Sp1-binding Elements in the Promoter of RAGE Are Essential for Amphoterin-mediated Gene Expression in Cultured Neuroblastoma Cells*

(Received for publication, February 13, 1998, and in revised form, July 13, 1998)

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The receptor for AGE (RAGE), a member of the immunoglobulin superfamily of cell-surface molecules, interacts with a range of ligands (1–2). In pathophysiologic circumstances, advanced glycation end products (AGEs) result from nonenzymatic glycation and oxidation of proteins and lipids and accumulate in conditions such as diabetes, renal failure, and amyloidoses (3–4). AGEs interact with RAGE in a manner linked to perturbation of vascular and inflammatory cells (5–9). In addition, RAGE interacts with amyloid β-peptide, consequences of which include enhanced neuronal and microglial oxidant stress and dysfunction (10–11). These processes may be important in the pathogenesis of neurodegenerative disorders such as Alzheimer’s disease. The expression of RAGE is strikingly enhanced in cells affected in settings associated with increased accumulation of AGEs and amyloid β-peptide (12–14). We recently identified that an important means by which the expression of RAGE might be regulated in inflammatory-type milieu such as diabetic vasculature and wounds, and Alzheimer brain, is via two functional NF-kB-binding elements located with the 5′-flanking region of the RAGE gene (15). Transfection of 5′-deletion luciferase reporter gene constructs containing two mutated NF-kB-binding sites into either endothelial cells or vascular smooth muscle cells significantly diminished RAGE promoter responsiveness to prototypic inflammatory stimuli such as lipopolysaccharide (15).

Our studies have also identified that the expression of RAGE is enhanced in the developing central nervous system, a setting in which excessive accumulation of AGEs and amyloid β-peptide is not likely (16). Indeed, the increased expression of RAGE in neurons of the cerebrum, hippocampus, and cerebellum colocalized with that of the polypeptide amphoterin. In in vitro studies, amphoterin bound neuronal RAGE in a dose-dependent manner and mediated neurite outgrowth. In the presence of blockade of neuronal RAGE, employing either anti-RAGE F(ab′)2 or soluble RAGE, the extracellular two-thirds of RAGE (17–18), amphoterin-mediated neurite outgrowth was inhibited (16).

We hypothesized that ligation of neuronal RAGE by amphoterin in the developing nervous system might be, at least in part, one means by which the expression of RAGE is enhanced in that setting. Such findings would suggest contributory mechanisms by which amphoterin-mediated regulation of RAGE promotes outgrowth of neurites. Since examination of the promoter of RAGE revealed multiple putative binding elements for Sp1, a transcription factor essential for early embryonic development (19–20), we tested the hypothesis that amphoterin-mediated activation of Sp1 might be important in regulation of RAGE expression in developing neurons.

In this study, we demonstrate that interaction of amphoterin with RAGE on cultured neuroblastoma cells up-regulates tran-
scription and translation of RAGE. The presence of functional Sp1-binding elements within the promoter of RAGE appears necessary for this outcome. These findings further suggest a role for amphoterin-RAGE interaction in the development of the nervous system and implicate RAGE as a gene whose regulation in the process of neuronal development is, at least in part, mediated by Sp1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**SK-N-SH cells were obtained from American Type Culture Collection (Rockville, MD). SK-N-SH cells are derived from a human neuroblastoma and their neuroendocrine characteristics have been extensively characterized (21). Cells were grown in Medium 199 containing fetal bovine serum (FBS) (10%) (Life Technologies, Inc.) in the presence of penicillin (100 units/ml) and streptomycin (100 μg/ml) in humidified incubators containing CO2 (5%).

**Preparation of Nuclear Extracts from Cultured Neuroblastoma Cells—**SK-N-SH cells were washed once with PBS and cultured in Medium 199 containing FBS (1%) for 16 h. After complete removal of serum for another 24 h. At the end of that time, cells were exposed to either recombinant rat amphoterin (40 μg/ml) or BSA (40 μg/ml) (Sigma) for 24 h. After washing with PBS (phosphate-buffered saline), cells were resuspended in PBS containing EDTA (final concentration, 0.05 M). After further washing to remove EDTA, cells were incubated with monospecific, polyclonal rabbit anti-human RAGE IgG for 1 h at 37 °C .

**Flow Cytometry Studies—**SK-N-SH cells were cultured in Medium 199 containing FBS (1%) for 16 h prior to complete removal of serum for another 24 h. At the end of that time, cells were exposed to either recombinant rat amphoterin (40 μg/ml) or BSA (40 μg/ml) (Sigma) for 24 h. After washing with PBS (phosphate-buffered saline), cells were resuspended in PBS containing EDTA (final concentration, 0.05 M). After further washing to remove EDTA, cells were incubated with monospecific, polyclonal rabbit anti-human RAGE IgG for 1 h at 37 °C. The suspension was then washed in PBS, and cells were resuspended in fluorescent-labeled goat anti-rabbit IgG antibody for 1 h at 37 °C. After washing with PBS, cells were resuspended in PBS and subjected to flow cytometry (FACS Star Plus, Becton-Dickinson, Franklin Lakes, NJ). Control incubations were performed using rabbit nonimmune IgG (Sigma).

**Immunohistochemistry—**Immunohistochemistry for detection of cell-surface RAGE on SK-N-SH cells was performed as described previously (16).

**Northern Blot Analysis—**SK-N-SH cells were grown in Medium 199 (1% FBS) for 16 h and then completely starved of serum for another 24 h. At the end of that time, cells were exposed to the indicated amount of recombinant rat amphoterin purified, prepared, and characterized as described previously (16) or bovine serum albumin (BSA) (Sigma) for 24 h. Cells (1.5 × 10^6) were washed twice in buffer containing Tri, NaCl, 0.1 M; phenylmethlysulfonfluoride, 0.002 μM; and octyl-β-glucoside, 1% at 4 °C for 4 h and then centrifuged at 11,000 × g for 20 min. The supernatant was collected and protein concentration determined using the Bradford reagent (Bio-Rad). The supernatant was then diluted with appropriate amounts of sodium bicarbonate/carbonate buffer, pH 9.5, in order to coat equal amounts of protein (0.1 ml) onto each well of a plastic dish (Nunc Maxisorp). After 16 h at 4 °C, unbound sites on the plate were blocked in PBS buffer containing 1% BSA. Wells were then incubated with rabbit anti-RAGE IgG (34 μg/ml) (prepared and characterized as described previously; see Ref. 16) and sites of primary antibody binding visualized using goat anti-rabbit IgG with peroxidase conjugate (Sigma). A standard curve was prepared using human RAGE previously prepared and purified from a baculovirus expression system. The limit of detection in the assay is <0.001 ng/ml.

**Electrophoretic Mobility Shift Assay (EMSA)—**Based on the results of DNase I footprinting assays, four double-stranded oligonucleotide probes were designed (see Fig. 5A). (23). Approximately 150 pg of (-32P)ATP-labeled probes was incubated with nuclear extract protein (5 μg) for 30 min. For competition assays, nuclear extracts were incubated with 100-fold molar excess of the indicated unlabeled nucleotides

**Nuclear Run-on Transcription Activity—**Nuclear run-on transcription assay was performed as described previously (8, 22). After incubation of SK-N-SH cells with amphoterin (40 μg/ml) or BSA (40 μg/ml) for 24 h at 37 °C, cells were washed twice with cold PBS, scraped, and pelleted by centrifugation. Pellets were then resuspended in lysis buffer (10 ml) containing Tris, 0.01 M, pH 7.4; NaCl, 0.01 M; MgCl2, 0.003 M; Nonidet P-40 (0.5%); leupeptin, 50 μg/ml; diithiothreitol, 0.001 M; phenylmethylsulfonyl fluoride, 0.0005 M; and aprotinin, 100 μg/ml for 15 min on ice, and nuclei were collected at 50 × g for 5 min at 4 °C. The supernatants were discarded, and nuclei were resuspended in 0.2 ml of reaction buffer containing Tris, 0.01 M, pH 8.0; MgCl2, 0.005 M; KCl, 0.3 M; diithiothreitol, 0.001 M; ATP, 0.0005 M; CTP, 0.0005 M; GTP, 0.0005 M; and 200 microcuries of [32P]UTP (ICN Pharmaceuticals, Aurora, OH) for 4 h at 37 °C. The RNA was extracted as above, and equal amounts of sample were resuspended in hybridization buffer containing PIPES, 0.05 M, pH 6.8; EDTA, 0.01 M; NaCl, 0.6 M; SDS, 0.2%; and denatured salmon test DNA, 100 μg/ml. Hybridization to denatured human RAGE and β-actin DNA slot-blotted onto nylon filters was performed at 42 °C for 72 h after pre-hybridization at 80 °C for 2 h in hybridization buffer as above containing SDS (0.1%) for 1 h three times, briefly air-dried, and exposed to autoradiographic film. For binding to the nylon filter, the human RAGE cDNA and β-actin cDNA were denatured by incubation with NaOH (0.3 M) for 30 min at 65 °C, and cDNA was spotted on the nylon filter and cross-linked with a UV cross-linker.

**DNase I Footprinting Assay—**DNase I footprinting assay was employed to determine the binding affinities of nuclear extract proteins to specific DNA sites located within the 5′-flanking region of the human RAGE gene as revealed by computer analysis (Genetics Computer Group; see Ref. 23). DNase I footprinting assay was performed with Core Footprinting System (Promega, Madison, WI) with modifications. An Sp1 probe spanning the region from −720 to +11 of the human RAGE gene was labeled at the 3′-end of the antisense strand with [32P]dCTP by the Klenow fragment (Stratagene). For each DNase I nicking reaction, 35 ng of the 32P-labeled DNA fragment was mixed in binding buffer (2×; 0.025 ml) containing BSA (200 μg/ml) in the presence or absence of the indicated amount of the purified human Sp1 (Promega) and incubated on ice for 4 h. To this was then added calcium/magnesium solution (0.05 ml) for 1 min at room temperature, followed by RQ 1 RNase-free DNase (0.003 ml), previously diluted 1:40 in dilution buffer, for 1 min at room temperature. The reaction was terminated by the addition of Stop Solution (prewarmed to 37 °C) (0.09 ml). The extraction reaction was then performed with phenol:chloroform:isoamyl alcohol (0.2 ml) one time. The upper aqueous phase was transferred to fresh tubes, and ethanol (100%; 0.5 ml) was added for precipitation on ice for 20 min. The tubes were then subjected to centrifugation at 14,000 × g for 15 min; supernatants were removed with ethanol (70%), and vacuum-dried. The pellet was resuspended in Loading Solution (0.004 ml), heated to 95 °C for 2 min, and then quickly chilled on ice for at least 2 min. The samples were then electrophoresed onto polyacrylamide sequencing gels (6%). Gels were then placed on x-ray film at −70 °C with an intensifying screen.
for 15 min at room temperature prior to incubation with the end-labeled oligonucleotide probes. Consensus sequence oligonucleotides for Sp1 were obtained from Promega; and consensus oligonucleotides were synthesized (Life Technologies) for the following: NF-IL6 (5'-GGGAAAAT- GCAGATTGCGCAATCTGCAAAAGGG-3'); AP-2 (5'-GGGAATCGAATCTGACCAGCCCGCCGCCGGCGTAGGG-3'); and NF-kB (5'-GGGAAAAAGGGAGGACTTTCCTCAGGGAAAGG-3') (Santa Cruz). For super-shift assays, nuclear extract was incubated with antibody prepared against human Sp1, NF-kB p50/p65, NF-IL6, or AP2 (Santa Cruz) for 4 h prior to the addition of the indicated probes.

5'-Deletion Plasmids of the RAGE Promoter and PCR-directed Mutagenesis—5'-Deletion fragments of the RAGE promoter and PCR-directed mutagenesis constructs of the RAGE promoter were created by subcloning into the pGL3-Basic vector (Promega) different fragments of DNA PCR-amplified from the 5'-flanking region of the human RAGE gene (Figs. 6 and 7). Due to the close proximity of Sp1 II/III, a single mutation construct encompassing these two potential binding sites was generated. All constructs employed for transfection were confirmed by DNA sequencing using ALFexpress DNA sequencing system (Amersham Pharmacia Biotech).

Transfection Experiments—SK-N-SH cells were grown and maintained as described above. Transfection of the cells was performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, 2 x 10^5 cells were plated onto the wells of tissue culture plates (6-wells, Corning, Corning, NY) 1 day prior to transfection. The DNA mixture for transfection was composed of test plasmid (2 μg) and pSV-β-galactosidase (0.5 μg) which served as internal control to normalize activities of luciferase. Cells were exposed to the mixture of LipofectAMINE and plasmid for 5 h. Following removal of the LipofectAMINE/plasmid mixture, fresh medium was added and the incubation continued for 48 h. Prior to studies of basal-stimulated function of the RAGE promoter, cells were washed with PBS and placed in medium as above, except that serum was omitted for 24 h prior to harvesting. Luciferase activity was determined using standard reagents (Promega) and measured in a luminometer (Wallac, Gaithersburg, MD) as directed, and results were standardized for β-galactosidase activity.

Statistical analysis was performed using ANOVA (analysis of variance).

RESULTS

Enhanced Expression of RAGE mRNA and Protein on Neuroblastoma Cells by Amphoterin—Increased expression of RAGE in the developing neurons of the central nervous system co-localizes with high levels of expression of amphoterin. In order to determine if interaction of amphoterin with RAGE, at
The enhanced mRNA for RAGE by amphoterin was dose-dependent. Maximal levels (6.1-fold enhancement) of mRNA for RAGE were noted in the presence of amphoterin, 40 μg/ml (Fig. 1A). Nuclear run-on transcription assay revealed that amphoterin-mediated increase in mRNA for RAGE was due to enhanced transcription (Fig. 1C).

Consistent with enhanced mRNA for RAGE in amphoterin-treated cultured neuroblastoma cells, increased cellular RAGE antigen was detected by ELISA in a dose-dependent manner (Fig. 2A). Compared with cells treated with BSA, an ~5.2-fold increase (p < 0.01) in RAGE protein was noted in the presence of amphoterin. That these effects were mediated by RAGE was demonstrated by inhibition of amphoterin-mediated expression of RAGE in the presence of anti-RAGE IgG, but not nonimmune IgG (Fig. 2B). Incubation of SK-N-SH cells with anti-RAGE IgG alone was without effect. Increased cell-surface RAGE in the presence of amphoterin was further confirmed by flow cytometry and immunohistochemistry (data not shown). Taken together, these data indicated that amphoterin modulated expression of RAGE in cultured neuroblastoma cells in a RAGE-dependent manner.

Identification of Sp1-binding Elements within the Promoter of RAGE—In our previous studies, we identified two putative binding sites for the transcription factor Sp1 (designated Sp1 II and Sp1 V) (Fig. 3) in the promoter of RAGE using computer analysis (Genetics Computer Group; 23) by matching consensus sequences for Sp1 with those in the RAGE promoter.

To determine if the putative Sp1-binding sites within the promoter of RAGE were functional, DNase I footprinting analysis was performed. By using purified human Sp1 and 32P-labeled RAGE promoter fragment from −720 to +11, protection of Sp1 sites (II and V) was demonstrated (Fig. 4A). Surprisingly, three more protected areas were noted (Sp1 I, Sp1 II, Sp1 III, and Sp1 IV). Comparison of the core nucleotide sequences comprising these footprints with the Sp1 consensus sequences reported by Bucher (25) revealed nearly 100% identity, or one nucleotide difference (Fig. 4B).

Activation of Sp1 DNA Binding Activity by Amphoterin in Cultured Neuroblastoma Cells—In order to determine if amphoterin induced activation of Sp1 DNA binding activity in neuroblastoma cells, SK-N-SH cells were treated with BSA, amphoterin, or amphoterin in the presence of anti-RAGE IgG or nonimmune IgG. Nuclear extracts were prepared and EMSA performed employing either Sp1 consensus probe or specific Sp1-binding site probes synthesized as indicated in Fig. 5A. Compared with treatment of neuroblastoma cells with BSA (Fig. 5B, lane 3), incubation with amphoterin resulted in increased Sp1 DNA binding activity (Fig. 5B, lane 1). Amphoterin-mediated increases in Sp1 were significantly decreased in
The presence of anti-RAGE IgG (Fig. 5B, lane 7) but not in the presence of nonimmune IgG (Fig. 5B, lane 9). Incubation of cells with anti-RAGE IgG alone was without effect (Fig. 5B, lane 5). Specificity for Sp1 was confirmed in competition experiments in which a marked attenuation of the respective bands was noted in the presence of excess unlabeled Sp1 (Fig. 5B, lanes 2, 4, 6, 8, and 10). Furthermore, the effects of amphoterin in increasing nuclear binding activity of Sp1 were dose-dependent; maximal activation of Sp1 DNA binding activity was noted in the presence of amphoterin, 40 μg/ml (Fig. 5C). In the presence of higher concentrations of amphoterin, no further enhancement in activation of Sp1 DNA binding activity was observed (not shown).

In order to determine if other transcription factors were involved in amphoterin-mediated increases in RAGE expression, NF-kB, AP2, and NF-IL6 were studied, since putative binding sites for these transcription factors were identified in the RAGE promoter by computer analysis. In amphoterin-stimulated cells, there was no evidence of activation of NF-IL6, AP2, or NF-kB (Fig. 5D, lanes 8, 9, and 10, respectively). In contrast, Sp1 was activated in amphoterin-stimulated cells (Fig. 5D, lanes 11–14). Consistent with these data, supershift assays employing anti-Sp1 IgG indicated a shift of the Sp1 band in amphoterin-treated cells (Fig. 5E, lane 7 and 8); no shift was observed in the presence of anti-NF-IL6 IgG, anti-NF-kB p50/−65 IgG, anti-AP2 IgG (Fig. 5E, lanes 1–6), or nonimmune IgG (data not shown). Incubation of radiolabeled consensus Sp1 probe with excess unlabeled Sp1 probe resulted in disappearance of the band (Fig. 5E, lane 9). In contrast, incubation with excess unlabeled NF-IL6, AP2, or NF-kB was without effect (data not shown). Taken together, these data suggested that Sp1 was an important transcription factor in amphoterin-mediated increases in RAGE expression in SK-N-SH cells.

**Functional Analysis of Sp1-binding Sites in the Promoter of RAGE in Basal and Amphoterin-treated Cultured Neuroblastoma Cells**—In order to delineate regulatory elements within the promoter of RAGE responsive to amphoterin, a series of 5′-deletion luciferase-reporter gene plasmids of the RAGE promoter was generated in a pGL3-basic vector as indicated in Fig. 6. The six constructs were then transfected into neuroblastoma cells. In the absence of amphoterin (basal; exposure of cells to BSA), the constructs containing one or more Sp1-binding sites contributed to basal promoter function (pGL-245, pGL-202, pGL-118, and pGL-60), but generally not in a manner significantly greater than construct pGL-40, which contains no putative Sp1-binding sites. From these data, it is not possible to discern if Sp1-binding elements were central in basal promoter function, as it is possible that other elements contained within these individual fragments contribute to basal activity (either enhancing or silencing elements). No luciferase activity was noted upon transfection with pGL-245R (construct containing reverse orientation of that in pGL-245).

In the presence of amphoterin, relative luciferase activity was increased ~3.1-fold in amphoterin-treated cells transfected with pGL-245 or pGL-202 compared with basal conditions (p < 0.01 in each case). Relative luciferase activity was increased ~3.8-fold in amphoterin-stimulated cells transfected with pGL-118 or pGL-60 compared with basal conditions (p < 0.01 in each case). However, in cells transfected with pGL-40, no increase in relative luciferase activity was noted upon incubation of the cells with amphoterin (p > 0.05). In cells transfected with pGL-245, pGL-202, pGL-118, or pGL-60, preincubation of the cells with anti-RAGE IgG prior to treatment with amphoterin significantly attenuated the amphoterin-mediated increase in luciferase activity (p < 0.01 in each case compared with amphoterin stimulation), such that relative luciferase activity was not significantly different than that observed under basal conditions (BSA). In contrast, treatment of the cells with nonimmune IgG was without effect (data not shown). Similarly, incubation with anti-RAGE IgG did not affect basal luciferase activity (data not shown).

In order to determine the importance of individual putative...
Sp1-binding elements in basal promoter function and responsiveness to amphoterin, PCR-directed mutagenesis was performed as indicated in Fig. 7. Under basal conditions (treatment with BSA), single or multiple mutational inactivation of Sp1-binding sites had no effect on relative luciferase activity compared with wild-type pGL-245 (containing all putative Sp1-binding sites), p > 0.05 in each case (Fig. 7). These data supported the concept that Sp1-binding sites are not central for maintenance of basal RAGE promoter function. In the setting of cell stimulation with amphoterin, no effect on relative luciferase activity from single (Fig. 7, lines 2, 7, and 11) or double mutations (Fig. 7, lines 3, 4, and 8) of Sp1-binding sites was detected. However, when Sp1-binding site V was mutated in either single (Fig. 7, line 13) or double mutations (Fig. 7, lines 5, 9, and 12), relative luciferase activity decreased ~23% in the presence of amphoterin (p < 0.01 in each case). In the setting of three simultaneous mutations (Fig. 7, lines 6, 10, 14, and 15), an ~46% decrease was noted only in the case of mutations including that of Sp1 V (Fig. 7, lines 10, 14, and 15). If wild-type Sp1 V was present, luciferase activity was only slightly decreased (Fig. 7, line 6), p > 0.05. Upon simultaneous mutation of all Sp1-binding sites identified within this region, complete loss of promoter responsiveness to amphoterin resulted (Fig. 7, line 16). These data indicate that Sp1 V is important in mediating promoter responsiveness to amphoterin; however, mutual cooperation of Sp1-binding sites appears essential for optimal promoter responsiveness.

**DISCUSSION**

We have reported that amphoterin may interact with neuronal RAGE to mediate increased transcription and translation of RAGE in an Sp1-dependent manner. Amphoterin has been previously described to be highly and selectively expressed in embryonic rat cortical neurons; indeed, by a few days after birth its expression is markedly attenuated. These findings, along with the observation that amphoterin mediated neurite outgrowth in vitro, suggested a role for amphoterin in the development of the nervous system (26–28).

Our laboratory first identified RAGE as a potential neuronal cell-binding site for amphoterin as application of agents to block access to RAGE, such as anti-RAGE F(ab')2 or soluble RAGE, the extracellular two-thirds of RAGE, inhibited neurite outgrowth in vitro; indeed, by a few days after birth its expression is markedly attenuated. These findings, along with the observation that amphoterin mediated neurite outgrowth in vitro, suggested a role for amphoterin in the development of the nervous system (26–28).

**FIG. 6. Development of luciferase chimeras from the 5'-flanking region of the human RAGE gene: functional analysis under basal and amphoterin-stimulated conditions in cultured SK-N-SH cells.** The properties of the 5'-flanking region of the RAGE gene luciferase (Luc) reporter gene constructs are shown on the right. The start site of transcription is denoted as +1. The five Sp1-binding sites identified in these areas are represented by symbols located in the black boxes. The constructs were then transfected into SK-N-SH cells as described above. Cells were serum-starved prior to incubation with either amphoterin (40 μg/ml top black bar in each case) or BSA (40 μg/ml middle bar in each case) for 24 h. The third bar represents results from cells pretreated with anti-RAGE IgG (50 μg/ml) for 1 h prior to incubation with amphoterin. Relative luciferase activity was obtained by comparison of the luciferase activity of cells transfected with pSV-β-galactosidase control vector. Data represent the mean ± S.E. of the mean obtained from three individual experiments. Incubation of transfected cells with nonimmune IgG had no effect on relative luciferase activity after stimulation with amphoterin (data not shown). Statistical analyses (ANOVA) of basal conditions (BSA) are as follows: pGL-245 and pGL-202 versus pGL-40, p > 0.05; and pGL-118 and pGL-60 versus pGL-40, p < 0.05. Amphoterin stimulation is as follows: pGL-245 versus pGL-40, p < 0.01; pGL-202 versus pGL40, p < 0.01; pGL-118 versus pGL40, p < 0.01; and pGL-60 versus pGL40, p < 0.01. Amphoterin stimulation versus basal for each construct is as follows: pGL-245, p < 0.01; pGL-202, p < 0.01; pGL-118, p < 0.01; pGL-60, p < 0.01; and pGL-40, p > 0.05. The presence or absence of anti-RAGE IgG for each construct is as follows: pGL-245, p < 0.01; pGL-202, p < 0.01; pGL-118, p < 0.01; pGL-60, p < 0.01; and pGL-40, p > 0.05.

**FIG. 5. Identification of Sp1-binding sites in the 5'-flanking region of the human RAGE gene by EMSA.** A, the indicated probes were synthesized and employed in labilities below. B, effect of amphoterin. SK-N-SH cells were cultured in Medium 199 containing FBS (1%) for 16 h and then deprived of serum for 24 h. Cells were then incubated with amphoterin (40 μg/ml; lanes 1 and 2), BSA (40 μg/ml; lanes 3 and 4), or anti-RAGE IgG (50 μg/ml) (lanes 5 and 6) for 8 h. Certain cells were preincubated for 1 h with either anti-RAGE IgG (50 μg/ml) (lanes 7 and 8) or nonimmune IgG (50 μg/ml) (lanes 9 and 10) prior to treatment with amphoterin. Nuclear extracts were prepared as described from cultured neuronal SK-N-SH cells, and EMSA was performed using consensus probes for Sp1. Each lane contains 5 μg of nuclear protein. In competition experiments, a 100-fold excess of unlabeled consensus probe for Sp1 was preincubated with the nuclear extract (lanes 2, 4, 6, 8, and 10). The protein-DNA complexes were analyzed by polyacrylamide gels (6%). C, dose response. The dose-dependent effects of amphoterin are shown in EMSA using the four synthesized Sp1 probes in A. In each case, concentrations of amphoterin are shown in μg/ml. EMSA was performed as in B. Densitometric analysis is shown in the inset. D, specificity studies. Nuclear extract was prepared from BSA- or amphoterin-treated cells (40 μg/ml), and EMSA was performed using the indicated 32P-labeled consensus or synthesized Sp1 probes. E, supershift assays. Nuclear extract (5 μg) was prepared as above from amphoterin (40 μg/ml)-treated cells and preincubated for 4 h at 4°C with the indicated antibodies (all antibodies were employed at final concentration, 2,000 μg/ml). Extracts were then incubated with 32P-labeled consensus probes for NF-IL6 (lane 1), NF-kB (lane 2), AP2 (lane 3), or consensus probe for Sp1 (lanes 4–9) in the presence of the indicated antibodies or excess (100-fold) unlabeled oligonucleotide probe. DNA-protein complex was then detected on a 6% polyacrylamide gel.
Sp1-binding Elements and Expression of RAGE

Relative luciferase activity

![PCR-directed mutagenesis and relative luciferase activities of chimera from the 5′-flanking region of the RAGE gene expressed in cultured SK-N-SH neuroblastoma cells.](image)

outgrowth selectively on amphoterin-coated matrices. Consistent with a possible role for the interaction of these two polypeptides in neuronal migration in the developing nervous system, increased expression of RAGE was noted by both immunohistochemistry and in situ hybridization in cerebral, hippocampal, and cerebellar neurons that co-localized with amphoterin (16). Similar to the rapid decline in expression of amphoterin in postnatal rat neurons, the expression of RAGE in adult neurons in homeostasis, although not completely absent, is significantly diminished. In settings of enhanced neuronal perturbation, however, such as in amyloid β-peptide-enriched environments and co-incident oxidant stress, potent stimuli mediate striking increases in RAGE expression (10–11). Our previous findings implicated an important role for two functional binding elements for NF-κB within the promoter of RAGE in this process (15).

The present studies implicate an important role for Sp1 in amphoterin-mediated increases in RAGE expression in cultured neuroblastoma cells. Sp1, a ubiquitous transcription factor, has been implicated both in maintenance of homeostasis and cellular responsiveness to environmental perturbation (29–31). Sp1 has certainly been linked intimately to the process of normal development. Marin and colleagues (19) recently reported that mice homozygous for deletion of the gene for Sp1 demonstrate lethality early in gestation. Whereas a broad range of phenotypic abnormalities was demonstrated in these mice, earlier clues suggested the critical role of Sp1 in development of the nervous system. Saffer and colleagues (20) described that in early mouse fetuses, the most striking immunoreactivity for Sp1 was found in neural tissue.

Our findings do not preclude other mechanisms involved in enhanced RAGE expression in developing neurons. Other, as yet unidentified, cues from the environment may act together with amphoterin to modulate transcription and translation of RAGE. In this context, other cell surface interaction sites for amphoterin, such as syndecam (32) and sulfoglycolipids (33), have been identified. It is possible that ligation of such sites may initiate the generation of other mediators important in enhancing RAGE expression. Certainly, in vivo, the complex interplay of multiple cell-cell and cell-matrix interactions converge to eventuate in expression of many genes important in neuronal growth, adhesion, and migration. In that setting, therefore, it is likely that a number of exogenous and intracellular stimuli may contribute to increased expression of RAGE in developing neurons.

Taken together, our studies suggest that regulation of RAGE in neurons is mediated, at least in part, by its ligation by amphoterin, a polypeptide selectively expressed in develop-
ment. Subsequent activation of Sp1 and its binding to specific elements within the promoter of RAGE appear necessary for modulation of RAGE expression. Given the importance of Sp1 in embryonic development, these findings further suggest a role for amphoterin-RAGE interaction in the development of the nervous system.

Acknowledgment—We gratefully acknowledge the suggestions of Dr. Osamu Hori in the performance of these studies.

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