# Extracellular processing of amphoterin generates a peptide active on erythroleukaemia cell differentiation

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The release of amphoterin by murine erythroleukaemia cells exposed to the chemical inducer hexamethylenebisacetamide represents an essential step for the process of their terminal differentiation. Once exported in the culture medium, amphoterin undergoes limited proteolysis, catalysed by a serine proteinase also secreted by stimulated cells. The isolated proteinase is responsible for degradation of amphoterin, with the production of a 10-amino-acid-residue fragment, specifically retaining the cell-differentiation-stimulating activity of the native protein molecule. This peptide does not express other properties of amphoterin, such as protein kinase C-stimulating activity or systemic toxicity. These findings define a selective mechanism accounting for extracellular amphoterin functional maturation.

Key words: PMA-induced secretion, proteolysis, serine proteinase.

## INTRODUCTION

Amphoterin, also named HMG1 protein, is a polypeptide belonging to the high-mobility group (HMG) protein family [1] and is abundantly expressed in immature and malignant cell phenotypes [2]. This protein shows an evolutionary conserved primary structure, indicating its involvement in fundamental cell functions that, despite intensive studies, have yet to be fully elucidated. The presence of two HMG box domains confers to amphoterin the ability to interact with chromatin, recognizing specific DNA structures with modulation of their architecture [3]. A gene regulation function has been also proposed [4]; however, identification of amphoterin both in the cytoplasm and on the external surface of the plasma membrane has suggested possible alternative functional roles [2]. It has been demonstrated that in glioma and neuroblastoma cells grown on laminin, amphoterin localizes peripherally, and is released in the absence of any cell damage and without entering the endoplasmic reticulum–Golgi secretory pathway [5]. A similar result has also been obtained with murine erythroleukaemia (MEL) cells, which export amphoterin using a calcium-activated mechanism and independently from the classical secretory system [6]. Extracellular amphoterin behaves as an adhesive protein factor, active in peripheral and central nervous system through a neurite outgrowth-promoting activity [7]. Recent observations [5] indicate that amphoterin, localized at the leading edge of immature and transformed cells of nerve origin, also promotes cell motility. Moreover, an inducible release of amphoterin has been measured in neonatal rat type I astrocytes [8], MEL cells [9] stimulated with chemical inducers of cell differentiation, human primary peripheral blood mononuclear cells and mouse macrophages stimulated with lipopolysaccharide [10], and in rat pituicytes treated with proinflammatory cytokines [11], indicating the presence of different amphoterin-releasing cells among normal and neoplastic mammalian phenotypes. It has been demonstrated that extracellular amphoterin represents a crucial signal molecule positively affecting the differentiation cell programme, both in nervous and erythroid cell models [8,12]. However, it has been reported that amphoterin also stimulates acute lung inflammation [13] and proinflammatory cytokine synthesis in monocytes [14], and behaves as a systemic mediator of endotoxin lethality in mice and in septic patients [10]. These results suggest the requirement for a tight control of the extracellular concentration of this protein molecule involved in multiple physiological and pathological signalling pathways.

In MEL cells the export of amphoterin is triggered by different chemical agents such as PMA, the most potent stimulator of amphoterin release, and hexamethylenebisacetamide (HMBA), an inducer of erythroid differentiation [6,12]. In the present study we demonstrate that these stimuli also promote MEL cell secretion of a proteolytic enzyme which recognizes amphoterin as a substrate and catalyses its limited proteolysis. Moreover, one of the peptides produced by this processing of amphoterin maintains the erythroid-differentiation-stimulating activity of the native protein molecule. This extracellular proteolytic activity, which reduces the concentration of native amphoterin outside cells, without a complete loss of its functional properties, represents a new component of the extracellular mechanisms involved in the control of amphoterin signalling.

## EXPERIMENTAL

### Purification and radiolabelling of amphoterin

Eukaryotic recombinant amphoterin was obtained and purified as described previously [12]. Where indicated, the purified protein was labelled with Na[$^{125}$I] [6], and its specific radioactivity was $1.5 \times 10^6$ c.p.m./$\mu$g. SDS/PAGE of amphoterin was carried out

Abbreviations used: HMBA, hexamethylenebisacetamide; HMG, high-mobility group; MEL, murine erythroleukaemia; PKC, protein kinase C; Amph(129–138), amphoterin peptide (spanning amino acid residues 129–138).

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on 10% polyacrylamide gels under reducing conditions [15]. Partial purification of extracellular amphoterin was carried out by affinity chromatography of 10 ml of conditioned medium on 1 ml heparin–agarose columns (Bio-Rad Laboratories) [8]. The adsorbed proteins were eluted with 0.8 M NaCl. The eluted proteins were combined (2 ml), aliquots of 100 $\mu$l were submitted to SDS/PAGE and Western blotting using an anti-amphoterin antipeptide antibody raised against the C-terminal acidic tail. The bound antibody was detected by incubation with an anti-rabbit secondary antibody conjugated with horseradish peroxidase followed by ECL® reaction (Amersham Pharmacia Biotech). The amount of amphoterin in each lane was evaluated by densitometric analysis of the signals using a Chemi Doc Gel Documentation System (Bio-Rad Laboratories).

### Isolation of proteolytic activities in MEL cell conditioned media

The C44 MEL cell clone was obtained and cultured as previously described [16]. The cell cultures were free of mycoplasma contamination, as assayed by a routine PCR test with a Mycoplasma Detection Kit (EuroClone, Wetherby, West Yorkshire, U.K.). Cells ($10^7$/ml) were incubated for 30 min in serum-free medium containing 10 mM Hepes buffer, pH 7.4, 5 mM KCl, 140 mM NaCl, 5 mM glucose and 100 ng/ml PMA. The conditioned medium (10 ml) was collected, concentrated to 1 ml over a 10 kDa cut-off membrane (Amicon), and again diluted to 10 ml by adding 50 mM $Na_2B_4O_7$ buffer, pH 7.5 (buffer A). These procedures and the following anion-exchange chromatography were performed at 4 °C. The conditioned medium was then loaded on to a DEAE 53 (Whatman, Maidstone, Kent, U.K.) column (1 cm × 3 cm) equilibrated in buffer A, and the adsorbed proteins were eluted with a 40 ml linear gradient of NaCl from 0–0.4 M. Fractions of 0.5 ml were collected, and the proteolytic activity was evaluated by adding 50 $\mu$l of the eluted fractions to the assay mixtures. Fractions 35–45 were collected, concentrated by ultrafiltration, and used as partially purified serine proteinase.

### Assay of proteolytic activities

The assay was performed in 0.5 ml of 50 mM buffer (sodium citrate, pH from 3.0 to 4.0; sodium acetate, pH from 5.0 to 6.0 or $Na_2B_4O_7$, pH from 7.0 to 9.0) containing 1 mg of acid-denatured globin [17] and the proteinase sample. The mixtures were incubated at 37 °C for 60 min, and the proteolytic activity was determined as described [18]. One unit of proteinase activity is defined as the amount causing the production of 1 $\mu$mol acid-soluble $NH_2$ groups/h in the specified conditions. Alternatively, the following synthetic peptides (from Sigma) were used as substrates: succinyl-Ala-Ala-Ala-*p*-nitroanilide (substrate of leucocyte elastase) [19], succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (substrate of chymotrypsin and cathepsin G) [20], benzoyl-D,L-Arg-*p*-nitroanilide (substrate of trypsin) [21] and L-Ala-Phe-Lys-*p*-nitroanilide (substrate of plasmin) [22]. The hydrolysis of these *p*-nitroanilide peptides was assayed at 37 °C in a 0.1 ml reaction mixture containing 50 mM $Na_2B_4O_7$ buffer, pH 7.5, 1.5 mM substrate, 4% DMSO and the enzyme solution. The increase in the absorbance at 405 nm was followed with a Beckman model 530 spectrophotometer for at least 1 h.

### Assay of protein kinase C (PKC) activity

Rat brain PKC was partially purified by anion-exchange chromatography and assayed as described [23].

### Production of amphoterin anti-peptide antibody

The amphoterin peptide, corresponding to the last 34 amino acid residues of amphoterin, was chemically synthesized and coupled through keyhole-limpet (*Diodora aspera*) haemocyanin (Sigma) [24]. Rabbit immunization and affinity-purification of the antibody were carried out as described in [24]. The detection limit of this anti-peptide antibody was 50 pg of native amphoterin, and 50 ng of denatured amphoterin in Western blotting performed as specified above. The specificity of this antipeptide antibody was established by its recognition of a single protein band in Western-blotting analyses carried out using total MEL cell lysates.

### Isolation and sequencing of amphoterin peptides

Recombinant amphoterin was obtained as described [12], and 0.1 mg of purified protein were incubated in 0.2 ml of buffer A, containing 0.2 unit of the partially purified serine proteinase obtained from the extracellular medium of MEL cells. After 18 h at 30 °C, the reaction was stopped by adding 2 mM PMSF and loaded on a Superdex Peptide column (Amersham Pharmacia Biotech) equilibrated in buffer A containing 0.1 M NaCl. Fractions of 50 $\mu$l were collected and 0.01 $\mu$l used to assay MEL cell differentiation stimulating activity. The active fractions were loaded on a LUNA C18 column (1 mm × 100 mm) (Phenomenex, Torrance, CA, U.S.A.) equilibrated in distilled water containing 0.15% trifluoroacetic acid. The adsorbed peptides were eluted by increasing the acetonitrile concentration to 80% with a linear gradient program. Fractions of 30 $\mu$l were collected and assayed for the presence of MEL cell-differentiation-stimulating activity as described above. The active peptide was directly applied to a Beckman LF3000 protein sequencer.

### Assay of MEL cell differentiation stimulating activity

N23 MEL cells were induced as described previously [16]. To assay MEL cell-differentiation-stimulating activity, samples of native amphoterin or amphoterin peptides were added to the induction mixture. One unit of differentiation-stimulating activity is defined as the amount which doubles the proportion of benzidine-reactive cells evaluated after 72 h from induction.

## RESULTS

MEL cells induced with HMBA, in the period preceding the acquirement of the committed phenotype, release a differentiation-stimulating protein, identified previously with the heparin-binding protein amphoterin. Experimental evidence has been collected suggesting an extracellular processing of amphoterin in MEL cell cultures: (i) as compared with the total amount of differentiation-stimulating activity, very low amounts of the native 29 kDa amphoterin molecule were recovered by purification procedures starting from the conditioned medium of stimulated MEL cells; (ii) a large fraction of this activity did not bind to immobilized-heparin columns. These data indicate an irreversible modification of amphoterin occurring following its export from differentiating cells.

To verify this hypothesis, MEL cells were exposed to the phorbol ester PMA, which produces a very rapid release of large amounts of amphoterin in the extracellular environment [6]. Both the differentiation-stimulating activity and the appearance of the native 29 kDa amphoterin were assayed. As shown in Figure 1, the level of differentiation-stimulating activity increased in the first 2 h of incubation and then it remained almost unchanged. In contrast, the extracellular amount of native

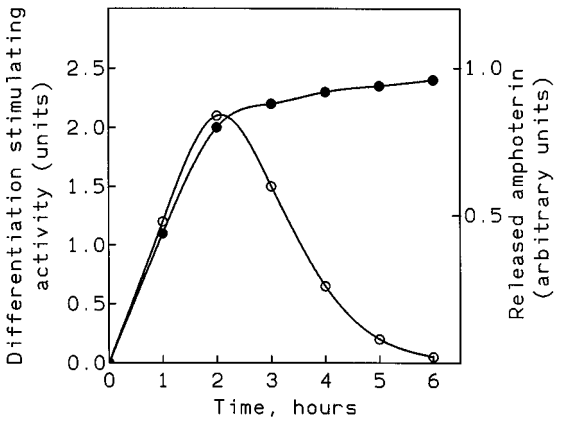


**Figure 1 Level of amphoterin and differentiation-stimulating activity in the extracellular medium of PMA-treated cells**

C44 MEL cells ($10^6$ cells/ml) were cultured in the presence of 100 ng/ml PMA. At the indicated times, 10 ml aliquots of the culture medium were collected, submitted to affinity chromatography on a heparin–agarose column, and the amount of native amphoterin (○) recovered was evaluated by Western blotting followed by ECL® and densitometric analysis of the detected signals. At the same times indicated, aliquots of the conditioned medium were assayed for the presence of MEL cell-differentiation-stimulating activity (●).

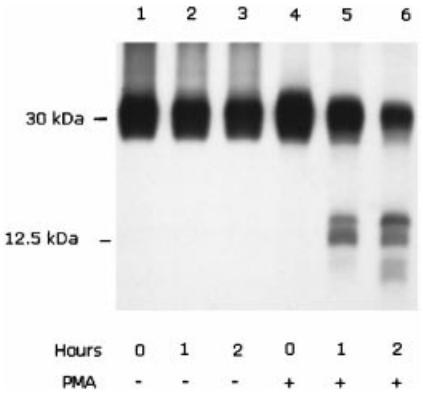


**Figure 2 Processing of amphoterin in the conditioned medium of MEL cells stimulated with PMA**

$^{125}$I-labelled amphoterin (5 $\mu$g) was added to 1 ml of a MEL cell culture containing $10^6$ cells/ml, in the presence or absence of 100 ng of PMA. At the indicated times, 30 $\mu$l of conditioned medium were submitted to SDS/PAGE and autoradiography. The radioactivity added to the cell culture did not modify cell viability, as measured by Trypan Blue staining.

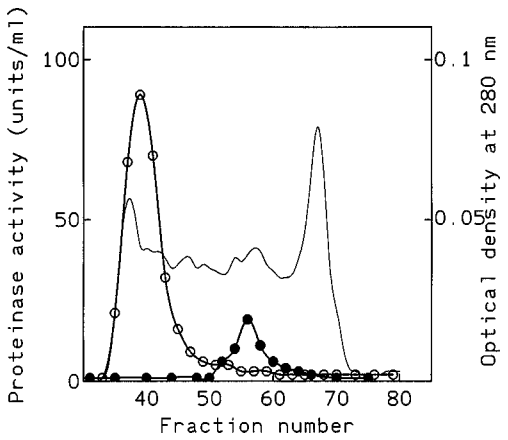


**Figure 3 Identification of proteolytic activities in the conditioned medium of PMA-stimulated cells**

MEL cells ($10^7$ cells/ml) were incubated with 100 ng/ml PMA for 30 min. The conditioned medium was collected and submitted to anion-exchange chromatography. Proteolytic activity was assayed at pH 3.0 (●) and pH 7.5 (○) using acid-denatured globin as the substrate.

**Table 1 Effect of proteinase inhibitors and metal ions on the MEL cell neutral proteinase**

Partially purified neutral proteinase was assayed using Suc-Ala-Ala-Pro-Phe-*p*-NA as a substrate. SBTI, soya-bean trypsin inhibitor.

| Addition | Concentration | Activity (% of control) |
|---|---|---|
| PMSF | 2 mM | 4 |
| Leupeptin | 0.1 mg/ml | 61 |
| Aprotinin | 0.1 mg/ml | 17 |
| SBTI | 0.1 mg/ml | 100 |
| Tos-Lys-$CH_2Cl$ | 0.1 mM | 100 |
| Tos-Phe-$CH_2Cl$ | 0.1 mM | 100 |
| EDTA or EGTA | 1 mM | 100 |
| $ZnCl_2$ | 0.01 mM | 100 |
| $CaCl_2$ | 1 mM | 100 |
| $MgCl_2$ | 5 mM | 98 |
| $MnCl_2$ | 5 mM | 67 |
| NaCl | 50 mM | 100 |

29 kDa amphoterin reached a maximum after 2 h, after that it was reduced within 6 h to undetectable amounts. These data suggest that, following the release in the culture medium of PMA-treated cells, the protein molecule undergoes structural modification which do not abolish its signalling properties. To evaluate the molecular changes of extracellular amphoterin in stimulated cells, $^{125}$I-labelled amphoterin was added to a MEL cell culture in the presence of PMA. Aliquots of the conditioned medium were submitted to SDS/PAGE and the radiolabelled protein bands were detected by autoradiography. As shown in Figure 2, amphoterin was recovered as a single protein band (29 kDa), also after 2 h of incubation with unstimulated cells. In contrast, in the presence of PMA, the native protein was gradually modified with appearance of faster migrating radioactive species, indicating that amphoterin is degraded by a proteinase specifically released in these conditions.

In order to isolate the proteinase responsible for amphoterin degradation, the conditioned medium of cells treated with PMA was submitted to anion-exchange chromatography. From this chromatographic analysis two peaks of proteolytic activity were eluted at approx. 0.1 M and 0.15 M NaCl respectively (Figure 3). The first peak contained a serine proteinase, as indicated by its susceptibility to typical inhibitors of these enzymes, which was active at neutral pH and not affected by chelating agents or bivalent metal ions (Table 1). Moreover, this enzyme was unable to hydrolyse synthetic substrates specific for plasmin, trypsin and elastase, but was active against a peptide known as a substrate for chymotrypsin and cathepsin G with a specific activity of 0.144 nmol/min per $\mu$g. The second peak contained a proteolytic activity detectable at below pH 5, with a maximum at pH 3; this proteinase was inhibited by pepstatin and identified as a cathepsin

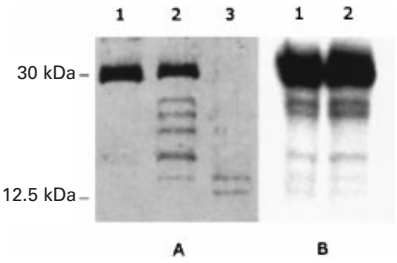


**Figure 4 Degradation of amphoterin by a serine proteinase secreted by MEL cells**

(**A**) Purified amphoterin, 20 $\mu$g, was diluted in 100 $\mu$l of 50 mM sodium borate buffer, pH 7.5, and incubated at 37 °C in the presence of 0.2 unit of partially purified serine proteinase. Aliquots (30 $\mu$l) were collected at 0 h (lane 1), 1 h (lane 2) and 6 h (lane 3), and submitted to SDS/PAGE followed by protein staining with Coomassie Blue. (**B**) $^{125}$I-labelled amphoterin was added to an MEL cell culture containing 100 ng/ml PMA, as specified in the legend to Figure 2, in the absence (lane 1) or presence (lane 2) of 0.1 mg/ml bacitracin. After 30 min, 30 $\mu$l of conditioned medium were submitted to SDS/PAGE, and the radioactive bands were detected by PhosphorImager (Packard, Meriden, CT, U.S.A.) using OptiQuant Image Analysis software.

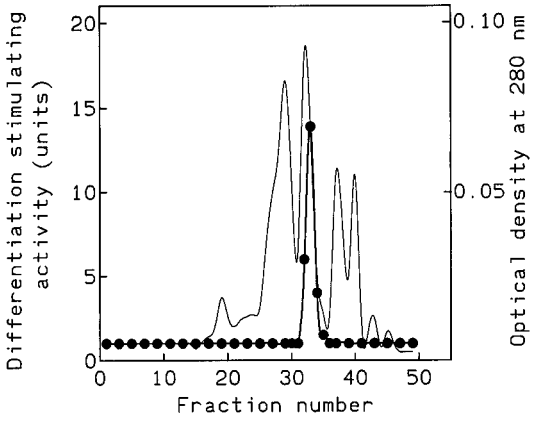


**Figure 5 Identification of a bioactive peptide of amphoterin**

Purified amphoterin (100 $\mu$g) was diluted in 500 $\mu$l of 50 mM sodium borate buffer, pH 7.5, and incubated for 18 h at 30 °C in the presence of 1 unit of partially purified serine proteinase. The products of digestion were resolved by gel chromatography as described in the Experimental section. MEL cell-differentiation-stimulating activity (●) was assayed using 0.01 $\mu$l of the eluted fractions.

D-like enzyme. The acidic proteinase was found to be secreted from growing cells, and accumulated in the conditioned medium independently of any stimulus. This proteinase was not considered an amphoterin-processing enzyme in physiological conditions, since (i) its catalytic activity is restricted at very low pHs; and (ii) unstimulated cells, although containing this enzyme in the culture medium, were unable to degrade extracellular amphoterin (Figure 2). Conversely, purified amphoterin was highly sensitive to digestion by the serine proteinase at neutral pH. As shown in Figure 4(A), the proteinase converted the 29 kDa amphoterin molecule into fragments, some of which were further processed and disappeared at prolonged times of incubation. The intermediate products of amphoterin degradation obtained in this condition showed an elecrophoretic mobility comparable with those identified in the extracellular culture medium after a brief exposure of cells to PMA (Figures 4A and 4B, lane 1), indicating that this degradation process was catalysed by a single proteinase. Moreover, an identical pattern and extent of degradation of radiolabelled amphoterin by stimulated cells was observed in the presence of an inhibitor of cell endocytosis (Figure 4B, lanes 1 and 2), supporting the idea that this proteolysis occurs extracellularly.

After exhaustive degradation of purified amphoterin with the MEL cell serine proteinase, no appreciable loss of differentiation-stimulating activity was observed (results not shown), indicating that one or more fragments retained the biological activity expressed by the native protein molecule. The products of an overnight amphoterin digestion were then separated by gel filtration and their differentiation-stimulating activity was assayed. As shown in Figure 5, a number of peaks were collected in an $M_w$ range from 7000 to 100 Da. However, a single peak of differentiation-stimulating activity was identified at an elution volume corresponding to a mass of 1000–1400 Da. Fractions containing active fragment(s) were then submitted to a further step of purification by reverse-phase chromatography. A single peptide was found to express the biological activity, and its sequence corresponded to the 10-residue peptide, Gly-Glu-Met-Trp-Asn-Asn-Thr-Ala-Ala-Asp, spanning from amino acids 129–138 in the HMG B domain of amphoterin. This peptide, named Amph(129–138), was also chemically synthesized and tested with MEL cells to determine its differentiation-stimulating activity. As shown in Figure 6, Amph(129–138) showed a dose-dependent differentiation-stimulating activity, and its maximal effect was identical with that obtained with equimolar amounts of native amphoterin. To determine if the same peptide maintains other properties, typical of intact amphoterin, we assayed its effect on PKC activity. As shown in Figure 7, native amphoterin-activated PKC produced $>2$-fold increase of its maximal catalytic activity, whereas the Amph(129–138) peptide was ineffective.

Furthermore, it has been recently reported that amphoterin displays toxic effects when injected in Balb C mice [10]. However, intraperitoneal injection in mice of the Amph(129–138) peptide, in amounts from 10 $\mu$g to 100 $\mu$g, was totally ineffective. These

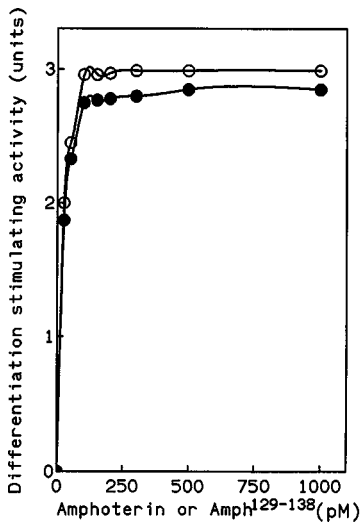


**Figure 6 Efficiency of amphoterin and Amph(129–138) peptide on stimulation of MEL cell differentiation**

Purified amphoterin (●) or chemically synthesized Amph(129–138) peptide (○) was added to the induction mixture of an MEL cell culture and the cell-differentiation-stimulating activity assayed after 72 h from induction.

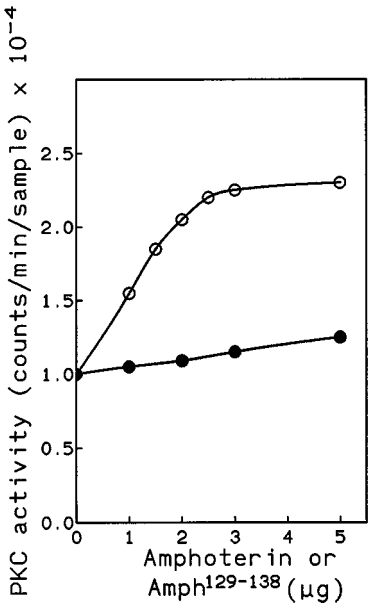


**Figure 7 Effect of amphoterin and Amph(129–138) peptide on PKC activity**

Rat brain PKC was assayed in the presence of the indicated amounts of amphoterin (○) or Amph(129–138) peptide (●).

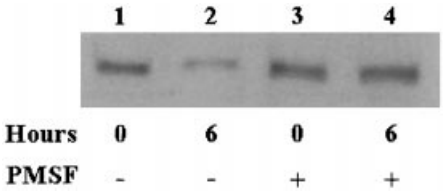


**Figure 8 Evaluation of extracellular amounts of amphoterin in MEL cell cultures induced with HMBA**

Purified amphoterin (5 $\mu$g/ml) was added to a C44 MEL cell culture containing $10^6$ cells/ml, 5 mM HMBA and, where indicated, 2 mM PMSF. At the indicated times, the amount of native amphoterin was evaluated by Western blotting, using 30 $\mu$l of conditioned media.

results are consistent with the concept that the Amph(129–138) peptide retains some amphoterin activities, although having lost other functions typical of the native molecule.

To analyse whether a proteolytic modification of amphoterin also occurs in the extracellular medium of MEL cells stimulated with agents promoting erythroid differentiation, native amphoterin was added to a cell suspension containing HMBA and, 6 h later, its amount was determined by Western blotting. As shown in Figure 8, the level of amphoterin decreased more than four times in the extracellular medium of cells exposed for 6 h to the inducer. However, amphoterin digestion was completely prevented by adding the serine proteinase inhibitor PMSF, confirming that a serine proteinase is involved in this degradation process.

## DISCUSSION

Several experiments [8,11] have demonstrated that amphoterin, released by different types of stimulated cells, operates as an autocrine or paracrine effector. This signal molecule displays different physiological functions, promoting brain development and erythroid differentiation [9,12]. However, interaction of extracellular amphoterin with tumour cell lines has also been found to enhance cell motility and hence metastatic invasiveness [25]. Furthermore, an increased plasma concentration of amphoterin has been described in septic patients, and this protein molecule is also considered as a late mediator of lethality [10]. These latter findings suggest that high extracellular amounts of the native molecule can produce persistent cell activation leading ultimately to pathological effects. The ability of amphoterin to activate plasminogen at the leading edge of outgrowing axon-like processes, with the production of active plasmin in the culture medium of neuroblastoma cell cultures, has been proposed as a mechanism for the degradation of extracellular amphoterin with loss of its adhesive properties [26]. In the present study we describe an alternative proteolytic system operating through a serine proteinase secreted from MEL cells following stimulation with agonists which have been demonstrated previously to trigger the cell export of amphoterin [6]. We can exclude that plasmin is involved in this limited processing of amphoterin since: (i) contamination of plasmin present in the cell culture medium has been avoided using serum-free conditioned media to isolate the enzyme; (ii) MEL cell cDNA, obtained from uninduced or induced cells, does not contain detectable amounts of plasminogen transcripts evaluated by PCR analyses (M. Pedrazzi, B. Sparatore and E. Melloni, unpublished work); (iii) the serine proteinase secreted by stimulated MEL cells does not belong to the family of trypsin-like proteinases [27]; and (iv) although not purified to homogeneity, the preparation obtained from the MEL cell medium contains a single proteolytic activity, as revealed by gelatin zymography (results not shown). The enzyme prefers substrates of chymotrypsin, but it is not inhibited by TPCK (Table 1), indicating that it is not chymotrypsin, and possibly another serine proteinase with somewhat similar specificity. Further studies are required to obtain a conclusive identification of this proteinase, as well as of the mechanisms triggering its secretion and activation.

The cathepsin D-like enzyme, secreted by growing MEL cells, seems not to be involved in the partially conservative degradation of amphoterin, being inactive at neutral pH and producing only inactive fragments of this substrate at acidic pHs (results not shown).

Conversely, the serine proteinase isolated in the conditioned medium of MEL cells catalyses a limited proteolysis of the amphoterin molecule, leading to accumulation of resistant peptides. One of these fragments of amphoterin has been found to be active in stimulating differentiation of MEL cells induced with HMBA. The concomitant release of amphoterin and of the proteinase in the culture medium of cells demonstrates for the first time the existence of an enzymic system which is not simply devoted to the control of the extracellular concentration of amphoterin, but also to the production of a novel active protein fragment. The cell-differentiation-stimulating peptide, produced by digestion of amphoterin with the extracellular serine proteinase, is localized in the HMG box B of the molecule, at the end of the second helix, in the protein segment connecting the two arms of the L-shaped domain [28], and is particularly exposed to proteolysis. At a functional level this peptide stimulates MEL cell differentiation showing an optimal effect at a concentration comparable with that of native amphoterin [9]. Conversely, the 10-residue peptide lacks other molecular and functional properties of amphoterin, such as heparin affinity, PKC-stimulating activity, systemic toxicity and the adhesive properties attributed to the peculiar distribution of basic and acidic amino acid residues along the native molecule [29]. The present data suggest

the existence of a proteolytic pathway promoting a conservative degradation of amphoterin, which can be considered as a molecular maturation of the protein. The process could be figured as a mechanism for the removal of redundant protein regions, involved in the pleiotrophic functions described previously for extracellular amphoterin, but leaving intact a fragment which retains the cell-differentiation-stimulating activity.

It has been reported that amphoterin interacts with the receptor for the advanced glycation end-products [30]. This receptor is expressed by MEL cells (M. Patrone, D. Gaggero and B. Sparatore, unpublished work), but its involvement in the process of MEL cell differentiation has not yet been explored. The cell-binding site(s) and the signal-transduction cascades, activated by interaction of the Amph(129–138) peptide or of amphoterin with MEL cells, remain to be identified. Studies are in progress to establish whether amphoterin and the active peptide derived from its extracellular proteolysis bind the receptor for the advanced glycation end-products, and to determine the effect of this binding on the differentiation process of different cell lines.

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## REFERENCES

1 Bianchi, M. E., Beltrame, M. and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1. Science (Washington, D.C.) **243**, 1056–1059
2 Parkkinen, J., Raulo, E., Merenmies, J., Nolo, R., Kajander, E. O., Baumann, M. and Rauvala, H. (1993) Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. J. Biol. Chem. **268**, 19726–19738
3 Ellwood, K. B., Yen, Y. M., Johnson, R. C. and Carey, M. (2000) Mechanism for specificity by HMG-1 in enhanceosome assembly. Mol. Cell. Biol. **20**, 4359–4370
4 Landsman, D. and Bustin, M. (1991) Assessment of the transcriptional activation potential of the HMG chromosomal proteins. Mol. Cell. Biol. **11**, 4483–4489
5 Fages, C., Nolo, R., Huttunen, H. J., Eskelinen, E.-L. and Rauvala, H. (2000) Regulation of cell migration by amphoterin. J. Cell Sci. **113**, 611–620
6 Passalacqua, M., Zicca, A., Sparatore, B., Patrone, M., Melloni, E. and Pontremoli, S. (1997) Secretion and binding of HMG1 protein to the external surface of the membrane are required for murine erythroleukemia cell differentiation. FEBS Lett. **400**, 275–279
7 Merenmies, J., Pihlaskari, R., Laitinen, J., Wartiovaara, J. and Rauvala, H. (1991) 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. J. Biol. Chem. **266**, 16722–16729
8 Passalacqua, M., Patrone, M., Picotti, G. B., Del Rio, M., Sparatore, B., Melloni, E. and Pontremoli, S. (1998) Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. Neuroscience (Oxford) **82**, 1021–1028
9 Melloni, E., Sparatore, B., Patrone, M., Pessino, A., Passalacqua, M. and Pontremoli, S. (1995) Extracellular release of the 'Differentiation enhancing factor', a HMG1 protein type, is an early step in murine erythroleukemia cell differentiation. FEBS Lett. **368**, 466–470
10 Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J. M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L. et al. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science (Washington, D.C.) **285**, 248–251
11 Wang, H., Vishnubhakat, J. M., Bloom, O., Zhang, M., Ombrellino, M., Sama, A. and Tracey, K. J. (1999) Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. Surgery **126**, 389–392
12 Sparatore, B., Passalacqua, M., Patrone, M., Melloni, E. and Pontremoli, S. (1996) Extracellular high-mobility group 1 protein is essential for murine erythroleukaemia cell differentiation. Biochem. J. **320**, 253–256
13 Abraham, E., Arcaroli, J., Carmody, A., Wang, H. and Tracey, K. J. (2000) HMG-1 as a mediator of acute lung inflammation. J. Immunol. **165**, 2950–2954
14 Andersson, U., Wang, H., Palmblad, K., Aveberger, A. C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H. and Tracey, K. J. (2000) High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokines synthesis in human monocytes. J. Exp. Med. **192**, 565–570
15 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
16 Melloni, E., Pontremoli, S., Viotti, P. L., Patrone, M., Marks, P. A. and Rifkind, R. A. (1989) Differential expression of protein kinase C isozymes and erythroleukemia cell differentiation. J. Biol. Chem. **264**, 18414–18418
17 Winterhalter, K. H. and Huehns, E. R. (1964) Preparation, properties and specific recombination of $\alpha\beta$-globin subunits. **239**, 3699–3705
18 Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E. and Pontremoli, S. (1996) Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localization. FEBS Lett. **392**, 11–15
19 Bieth, J., Spiess, B. and Wermuth, C. G. (1974) The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem. Med. **11**, 350–357
20 Nakajima, K., Powers, J. C., Ashe, B. M. and Zimmerman, M. (1979) Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the alpha 1-protease inhibitor reactive site. J. Biol. Chem. **254**, 4027–4032
21 Gaertner, H. F. and Puigserver, A. J. (1992) Increased activity and stability of poly(ethylene glycol)-modified trypsin. Enzyme Microb. Technol. **14**, 150–155
22 Collen, D., Lijnen, H. R., De Cock, F., Durieux, J. P. and Loffet, A. (1980) Kinetic properties of tripeptide lysyl chloromethyl ketone and lysyl p-nitroanilide derivatives towards trypsin-like serine proteinases. Biochim. Biophys. Acta **615**, 158–166
23 Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Cakiroglu, A. G., Jackson, G. F., Rifkind, R. A. and Marks, P. A. (1987) Protein kinase C activity and hexamethylenebisacetamide-induced erythroleukemia cell differentiation. Proc. Natl. Acad. Sci. U.S.A. **84**, 5282–5286
24 Maloy, W. L., Coligan, J. E. and Paterson, Y. (1997) Production of antipeptide antisera. In Current Protocols in Immunology (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W., eds.), pp. 1–11, John Wiley and Sons, Inc., New York
25 Taguchi, A., Blood, D. C., Del Toro, G., Canet, A., Lee, D. C., Qu, W., Tanji, N., Lu, Y., Lalla, E., Fu, C. et al. (2000) Blockade of RAGE-amphoterin signalling suppresses tumor growth and metastases. Nature (London) **405**, 354–359
26 Parkkinen, J. and Rauvala, H. (1991) Interaction of plasmin and tissue plasminogen activator (t-Pa) with amphoterin. J. Biol. Chem. **266**, 16730–16735
27 Rawlings, N. D. (1998) Serine and threonine peptidases. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 3–12, Academic Press, San Diego
28 Weir, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R. C., Laue, E. D. and Thomas, J. O. (1993) Structure of the HMG box motif in the B-domain of HMG1. EMBO. J. **12**, 1311–1319
29 Rauvala, H. and Pihlaskari, R. (1987) Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. J. Biol. Chem. **262**, 16625–16635
30 Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J. X., Nagashima, M., Lund, E. R., Vijay, S., Niteki, D., Morser, J., Stern, D and Schmidt, A. M. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. J. Biol. Chem. **270**, 25752–25761

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