ABSTRACT

Amphoterin has been suggested to regulate invasive process extension and cell migration in tumor cells and embryonic neurons through binding to receptor for advanced glycation end products (RAGE), a multiligand transmembrane receptor belonging to the immunoglobulin superfamily. In this study, we identify a COOH-terminal motif in amphoterin (amino acids 150–183) that is responsible for RAGE binding. We show that as a surface-bound ligand, this part of amphoterin is sufficient to induce RAGE-dependent process extension, suggesting a role in the regulation of cell motility. When applied in solution, the RAGE-binding COOH-terminal motif of amphoterin efficiently inhibits process extension and transendothelial migration of tumor cells. Furthermore, in an in vivo model, the corresponding synthetic peptide significantly suppresses formation of lung metastases. Taken together, these results suggest that amphoterin binds to RAGE through a COOH-terminal motif that can be used as an efficient inhibitor to block invasive migration of tumor cells.

INTRODUCTION

The acquisition of invasive motile phenotype is a key step in the development of malignant tumors and formation of metastases. Alteration of the interactions between the neoplastic cells and their surrounding extracellular matrix plays an important role in the detachment of tumor cells from the primary tumor and in the migration of invasive cells through the endothelium to the vasculature, through which they can reach distant locations throughout the body (reviewed in Ref. 1).

Amphoterin and RAGE1 have been identified as a ligand-receptor pair that plays an important role in regulating the invasiveness of tumor cells (2). Amphoterin (also known as HMGB1) is expressed in a large variety of transformed cells (3) and has been shown to be a general regulator of cell migration (4). On the other hand, RAGE, an immunoglobulin superfamily member (5), is known to stimulate multiple signal-regulated kinase 1/2 (6), Cdc42/Rac (7), stress-activated protein kinase family members (8), and various amyloids [amyloid-β peptide and amyloid A (17, 18)]. Rational design of RAGE antagonists for practical applications, including inhibition of tumor cell invasion, would require insight into the structural basis of RAGE-ligand interactions, which is currently poorly understood. In this study, we identify a COOH-terminal motif of amphoterin that is responsible for RAGE binding. We show that when immobilized to a surface, this RAGE-binding motif can induce RAGE-mediated process outgrowth, but in solution, it can efficiently block transendothelial migration of tumor cells. Furthermore, a synthetic peptide corresponding to the RAGE-binding region in amphoterin significantly suppressed formation of lung metastases in an in vivo model. Interestingly, we found that the sequence of the RAGE-binding COOH-terminal motif of amphoterin is homologous with S100 proteins.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND PROTOCOLS. The production and purification of baculovirus-expressed wild-type amphoterin have been described previously (3). Amphoterin fragments (Fig. 1A) were synthesized by PCR and cloned in-frame with the GST of pGEX-6P1 vector (Amersham Biosciences). The following oligonucleotides (Sigma-Genosys) were used: amphoterin 1–185 For, AGAGGATCCATGGGCAAAG-GGAGATCCT; amphoterin 1–185 Rev, GGCGAATTCTCACTTCTTTTTCT-TGCT; amphoterin 1–140 For, AGAGGATCCATGGGCAAAG-GGAGATCCT; amphoterin 1–140 Rev, GGCGAATTCTCACTTCTTTTTCT-TGCT; amphoterin 1–105 For, AGAGGATCCATGGGCAAAG-GGAGATCCT; amphoterin 1–105 Rev, GGCGAATTCTCACTTCTTTTTCT-TGCT.

The authenticity of cloned constructs was verified by sequencing. GST-amphoterin fragment fusion proteins were expressed in Escherichia coli and purified on glutathione-Sepharose columns (Amersham Biosciences). Some of the proteins were used as GST fusion proteins, whereas some were used with the GST moiety cleaved off. To obtain amphoterin fragments without GST, fusion proteins were cleaved with PreScission Protease (Amersham Biosciences), and the cleaved GST was then removed with a glutathione-Sepha-

Received 3/27/02; accepted 6/7/02.

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1 Supported by grants from the Academy of Finland (Finnish Center of Excellence Programme 2000-2005), Finnish Cancer Society, Sigrid Juselius Foundation, Emil Aaltonen Foundation, and European Community Grant QLG1-CT-1999-01036.

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3 The abbreviations used are: RAGE, receptor for advanced glycation end products; HMG, high mobility group; MMP, matrix metalloproteinase; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; sRAGE, soluble ectodomain of RAGE.
the columns was detected by Western blotting with an anti-RAGE antibody. Not Nhe I site; R&D Systems) to generate a fusion protein with a COOH-
TGGAGAGCCACTTG; and RAGE Rev, ATAGTTTAGCGGCCGCCCA-
duction of recombinant protein: RAGE For, TACTAGCTAGCGCCCGGAT-
rose column. Recombinant proteins were characterized on SDS-PAGE gels
and Western blots (Fig. 1

\[ \text{A} \]

\begin{align*}
\text{Amphoterin} & \quad 50 \text{ kDa} \\
\text{Amphoterin 1-185} & \quad 37 \text{ kDa} \\
\text{Amphoterin 1-140} & \quad 25 \text{ kDa} \\
\text{Amphoterin 141-190} & \quad 9 \text{ kDa}
\end{align*}

\[ \text{B} \]

\begin{align*}
\text{C} \quad \text{Blot: RAGE}
\end{align*}

**In Vitro Binding Assay.** Glutathione-Sepharose (Amersham Biosciences) was washed in 0.2-ml columns to which 50 μg of GST-amphoterin fragment fusion proteins were attached by a 1-h incubation at 4°C. Columns were washed with 5 volumes of cold PBS and then incubated with 10 μg of sRAGE-Fc protein for 2 h at 4°C. The columns were washed with 5 volumes of cold PBS and eluted with 2 × 200 μl of 1.0 M NaCl. Eluates were run on 10–20% SDS-PAGE gels and electrotransferred on nitrocellulose filters. Filters were blocked with 2% BSA, and RAGE was detected with a primary antibody (Chemicon) followed by incubation with a peroxidase-conjugated secondary antibody (Amersham Biosciences). The bands were visualized with enhanced chemiluminescence (Amersham Biosciences).

**Neurite Outgrowth Assay.** Stably transfected clones of N18 mouse neuroblastoma cells were obtained as described previously (16). Cells were cultured in DMEM supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin G, 10% FCS, and 100 μg/ml G418 (Invitrogen) in 5% CO2 atmosphere. For neurite outgrowth assays, the cells were detached from culture plates by incubation in PBS + 0.5 mM EDTA, washed with serum-free DMEM supplemented with 1% BSA (Sigma), and plated without serum on 96-well plates that had been coated with either 20 μg/ml recombinant amphoterin fragments or 50 μg/ml synthetic peptides. Cells were fixed after 24 h of incubation, and neurite outgrowth was quantified by counting cells bearing neurites longer than 1 diameter of cell soma in five randomly selected fields. Phase-contrast micrographs were taken with an Olympus IX-70 reversed microscope with Hoffman Modulation phase-contrast optics and an Olympus DP-10 digital camera.

**Transendothelial Migration Assay.** HUVECs were prepared from umbilical veins as follows. The umbilical cords were flushed twice with PBS, filled with 0.1% collagenase (Sigma) in PBS prewarmed to 37°C, and incubated for 20 min at 37°C. The collagenase solution was then collected, and the veins were washed twice with 20 ml of Medium 199 (Invitrogen) containing 20% FCS to collect the remaining cells. The collagenase solution and the washes were pooled and centrifuged. The cell pellet was resuspended in 20 ml of Medium 199/20% FCS, and the cells were plated into a 75-ml culture flask (Corning Life Sciences) coated with 10 μg/ml fibronectin. After 3 h, the medium was changed to remove nonadherent cells. HUVECs were cultured in Medium 199 containing 20% FCS, 100 μg/ml endothelial cell growth supplement (Sigma), 100 μg/ml heparin (Sigma), and 1% Nutridoma NS (Boehringer Mannheim) in flasks coated with 10 μg/ml human fibronectin.

For transendothelial migration assays, HUVECs were grown on fibronectin-coated polyester Transwell-Clear filters (3 mm, pore size; 12 mm, diameter; Corning Life Sciences). The cells were plated at a density of 3 × 10^5 cells/well, and after 4 h, nonadherent cells were removed. Filters were used for experiments 4 days after plating. HUVEC monolayers grown on filters were examined by microscopy for confluence before the experiment. HT1080 human fibrosarcoma cells or stably transfected clones of N18 cells (16) were serum-starved and labeled by incubation for 15 min with 6.7 μM succinimidyl ester of carboxyfluorescein diacetate (CFDA SE; Molecular Probes, Inc.). After a 30-min preincubation with the amphoterin peptides, HT1080 cells were plated on HUVEC monolayers. Cells were incubated for 5 h before they were fixed and stained with TRITC-phalloidin (Molecular Probes, Inc.). Migrated cells were counted in four randomly selected fields in three layers with an Olympus IX-70 reversed fluorescence microscope.

**Experimental Pulmonary Metastasis Assay.** B16-F1 mouse melanoma cells were grown in DMEM supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin G, and 10% FCS in 5% CO2 atmosphere for 1 week to collect a sufficient number of cells. The cells were detached from culture plates by incubation in PBS + 0.5 mM EDTA and washed with serum-free DMEM. A total of 3.0 × 10^6 cells exhibiting >90% viability were suspended in 250 μl of serum-free DMEM and preincubated with the amphoterin peptides for 30 min before injection into the tail veins of isogenic, immunocompromised C57BL/6-scid-beige mice (6–8-week-old female mice; Harlan). After 14 days, the animals were sacrificed, and the lungs were collected and analyzed for the presence of metastatic foci. Stably transfected B16-F1 cells expressing either full-length RAGE or the cytoplasmic domain deletion mutant of RAGE (RAGEcyto) were produced as described for N18 cells (16). Photomicrographs were taken with a Leica stereomicroscope and an Olympus DP-10 digital camera.

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Sequence Analysis. Amino acid sequence alignments were calculated using Genetics Computer Group Wisconsin Package Version 10.2 (19). Multiple sequence alignment was constructed using the ClustalX program (20).

Statistical Analysis. Student’s t test was used to analyze the statistical significance of the results. P values are shown in the Figs. 2–5.

RESULTS

Identification of RAGE-binding Motif in Amphoterin. Our previous studies have shown that an antibody against a COOH-terminal peptide of amphoterin (amino acids 166-75) inhibits haptotactic cell migration to laminin (4). To study whether this region of amphoterin was involved in receptor binding, we produced and purified GST fusion proteins with various amphoterin fragments (Fig. 1, A and B). Amphoterin 1–185 consisted of the major part of the molecule lacking the 31 last amino acids that make up the highly acidic tail of amphoterin. Amphoterin 1–140 consisted of HMG box 1 and the first two \( \alpha \)-helices of HMG box 2, whereas amphoterin 141–190 consisted of the third \( \alpha \)-helix of HMG box 2 and the region between the HMG box 2 and the acidic tail of amphoterin (21, 22).

To study the binding of the amphoterin fragments to RAGE, the receptor of amphoterin, GST fusion proteins were attached to glutathione-Sepharose columns through which purified soluble ectodomain of RAGE was passed. After extensive washing, the columns were eluted with 1.0 M NaCl, and bound RAGE was identified by Western blotting. As seen in Fig. 1C, amphoterin 1–185 and amphoterin 141–190 were able to bind to RAGE, whereas amphoterin 1–140 columns did not bind RAGE. This result suggests that RAGE binds to a COOH-terminal region of amphoterin that consists partly of the HMG box 2 and partly of the intermediate region between the HMG box 2 and the acidic tail of the molecule.

RAGE-binding Motif of Amphoterin Induces Neurite Outgrowth. To further characterize the RAGE-binding motif of amphoterin, we tested the amphoterin fragments in a simple bioassay previously used to study the role of RAGE signaling in process outgrowth (7, 16). N18 neuroblastoma cells expressing either full-length RAGE or a signaling-incapable, cytoplasmic domain deletion mutant of RAGE (RAGE\textsubscript{Acyo}) were grown on surfaces coated with amphoterin fragments (the GST moiety had been cleaved off), and neurite outgrowth response was followed for 24 h.

Roughly 25% of RAGE-expressing cells and 5% of RAGE\textsubscript{Acyo}-expressing cells grew neurites on amphoterin surface (Fig. 2G). Amphoterin 1–185 had a similar capacity to promote neurite outgrowth as the full-length protein (Fig. 2, A and G). Remarkably, RAGE-expressing cells plated on amphoterin 1–140 were unable to grow neurites (Fig. 2, B and G), whereas on amphoterin 141–190, \( \approx \)20% of RAGE-expressing cells grew neurites (Fig. 2, C and G). The neurite outgrowth-promoting effect of both amphoterin 1–185 and 141–190 depends on RAGE signaling because RAGE\textsubscript{Acyo}-expressing cells did not display neuritic morphology when grown on these amphoterin fragments (Fig. 2, D, F, and G).

A peptide corresponding to a slightly shorter region of the RAGE-binding motif of amphoterin (amino acids 150–183) was synthesized for in vivo assays. In addition, the same sequence was used in a scrambled order to synthesize a control peptide. The amphoterin 150–183 peptide was tested in the neurite outgrowth assay (coated at 50 \( \mu \)g/ml) and showed a similar capacity to promote RAGE-dependent neurite outgrowth as did the longer recombinant amphoterin 141–190 fragment, whereas cells grown on the scrambled amphoterin 150–183 peptide displayed a significantly lower number of neurites (Fig. 2H). Next we tested whether these peptides could be used to inhibit amphoterin-RAGE interaction when applied in solution (at 500 \( \mu \)g/ml, \( \sim \)130 \( \mu \)m). As seen in Fig. 2I, when cells were grown on an...
amphoterin-coated surface in the presence of soluble amphoterin 150–183 peptide, the amount of neurites was reduced more than 2-fold in comparison with the scrambled peptide.

These results confirm that the COOH-terminal motif of amphoterin is responsible for RAGE binding and that the RAGE-binding region in amphoterin can be shortened to residues 150–183. In addition, these results show that the RAGE-binding motif of amphoterin can mimic the full-length protein, serving as a RAGE agonist when presented to the cells in a surface-bound form, but when applied in solution, this motif serves as a RAGE antagonist.

**RAGE-binding Peptide Inhibits Transendothelial Migration of Tumor Cells.** Because previous results have suggested that both amphoterin and RAGE are involved in invasive migration and formation of metastasis (2, 4), we decided to determine whether the RAGE-binding COOH-terminal motif of amphoterin could be used to block migration of tumor cells through the endothelium. We set up an *in vitro* transendothelial migration assay using labeled HT1080 fibrosarcoma cells migrating through an endothelial cell monolayer prepared from HUVECs. A synthetic peptide corresponding to region 150–183 of amphoterin was used as an inhibitor in solution to block the endogenously occurring RAGE in HT1080 fibrosarcoma cells (data not shown). Scrambled sequence of amphoterin 150–183 was used as the control peptide.

Fig. 3. Effect of amphoterin 150–183 peptide on transendothelial migration. HUVEC monolayers were prepared on fibronectin-coated (10 μg/ml) polycarbonate filters in the transfilter system. HT1080 fibrosarcoma cells were serum-starved and loaded with a fluorescent tracer (succinimidyl ester of carboxyfluorescein diacetate). After a 30-min preincubation with the peptides, HT1080 cells were plated on HUVEC monolayers. Cells were incubated for 5 h before they were fixed and stained with TRITC-phalloidin. Migrated cells were counted in four randomly selected fields in three layers. The number of cells that migrated through the endothelial monolayer in three replicate experiments ± SD is shown. Scrambled sequence of amphoterin 150–183 was used as the control peptide.

**RAGE-binding Peptide Suppresses Formation of Pulmonary Metastasis.** The metastatic potential of tumor cells can be studied *in vivo* in several animal models using immunocompromised mice. We tested the effect of the amphoterin peptides in an experimental pulmonary metastasis model using B16-F1 melanoma cells injected into the tail veins of isogenic C57BL/6-scid-beige mice (23, 24). After 2 weeks, the animals were sacrificed, and the lungs were analyzed for metastatic foci. The number of lung colonies ± SD is shown.

**Fig. 4. Blockade of RAGE signaling inhibits transendothelial migration and metastasis of tumor cells.** A, N18/RAGE and N18/RAGEΔcyto were tested in the transendothelial migration assay as described in Fig. 3. The proportion of cells that migrated through the endothelial cell monolayer and the proportion of nonmigrated cells attached to the upper surface of the endothelial cell layer are shown for both cell lines. B–E, parental B16-F1 melanoma cells (B) and stably transfected B16-F1 cells expressing either RAGE (B16/RAGE; C) or the cytoplasmic domain deletion mutant of RAGE (B16/RAGEΔcyto; D) were injected (3 × 10⁶ cells in 250 μl) into the tail veins of C57BL/6-scid-beige mice. After 2 weeks, the animals were sacrificed, and the lungs were analyzed for metastatic foci. E, the number of lung colonies ± SD is shown.
weeks, lung colonies can be easily detected because the B16-F1 cell line is pigmented and has been selected on the basis of almost exclusive formation of lung metastasis.

When control B16-F1 cells were injected i.v. to mice, approximately 150 lung colonies formed during the 2-week period (Fig. 4, B and E). Mice receiving injection of B16/RAGEΔcyto cells displayed 71.6% fewer lung colonies than mice receiving injection of parental B16-F1 cells (Fig. 4, D and E), suggesting that blocking RAGE signaling can efficiently inhibit formation of tumor metastasis. Remarkably, competition of ligand binding to RAGE by injection of cells together with 500 μM amphoterin 150–183 peptide decreased the number of lung colonies by 64.3% (Fig. 5, B and D). Importantly, roughly 160 lung colonies were detected when cells were injected together with 500 μM scrambled control peptide (Fig. 5, C and D). These results show that inhibition of RAGE signaling is an effective way to suppress invasive migration and that the inhibitory activity of amphoterin 150–183 peptide on invasive migration can be extended to an in vivo situation.

The COOH-terminal RAGE-binding Motif of Amphoterin Shares Sequence Homology with S100 Proteins. Amphoterin is an extremely well-conserved protein between species, especially between vertebrates. For example, region 141–188 of amphoterin is identical in human, rat, mouse, and bovine sequences. In addition to amphoterin, RAGE is known to serve as a receptor for members of the S100 protein family (15, 16), advanced glycation end products (5), and various amyloids [amyloid-β peptide and amyloid A (17, 25)]. Because both amphoterin and S100 proteins are small RAGE-binding proteins with α-helical secondary structures, we did a sequence comparison to look for similarities between the newly identified COOH-terminal RAGE-binding region of amphoterin and S100 proteins. Multiple alignment of amphoterin residues 141–193 with various S100 proteins (Fig. 6) shows that the RAGE-binding region in amphoterin has remarkable resemblance with the NH₂ termini of S100 proteins.

DISCUSSION

Tumor cell invasion in general may be a deregulated form of a physiological invasion process required for neuronal wiring in the embryo because many mechanistic features seem to be common for these processes. Both amphoterin and RAGE seem to be involved in regulation of neurite outgrowth of embryonic neurons and invasive migration of tumor cells (reviewed in Refs. 26 and 27). The present study demonstrates that amphoterin can bind to RAGE through a COOH-terminal motif spanning residues 150–183 and that this part of amphoterin can be used to inhibit amphoterin-RAGE interaction-mediated invasive migration both in vitro and in vivo. Interestingly, a heparin binding consensus sequence can be found in the NH₂ terminus of amphoterin (residues 5–11; Ref. 28). Thus, it is possible that the NH₂ terminus of amphoterin is responsible for proteoglycan binding, whereas the COOH terminus of amphoterin binds to RAGE.

Our results show that the RAGE-binding COOH-terminal motif of amphoterin is sufficient to block transendothelial migration of tumor cells. However, the exact mechanism of RAGE-mediated transendothelial migration is not fully understood. Because RAGE is expressed in endothelial cells (29), it is likely that endothelial RAGE is involved in the transmigration process. In fact, it has been shown that inhibition of endothelial RAGE inhibits amyloid-β peptide-induced migration of monocytes across brain endothelial cells (30). It seems that RAGE activation in the migrating cells is important for invasion, provided that they express RAGE. Several reports have shown that the metastatic potential of tumor cells correlates with the expression level of RAGE (2, 10, 11). Interestingly, our results show that tumor cells expressing a signaling-incapable, cytoplasmic domain deletion mutant of RAGE adhere normally to endothelial cells but are unable to migrate through the endothelial cell monolayer. Furthermore, cells expressing the cytoplasmic domain deletion mutant of RAGE formed 70% fewer lung colonies in the pulmonary metastasis assay.

It is interesting to note that the RAGE-binding COOH-terminal region of amphoterin shares sequence homology with the NH₂ termini of S100 proteins. Structures of several S100 proteins have been resolved, whereas only separate structures of the two HMG boxes of amphoterin are currently known (21, 22). Our homology modeling studies of amphoterin 150–183 with S100A12 (31) and S100B (32) suggest that residues 173–183 of amphoterin could form a seventh α-helix (data not shown) and that, together with the third α-helix of HMG box 2 (residues 142–161), this α-helix could form a helix-loop-helix structure resembling the first EF-hand of the S100 proteins (as illustrated in Fig. 6). It seems likely that the RAGE-binding peptides form a structural motif that is a prerequisite for RAGE binding because two shorter amphoterin peptides (residues 162–177 and 160–183) had greatly reduced activity in our bioassays (data not shown). Interestingly, our preliminary results with the EN-RAGE (S100A12) 3–39 peptide suggest that this peptide possesses even stronger anti-invasive capacity than the amphoterin 150–183 peptide (data not shown), supporting our working hypothesis.

Our present results show that intervening with the amphoterin-RAGE interaction using a synthetic peptide can efficiently suppress formation of metastasis in an in vivo model. Blocking amphoterin-RAGE interaction might be an efficient way to inhibit tumor cell invasion for several reasons. Ligation of RAGE is known to activate multiple signaling pathways, including Ras-extracellular signal-regulated kinase 1/2 (6), Cdc42/Rac (7), stress-activated protein kinase c-Jun-NH₂-terminal kinase, and p38 mitogen-activated protein kinase.
Fig. 6. Sequence comparison of amphoterin COOH terminus with NH2 termini of S100 family proteins. Alignment of amino acids 141–193 of amphoterin with tenasin-R, amphoterin, and amino acids that are identical in 50% of the sequences are shown in black, amino acids that are identical in 50% of the sequences are shown in dark gray. Amino acids that are similar by nature are shown in light gray.

ACKNOWLEDGMENTS

The excellent technical assistance of Nina Nevalainen and Eeva-Liisa Saarikalle is gratefully acknowledged.

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Receptor for Advanced Glycation End Products-binding COOH-terminal Motif of Amphoterin Inhibits Invasive Migration and Metastasis

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