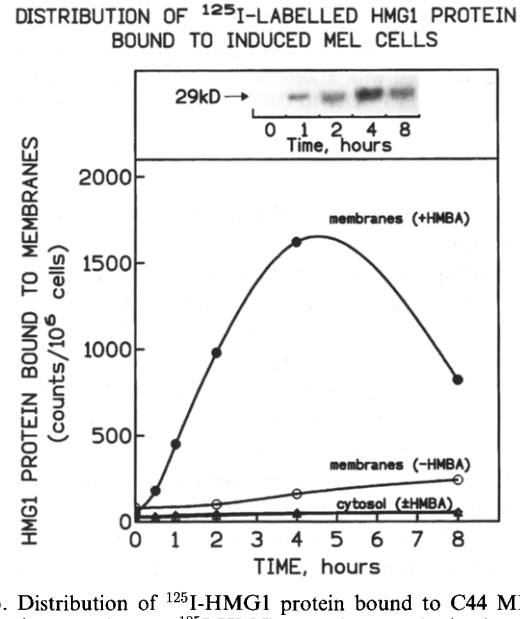

Fig. 3. Distribution of ^{125}I-HMG1 protein bound to C44 MEL cell during the commitment. ^{125}I-HMG1 protein was obtained and incubated with C44 MEL cells in the presence or absence of 5 mM HMBA as reported in Section 2. At the indicated times, aliquots of the cell suspension, containing 3×10^7 cells, were collected and loaded on a Ficoll gradient, to remove unbound labelled HMG1 protein. One tenth of the cells were then utilized for the preparation of the soluble and membrane fractions (see Section 2). The subcellular fractions were analyzed on SDS-PAGE. The dried slab gels were exposed to X-ray film for autoradiography, the radioactive protein bands were excised from the gel and the associated radioactivity determined using a γ-counter. The inset shows the autoradiogram obtained with the membrane cell fraction, following induction for the indicated times.

4. Discussion

In recent years, several data have been obtained demonstrating that HMG1 protein is an intracellular protein expressed by MEL cells and released into the culture medium following cell stimulation with the inducer HMBA [8]. The data presented in this paper are intended to provide a plausible understanding of the mechanism involved in this release since HMG1 protein lacks the typical hydrophobic secretory signal [22]. It has been preliminarily established that the export of this protein cannot be ascribed to aspecific leakage. Moreover, it has been observed that HMBA induced export of HMG1 protein is inhibited by methylamine or by lowering the temperature of incubation [23,24], but is unaffected by the addition of monensin [20]. Thus, it can be postulated that in MEL cells the mechanism for the release of this protein does not involve the ER-Golgi system.

Extracellular release of HMG1 protein in significant amounts by intact uninduced MEL cells has been reproduced by cell treatment with A23187 Ca^{2+} ionophore or with a phorbol ester. In the first case, an increase in the intracellular concentration of Ca^{2+} occurs, in the second one the implication of PKC activity can be postulated [25]. Both observations deserve additional experiments but it might be relevant to recall that variation in Ca^{2+} influx and activation of PKC have been demonstrated to be involved in the early steps of MEL cell differentiation [19,26,27]. Direct experimental evi-

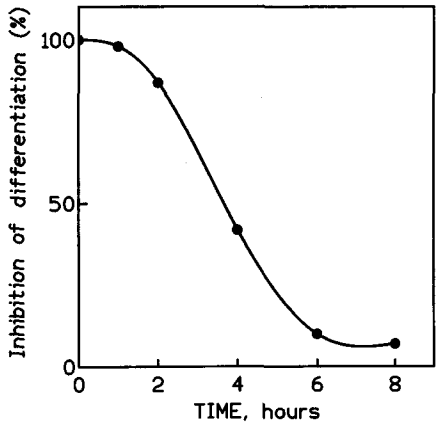

Fig. 4. Changes in the sensitivity of MEL cells to the inhibitory effect of anti-HMG1 protein mAb 23.38 during HMBA induced differentiation. C44 MEL cells were induced as reported under Section 2. At the indicated times, also the purified anti-HMG1 protein mAb 23.38 (40 μg/ml) was added to the cell culture. The number of differentiated cells was determined following 36 h of exposure to HMBA. The data are expressed as percentage of inhibition exerted by the antibody.

dence has been obtained indicating that following induction with HMBA, MEL cells secrete and accumulate HMG1 protein on the external cell surface in a membrane-associated form. The kinetics of this phenomenon are correlated with the rate of MEL cell differentiation being faster in the rapidly responding clone as compared to the slowly responding one. However, in both cases, maximum association occurs at a time that roughly corresponds to the onset of irreversible cell commitment [14].

The use of radiolabelled HMG1 protein added to the culture medium together with the inducer has provided the following important information: (1) HMG1 protein associates almost exclusively at the external cell surface; (2) the kinetics of this binding confirm that maximum association coincides with cell commitment; (3) within the time considered, and until the HMG1 protein effect is completed, its association with the cell membrane occurs without extensive modification of the native molecular structure.

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