THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 51, Issue of December 22, pp. 40096–40105, 2000
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Coregulation of Neurite Outgrowth and Cell Survival by Amphoterin and S100 Proteins through Receptor for Advanced Glycation End Products (RAGE) Activation*

Received for publication, August 3, 2000, and in revised form, August 30, 2000
Published, JBC Papers in Press, September 27, 2000, DOI 10.1074/jbc.M006993200

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Amphoterin is a protein enhancing process extension and migration in embryonic neurons and in tumor cells through binding to receptor for advanced glycation end products (RAGE), a multiligand transmembrane receptor. S100 proteins, especially S100B, are abundantly expressed in the nervous system and are suggested to function as cytokines with both neurotrophic and neurotoxic effects. However, the cell surface receptor for the cytokine function of S100B has not been identified. Here we show that two S100 family proteins, S100B and S100A1, activate RAGE in concert with amphoterin inducing neurite outgrowth and activation of transcription factor NF-κB. Furthermore, activation of RAGE by amphoterin and S100B promotes cell survival through increased expression of the anti-apoptotic protein Bcl-2. However, whereas nanomolar concentrations of S100B induce trophic effects in RAGE-expressing cells, micromolar concentrations of S100B induce apoptosis in an oxidant-dependent manner. Both trophic and toxic effects are specific for cells expressing full-length RAGE since cells expressing a cytoplasmic domain deletion mutant of RAGE are unresponsive to these stimuli. These findings suggest that activation of RAGE by multiple ligands is able to promote trophic effects whereas hyperactivation of RAGE signaling pathways promotes apoptosis. We suggest that RAGE is a signal-transducing receptor for both trophic and toxic effects of S100B.

Receptor for advanced glycation end products (RAGE)[1] is a member of the immunoglobulin superfamily of cell surface proteins interacting with a range of ligands, including advanced glycation end products (AGE) (1), amyloid-β peptide (2), amphoterin (3), and members of the S100 family (4). Whereas AGE and amyloid-β peptide are known to induce cellular perturbation through their interaction with RAGE, amphoterin and S100 proteins are considered to be physiological ligands of RAGE in migratory and inflammatory cellular responses. However, mechanistically it is not understood how RAGE-mediated cellular responses can change from trophic to toxic.

Amphoterin is a heparin-binding, neurite outgrowth-promoting protein that is highly expressed in embryonic and transformed cells (5–7). RAGE has been shown to mediate neurite outgrowth of cortical neurons and neuroblastoma cells on amphoterin-coated substrates (3, 8). Furthermore, amphoterin and RAGE co-localize at the leading edge of advancing neurites in the developing central nervous system (3). Our previous results suggesting that amphoterin might be a more general regulator of cell migration (reviewed in Ref. 9) are supported by the recent findings showing that blockade of amphoterin-RAGE interaction decreases invasion and growth of both implanted and spontaneously developing tumors (10).

S100B is a member of a multigenic family of Ca^{2+}-regulated proteins of the EF-hand type that has been implicated in the regulation of protein phosphorylation, the dynamics of cytoskeleton constituents, the cell cycle, and some enzymes (11, 12). S100B is abundant in the nervous system where it is mainly expressed in astrocytes, oligodendrocytes, and Schwann cells. S100B has been shown to be released by astrocytes to the extracellular space and to affect astrocytes in an autocrine manner and neurons in a paracrine manner (13–15). Once released, effects of S100B on target cells depend on its concentration. At nanomolar concentrations S100B is trophic in that the protein stimulates neurite outgrowth (13–15), enhances survival of neurons during development (16) and after injury (17), prevents motor neuron degeneration in newborn rats after sciatic nerve section (18), and local administration of S100B stimulates regeneration of injured rat sciatic nerve *in vivo* (19). All these observations point to a physiological role of secreted S100B as a neurotrophic factor, which could be important during both development and nerve regeneration. However, at micromolar concentrations S100B causes astrocytic and neuronal death via enhanced expression of inducible nitric oxide synthase in astrocytes and secretion of nitric oxide (20). Owing to these effects, S100B is currently viewed as a brain cytokine (21, 22). Until now, however, the cell surface receptor mediating the cytokine function of S100B has remained unidentified.

Recently, nanomolar concentrations of extracellular S100B have been shown to enhance neuronal survival and neurite outgrowth via nuclear translocation of NF-κB (23) and to increase the activity of extracellular signal-regulated kinases (ERK) in astrocytes (24). These findings strongly suggest that

* This work was supported by grants from the Academy of Finland Finnish Center of Excellence Program 2000–2005, the Sigrid Jusélius Foundation (H. R.), and the Italian Ministry for University and Technological Research-University of Perugia (COFIN) (to R. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RAGE, receptor for advanced glycation end products; AGE, advanced glycation end products; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SAPK/JNK, stress-activated protein kinase/c-Jun-NH_2-terminal kinase; MEK, MAPK/ERK kinase; MEKK, MEK kinase; PBS, phosphate-buffered saline.

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S100B acts on neurons and astrocytes by interacting with a cell surface receptor activating the Ras/MAP kinase/NF-κB signaling pathway. RAGE has been reported to bind both amphoterin (3) and S100B (4). On the other hand, RAGE ligation has been shown to activate multiple signaling pathways, such as activation of the transcription factor NF-κB through a redox-dependent activation Ras-ERK1/2 pathway (25). In addition, RAGE ligation is known to activate Cdc42/Rac pathway (8), p38, and SAPK/JNK MAP kinase pathways (10).

In the present study, we demonstrate that two S100 family proteins, S100B and S100A1, interact with RAGE on the cell surface in concert with amphoterin inducing neurite outgrowth and increased cell survival. Furthermore, RAGE ligation by amphoterin or S100 proteins results in activation of NF-κB and increased expression of the anti-apoptotic protein Bcl-2. However, when RAGE expressing cells are exposed to micromolar concentrations of S100 proteins cell viability is decreased in an oxidant- and MAP kinase-dependent manner. These results identify RAGE as a receptor capable of mediating both trophic and toxic effects of S100 proteins, and Bcl-2 as a RAGE-induced gene target capable of regulating cellular susceptibility to RAGE-mediated toxic effects.

MATERIALS AND METHODS

Cell Culture and Transfection—N18 mouse neuroblastoma cells and C6 rat glioma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin G, and 10% fetal calf serum. N18 neuroblastoma cells were transfected with Fugene 6 reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). To obtain stably transfected clones the cells were first incubated with 1.2 mg/ml G418 (Life Technologies, Inc.) for 2 weeks. Then single cell clones were picked, seeded on 96-well plates, and incubated in the presence of G418 for another 2 weeks. High expression clones were then selected based on mRNA expression levels detected on Northern blots as described previously (8).

Production and Purification of Recombinant Proteins—The production and purification of baculovirus-expressed rat amphoterin has been described previously (26). Recombinant S100B was expressed in *Escherichia coli* BL21 using the S100B expression vector pVUSB-1 plasmid containing the bovine brain S100B sequence (15). Recombinant S100A1 or a truncated form of S100A1 lacking the last six residues (Phe^{88}-Ser^{93}) (S100A1Δ88–93) was expressed in *E. coli* strain BL21(DE3)-pLysS (Novagen) transformed with an expression plasmid containing the human *S100A1* gene (27). Proteins were purified from bacterial extracts as described (28). The purity of individual S100s was checked by SDS-polyacrylamide gel electrophoresis (15% acrylamide). The S100B construct was kindly supplied by Linda J. Van Eldik (Northwestern University Medical School, Chicago, IL) and the S100A1 and S100A1Δ88–93 constructs by Volker Gerke (Institute for Medical Biochemistry, University of Münster, Münster, Germany). The peptide TRTKIDWNKILS (TRTK-12), derived from the S100A1- and S100B-binding region of the α-subunit of actin capping protein CapZ, was custom synthesized by Genosys.

Neurite Outgrowth Assay—96-Well enzyme-linked immunosorbent assay plates were coated with the indicated amounts of recombinant amphoterin or one of the S100 proteins in PBS for 30–60 min. The wells were then washed twice with PBS and blocked with Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin (Sigma). Stably transfected N18 cells were serum-starved for 16–24 h before the assays and then detached with PBS containing 0.5 mM EDTA. Cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin, pretreated for 30 min with recombinant proteins in solution, and plated either on plates coated with recombinant proteins or regular tissue culture-treated 96-well plates in the presence of the recombinant proteins. After 20 h in culture the cells were fixed with 4% paraformaldehyde, stained with 0.5% toluidine blue (Sigma), and the proportions of neurite-bearing cells (processes longer than one diameter of the cell soma) of all cells were counted under an Olympus IX-70 inverted microscope. Photomicrographs were taken with Olympus DP-10 CCD camera.

NF-κB Activation Assay—Path-Detect Luciferase cis-Reporter-Plasmid system (Promega) was used to detect activation of NF-κB. C6 rat glioma cells were used in order to reach high levels of transfection efficiency in a transient transfection assay. Briefly, cells were transfected (in a 4:2:4 proportion) with a plasmid encoding luciferase cDNA under an enhancer element containing five NF-κB-binding sites, a plasmid containing β-galactosidase and a plasmid containing either wild-type RAGE or a dominant negative RAGE mutant lacking the cytoplasmic domain. Cells were transfected by electroporation as described previously (8). After a 6-h recovery in the presence of serum the cells were serum-starved for 16 h. The cells were then detached with PBS containing 0.5 mM EDTA and resuspended in Dulbecco's modified Eagle's medium supplemented with 1% bovine serum albumin, pretreated for 30 min with recombinant S100B in solution in the case of S100B stimulation, and plated either on non-tissue culture-treated plates coated with recombinant amphoterin or regular tissue culture-treated plates in the presence of recombinant S100B. After 24 h in culture the cells were lysed in Reporter Lysis Buffer (Promega) and luciferase and β-galactosidase activities were quantitated with either β-galactosidase Enzyme Assay System or Luciferase Assay System (Promega) using Wallac Victor^2 1420 Multilabel Counter. Luciferase activity was normalized to β-galactosidase activity.

Assessment of Cell Survival, Cytochrome c Release, and Caspase-3 Activation—Stably transfected N18 neuroblastoma cells were treated and plated similarly as in the neurite outgrowth assays and cultured for up to 6 days. The viability of cells was assessed by staining with 0.4% trypan blue (Sigma) before plating and after 2, 4, or 6 days in culture. The variation in total cell numbers was monitored and was less than 8% between different groups. To visualize intracellular localization of cytochrome *c* similarly treated RAGE-transfected cells were fixed with 4% paraformaldehyde and stained first with monoclonal anti-cytochrome *c* antibody (Pharmingen) followed by tetramethylrhodamine B isothiocyanate-conjugated rabbit anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.). Nuclear morphology was visualized by staining the same cells with 4,6-diamidino-2-phenylindole (Molecular Probes, Inc.). The cells were examined with an Olympus Provis AX-70 fluorescence microscope and photomicrographs were obtained with Photometrics SenSys CCD camera. Caspase-3 activity was measured from cell lysates using EnzChek Caspase-3 Assay Kit (Molecular Probes, Inc.) based on the fluorometric detection of 7-amino-4-methylcoumarin after proteolytic cleavage of the synthetic substrate Z-DEVD-amino-4-methyl coumarin (Z-DEVD-AMC) with Wallac Victor^2 1420 Multilabel Counter. Cells were treated as above and lysed after 48 h. In inhibition assays the cells were pretreated for 30 min with the antioxidants ascorbic acid (Sigma), α-tocopherol (Calbiochem), or a specific MEK-inhibitor U0126 (Calbiochem) before stimulation with 5 μM S100B.

Western Blotting and Quantitation of Bcl-2 Expression—Stably transfected N18 neuroblastoma cells were treated as above. After 2 days in culture the cells were lysed in RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin) and protein concentrations were measured with a modified Bradford assay as described previously (29). Equal amounts of proteins were separated on SDS-polyacrylamide gel electrophoresis gels and electrotransferred on nitrocellulose filters. Bcl-2 was detected with an antibody purchased from Santa Cruz Biotechnology and with peroxidase-conjugated secondary antibodies obtained from Amersham Pharmacia Biotech. Filters were reprobed with anti-β-tubulin antibodies (Sigma) to confirm equal protein loading. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech) and densitometric analysis was used to measure relative band intensities.

RESULTS

Coregulation of Neurite Outgrowth by Amphoterin and S100 Proteins through RAGE Activation—RAGE has been previously shown to mediate neurite outgrowth on amphoterin-coated matrices (3). Furthermore, we have shown that RAGE-mediated neurite outgrowth can be abolished by deleting the cytoplasmic domain of RAGE (8). Since RAGE was recently shown to be a receptor for S100 family proteins in leukocytes and endothelial cells (4) and on the other hand S100B has been shown to be a potent activator of neurite extension (13, 15), it seemed plausible that RAGE could be the receptor responsible for S100B-induced neurite outgrowth.

In order to investigate this we stimulated N18 mouse neuroblastoma cells stably transfected with either full-length human RAGE or a cytoplasmic deletion mutant of RAGE (8) with amphoterin and S100B or another S100 family protein S100A1. Serum-starved N18 cells were stimulated with either sub-

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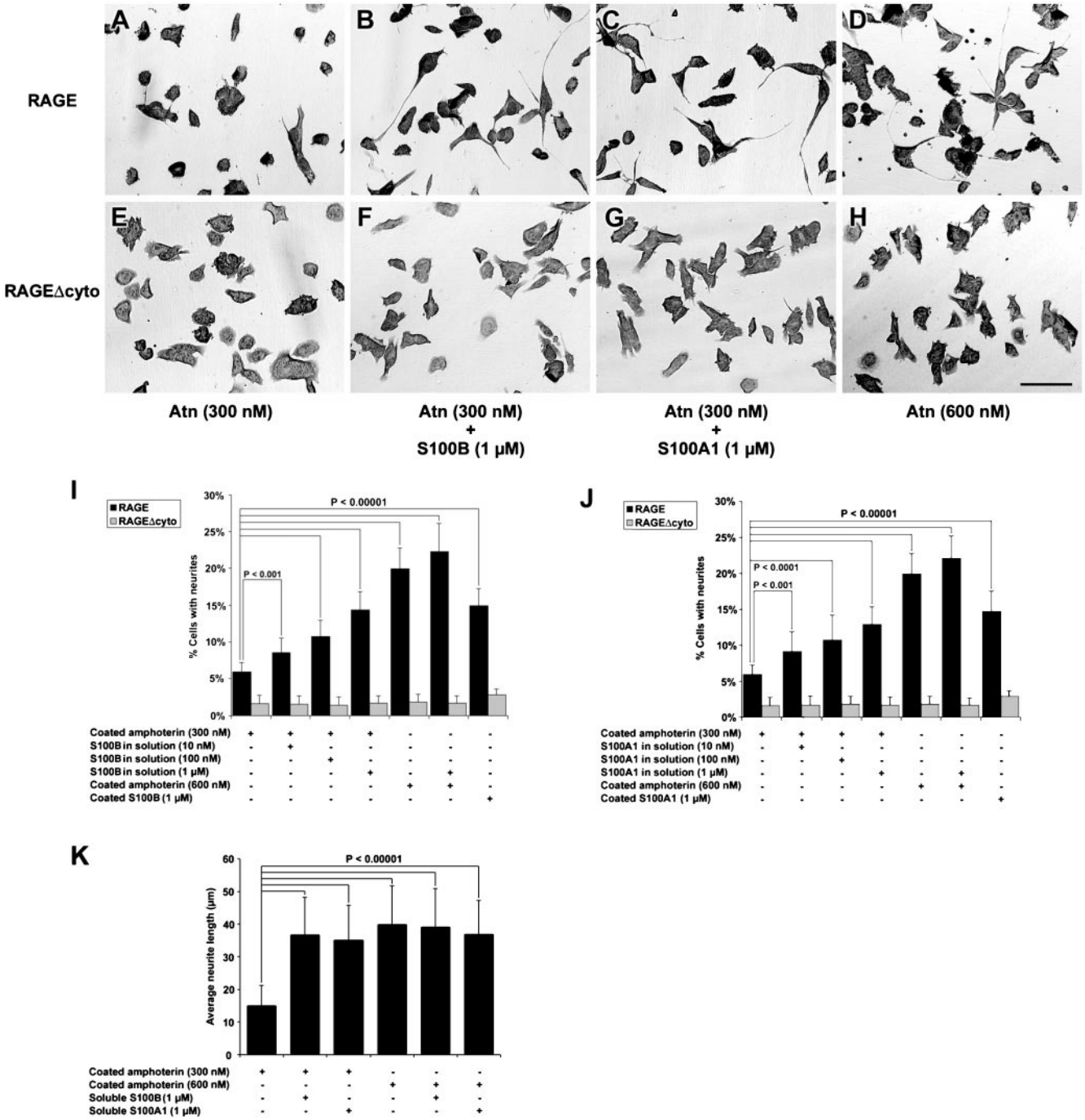

FIG. 1. **Coregulation of neurite outgrowth by amphoterin and S100 proteins through RAGE activation.** Serum-starved N18 neuroblastoma cells expressing either wild-type RAGE (*A-D*) or cytoplasmic domain deletion mutant of RAGE (*E-H*) were grown on amphoterin-coated (300 nM in *A-C* and *E-G*; 600 nM in *D* and *H*) surfaces with two S100 family proteins, S100B or S100A1 (1 μM). Morphological quantitation of S100B (*I*) and S100A1 (*J*)-stimulated cells reveals an additive nature of amphoterin and S100 treatment. The effect of these stimuli on average neurite length (*K*) is also shown. An average of three replicate experiments plus standard deviation is shown. Statistical significance was determined by Student's *t* test and *p* values are shown in the graphs. *Bar* = 40 μm.

strate-bound amphoterin or S100B or S100A1 in solution. Different combinations of the proteins were also tested. RAGE expressing N18 cells grew few neurites on plates coated with 300 nM amphoterin (approximately 6% neurite bearing cells; Fig. 1*A*) in comparison to the cells grown on plates coated with 600 nM amphoterin (approximately 20% neurite bearing cells; Fig. 1*D*). However, when either S100B (Fig. 1*B*) or S100A1 (Fig. 1*C*) was added to the medium, neurite outgrowth was enhanced in a concentration-dependent manner (*black bars* in Fig. 1, *I* and *J*). This effect seems to be strictly RAGE-dependent as cells expressing the cytoplasmic deletion mutant of RAGE grew hardly any neurites (<3% neurite bearing cells; Fig. 1, *E-H,* and *gray bars* in Fig. 1, *I* and *J*). Both S100B and S100A1 were capable of inducing RAGE-mediated neurite outgrowth when either coated or applied in solution without the presence of amphoterin. Thus it seems that RAGE can mediate neurite outgrowth induced by amphoterin or S100 proteins alone but is also able to promote neurite outgrowth in an additive manner when both amphoterin and S100B or S100A1 are present. Interestingly, S100B and S100A1 at concentra-

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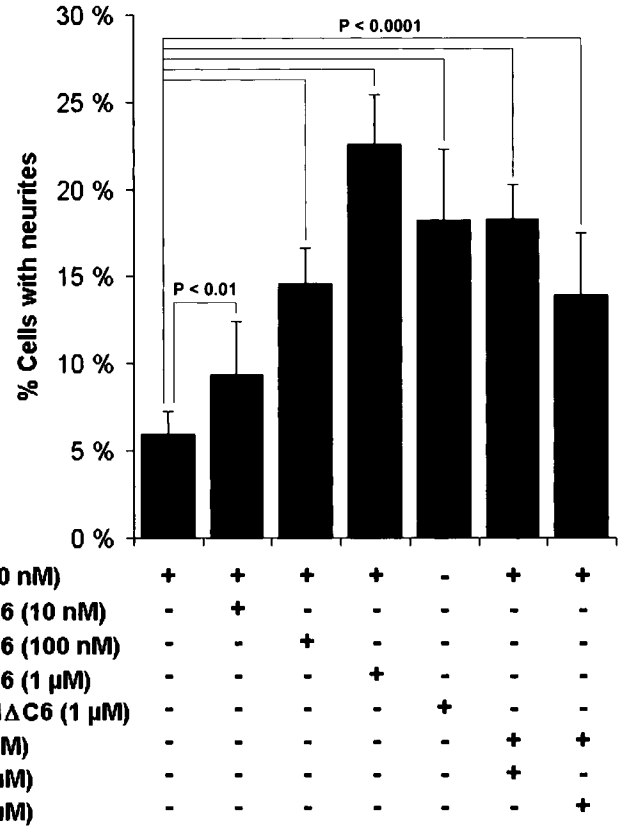

FIG. 2. **Neurite outgrowth promoting activity of S100A1 does not require the C-terminal extension of S100A1.** A deletion mutant of S100A1 lacking six C-terminal amino acids was used in different combinations with amphoterin to stimulate N18 neuroblastoma cells expressing wild-type RAGE. A peptide TRTK-12 known to bind to six C-terminal acids of S100 proteins was also tested together with amphoterin and S100B stimulation. An average of three replicate experiments plus standard deviation is shown. Statistical significance was determined by Student's *t* test and *p* values are shown in the graphs.

tions above 1 μM did not enhance neurite outgrowth but rather led to the decline of morphology of the cells suggesting that these molecules can exert also toxic effects on the cells.

Amphoterin and S100 proteins had also a stimulating effect on neurite length. The average length of neurites in cells grown on plates coated with 600 nM amphoterin or in the presence of either S100B or S100A1 was approximately 40 μm whereas the neurites in cells grown on 300 nM amphoterin-coated plates were only 15 μm long on average (Fig. 1*K*).

The results showing that both S100B or S100A1 are capable of inducing neurite outgrowth in a RAGE-dependent manner suggest that different S100 proteins share a similar structure involved in RAGE binding. Previously it has been shown that the C-terminal extension of S100 members is responsible for binding of many intracellular ligands of S100 proteins such as annexin I, annexin II, GFAP, tubulin, p53, and the α-subunit of actin capping protein CapZ (27, 30–34). To investigate the role of the C-terminal extension of S100A1 in RAGE binding we used a deletion mutant of S100A1 lacking the last six residues (Phe^{88}-Ser^{93}). As shown in Fig. 2, S100A1Δ88–93 displayed similar capability to induce neurite outgrowth on RAGE expressing cells as the wild-type S100A1. Furthermore, a synthetic peptide TRTK-12, derived from the S100A1- and S100B-binding region of actin capping protein CapZ (31), did not significantly inhibit neurite outgrowth induced by S100B (the *last two bars* in Fig. 2), confirming that a structure distinct from the C-terminal extension of S100A1 and S100B is responsible for RAGE binding.

RAGE-dependent Activation of NF-κB by Amphoterin and S100 Proteins—A hallmark of RAGE ligation is activation of the transcription factor NF-κB (4, 8, 35). We used a luciferase reporter plasmid system to investigate changes in NF-κB activity induced by amphoterin and S100 proteins. Cells were transfected with full-length RAGE or the cytoplasmic domain deletion mutant of RAGE together with the NF-κB-luciferase reporter plasmid. Also a plasmid encoding β-galactosidase was included as an internal control for transfection efficiency. Non-treated mock-transfected cells were used as a control of basal transcriptional activity. RAGE expressing cells grown on amphoterin-coated (300 nM) matrix showed an activation to 147.1 ± 3.0% of the control level in NF-κB-dependent transcription. The corresponding values in the cells treated with 100 nM S100B, 100 nM S100A1, 300 nM coated amphoterin with 100 nM S100B, 300 nM coated amphoterin with 100 nM S100A1, and 600 nM coated amphoterin were 186.8 ± 4.0%, 180.9 ± 4.8%, 206.1 ± 10.9%, 203.9 ± 13.4%, and 199.4 ± 11.3%, respectively (*black bars,* Fig. 3). Cells expressing the cytoplasmic domain deletion mutant did not show significant changes in NF-κB-dependent transcription in response to any of these stimuli (*gray bars* in Fig. 3). These results show that both S100B and S100A1 are able to activate NF-κB either alone or in a noncompetitive manner together with amphoterin through binding to RAGE on the cell surface.

Effects of Amphoterin and S100B on Cell Survival—In addition to neurite outgrowth, S100B has also been shown to promote cell survival in specific neuronal populations (16–19). To test whether the survival promoting effect of S100B is mediated by RAGE, N18 neuroblastoma cells stably transfected with full-length RAGE or the cytoplasmic deletion mutant were treated similarly as above and cell survival was assessed by trypan blue staining after 2, 4, or 6 days after plating and compared with mock-treated RAGE-transfected control cells. Serum deprivation gradually decreased cell survival during the incubation period and all the following experiments were performed without the presence of serum. However, cells growing on amphoterin-coated (300 nM) matrix or in the presence of 100 nM S100B showed significantly increased cell survival (*left panel* in Fig. 4*A*). After 6 days in culture 33.7 ± 1.5% of mock-treated stained negative in trypan blue assay compared with 47.8 ± 7.8, 53.5 ± 6.0, and 62.3 ± 4.7% of amphoterin (300 nM), S100B (100 nM), or both amphoterin (300 nM) and S100B (100 nM) stimulated cells, respectively ($p < 0.05$). Again am-

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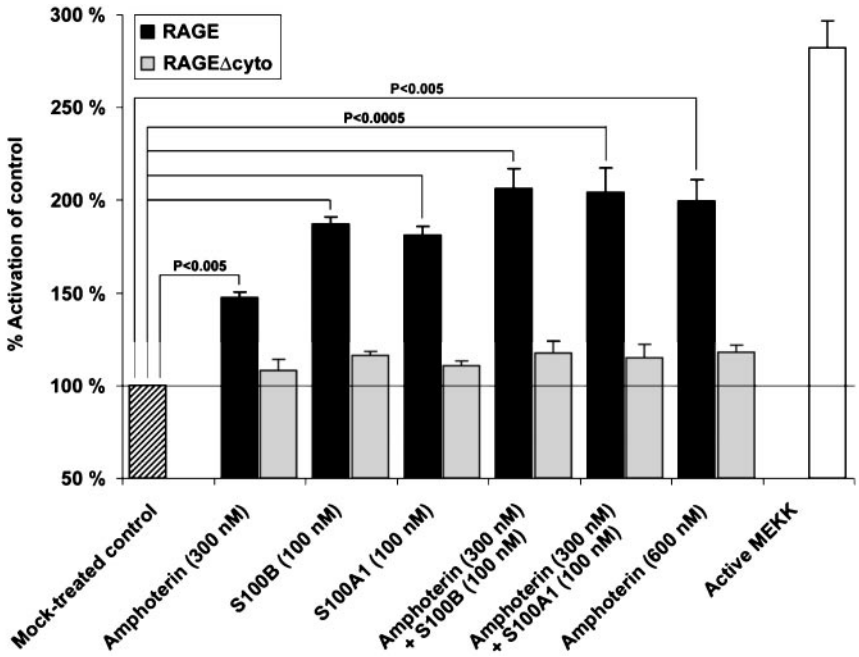

FIG. 3. **RAGE-dependent activation of NF-κB by amphoterin and S100 proteins.** C6 glioma cells were transiently transfected with an NF-κB-responsive *cis*-reporter gene construct, β-galactosidase expression construct, and with either full-length RAGE or the cytoplasmic domain deletion mutant RAGEΔcyto. Serum-starved cells were grown overnight on amphoterin-coated surface (300 or 600 nM) with or without S100B or S100A1 (100 nM) present. Construct encoding the catalytic domain of MEKK (360–672; Stratagene) was used as a positive control. After the cells were lysed luciferase activity was quantitated and normalized to β-galactosidase activity. Non-stimulated mock-transfected cells were used as a control of basal transcriptional activity (*hatched bar*) and cells transfected with the catalytic domain of MEKK as a positive control (*white bar*). An average of relative luciferase activity from three replicate experiments plus standard deviation is shown.

photerin and S100B showed a concerted effect and this effect was also RAGE-dependent since RAGEΔcyto expressing cells showed virtually no differences in cell survival when treated similarly (*right panel* in Fig. 4*A*).

Whereas nanomolar concentrations of S100B can promote cell survival, micromolar concentrations of S100B have been shown to induce apoptosis (22, 36). When the above mentioned survival assay was performed in the presence of 5 μM S100B cell survival was significantly decreased. After 6 days in culture only 12.6 ± 1.8% of cells grown in the presence of 5 μM S100B stained negative in the trypan blue assay compared with 33.7 ± 1.5% of the mock-treated control (*left panel* in Fig. 4*B*, $p < 0.0005$). Interestingly, 5 μM amphoterin either coated or in solution had no such effect (45.3 ± 1.5 and 35.4 ± 3.7% living cells, respectively). Also the toxic effect of S100B seems to be mediated by RAGE since cells expressing RAGEΔcyto did not show decreased cell survival in the presence of 5 μM S100B (*right panel* in Fig. 4*B*). Using nanomolar or micromolar S100A1 instead of S100B produced virtually identical results in the survival assay (data not shown). The variation in total cell numbers was monitored between different test groups and was less than 8% suggesting that these treatments did not affect cell proliferation (data not shown).

Cytochrome c Release and Caspase-3 Activation upon Serum Deprivation or Micromolar S100B—Cytochrome *c* release from mitochondria to cytoplasm has been shown to be a central event in stress-induced apoptosis leading to activation of the caspase cascade and the execution phase of apoptosis (37–39). To confirm that cell death induced by serum deprivation or micromolar S100B was due to apoptosis, cells expressing full-length RAGE were treated with amphoterin or S100B for 48 h and immunostained with cytochrome *c* antibodies. Among serum-starved control cells several cells displayed a diffuse cytoplasmic cytochrome *c* staining compared with the cells grown on amphoterin-coated matrix or in the presence of 100 nM S100B (Fig. 5, *A, C, E,* and *G*). Cells treated with amphoterin (Fig. 5C) or 100 nM S100B (Fig. 5*E*) displayed a typical punctate mitochondrial cytochrome *c* staining indicating that no ongoing release of cytochrome *c* to the cytoplasm was taking place. In contrast, after treatment with 5 μM S100B a large number of cells displayed cytoplasmic cytochrome *c* staining (Fig. 5*G*). Counterstaining with nuclear stain 4,6-diamidino-2-phenylindole showed that the same cells displaying cytoplasmic cytochrome *c* staining had also started to display pyknotic nuclei, a typical feature of apoptosis (Fig. 5, *B, D, F*, and *H*).

Another typical feature of stress-induced apoptosis is activation of the caspase cascade (reviewed in Ref. 40). We used a fluorometric caspase-3 activation assay to quantitate caspase-3 activity from cell lysates. Cells expressing either full-length RAGE or the cytoplasmic deletion mutant were stimulated as above, lysed after 48 h in culture, and probed for caspase-3 activity. Full-length RAGE expressing cells growing on amphoterin-coated matrix or in the presence of 100 nM S100B showed decreased caspase-3 activity compared with mock-treated control (*black bars* in fig. 5*I*). In contrast, cells treated with 5 μM S100B showed an increase in caspase-3 activity. Cells expressing the cytoplasmic domain deletion mutant of RAGE did not respond to these stimuli with changes in caspase-3 activity (*gray bars* in Fig. 5*I*).

Antioxidants and MEK Inhibition Protect from the Toxicity of Micromolar S100B—RAGE ligation by AGE or amphoterin has been shown to induce an oxidant stress-dependent activation of ERK1/2, p38, and SAPK/JNK MAP kinase pathways (10, 25). Furthermore, RAGE-mediated deleterious effects of AGE on cells have been shown to be attenuated by antioxidants (35, 41). To determine whether RAGE-mediated toxicity of S100B was also due to the activation of the same intracellular signaling pathways, we pretreated the cells with a hydrophilic antioxidant ascorbic acid, a lipophilic antioxidant α-tocopherol, or a specific MEK inhibitor U0126 (42) before treatment with 5 μM S100B. 100 μM ascorbic acid, 100 μM α-tocopherol, and 1 μM U0126 were all capable of conferring protection against the toxicity of 5 μM S100B (Fig. 6, *A* and *B*).

Activation of RAGE Induces Bcl-2 Expression—Activation of NF-κB was recently shown to be crucial for up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X_L required for tumor necrosis factor-induced neuroprotection (43). Interestingly, Bcl-2 expression level has been shown to regulate cell sensitivity to S100B-induced apoptosis (44). As RAGE ligation is known to be able to activate NF-κB, we next determined whether RAGE activation is able to regulate Bcl-2 expression. Cells expressing RAGE were first serum starved and then treated with amphoterin or S100B for 48 h followed by immunoblotting of the cell lysate with Bcl-2 antibodies. Serum dep-

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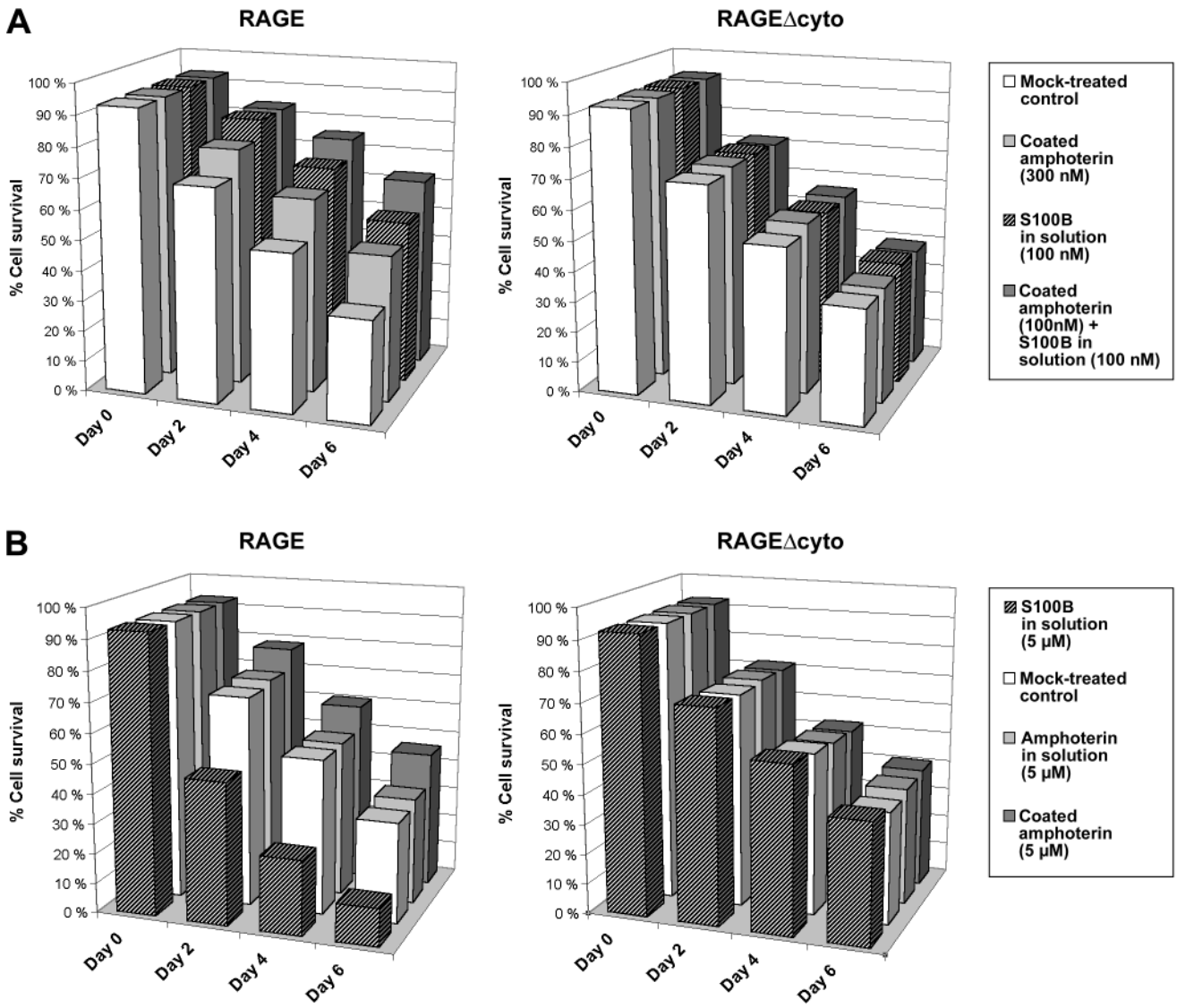

FIG. 4. **Effects of amphoterin and S100 proteins on cell survival.** Serum-starved N18 neuroblastoma cells expressing either wild-type RAGE (*left panels*) or cytoplasmic domain deletion mutant of RAGE (*right panels*) were grown without serum with either nanomolar (*A*) or micromolar concentrations (*B*) of amphoterin and S100B for up to 6 days. Dead cells were counted after staining with 0.2% trypan blue at the indicated time points. An average of three replicate experiments is shown.

rivation down-regulated Bcl-2 expression but stimulation with either 300 nM amphoterin or 100 nM S100B increased Bcl-2 expression back to the levels before serum deprivation (Fig. 7). Compared with the serum-starved control cells, an increase of 228.6 ± 33.4 and 219.9 ± 35.0% was achieved with 300 nM amphoterin and 100 nM S100B stimulation, respectively. Interestingly, S100B-induced increase in Bcl-2 expression level was concentration dependent since only 100 nM but not 5 μM S100B was able to increase Bcl-2 expression.

Pretreatment with Amphoterin or Nanomolar S100B Protects from the Toxicity of Micromolar S100B—Preactivation of NF-κB in cells is known to be protective against various apoptosis-inducing stimuli. For example, pretreatment of neurons with nanomolar concentrations of amyloid-β peptide protects neurons from later stimulation with toxic concentrations of amyloid-β peptide through activation of NF-κB (45). Since amphoterin and nanomolar S100B both activate NF-κB and increase the expression of Bcl-2 we tested whether pretreatment with nanomolar concentrations of amphoterin or S100B is sufficient to protect cells from the toxicity of micromolar S100B. N18 neuroblastoma cells stably transfected with RAGE were pretreated by growing them on amphoterin-coated matrix or in the presence of 100 nM S100B for 24 h before addition of 5 μM S100B. Five days after the addition of 5 μM S100B only 6.6 ± 3.0% of the cells were alive compared with 23.3 ± 1.3% in mock-treated control cells (Fig. 8). However, pretreatment with either amphoterin or 100 nM S100B increased cell survival to 34.8 ± 7.9 and 32.1 ± 0.3%, respectively. This suggests that the RAGE-ligation induced increase in Bcl-2 expression might be sufficient to protect cells from apoptotic stimuli.

DISCUSSION

Since its isolation in 1965 (46) many functions have been attributed to S100B both inside and outside the cell (reviewed in Ref. 11). For years S100B has been known to be a potent glia-derived neurotrophic factor promoting neurite outgrowth and cell survival. On the other hand S100B has been reported to be toxic at higher concentrations, which is a common feature for many cytokines. However, no receptor had been identified mediating these neurotrophic and neurotoxic effects of S100B. Recently, Hofmann *et al.* (4) reported that RAGE, a multiligand receptor of the immunoglobulin superfamily, is a signal trans-

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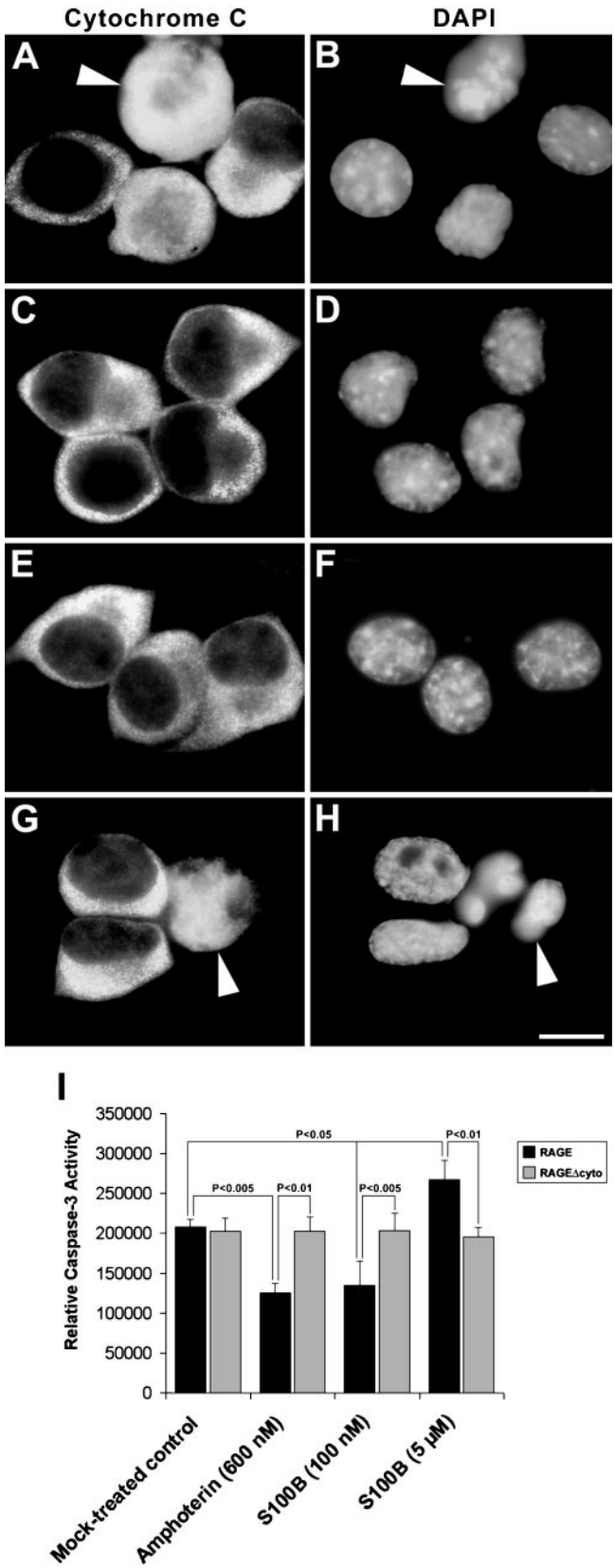

FIG. 5. **Effect of RAGE activation on cytochrome *c* release and caspase-3 activity.** Serum-starved RAGE expressing N18 neuroblastoma cells were left untreated (*A* and *B*), grown on amphoterin-coated substrate (*C* and *D*), with 100 nM S100B in solution (*E* and *F*) or with 5 μM S100B in solution (*G* and *H*). After 2 days in culture the cells were fixed and double-stained with cytochrome *c* antibodies (*A, C, E,* and *G*) and nuclear stain 4,6-diamidino-2-phenylindole (*B, D, F,* and *H*). Apoptotic cells are indicated with *arrowheads. I,* cells treated as above were lysed after 48 h stimulation and caspase-3 activity was monitored in cell lysates with fluorometric caspase-3 assay using Z-DEVD-AMC as a substrate. An average of three replicate experiments plus standard deviation is shown. Statistical significance was determined by Student's *t* test and *p* values are shown in the graphs. *Bar* = 10 μm.

ducing receptor for S100/calgranulin proteins. They characterized a novel RAGE-binding protein EN-RAGE as a human homologue of bovine calgranulin C (S100A12) and suggested that RAGE-S100A12 interaction might play a central role in chronic inflammation and tissue injury. Interestingly, other RAGE ligands were shown to compete for receptor binding with S100A12. They showed that in addition to S100A12, S100B was also capable of activating NF-κB in endothelial cells in a RAGE-dependent manner.

In the present study, we have compared different RAGE ligands amphoterin, S100B and S100A1 in a context related to neuronal development. S100B or S100A1 do not seem to compete with amphoterin in a neurite outgrowth or survival assays but instead S100B or S100A1 can induce trophic effects in a concerted manner with amphoterin. However, there seem to be some differences in the modes of amphoterin and S100B action. Whereas S100B and S100A1 can exert their effects either in solution or when bound to a substrate, amphoterin can only act as a substrate-bound protein. This could be due to the structural nature of the proteins. S100B functions as a stable dimer (13, 15) and although amphoterin also has a tendency to form dimers and multimers (6), it probably has to be attached to a substrate in order to effectively activate the receptor. Amphoterin is known to bind to some other cell surface components in addition to RAGE, including syndecan-1 (47), receptor-type tyrosine phosphatase β/ζ (48), and some sulfated glycolipids (49). How these molecules are involved in this whole scenario remains to be seen. Nevertheless, since both neuritogenic and prosurvival functions of S100B, S100A1, and amphoterin are abolished when a dominant negative form of RAGE is expressed, it seems that RAGE functions as a crucial signal transducing receptor for all of these ligands.

One unexpected observation made in this work is that S100A1 has both neuritogenic and prosurvival effects. It has been long known that S100B has to be in its disulfide cross-linked dimer conformation to exert trophic effects on neurons and that the presence of *both* Cys^{68} and Cys^{84} in each S100B monomer is essential to the neurotrophic effects of the protein (15). However, recent observations indicate that: (i) S100A12, the human analogue of EN-RAGE, which activates inflammatory cells (4), does not contain Cys residues; (ii) a mutant of S100B lacking the last nine residues (and hence Cys^{84}) stimulates nitric oxide production and secretion by astrocytes (50); and (iii) oxidized and non-oxidized S100B cause an identical activation of ERK1/ERK2 in astrocytes at concentrations higher than 10 nM (24). Thus, the S100B effects described here seem to be independent of the oxidation state of S100B, and interaction of S100 proteins with RAGE does not require the previous formation of disulfide cross-linked dimers. Additionally, our data indicate that the C-terminal extension, that was shown to be essential for the Ca^{2+}-dependent interaction of a number of intracellular target proteins as well as S100A1 and S100B regulatory effects on intermediate filament assembly (11, 32), plays no major role in the S100 interaction with RAGE and S100 effects on neuroblasts.

Another functional difference between amphoterin and S100B is the fact that micromolar concentrations of S100B induce apoptosis in a RAGE-dependent manner whereas similar concentrations of amphoterin have no such effect. An explanation for this might be the interaction with tissue plasminogen activator/plasminogen. Amphoterin has been shown to

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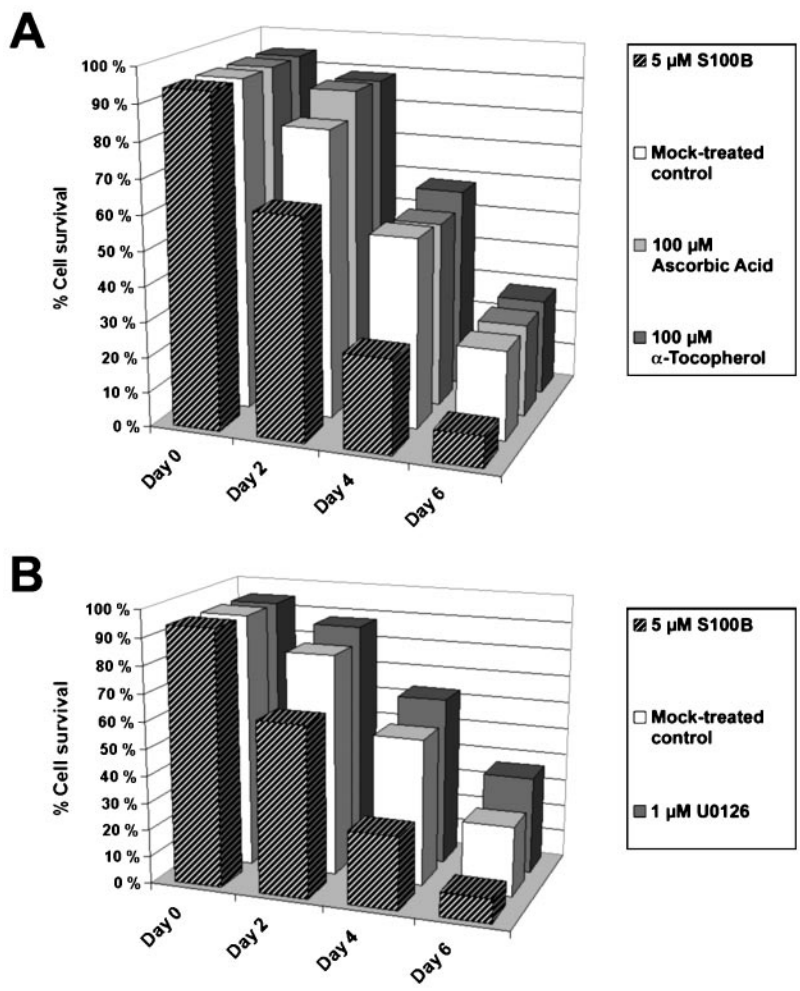

FIG. 6. **Pharmacological protection from the toxicity of micromolar S100B.** Serum-starved N18 neuroblastoma cells expressing wild-type RAGE were pretreated with antioxidants ascorbic acid or α-tocopherol (*A*) or a specific MEK inhibitor U0126 (*B*) and then exposed to 5 μM S100B. Cells were cultured for up to 6 days and the number of dead cells was monitored as in Fig. 4. An average of three replicate experiments is shown.

enhance its own degradation in the extracellular space by enhancing plasminogen activator-catalyzed plasmin production (26). On the other hand, for S100B such an interaction has not been reported. It is thus possible that amphoterin activates RAGE only transiently whereas the presence of a slowly degradable ligand in micromolar concentrations could result in a chronic activation of RAGE. Most likely this is the case with two other toxic ligands of RAGE, AGE and amyloid-β peptide (2, 35). The fact that inhibition of RAGE signaling pathways by antioxidants and inhibition of the ERK1/2 pathway has been shown to block the detrimental effects of AGE and amyloid-β peptide and that they also protect from the toxicity of micromolar S100B (Fig. 6), supports this conclusion.

How is it possible that the same ligand-receptor interaction can induce both pro- and anti-apoptotic signals? Persistent activation of RAGE by micromolar concentrations of S100B is capable of producing increased amounts of oxyradicals perturbing the delicate redox balance within the cell. This could lead to mitochondrial dysfunction and induction of apoptosis *per se*. Chronic activation of the RAGE signaling pathways could contribute by activating transcription of pro-apoptotic genes. Persistent ERK1/2 activation has been shown to be a crucial factor, *e.g.* for oxidant stress-dependent glutamate neurotoxicity (51). On the other hand, RAGE ligation was recently shown to activate SAPK/JNK and p38 MAP kinase pathways (10) both of which have been classically linked to cell stress and induction of apoptosis (reviewed in Ref. 52). The relative contribution of these different signaling pathways to RAGE-induced cell stress remains to be elucidated.

The increase in Bcl-2 expression after amphoterin or nanomolar S100B treatment provides a clear mechanistic explanation for the anti-apoptotic effects. NF-κB activity has been intimately linked to the regulation of cell survival (reviewed in Ref. 53), and the survival promoting activity of S100B has been shown to depend on NF-κB activity (23). Interestingly, it has also been reported that NF-κB activation is required for increased expression of Bcl-2 in neuronal cells (43). Thus Bcl-2 expression might be simply increased after RAGE ligation through activation of NF-κB. Unfortunately we were unable to address the question about the specific requirement of NF-κB for RAGE-promoted cell survival since inhibition of NF-κB activity in these cells led to massive cell death (data not shown). However, induction of Bcl-2 expression by pretreatment with amphoterin or nanomolar S100B seems to be involved in protection against toxicity of micromolar S100B. Interestingly, only nanomolar S100B but not micromolar S100B was capable of increasing Bcl-2 expression. These results are in consensus with the report by Wang *et al.* (44) showing that Bcl-2 expression levels regulate cell sensitivity to S100B-mediated apoptosis. Although our results do not exclude involvement of other anti-apoptotic proteins (such as Bcl-X_L), they suggest that the Bcl-2 expression level dictates the cellular susceptibility to the toxicity of micromolar S100B. Recently translocation of Bax, a pro-apoptotic member of the Bcl-2 family, from cytosol to mitochondria was shown to be a critical event in neuronal apoptosis (54). It is possible that regulation of Bax or related pro-apoptotic proteins is also involved in the RAGE-induced apoptosis.

Survival promoting function has not previously been described for amphoterin. Considering the recent results by

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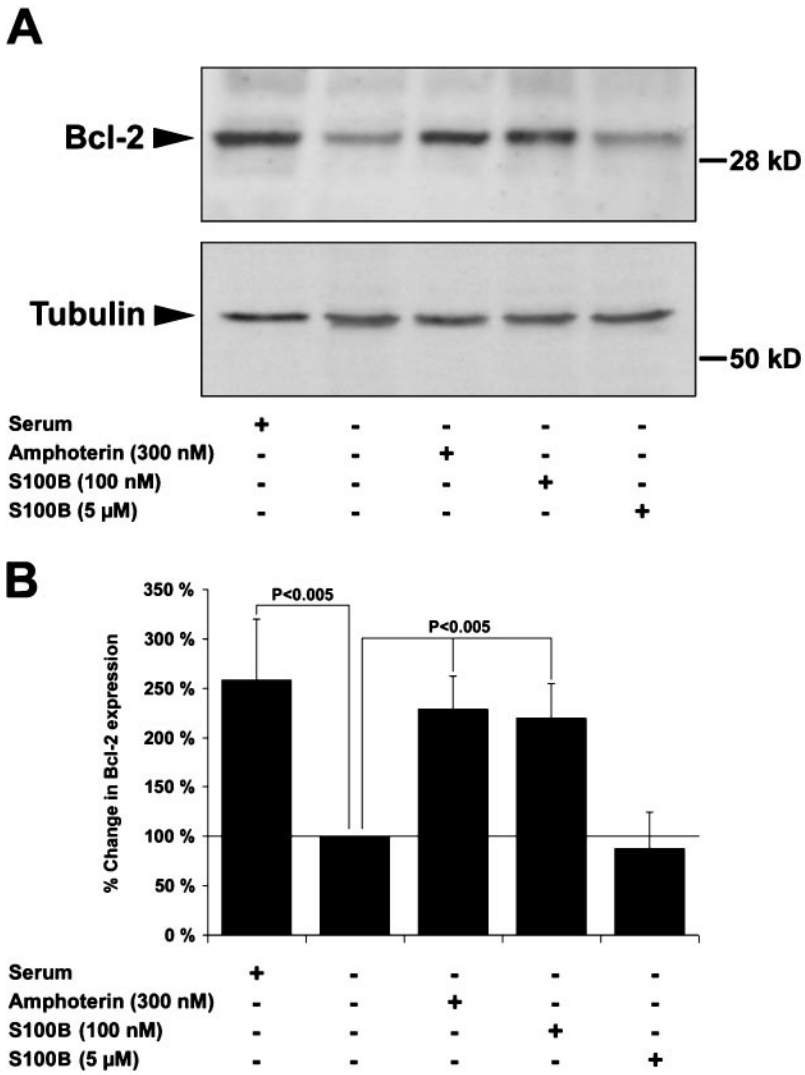

FIG. 7. **Effect of amphoterin and S100B stimulation on the expression of Bcl-2.** N18 neuroblastoma cells expressing wild-type RAGE were left untreated or serum-starved and stimulated with coated amphoterin (600 nM) or 100 nM S100B or 5 μM S100B in solution for 48 h. *A,* equal amounts of total protein from cell lysates was immunoblotted with anti-Bcl-2 and reprobed with anti-β-tubulin antibodies to confirm equal protein loading. *B,* densitometric analysis of Bcl-2 expression. An average plus standard deviation of three replicate experiments is shown. Results are reported as percentages of serum-starved control cells.

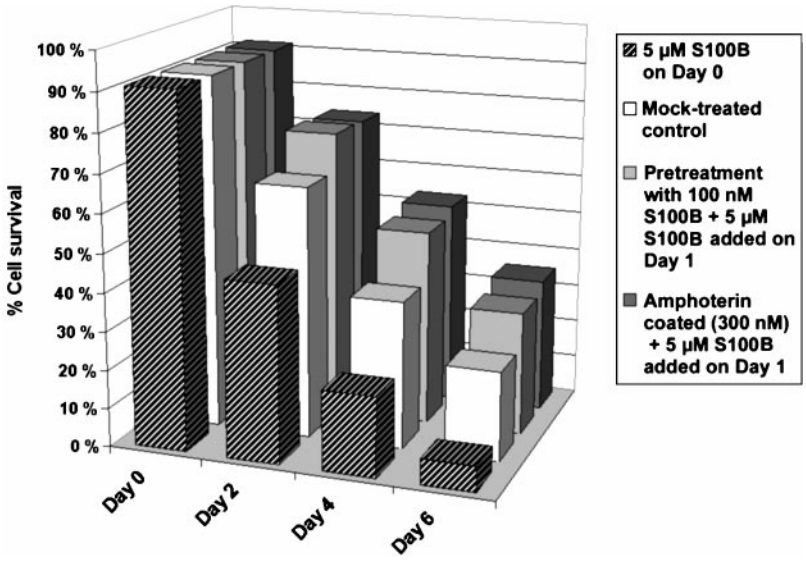

FIG. 8. **Pretreatment with amphoterin or nanomolar S100B protects from the toxicity of micromolar S100B.** Serum-starved N18 neuroblastoma cells expressing wild-type RAGE were pretreated either by growing the cells on amphoterin-coated (300 nM) matrix or in the presence of 100 nM S100B for 24 h and then exposed to 5 μM S100B. The cells were cultured for up to 6 days and the number of dead cells was monitored as in Fig. 4. An average of three replicate experiments is shown.

Taguchi *et al.* (10) showing that amphoterin-RAGE interaction is crucial for tumor growth and invasive migration of tumor cells, the present results suggest that amphoterin-RAGE interaction could also promote tumor progression by enhancing cell survival. After amphoterin is secreted by transformed cells it binds to the cell surface receptors acting in an autocrine manner. Enhanced expression of Bcl-2 through amphoterin-RAGE interaction might suppress anoikis, a special type of apoptosis due to loss of cell-matrix contact (reviewed in Ref. 55), eventually contributing to anchorage-independent growth and increased metastatic properties of the tumor cells. In this scenario, the apparent lack of toxic effects of amphoterin as

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compared with other RAGE ligands is also noteworthy.

Amphoterin, S100B, and RAGE are present in the developing nervous system (3, 6, 16, 17, 56). Amphoterin secreted by the advancing growth cones themselves engages RAGE on the cell surface inducing neurite outgrowth in an autocrine manner. Secretion of S100B by surrounding glial cells might enhance this effect by additively activating RAGE. The neurite outgrowth promoting effect of S100B has been shown to be selective for specific neuronal populations (16, 17, 57, 58). It will be interesting to learn whether this reflects selective expression of RAGE and thus responsiveness to S100B in these neuronal populations. Interestingly, it seems that independently of its anti-apoptotic function Bcl-2 participates in the regulation of axonal extension (59, 60) and neuronal differentiation (61). Thus RAGE-mediated increase in Bcl-2 expression could also function as a differentiation inducing signal during embryonic development. However, it should be remembered that the results provided in this report were obtained from artificial transfection systems and it will be a matter of future studies to see how these results can be extrapolated to *in vivo* systems.

The fact that the human gene encoding S100B is located on chromosome 21q22.3 (62), and that elevated levels of S100B have been detected in brains from patients with Down's syndrome (trisomia 21) and Alzheimer's disease (63), suggests that S100B might have a role in the pathogenesis of these diseases. Our present results suggest that RAGE, already known to interact with amyloid-β peptide, can also mediate neurotoxicity due to elevated levels of S100B. This might shed new light on studies of molecular pathophysiology of Down's syndrome and Alzheimer's disease.

Acknowledgments—We thank Linda J. Van Eldik and Volker Gerke kindly providing reagents specified under "Materials and Methods." We thank Urmas Arumae (Institute of Biotechnology, University of Helsinki, Helsinki, Finland) for the valuable comments during preparation of this manuscript. The excellent technical assistance of Eeva-Liisa Saarikalle, Seija Lehto, and Nina Nevalainen is gratefully acknowledged.

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Coregulation of Neurite Outgrowth and Cell Survival by Amphoterin and S100 Proteins through Receptor for Advanced Glycation End Products (RAGE) Activation

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J. Biol. Chem. 2000, 275:40096-40105.
doi: 10.1074/jbc.M006993200 originally published online September 27, 2000

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