The Receptor for Advanced Glycation End Products (RAGE) Is a Cellular Binding Site for Amphoterin

MEDIATION OF NEURITE OUTGROWTH AND CO-EXPRESSION OF RAGE AND AMPHOTERIN IN THE DEVELOPING NERVOUS SYSTEM*

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The receptor for advanced glycation end products (RAGE), a newly-identified member of the immunoglobulin superfamily, mediates interactions of advanced glycation end product (AGE)-modified proteins with endothelium and other cell types. Survey of normal tissues demonstrated RAGE expression in situations in which accumulation of AGEs would be unexpected, leading to the hypothesis that under physiologic circumstances, RAGE might mediate interaction with ligands distinct from AGEs. Sequential chromatography of bovine lung extract identified polypeptides with Mₐ values of ~12,000 (p12) and ~23,000 (p23) which bound RAGE. NH₂-terminal and internal protein sequence data for p23 matched that reported previously for amphoterin. Amphoterin purified from rat brain or recombinant rat p23 matched that reported previously for amphoterin. Amphoterin purified from rat brain or recombinant rat amphoterin bound to purified sRAGE in a saturable and dose-dependent manner, blocked by anti-RAGE IgG or a soluble form of RAGE (sRAGE). Cultured embryonic rat neurons, which express RAGE, displayed dose-dependent binding of [125I]amphoterin which was prevented by blockade of RAGE using antibody to the receptor or excess soluble receptor (sRAGE). A functional correlate of RAGE-amphoterin interaction was inhibition by anti-RAGE F(ab)₂ and sRAGE of neurite formation by cortical neurons specifically on amphoterin-coated substrates. Consistent with a potential role for RAGE-amphoterin interaction in development, amphoterin and RAGE mRNA/antigen were co-localized in developing rat brain. These data indicate that RAGE has physiologically relevant ligands distinct from AGEs which are likely, via their interaction with the receptor, to participate in physiologic processes outside of the context of diabetes and accumulation of AGEs.

Incubation of proteins or lipids with aldose sugars results in nonenzymatic glycation and oxidation (1–7). Following formation of the reversible early glycation products, Schiff bases and Amadori products, further complex molecular rearrangements result in irreversible advanced glycation end products (AGEs).¹ Factors favoring nonenzymatic glycation include delayed protein turnover, as in amyloidosis, accumulation of macromolecules with high lysine content, and situations with elevated glucose levels, as in diabetes. AGE formation occurs during normal aging, and at an accelerated rate in diabetes, in which their accumulation in the plasma and vessel wall has been speculated to underlie the pathogenesis of vasculopathy (1, 2, 4).

One of the principal means through which AGEs impact on cellular elements is through interaction with cellular binding proteins. Although there are several possible cell-associated polypeptides with which AGEs might interact (8, 9), our work has focused on the receptor for AGEs (RAGE), as its expression in endothelium, vascular smooth muscle, mononuclear phagocytes, and the central nervous system suggests strategic loci for interaction with the glycated ligands (10, 11). The potential pathophysiologic relevance of AGE-RAGE interaction was emphasized by studies demonstrating that blockade of RAGE prevented multiple AGE-induced perturbations of cellular functions. For example, AGE-stimulated mononuclear phagocyte migration and activation, endothelial expression of vascular cell adhesion molecule-1, and increased monolayer permeability were prevented by blocking AGE interaction with RAGE (12, 13). Consistent with the latter data in cell culture, in vivo studies have shown RAGE to mediate the early and rapid removal of AGEs from the intravascular space, AGE induction of vascular oxidant stress and vascular hyperpermeability in diabetic animals and rodents infused with AGEs (14–17).² These data emphasize a potential role for AGE-RAGE interactions in pathologic states, and have led us to assess RAGE expression in vasculopathies, especially that associated with diabetes.

During the course of tissue surveys to assess RAGE distribution, it became evident that expression of the receptor occurred in early development, especially in the central nervous system; i.e. in situations distinct from those in which AGEs might be present. RAGE is a member of the immunoglobulin superfamily of cell surface molecules (19), and the extracellular domain consists of one putative "V" type followed by two putative "C" type domains, bearing closest homology to neural cell adhesion molecule (19). In view of previous studies with other

¹ The abbreviations used are: RAGE, receptor for advanced glycation end product; AGE, advanced glycation end product; sRAGE, soluble RAGE; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

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immunoglobulin-like receptors, such as intercellular adhesion molecule-1, in which one receptor has several pathophysio-
logically relevant ligands (20). We considered the hypothesis that AGEs might be incidental ligands for a receptor which had other functions. By analogy with other immunoglobulin super-
family members, we speculated that RAGE might participate in cell-cell or cell-matrix interactions, or, perhaps, might func-
tion as a cytokine or growth factor receptor. Toward this end, we sought to define putative natural ligands for RAGE which were not AGEs. In this study we report the identification of ampho-
terin as a ligand for RAGE: amphoterin binds RAGE with higher affinity than AGEs; RAGE serves as the binding site for amphoterin on rat embryonic cortical neurons; ampho-
terin-RAGE interaction promotes neurite outgrowth in cell cul-
ture; and, RAGE and amphoterin are co-expressed in develop-
mentally relevant ligands (20), we considered the hypothesis that RAGE might participate in neuronal development, these studies provide a first step in identifying novel functions for RAGE distinct from its role as a receptor for AGEs in the vasculature.

EXPERIMENTAL PROCEDURES

Preparation of AGEs—Bovine serum albumin (fraction V; Sigma) was glycated by incubation with glucose (0.5 M) for 6 weeks at 37 °C. Glycated protein was characterized based on fluorescence and binding to cultured endothelial cells and mononuclear phagocytes, as described previously (10). Nonglycated albumin consisted of the same initial preparations of albumin incubated at 37 °C in the same manner, except that no aldose was present.

Purification of RAGE Binding Proteins—Bovine lung powder (100 g; Sigma) was extracted with buffer containing Tris (0.02 M; final pH 7.4), NaCl (0.1 M), octyl-β-D-glucoside (1.0%), and phenylmethylsulfanyl fluo-
ride (0.001% v/v) over 16 h at 4 °C, followed by centrifugation for 20 min at 13,000 × g. The supernatant was filtered (0.45-μm) and applied to heparin-Sepharose (bed volume, 25 ml; Sepharose, Marlborough, MA) equili-
brated with Tris (0.02 M; final pH 7.4)/NaCl (0.1 M) containing octyl-β-D-glucoside (0.1%). The column was washed extensively with the same buffer until the OD 280 was <0.01, and eluted sequentially with increasing NaCl in the equilibration buffer (0.25, 0.5, 0.75, and 1.0 M). One-milliliter fractions were collected and protein content determined by adsorption at OD 280 (Corning, Oberlin, OH).

Fractions from the heparin column were screened for RAGE binding activity following their adsorption to MaxiSorp microtiter wells (Nunc, Naperville, IL) in bicarbonate/bicarbonate buffer (pH 9.6) for 16 h at 4 °C. After blocking excess binding sites in the wells with phosphate-buffered saline containing bovine serum albumin, a binding assay for RAGE was performed. For these studies, RAGE was purified to homogeneity from bovine lung, as described (10), and radiolabeled by the lactoperoxidase method using Enzymobeads (Bio-Rad) according to the manufacturer’s instructions. The final specific radioactivity was 1,000 cpm/μg, and 125I-RAGE was >95% precipitable in trichloroacetic acid (20%), migrated as a single band on SDS-PAGE (comigrating with the unlabeled protein), and bound AGEs. 125I-RAGE was added to wells previously coated with fractions from the heparin column for 3 h at 37 °C in phosphate-buffered saline containing bovine serum albumin (0.2%). At the end of the incubation period, wells were washed rapidly (10 s/wash and 0.3 ml/wash) with Hank’s balanced salt solution (Life Technologies, Inc.). Bound radioactivity was eluted with NaCl (2 M) and counted in a γ-counter (Pharmacia LKB, Gatlithersburg, MD). Fractions containing RAGE binding activity, identified in the 0.5 M NaCl eluate, were dialyzed versus phosphate-buffered saline containing octyl-β-D-glucoside (0.1% final pH 7.4) and applied to Affi-Gel 10 (Bio-Rad), to which had been bound purified bovine RAGE (2 mg/ml resin), according to the manufacturer’s instructions. After 16 h of incu-
bation at 4 °C, the resin was washed with 10 bed volumes of buffer (phosphate-buffered saline containing octyl-β-D-glucoside, 0.1%), and eluted by high salt (NaCl, 2 M). Fractions containing binding activity for 125I-RAGE, based on the competitive binding assay above, were dia-
lyzed versus phosphate-buffered saline containing octyl-β-D-glucoside (0.1%) and subjected to nonreduced SDS-PAGE (10%). Protein on the gel was either visualized by silver staining (Bio-Rad), or gels were sliced (2 mm) and individual slices were eluted by incubation in acetate buffer (pH 8.0) for 4 h at 4 °C. The mixture was then centrifuged to pellet debris, and supernatants were tested for RAGE binding activity. Two positive fractions were identified, eluted from the gel, and sub-
jected to nonreduced SDS-PAGE (12%). Protein on the gel was visual-
ized by either silver or Coomassie Blue staining to identify bands. Two protein bands with Mr = 23,000 and ~12,000 were visualized and sequence analysis performed as follows.

Amino-terminal Sequence Analysis—The gel bands were eluted from the 3% SDS-PAGE gels using previously described methods (21). This method was modified by increasing buffer volumes to accommodate the larger gel volume, and adding a final wash with two 0.1 ml aliquots of guanidine (5.0 M), urea (5.0 M), trifluoroacetic acid (0.2%), acetonitrile (10%), and triethylamine-3·08 (1.0%) (Calbiochem, San Diego, CA) to insure that protein was completely washed from the filter. Automated Edman degradation was carried out using either an HP-G1006A sequenator (Hewlett Packard Analytical Instruments, Palo Alto, CA) or a 477A sequencer (Perkin Elmer-Applied Biosystems, Foster City, CA).

Internal Sequence Analysis—To obtain internal sequence, the gel bands were treated as above for elution, except that the extraction buffer had half the usual amount of SDS (21). One microgram of endoproteinase Lys-C (Boehringer Mannheim) was added and the sam-
ples incubated overnight. Separation of the fragments was carried out by HPLC on a Yvdac C18 column. A linear gradient of 10-40% acetonitrile in trifluoroacetic acid (0.1%) over 35 min was used to elute the frag-
ments. Fractions were collected at 2-min intervals and absorbance monitored at 214 nm. Fractions that corresponded to chromatographic peaks were then subjected to sequence analysis. In order to obtain additional internal sequence, small fractions were eluted rather than 500 mM acetonitrile in trifluoroacetic acid (0.1%) to elute the material from a Vydc C8 column. Fractions were collected at 1-min intervals. Initially, one-fourth of the available sample was run and peaks identi-
fied by NH2-terminal sequence analysis. The remainder of the material was then run and the 234Da containing fraction digested with cyanog-
ogen bromide to obtain the internal sequence. To digest with cyanogen bromide a 100 mM sodium carbonate buffer (pH 10.5) was used and the sample incubated overnight. Separation of the fragments was carried out by HPLC on a Vydc C18 column. A linear gradient of 10-40% acetonitrile in trifluoroacetic acid (0.1%) over 35 min was used to elute the frag-
ments. Fractions were collected at 2-min intervals and absorbance monitored at 214 nm. Fractions that corresponded to chromatographic peaks were then subjected to sequence analysis.

Data Base Search—The NH2-terminal sequence was used to query the PIR and SWISS-PROT protein data bases using the Genetics Com-
puter Group Sequence Analysis Software package (22).

Purification of Rat Amphoterin, Rat RAGE, and Preparation of An-
tiserum—Amphoterin was purified from the brains of 10-day-old Wistar rats as described (23) by sequential chromatography on heparin-Sepha-
rose and Affi-Gel Blue (Bio-Rad). A single band, M, = 30,000, was identified on nonreduced SDS-PAGE (12%), eluted and subjected to NH2-terminal sequence analysis and identified as amphoterin. Rat RAGE was purified to homogeneity from lung powder (Sigma) using similar methods described for bovine RAGE (10). Immunoreactivity with anti-RAGE IgG (see below) and NH2-terminal and internal se-
quencing indicated identity to the predicted sequence for rat RAGE based on that deduced from the rat cDNA. Based on M, = 30,000 and comparision with RAGE purified from bovine lung, rat lung RAGE also most likely represents the amino-terminal two-thirds of the molecule (i.e. the extracellular domain; Ref. 19) and is identified as soluble RAGE or sRAGE. Rat sRAGE bound AGE albumin in a dose-dependent and specific manner analogous to bovine sRAGE (data not shown). Polyclonal antiserum to rat RAGE was prepared in rabbits by standard protocols. Nonimmune IgG and anti-rat uRAGE IgG were subjected to chromato-
ography on Protein A (Schleicher and Schuell) according to the manufacturer’s instructions. F(ab)2 fragments were prepared from IgG using a kit from Pierce according to the manufacturer’s instructions. Nonimmune IgG and F(ab)2 fragments were similarly made from sera derived from rabbits not immunized with RAGE. Polyclonal antiserum to rat and chicken was generated in chickens and anti-cRAGE IgG was purified by ammonium sulfate precipitation (25). Enzyme-linked immu-
osorbent assay for AGE antigen was performed with affinity purified anti-AGE antibody as described previously (14).

Cloning and Expression of Rat Amphoterin—A baculovirus expres-
sion plasmid coding for rat amphoterin was prepared as follows. A DNA fragment coding for amphoterin was obtained from rat lung cDNA by

3 A. M. Schmidt, O. Horii, and D. Stern, unpublished observation.
polymerase chain reaction (GeneAmp, Perkin-Elmer); primers used were 5'-CTAACATGGGCAAGAGCATG-3' and 5'-CTGAGACCAAATTTTACC-3'. Sequencing of the polymerase chain reaction product by the dideoxy chain termination method (26) revealed the same sequence as that published for rat brain amphoterin (27). The polymerase chain reaction product was subcloned into the pCR® II vector (Invitrogen, San Diego, CA) and the EcoRI-film fragment of the resultant plasmid was cloned into the pBacPAK8 vector (Clontech, Palo Alto, CA) under control of AcMNPV polyhedrin promoter. Baculovirus expression of recombinant rat amphoterin was performed by co-transfecting the plasmid pBacPAK/Ramphoterin with a linearized pBacPAK viral DNA (Clontech) into Spodoptera frugiperda (Sf9) cells according to the manufacturer’s instructions. Recombinant viruses were identified and purified by their β-galactosidase-negative phenotype. Recombinant amphoterin was then purified to homogeneity by sequential chromatography on heparin-Sepharose and Affi-Gel blue. Recombinant amphoterin was identified by immunoblotting with monospecific anti-rat amphoterin IgG and mobility on SDS-PAGE.

Binding of Radiolabeled Amphoterin to Purified RAGE—Rat RAGE (2.5 μg) dissolved in bicarbonate carbonate buffer (pH 9.6) was incubated for 16 h at 4°C in MaxiSorp (Nunc) microtiter wells. Wells were washed four times with washing buffer (phosphate-buffered saline containing Tween 20, 0.02%), and blocked with phosphate-buffer saline containing bovine serum albumin (0.5%). Slides were incubated for 2 h at 37°C with the indicated concentration of 125I-amphoterin either alone or in the presence of an 100-fold excess of unlabeled material. Following the incubation period, wells were washed four times rapidly in washing buffer, as above, and eluted with NaCl (2 M) for 10 min. Specific binding was defined as the total binding minus binding in the presence of excess unlabeled material (i.e., nonspecific binding) and analyzed by the methods of Kidtz and Hunston (28). Where indicated, wells were preincubated with anti-RAGE (2%), or 125I-amphoterin prior to incubation with 125I-amphoterin (2–3 nM) or 125I-amphoterin was preincubated with various concentrations of rat sRAGE for 2 h at 37°C in phosphate-buffered saline containing bovine serum albumin (1%), either alone or in the presence of an 100-fold excess of unlabeled material. Following the incubation period, wells were washed four times rapidly in washing buffer, as above, and eluted with NaCl (2 M) for 10 min. Specific binding was defined as the total binding minus binding in the presence of excess unlabeled material (i.e., nonspecific binding) and analyzed by the methods of Kidtz and Hunston (28). Where indicated, wells were preincubated with anti-RAGE (2%), or 125I-amphoterin prior to incubation with 125I-amphoterin (2–3 nM) or 125I-amphoterin was preincubated with various concentrations of rat sRAGE for 2 h at 37°C in phosphate-buffered saline containing bovine serum albumin (1%) prior to incubation with RAGE adsorbed to the wells. In certain experiments, wells were preincubated with AGE albumin or native albumin for 2 h at 37°C prior to the addition of 125I-amphoterin.

Binding of Radiolabeled Amphoterin to Rat Cortical Neurons—Cortical neurons were isolated from the brains of 17-day-old embryonic Wistar rats according to established methods (23). Cells (10^5 cells/well) in Dulbecco's minimal essential medium (Life Technologies, Inc.), containing fetal bovine serum (10% Gemini, Calabasas, CA) were plated in 96-well plates and cultured for at least 12 h in serum-free medium, with poly-L-lysine (50 μg/ml) to enhance attachment (Sigma). The cell population was >90% neuronal based on immunostaining with anti-neuronal filament antibody (Sigma). Two days later, cells were washed extensively in phosphate-buffered saline, fixed with paraformaldehyde (2% fixation was required to prevent detachment of cells during the binding assay), and again washed four times with phosphate-buffered saline. Fixed cultures were then placed in phosphate-buffered saline containing bovine serum albumin (1%) and incubated for 2 h at 37°C with 125I-amphoterin either alone or in the presence of an 100-fold excess of unlabeled amphoterin. Wells were then washed four times with phosphate-buffered saline fixed cultures were then placed in phosphate-buffered saline then incubated with 125I-amphoterin (2–3 nM) or 125I-amphoterin was preincubated with anti-RAGE IgG prior to incubation with 125I-amphoterin (2–3 nM) or 125I-amphoterin was preincubated with various concentrations of rat sRAGE for 2 h at 37°C in phosphate-buffered saline containing bovine serum albumin (1%) prior to incubation with RAGE adsorbed to the wells. In certain experiments, wells were preincubated with AGE albumin or native albumin for 2 h at 37°C prior to the addition of 125I-amphoterin.

Assessment of Neurite Outgrowth—Eight-chamber slides (Nunc Lab-Tek) were coated with either purified brain amphoterin (20 μg/ml), recombinant amphoterin (10 μg/ml), or bovine serum albumin (50 μg/ml) for 16 h at 37°C. Cortical neurons were isolated from the cerebral hemispheres of day 17 rat embryos, plated on the latter precoated slides, and incubated for 18 h at 37°C in Dulbecco’s minimal essential medium containing bovine serum albumin (0.5%). Slides were subsequently fixed with paraformaldehyde (4%) containing Nonidet P-40 (0.1%) and stained with anti-tubulin antibody (Sigma) according to the manufacturer's instructions. Where indicated, isolated neuronal cells in medium containing bovine serum albumin (0.5%) were preincubated for 1 h at 4°C with anti-RAGE F(ab')2 or nonimmune F(ab')2, or amphoterin-coated wells were pretreated for 1 h at 37°C with sRAGE in medium containing bovine serum albumin (0.5%) prior to the addition of the cortical neurons.

In Situ Hybridization for RAGE and Amphoterin mRNA—In situ hybridization for RAGE was performed according to previously published protocols (11). Cortical neurons were prepared as described above, fixed with paraformaldehyde (4%) containing Nonidet P-40 (1%), or rat brains were harvested, fixed in formalin (10%), and thin sagittal sections were prepared.Digoxigenin-labeled riboprobes were transcribed from the plasmid B279–2A containing a 1406-base pair fragment of bovine RAGE (nucleotides 1–1406) cloned into the EcoRI site of pBluescript II SK. Antisense probe was transcribed from the T3 promoter with the plasmid linearized by XbaI. Transcription was performed using an RNA labeling kit (Boehringer Mannheim) with digoxigenin-UTP by in vitro transcription with either T3 or T7 polymerase. The digoxigenin-labeled RNA probe was hybridized to cellular mRNA for 16 h at 55°C and detected with anti-digoxigenin conjugated antibody (Sigma). Antibody was visualized after 6 h incubation with X-phosphate and nitro blue tetrazolium salt. In situ hybridization for amphoterin was performed using a plasmid containing 936 base pairs of rat amphoterin spanning nucleotides 32–967. The antisense probe was transcribed from the SP6 promoter with the plasmid linearized by XbaI. The sense probe was transcribed from the T7 promoter with the plasmid linearized by HindIII. Transcription, labeling, and detection were carried out as above and the antibody visualized for 10 h with X-phosphate and nitro blue tetrazolium salt.

Immunochemistry for RAGE and Amphoterin—Cortical neurons, prepared as described above, were fixed with paraformaldehyde (2%) or rat brains, harvested and prepared as above, were fixed in formalin (10%). Immunohistochemistry for RAGE was performed using rabbit anti-RAGE IgG and nonimmune rabbit IgG as above and immunohistochemistry for amphoterin was performed using chicken anti-amphoterin IgG and nonimmune chicken IgG. Peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-chicken IgG (Sigma) were used, respectively, as secondary antibodies, according to the manufacturer's instructions.

RESULTS

Purification of Ligands for RAGE from Bovine Lung—Detergent extract of bovine lung acetone powder was applied to heparin-Sepharose and the column was step-eluted with ascending concentrations of salt (Fig. 1A). Fractions were evaluated for the presence of RAGE binding activity following adsorption to microtiter wells using a competitive binding assay measuring specific binding of bovine 125I-RAGE. Only material eluting from the heparin column at an NaCl concentration of 0.5 M bound RAGE, and the latter fractions (50–100) were pooled, dialyzed, and applied to a column with immobilized RAGE (Fig. 1B). Following extensive washing, the column was eluted with high salt buffer (2 M NaCl), and fraction numbers 10–20 demonstrated the capacity to bind RAGE. The latter pool of active fractions from the immobilized RAGE column was subjected to nonreduced SDS-PAGE. Two protein bands were visualized on the gel by silver staining, corresponding to approximate M, values of 12,000 and 23,000. Material eluted from these bands was re-run on SDS-PAGE, appeared homogenous by silver staining with M, ~23,000 and M, ~12,000 (Fig. 1C, p23 and p12, respectively) and bound 125I-RAGE, based on subsequent gel elution (Fig. 1D). Because of limited amounts of purified protein which could be prepared, the current studies are limited to further characterization of the ~23,000 polypeptide which binds to RAGE.
sequence of p23 had 17 identifiable residues of the first 20. The 17 identified residues matched exactly the NH₂ terminus of bovine amphoterin (Table I). Five internal sequence peptides generated by Lys-C and cyanogen bromide cleavage were found (Table I). In total, the residues identified from the NH₂-terminal and five internal peptides (including tentative assignments) matched 65 of 68 residues in amphoterin. All of the inconsistent amino acid assignments occurred in one fragment (number 3), possibly the result of a contaminating peptide that copurified with fragment 3 on the HPLC). Each of the internal peptides generally aligned with amphoterin in a location that would be predicted by the cleavage used to generate the peptide (Lys-C peptides follow lysine residues and cyanogen bromide peptides follow methionine). These data indicated that p23 and amphoterin were identical and suggested the hypothesis that RAGE might be a cell surface acceptor site for amphoterin.

Amphoterin Binds to RAGE—Microtiter wells with adsorbed RAGE bound ¹²⁵I-rat brain amphoterin in a dose-dependent and saturable manner, with \( K_d = 6.4 \pm 1.0 \text{ nM} \), and a capacity of 46.7 ± 2.4 fmol/well (Fig. 2A). To further characterize the interaction of amphoterin with RAGE, rat amphoterin was expressed recombinantly using a baculovirus expression system and was purified from neonatal rat brain. In this case, adsorbed RAGE bound ¹²⁵I-recombinant amphoterin in a similar manner, with \( K_d \) of 10.24 ± 2.84 nM, and a capacity of 43.01 ± 4.53 fmol/well (data not shown). Binding of ¹²⁵I-rat brain amphoterin to RAGE-coated wells was specific, as demonstrated by dose-dependent inhibition on addition of excess sRAGE (Fig. 2B) or by preincubation of immobilized RAGE with blocking antibody to RAGE (Fig. 2C). In contrast, nonimmune IgG was without effect (Fig. 2C), and wells coated with an irrelevant polypeptide, such as albumin, in place of RAGE showed no specific binding of ¹²⁵I-amphoterin (data not shown). Binding was also inhibited by excess amounts of AGE albumin, whereas native albumin was without effect (Fig. 2D). Similar results were observed using recombinant radiolabeled amphoterin (data not shown).

Cultured E17 Cortical Neurons Express RAGE and Bind Amphoterin—Previous studies have demonstrated amphoterin
to be highly expressed in embryonic rat neurons, and that amphoterin promotes neurite outgrowth (23). As these data suggest a physiologic role for amphoterin in neurons early in development, we sought to establish whether RAGE would be expressed as well. Immunostaining with anti-rat RAGE IgG displayed the antigen in embryonic cortical neurons (Fig. 3A), whereas cultures incubated with nonimmune IgG were negative (Fig. 3B). Similarly, in situ hybridization revealed the presence of RAGE mRNA in E17 neuronal cells using the antisense probe (Fig. 3C), whereas hybridization with sense probe was negative (Fig. 3D). Although neural cell adhesion molecule and RAGE share some sequence homology, as both are members of the immunoglobulin superfamily, there is no evidence that antibodies to either cross-react, and the pattern of neural cell adhesion molecule immunostaining on cultured rat cortical neurons was distinct from that for RAGE (data not shown). Additional data supporting the specificity of RAGE immunostaining was its disappearance on addition of sRAGE (data not shown). Since neonatal cortical neurons expressed RAGE, it was important to determine if they bound amphoterin, and if this was mediated by interaction with RAGE. Radioligand binding studies with 125I-amphoterin were performed on cortical neurons isolated from neonatal rat brain (E17) and cultured on poly-L-lysine-coated wells after brief fixation in paraformaldehyde. 125I-Rat brain amphoterin bound specifically to cultured

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**Table I**

| NH2 terminal sequence | G | K | G | D | P | K | K | P | R | G | K | M | S | S | Y | A | F | F | V | Q |
|-----------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Atn NH2-terminal       |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| p23                   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

**Lys-C fragment**

1. Atn 30–41
   - p23
     - K | H | P | D | A | S | V | N | F | S | E
   - Lys-C fragment
     - 1 Atn 30–41
       - K | H | P | D | A | S | V | N | F | S | E
     - 2 Atn 112–122
       - K | I | K | G | E | H | P | G | L | S | I
     - Cyanogen bromide fragments
       - 4 Atn 13–29
         - M | S | S | Y | A | F | F | V | Q | T | C | R | E | E | H | K
       - 5 Atn 75–81
         - S | X | (V) | A | F | F | V | Q | T | X | X | E | E | X | X

2. Atn 30–41
   - p23
     - K | H | P | D | A | S | V | N | F | S | E
   - Lys-C fragment
     - 1 Atn 30–41
       - K | H | P | D | A | S | V | N | F | S | E
     - 2 Atn 112–122
       - K | I | K | G | E | H | P | G | L | S | I
     - Cyanogen bromide fragments
       - 4 Atn 13–29
         - M | S | S | Y | A | F | F | V | Q | T | C | R | E | E | H | K
       - 5 Atn 75–81
         - S | X | (V) | A | F | F | V | Q | T | X | X | E | E | X | X

3. Atn 127–38
   - p23
     - K | K | L | G | E | H | W | N | N | T | A
     - Cyanogen bromide fragments
       - 4 Atn 13–29
         - M | S | S | Y | A | F | F | V | Q | T | C | R | E | E | H | K
       - 5 Atn 75–81
         - S | X | (V) | A | F | F | V | Q | T | X | X | E | E | X | X

The following abbreviations were used: Atn, bovine amphoterin published sequence; numbers indicate the residues shown; X, no residue identifiable at that cycle; parenthesis, tentative assignment.

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**Fig. 2.** Binding of amphoterin to immobilized RAGE: dose dependence (A), effect of sRAGE (B), blocking antibody to RAGE (C), and AGE albumin (D). A, dose-dependence. Rat brain amphoterin was prepared and purified as described and radiolabeled with 125I. Microtiter wells were coated with purified RAGE (2.5 μg/well) and a binding assay was performed as described by adding 125I-amphoterin alone (total binding) or in the presence of 100-fold excess unlabeled amphoterin (nonspecific). Specific binding of 125I-amphoterin is plotted versus free/added 125I-amphoterin. Parameters of binding for rat brain amphoterin were \( K_d = 6.4 \pm 1.0 \text{ nM} \) with capacity of 46.7 ± 2.4 fmol/well. B, effect of sRAGE. 125I-Amphoterin (3 nM) was preincubated with the indicated concentration of sRAGE and then binding to RAGE immobilized on microtiter wells was studied as above. Maximal binding was specific binding observed in the absence of added sRAGE. C, effect of anti-RAGE IgG. Rat brain amphoterin was preincubated with anti-RAGE IgG or nonimmune IgG for 2 h and then a radioligand binding assay performed with 125I-amphoterin and excess unlabeled amphoterin as above. D, effect of AGE albumin. RAGE-coated wells were preincubated with AGE albumin or native albumin for 2 h and then a radioligand binding assay performed as above.
neurons in a dose-dependent and saturable manner, with $K_d = 8.8 \pm 2.4 \text{ nM}$ and a capacity of $28.8 \pm 2.8 \text{ fmol/well}$ (Fig. 4A). The binding of $^{125}$I-recombinant amphoterin was very similar, with $K_d = 8.09 \pm 1.60 \text{ nM}$ and capacity of $34.66 \pm 2.34 \text{ fmol/well}$ (data not shown). Binding of $^{125}$I-rat brain amphoterin to cortical neurons was dependent on interaction with RAGE; addition of sRAGE blocked binding in a dose-dependent manner (Fig. 4B) and pretreatment of cells with increasing doses of anti-RAGE IgG also prevented binding, whereas nonimmune IgG was without effect (Fig. 4C). Binding of $^{125}$I-rat brain amphoterin to cortical neurons was also inhibited in the presence of AGE albumin, whereas native albumin was without effect (Fig. 4D). Similar results were observed using recombinant radiolabeled amphoterin (data not shown).

RAGE-Ampoterin Interaction Promotes Neurite Outgrowth in Vitro—Previous studies suggested a role for amphoterin in neuronal development, based on induction of neurite outgrowth in vitro. We sought to determine if amphoterin interaction with RAGE mediated the latter effect. Cortical neurons plated on amphoterin, or poly-L-lysine substrates demonstrated neurite outgrowth (Fig. 5A and G, respectively). When amphoterin-coated wells were pretreated with sRAGE, a dose-dependent inhibition of neurite outgrowth occurred (Fig. 5B, sRAGE at 50 $\mu$g/ml; and Fig. 5Cc, sRAGE at 5 $\mu$g/ml). Further evidence implicating amphoterin-RAGE interaction in neurite outgrowth was inhibition in the presence of F(ab')$_2$ prepared from anti-RAGE IgG. Pretreatment of the neurons with anti-RAGE F(ab')$_2$ inhibited neurite formation by neurons in amphoterin-coated wells in a dose-dependent manner (Fig. 5D, anti-RAGE F(ab')$_2$ at 40 $\mu$g/ml; and Fig. 5F, anti-RAGE F(ab')$_2$ at 4 $\mu$g/ml) whereas pretreatment with nonimmune F(ab')$_2$ was without effect (Fig. 5D). To demonstrate that blocking access to RAGE...
specifically inhibited neurite outgrowth observed on amphoterin-coated substrates, parallel experiments were performed with neurons plated on poly-L-lysine, which provides a suitable substrate for neurite outgrowth. Neurite outgrowth on poly-L-lysine-coated wells was not inhibited by sRAGE (Fig. 5a) or by anti-RAGE F(ab')2 (Fig. 5i).

Co-localization of Messenger RNA and Protein for Amphoterin and RAGE in Developing Rat Brains—These data indicated that the interaction of amphoterin with cultured rat neurons is mediated, at least in large part, by RAGE, and that neurite outgrowth is a consequence of amphoterin binding to RAGE. As a first step in understanding the potential implications of our findings for the developing nervous system in vivo, studies to identify RAGE and amphoterin in brains of E17 as well as 5- and 17-day-old rats (P5 and P17, respectively) were performed. In situ hybridization studies revealed the co-localization of RAGE and amphoterin mRNA in the cerebral cortex of E17 rats (Fig. 6: a, E17 RAGE antisense; and b, E17 amphoterin antisense). Sense control experiments were negative for RAGE and amphoterin in E17 cerebral cortex (Fig. 6: c, E17 RAGE sense; and d, E17 amphoterin sense). Similar co-localization of RAGE and amphoterin mRNA was demonstrated in P5 cerebral cortex (Fig. 6: e, P5 RAGE antisense; and f, amphoterin antisense). Sense controls for P5 RAGE and amphoterin were negative (data not shown). RAGE and amphoterin mRNA were also co-localized in developing areas of the hippocampus in P5 rats (Fig. 6: g, P5 RAGE antisense; and h, P5 amphoterin antisense) as well as the developing cerebellum of P17 rats (Fig. 6: i, P17 RAGE antisense; and j, P17 amphoterin antisense). In all cases, sense controls were negative (data not shown). In P17 cerebellum, the pattern of RAGE and amphoterin mRNA localization was similar in the granular layer, but the intensity of the RAGE mRNA signal was detected to a slightly greater degree in the Purkinje layer (Fig. 6, arrow).

Immunohistochemistry studies further supported the co-localization of amphoterin and RAGE in the developing nervous system of the rat. RAGE and amphoterin protein were both present in the cerebral cortex of P5 rat (Fig. 7: a, staining with anti-RAGE IgG; c, nonimmune rabbit IgG revealed no staining) and b, staining with anti-amphoterin IgG (d, nonimmune chicken IgG was negative)). Higher magnification views of the P5 cerebral cortex revealed that while the cell bodies of the developing neurons were intensely positive for RAGE and amphoterin, staining of the developing axonal processes was even more dramatic (Fig. 7, e and f, respectively, thick arrow). Similar results were observed in the hippocampus of P5 rats (Fig. 7, g and i, anti-RAGE IgG; and h and j, anti-amphoterin IgG).

Taken together, these data from in situ hybridization and immunohistochemistry studies suggest that cells likely to express RAGE and amphoterin in developing rat brain are in close proximity, potentially allowing RAGE-amphoterin interaction to mediate neurite outgrowth.

DISCUSSION

We initially anticipated that cellular binding proteins/receptors for AGEs would be analogous to the collagen-like heterotrimeric scavenger receptors for acetylated low density lipoprotein on mononuclear phagocytes which mediate cellular uptake of modified lipoproteins (29). In fact, recent studies have shown that such scavenger receptors can interact with AGEs (9). However, RAGE, the first AGE cellular binding protein to be characterized in detail, was most analogous to immunoglobulin-like receptors. Other functional properties of RAGE suggested it was not an effective scavenger: infused AGEs, removed from the circulation by endothelial RAGE, were, in large part, transported by a transcytotic mechanism to the subendothelium where they became associated with matrix and smooth muscle cell elements (16). Consistent with these data, following infusion of AGE albumin, induction of oxidant stress, assessed by appearance of malondialdehyde epitopes in the vasculature, was present in endothelium, subendothelium, and smooth muscle cells (14). Furthermore, since the interaction of AGEs with RAGE enhanced monocyte chemotaxis and activation, and increased expression of endothelial vascular cell adhesion molecule 1, it was plausible that under physiologic conditions, natural or non-AGE ligands might interact with RAGE to mediate, for example, adhesive functions critical for normal development (12, 13, 30).

As lung is a rich source of RAGE, we considered this tissue a logical place in which to identify natural ligands of RAGE. The data presented in this study demonstrate that two polypeptides, with M<sub>r</sub> values of ~12,000 and ~23,000 on SDS-PAGE, bind AGES. Whereas the ~12-kDa polypeptide has an unique NH<sub>2</sub>-terminal sequence and its initial characterization is still under way, the ~23-kDa polypeptide proved to be identical to amphoterin based on extensive protein sequence data. Although the more rapid migration of p23 purified from bovine lung is somewhat different from the M<sub>r</sub> reported for amphoterin purified from rat brain (~30,000; Ref. 23), in view of sequence identity and recognition of p23 by anti-peptide antisera made to either amino acids 2–21 or 94–101 (data not shown), it is most likely that p23 arises from cleavage of amphoterin during the tissue extraction or purification procedure. Consistent with this view, Northern analysis of rat lung RNA using amphoterin cDNA showed a single band, which is similar to that reported for amphoterin mRNA in rat brain (data not shown).

More detailed studies of amphoterin-RAGE interaction, performed with larger amounts of amphoterin purified from rat brain or produced recombinantly with baculovirus, showed that...
binding of amphoterin to RAGE was specific, saturable, and of higher affinity than that previously reported for AGEs ($K_d$ of ~6 nM for amphoterin versus $K_d$ of ~50 nM for AGE albumin). Domains in amphoterin mediating interaction with RAGE appear to be unrelated to AGE-like epitopes, as enzyme-linked immunosorbent assay of amphoterin preparations showed no detectable AGE antigen and anti-AGE IgG had no effect on amphoterin binding to RAGE (data not shown), although this antibody has been previously shown to block interaction of AGE albumin and AGEs immunosolated from diabetic plasma with RAGE (14).

Amphoterin was first identified by Rauvala and Pihlaskari (23), as an ~30-kDa polypeptide selectively and highly expressed in the developing rat central nervous system. Cultured embryonic neurons plated on amphoterin-coated matrices formed neuritic processes, suggestive of their differentiation, a critical step in the overall design and maturation of the nervous system. RAGE expression in cell bodies and axonal processes was first observed in the bovine nervous system in motor neurons and in certain populations of cortical neurons (11), and has been extended in this study to include the developing rat central nervous system. Consistent with these data, embryonic rat cortical neurons which have been show to produce amphoterin also expressed RAGE. Neuronal RAGE was functional, as indicated by its capacity to bind amphoterin and to mediate amphoterin-induced neurite outgrowth.

Previous studies have identified potential binding sites for amphoterin distinct from RAGE. Salmivirta and colleagues
nervous system based on solid phase binding assays. The present studies identify RAGE as a cellular binding site for amphoterin, based on binding and functional data, and provide the strongest evidence, thus far, for a putative amphoterin receptor in the developing central nervous system.

Expression of amphoterin has also been demonstrated in transformed cells (33). Parkkinen and colleagues (33) showed that C6 glioma cells, HL-60 promyelocytes, U937 promonocytes, HT1080 fibrosarcoma cells, and B16 melanoma cells produced amphoterin. These investigators also demonstrated that amphoterin strongly enhanced the rate of plasminogen activation and promoted the generation of surface-bound plasmin by both tissue-type and urokinase-type plasminogen activators, suggesting a role for amphoterin in invasive neoplastic lesions (18). Future studies will be required to determine if RAGE modulates any of these properties of amphoterin in the biology of neoplasia.

In summary, these data suggest possible roles for RAGE under circumstances in which the presence of AGEs is unlikely. Specifically, in the rat embryonic nervous system, RAGE is highly expressed, and co-localizes, at the level of antigen and mRNA, with the presence of amphoterin. As blocking access to RAGE, with either excess sRAGE or anti-RAGE IgG/F(ab')2, prevents amphoterin-induced neurite outgrowth in cell culture, it is tempting to speculate that RAGE mediates the potential role of amphoterin in neuronal development. These observations provide a first step in characterizing a novel aspect of the biology of RAGE, and emphasize the importance of future in vivo studies to address the physiologic significance of amphoterin-RAGE interaction, with respect to the development and maturation of the central nervous system.

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