S100B and S100A6 Differentially Modulate Cell Survival by Interacting with Distinct RAGE (Receptor for Advanced Glycation End Products) Immunoglobulin Domains*

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Received for publication, May 14, 2007, and in revised form, August 10, 2007 Published, JBC Papers in Press, August 28, 2007, DOI 10.1074/jbc.M703951200

S100 proteins are EF-hand calcium-binding proteins with various intracellular functions including cell proliferation, differentiation, migration, and apoptosis. Some S100 proteins are also secreted and exert extracellular paracrine and autocrine functions. Experimental results suggest that the receptor for advanced glycation end products (RAGE) plays important roles in mediating S100 protein-induced cellular signaling. Here we compared the interaction of two S100 proteins, S100B and S100A6, with RAGE by in vitro assay and in culture of human SH-SY5Y neuroblastoma cells. Our in vitro binding data showed that S100B and S100A6, although structurally very similar, interact with different RAGE extracellular domains. Our cell assay data demonstrated that S100B and S100A6 differentially modulate cell survival. At micromolar concentration, S100B increased cellular proliferation, whereas at the same concentration, S100A6 triggered apoptosis. Although both S100 proteins induced the formation of reactive oxygen species, S100B recruited phosphatidylinositol 3-kinase/AKT and NF-κB, whereas S100A6 activated JNK. More importantly, we showed that S100B and S100A6 modulate cell survival in a RAGE-dependent manner; S100B specifically interacted with the RAGE V and C1 domains and S100A6 specifically interacted with the C1 and C2 RAGE domains. Altogether these results highlight the complexity of S100/RAGE cellular signaling.

The S100 proteins possess various intra- and extracellular functions. Intracellular functions range from regulation of cell motility, protein phosphorylation, and calcium homeostasis to tumor progression or suppression. Most S100 proteins undergo a conformational change upon calcium binding allowing them to interact with target proteins, although some S100 proteins can also interact with their target in a calcium-independent way (4). Interestingly several members of the S100 protein family have been shown to interact with the same target. For instance, S100A4, S100A5, S100A11, and S100A13 regulate in vitro activity of aldolase A in a calcium-independent manner (4). The interaction of several S100 proteins with the same target can also result in different effects on the target: whereas S100A1 stimulates phosphoglucomutase activity in vitro, S100B inhibits this enzyme. Interestingly a few S100 proteins are secreted and exert cytocrine functions (4). S100B is secreted by astrocytes, and increased S100B concentration has been found in the cerebrospinal fluid of patients suffering from brain trauma, ischemia, or Alzheimer disease (6). S100A1 is released in the serum of patients suffering from acute myocardial damage and promotes neurite outgrowth at micromolar concentrations (7, 8). S100A4 secretion in culture medium was estimated to be about 10 μM, a concentration that induces neurtogenesis in primary rat neurons (9, 10). Similarly S100A12 is found in synovial fluid of patients suffering from rheumatoid arthritis and also triggers neurtogenesis of hippocampal neurons when added extracellularly at submicromolar to micromolar concentrations (11, 12). Finally S100A6 was found in the extracellular medium of breast cancer cells (13). S100B is mainly expressed in the brain and primarily in astrocytes of the human cortex and hippocampus but is also present in certain populations of oligodendrocytes and neurons (14–18). In contrast, S100A6 is mainly expressed in neurons in restricted areas of the brain (amygdala and entorhinal cortex) and found in a few astrocytes (19, 20). The expression of both S100A6 and S100B proteins has been shown to be modulated during human brain development (21). Higher levels of S100B have also been detected in the serum of patients after brain trauma or ischemia and of patients with Alzheimer disease or Down syndrome (6). Interestingly overexpression of S100A6 has also been observed in patients suffering from Alzheimer disease or amyotrophic lateral sclerosis (22, 23).

* This work was supported by Transregio-Sonderforschungsbereich Konstanz-Zurich Grant TR5PB 11 (to C. W. H. and Peter M. H. Kroneck). 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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S100-RAGE Interaction and Influence on Cell Survival

Recently the receptor for advanced glycation end products (RAGE) has been identified as a new target for several S100 proteins including S100B, S100A1, and S100A12 (7, 24—26). RAGE is a member of the immunoglobulin-like cell surface receptor superfamily composed of three extracellular domains, a “V” type immunoglobulin-like domain followed by two “C” type domains, a single transmembrane-spanning helix, and a short cytosolic domain (27). Additional RAGE isoforms lacking the transmembrane and cytosolic regions (soluble RAGE (sRAGE)) or the “V” immunoglobulin domain (NtRAGE) were identified in human brain suggesting isoform-specific functions for the receptor (28). RAGE is expressed at high levels during brain development, but its expression is low in adult human brain and restricted to specific population of neurons and glial cells (29). RAGE is also expressed in other cell types such as monocytes/macrophages, endothelial cells, mesangial cells, or smooth muscle cells (30). It is also overexpressed in pathologic states such as oxidative stress or inflammation (31).

In this study, we compared the interaction of S100A6 and S100B with sRAGE both in vitro by surface plasmon resonance (SPR) and in cell assays using human SH-SY5Y neuroblastoma cells. Our SPR experiments showed that both S100 proteins bind to RAGE and suggested that S100A6 and S100B do not recognize the same binding site. We showed that, at micromolar concentration, extracellular S100B and S100A6 trigger opposite effects on cells: whereas S100B activated cell proliferation, S100A6 enhanced apoptosis. More importantly, we showed that binding of S100A6 and S100B induces the formation of reactive oxygen species (ROS) but also triggers different signaling pathways: whereas S100B-RAGE interaction resulted in the activation of PI 3-kinase/AKT and NF-κB transcription factor, S100A6-RAGE interaction induced the activation/phosphorylation of JNK. Our results suggest that the two S100 proteins trigger distinct RAGE-dependent signaling pathways in vivo.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Human S100B and S100A6—Recombinant S100B and S100A6 were expressed in Escherichia coli and purified as described previously (32). The purity of the proteins was checked by SDS-PAGE, Western blot, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. After dialysis against ethanolamine prior to measurements. About 4,000 RU were obtained after immobilization with the C2 domain. A "V" type immunoglobulin-like domain followed by two "C" type domains were expressed in Pichia pastoris described previously (33). The purified RAGE V, VC1, and C2 domains were expressed in E. coli and purified as described previously (24).

Antibodies—The generation of rabbit polyclonal antibodies against the RAGE V, C1, and C2 domains has been described previously (33). The polyclonal IgGs were affinity-purified using a HiTrap protein A column (GE Healthcare) following the manufacturer’s protocol and were dialyzed against PBS. These RAGE polyclonal antibodies were either used individually or mixed together for the purpose of the experiments. Non-specific mouse and rabbit immunoglobulins (IgGs) and the mouse monoclonal antibody (MAB1145) directed against the C1 domain of RAGE were from R&D Systems and were resuspended in PBS. The rabbit polyclonal antibodies against S100B and S100A6 were from Dako, the monoclonal mouse anti-β-tubulin 1 was from Sigma, the goat polyclonal anti-flotillin 1 was from AbCam, and the rabbit polyclonal antibodies anti-p-JNK (Thr-183/Tyr-185), -p-AKT (Ser-473), and anti-AKT were from Cell Signaling Technology Inc.

Other Reagents—SN50, SP600125, and LY294002 were from Calbiochem; ionomycin, N-acetyl-l-cysteine (NAC), and 4',6-diamidino-2-phenyl-indole (DAPI) were from Sigma; and 2',7'-dichlorofluorescein diacetate was from Molecular Probes.

Surface Plasmon Resonance—sRAGE was purified from HEK293 cells as described previously (26). The V, VC1, and C2 domains were purified from E. coli as described previously (24). sRAGE and the RAGE domains were immobilized onto CM5 Biacore sensor chips according to procedures described previously (34). Briefly the proteins or fragments were diluted at 50 μg/ml in 20 mM sodium acetate, pH 5, and injected over the sensor chips that had been preactivated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide as recommended by the manufacturer. After injection of the proteins, the surface of the sensor chip was then blocked with ethanolamine prior to measurements. About 4,000 RU were obtained with sRAGE, and 5,000 RU were obtained for the V and VC1 domains. Due to difficulties in immobilization, only 300 RU were obtained after immobilization with the C2 domain. A series of increasing concentrations of S100B (from 0.78 to 12.6 μM) and S100A6 (from 0.125 to 10 μM) were then injected over the flow cells in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 5 mM CaCl2, and 0.005% P20. The sensorgrams were analyzed by global analysis using BiaEvaluation 3.1 software (35). Between each cycle of binding, the surface was regenerated by 1-min contact with 0.5 mM EDTA followed by 1-min contact with 50 mM sodium borate, pH 8.5, containing 1 mM NaCl.

Cell Culture and Treatment—The human U87-MG glioblastoma cells (ATCC) were cultivated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (BioConcept), 2 mM glutamine, and streptomycin/penicillin. After plating in a 12-well plate, U87-MG cells were cultivated for 24 h and were then serum-starved for an additional 24 h. Cells were used. Protein concentration was determined using the BCA protein assay kit (Pierce).

Expression and Purification of Soluble RAGE and the RAGE Domains—sRAGE was purified from the three extracellular immunoglobulin domains was expressed in Pichia pastoris and purified as described previously (33). The purified RAGE V, VC1, and C2 domains were expressed in E. coli and purified as described previously (24).
then stimulated without serum for 20 min with Me₂SO, H₂O, 2 mM EGTA, or 1 mM ionomycin in medium supplemented with 1.5 mM CaCl₂. The cell culture media were harvested after the different treatments and centrifuged at 3,000 × g for 5 min to remove cells.

The human neuroblastoma cell line SH-SY5Y (ATCC) was maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, and streptomycin/penicillin. After plating, cells were cultured with 10% fetal bovine serum for 24 h and were then serum-starved for an additional 24 h before treatment with S100B or S100A6 in serum-free medium. SP600125 and LY294002 were dissolved in Me₂SO, whereas NAC and SN50 were dissolved in water. Each inhibitor was added to the cells in serum-free medium simultaneously to the S100 proteins at the indicated concentration and for the indicated time periods and then incubated at 37 °C. Control cells were treated with a similar amount of Me₂SO or water or with the same concentration of inhibitor in the absence of S100 protein. Both control and treated cells were harvested at the same time.

Purified RAGE domains, sRAGE, and RAGE antibodies were all diluted in PBS. Each component was added to the cells in serum-free medium simultaneously to the S100 proteins at the indicated concentration and for the indicated period of time, and the incubation was performed at 37 °C. Control cells were treated either with PBS or with the same concentration of RAGE domain, sRAGE, or RAGE antibody in the absence of S100 protein. Both control and treated cells were harvested at the same time.

S100A6 Immunoassay—The amount of S100A6 present in the cell culture medium was determined by a “sandwich” ELISA method. Briefly an S100A6-specific rabbit antibody was coated onto an ELISA plate overnight at 4 °C in the presence of 100 mM NaH₂PO₄, 100 mM Na₂HPO₄, pH 8.1. After blocking with 10% bovine serum albumin in PBS for 3 h at room temperature and washing with PBS-T (PBS with 0.05% Tween 20), S100A6 standards and the supernatants of U87-MG cells (100 μl) were added to the wells in triplicate overnight at 4 °C. The wells were then washed three times with PBS-T, and a second S100A6-specific goat antibody (19) was added in the presence of 10% bovine serum albumin in PBS-T. After a 3-h incubation at room temperature, the wells were washed three times with PBS-T, and wells were then incubated with an anti-goat antibody conjugated with horseradish peroxidase (HRP) for 2 h at room temperature. After a final washing procedure, the HRP substrate tetramethylbenzidine (TMB Peroxidase ELA Substrate kit, Bio-Rad) was added to the wells, and the reaction was stopped by addition of H₂SO₄. The HRP products were read at 450 nm using an ELISA plate reader (TECAN). The absorbances obtained from cell culture medium were compared with a standard curve. Specificity of the ELISA test for S100A6 was assessed by the absence of cross-reactivity with S100B, S100A1, or S100A12.

Cell Viability Assays—Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science) as instructed by the manufacturer. Percentage of viability was determined by comparing the number of viable cells in treated cultures to the number of viable cells in a control culture treated in parallel with PBS for an equivalent time.

Cell cycle distribution analysis was determined by fluorescence-activated cell sorting. Isolation and staining of the SH-SY5Y cells were performed using the CycleTEST Plus DNA kit (BD Biosciences), and cell cycle analysis was then performed with a BD Biosciences FACSCalibur flow cytometer. A total of 10⁶ cells were analyzed for each sorting, and quantification of cell cycle distribution was performed with CellQuest Pro software.

Apoptotic cells were detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay using the In Situ Cell Death Detection kit with fluorescein (Roche Applied Science). Detection of apoptotic cells and counterstaining with DAPI were performed according to the instructions provided by the manufacturer. The slides were viewed and photographed by using a fluorescence Zeiss microscope, and cells stained with DAPI and by TUNEL assay were then counted on random fields and by scoring at least 1,000 cells per experimental group.

Caspase 3/7 activity was measured using the caspase 3/7 luminescence assay kit according to the directions of the manufacturer (Promega). Cells were incubated with the caspase-Glo 3/7 substrate for 1 h at room temperature, and caspase 3/7 was measured by luminescence quantification using a microplate reader (TECAN).

Incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) into newly synthesized DNA was performed as a cell proliferation assay. Cells were treated for 24 h with S100B in the presence of 10 μM BrdUrd. Immunofluorescence detection of BrdUrd incorporation was performed according to the manufacturer’s protocol (Roche Applied Science). Nuclei were counterstained with DAPI, and slides were visualized by fluorescence microscopy and photographed with a digital camera. Cells stained with DAPI and with incorporated BrdUrd were then counted on random fields and by scoring at least 1,000 cells per experimental group.

ROS Formation—SH-SY5Y cells were seeded in 96-well plates in RPMI 1640 containing 10% serum. After 24 h, cells were serum-starved for an additional 24 h and were then treated with or without S100B or S100A6 for the indicated time periods. 30 min prior to harvesting, cells were incubated with 10 μM cell-permeable fluorescent dye 2′,7′-dichlorofluorescein diacetate and washed with PBS, and fluorescence was measured with a microplate reader (excitation, 485 nm; emission, 520 nm).

Transfection and NF-κB Activation Assay—SH-SY5Y cells were co-transfected with the pNFκB-Luc construct (Clontech) and a plasmid containing the β-galactosidase gene using the FuGENE®HD transfection reagent (Roche Applied Science) for 24 h in the presence of serum according to the manufacturer’s instructions. Cells were serum-starved for 24 h before being treated with S100B or S100A6. After 24 h in culture, cells were washed with PBS and lysed in Reporter Lysis Buffer (Promega). Luciferase activity was monitored using the Luciferase Assay System (Promega) using a microplate reader. β-Galactosidase activity was quantified by mixing cell lysate with an equal volume of 2× β-galactosidase assay mixture (120 mM Na₂HPO₄, 80 mM NaH₂PO₄, 2 mM MgCl₂, 100 mM β-mercaptoethanol,
and 1.33 mg/ml O-nitrophenyl β-D-galactopyranoside) and incubation at 37 °C; the absorbance was read at 420 nm. Luciferase activity was normalized to β-galactosidase activity.

Western Blot Analysis—The cells were washed twice with PBS and scraped from the plates in lysis buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-10, 10 mM NaF, 10 mM β-glycerophosphate, and 1 mM Na3VO4) in the presence of a protease inhibitor mixture (Roche Applied Science). The membrane fraction and soluble proteins were prepared as described previously (36). SH-SY5Y cells were washed with PBS, scraped from the plates, and centrifuged, and then lysis buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, containing Complete protease inhibitor mixture (Roche Applied Science)) was added to the cell pellet. Cells were homogenized with a glass Dounce homogenizer and then centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was further centrifuged at 100,000 × g for 1 h at 4 °C. The membrane pellet was resuspended in sucrose homogenization buffer with protease inhibitors and stored at −80 °C. The supernatant contained the cytosolic fraction. The protein concentration of both fractions was determined using the BCA protein assay kit (Pierce). For SDS-PAGE, an equal amount of protein was loaded on a 10% bis-Tris gel (Invitrogen). The SDS gel was transferred onto a nitrocellulose membrane. The blot was blocked with 4% fat-free milk powder in TBS-T (Tris-buffered saline with 0.05% Tween 20) and incubated with the primary antibody dissolved in TBS-T containing 5% bovine serum albumin for 1 h at room temperature. After incubation, the blot was washed with TBS-T and incubated with the appropriate secondary antibody (diluted in TBS-T with 5% bovine serum albumin at 1:10,000 for the anti-mouse, 1:5,000 for the anti-goat, and 1:10,000 for the anti-rabbit secondary antibodies conjugated with HRP). Detection was performed using a chemiluminescent HRP substrate (Pierce). The dilution of the primary antibodies were as follow: anti-β-tubulin 1, 1:10,000; anti-flotillin 1, 1:1,000, anti-p-JNK, 1:1,000; anti-JNK, 1:1,000; anti-p-AKT, 1:1,000; and anti-AKT, 1:1,000.

Immunofluorescence Staining of SH-SY5Y Cells—Immunofluorescence staining of SH-SY5Y cells grown on coverslips was performed as follow. Serum-starved cells were washed with PBS, fixed in 4% formaldehyde for 15 min, and washed with PBS. The cells were then immunostained with the anti-C1 RAGE antibody diluted at 1:500 for 1 h, washed with PBS, and incubated with the Cy3-conjugated anti-rabbit secondary antibody diluted at 1:200 for 1 h. DNA was counterstained with DAPI. Coverslips were mounted on slides prior to analysis using a fluorescence microscope.

Statistical Analysis—Statistical analysis between the different groups was determined using the one-way analysis of variance, and where indicated individual comparison was performed by analysis of variance with posthoc analysis using SPSS software.

RESULTS
S100B and S100A6 Bind Differently to RAGE—We have shown previously by surface plasmon resonance that S100B binds to sRAGE (26) and to the RAGE V and VC1 domains (24), which had been expressed in E. coli. Consequently the purified proteins were not glycosylated. Here we show that both S100B and S100A6 interacted with glycosylated sRAGE purified from HEK293 cells (Fig. 1A). Analysis of the S100B sensorgrams, after fitting with a two-independent binding site model, revealed binding constants of $K_{D1} = 3.6 \mu M$ (62% species) and

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Binding of S100B and S100A6 to sRAGE and the RAGE V, VC1, and C2 domains. A, sRAGE (4,500 RU) was directly immobilized onto the CMS sensor chip as described under “Experimental Procedures.” A series of S100B (0.78–12.6 μM, in black) or S100A6 (0.125–10 μM, in red) concentrations were injected over the flow cells. B and C, RAGE V (black) and VC1 (red) domains (5,000 RU) were immobilized, and a series of S100B (0.78–12.6 μM) or S100A6 (C) (0.125–10 μM) concentrations were injected over the flow cells. D, as in A with the immobilized RAGE C2 domain (300 RU).
Although the binding signal was reduced with both proteins (5,000 RU with the V domain), a significant binding of S100A6 to the V domain directly immobilized on the sensor chip (Fig. 1C) and compared these with the binding of S100B to the same domains (Fig. 1B). We then decided to compare the effect of extracellularly added S100B and S100A6 on cellular functions and to explore the role of RAGE in these processes. Most of the previous studies on the role of extracellular S100 proteins were performed with cells that did not express endogenous RAGE but were instead realized in cells overexpressing the receptor, resulting in non-physiological conditions (7, 38–41). In addition, many cells express S100B or S100A6, and it was shown that addition of S100 protein in the extracellular medium results in the translocation of the corresponding endogenous protein (32). Such a phenomenon could therefore mislead our analyses and interpretations by creating potential interferences between endogenous and extracellularly added S100 proteins. We then screened several cell lines for the expression of RAGE, S100B, and S100A6, and we finally chose the human neuroblastoma SH-SY5Y cell line. SH-SY5Y cells have been shown previously to express RAGE (42). We show here by immunohistochemistry that RAGE was mainly present on the cell surface in SH-SY5Y cells (Fig. 2B). Western blot analysis of cytosolic and membrane fractions (Fig. 2C) revealed that most of RAGE was present in the membrane fraction. The weak band observed in the cytosolic fraction might correspond to newly translated RAGE. In addition, SH-SY5Y cells also did not express S100B and S100A6 as confirmed here by Western blot (Fig. 2D), making these cells a suitable system for our studies.

The human S100B and S100A6 were expressed in E. coli and purified. The purity of both recombinant human proteins was assessed by SDS gel electrophoresis (Fig. 2E), Western blot (Fig. 2F) and MALDI-TOF mass spectrometry (Fig. 2G). The mass spectra revealed a single peak for each protein with a molecular mass of 10,741 and 10,048 Da for S100B and S100A6, respectively (Fig. 2G). In addition, both S100B and S100A6 were stable in the SH-SY5Y culture medium during the time course of the experiment and did not show any sign of degradation even after 48 h (Fig. 2H).

Exposure of serum-starved SH-SY5Y cells to increasing concentrations of S100B or S100A6 for 48 h resulted in a significant alteration of cell death, evaluated by trypan blue exclusion after the different treatments, excluded that S100A6 could be released by damaged cells. Altogether these results suggest that the observed variations of extracellular S100A6 content resulted from active secretion of the protein by glioblastoma cells.

### TABLE 1

| Protein | K$_{D1}$ | Percent species | K$_{D2}$ | Percent species | R$_{max}$ | Target |
|---------|----------|----------------|----------|----------------|-----------|--------|
| S100B   | 3.6 µM   | 62             | 2.2 nM   | 38             | 1.2       | sRAGE  |
| S100A6  | 0.6 µM   | 51             | 0.5 µM   | 49             | 2.2       | sRAGE  |
| S100B   | 0.5 µM   | 75             | 0.6 µM   | 25             | 76        | V      |
| S100A6  | 13.5 µM  | 97             | 0.5 µM   | 3              | 14        | V      |
| S100B   | 11 nM    | 84             | 0.2 µM   | 16             | 14        | VC1    |
| S100A6  | 5.8 µM   | 82             | 0.6 µM   | 18             | 0.6       | VC1    |
| S100B   | ND*      | ND             | ND       | ND             | ND        | C$_2$  |
| S100A6  | 1 µM     | 55             | 28 nM    | 45             | 4         | C$_2$  |

* ND, not determined.

K$_{D1}$ = 2.2 nM (38% species) (Table 1). The sensorsgrams of S100A6 could also be fit with a two-binding site model with binding constants of K$_{D1}$ = 0.6 µM (51% species) and K$_{D2}$ = 0.5 µM (49%) (Table 1). In both cases, the level of maximal resonance estimated (R$_{max}$) was only 1% (S100B) and 2% (S100A6) of the theoretical response one should obtain if the S100 proteins would recognize all the immobilized sRAGE molecules, suggesting that immobilization of sRAGE on the sensor chip resulted in the modification of most of the S100 binding epitopes. The 2-fold difference in sRAGE recognition by S100B and S100A6 suggested slight differences in binding. We further investigated the binding of S100A6 to the V and VC1 domains (Fig. 1C) and compared these with the binding of S100B to the same domains (Fig. 1B). As described previously, S100B interacted with both V and VC1 domains with submicromolar affinity for the V domain (K$_{D1}$ = 0.5 µM; K$_{D2}$ = 0.6 µM) and a higher affinity for the VC1 domain (K$_{D1}$ = 11 nM (84%); K$_{D2}$ = 0.2 µM (16%) (24). The sensorsgrams obtained for S100A6 with the V domain were fitted with the two-binding site model and showed binding constant K$_{D1}$ = 13.5 µM (97% species) and K$_{D2}$ = 0.49 µM (3% species) (Table 1). Similarly the binding data of S100A6 with the VC1 domain were also best fitted with a two-binding site model and resulted in K$_{D1}$ = 5.6 µM (82% species) and K$_{D2}$ = 0.58 µM (18% species). However, although S100B recognized 76 and 14% of immobilized V and VC1 domains, respectively, S100A6 recognized only 14 and 0.6% of the bound V domain and VC1 domains, respectively (Table 1), again suggesting differences in binding between the two S100 proteins. We also compared the binding of S100B and S100A6 to the C$_2$ domain directly immobilized on the sensor chip (Fig. 1D). Although the binding signal was reduced with both proteins because of the lower amount of protein on the chip (300 versus 5,000 RU with the V domain), a significant binding of S100A6 to the C$_2$ domain was observed, whereas the interaction of S100B to this domain was negligible. The fitting of the sensorsgrams of S100A6 revealed two binding sites with K$_{D1}$ = 1 µM (55% species) and K$_{D2}$ = 28 nM (45% species) (Table 1).

S100B and S100A6 Modulate Survival of Human SH-SY5Y Cells—SH-SY5Y cells are, with S100A1, the most abundantly expressed members of the S100 protein family in the brain (37). S100B is actively secreted by cells, and previous studies have shown the effect of extracellular S100B on cellular functions. S100A6 was found in the extracellular medium of breast cancer cells (13), but to our knowledge, no data are available on the active secretion of S100A6 by cells and on the extracellular effect of the protein on cellular functions. To determine whether S100A6 was actively released from brain-related cells, we used human U87-MG glioblastoma cells, which endogenously express S100A6 (Fig. 2A). Quantification of extracellular S100A6 levels by ELISA analysis revealed the presence of 53.0 ± 3.7 ng/ml S100A6 in serum-free medium, whereas 195.1 ± 6.7 ng/ml S100A6 was found intracellularly. Interestingly, modulation of Ca$^{2+}$ homeostasis influenced S100A6 extracellular levels. The increase of intracellular Ca$^{2+}$ concentration following ionomycin treatment for 20 min enhanced S100A6 content in the extracellular medium from 53.0 ± 3.7 ng/ml in control cells to 98.4 ± 1.9 ng/ml in presence of ionomycin. In contrast, EGTA treatment drastically reduced the extracellular amount of S100A6 to 12.7 ± 1.5 ng/ml. The lack of significant alteration of cell death, evaluated by trypan blue exclusion after the different treatments, excluded that S100A6 could be released by damaged cells. Altogether these results suggest that the observed variations of extracellular S100A6 content resulted from active secretion of the protein by glioblastoma cells.
S100-RAGE Interaction and Influence on Cell Survival

A

B

C

D

E

F

G

H

10741

10713

10048

10179

10 KDa

50 KDa

10 KDa

50 KDa

MW S100B S100A6

αS100B αS100A6

S100B S100A6

relative intensity

mass (Da)

relative intensity

mass (Da)

S100B S100A6

Control S100 Control S100

0 48h

JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 43 • OCTOBER 26, 2007

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concentration-dependent increase and decrease of cell viability, respectively, as shown by MTT assay (Fig. 3, A and B). Although S100B already significantly increased cell viability at 50 nM, S100A6 was only active at 5 μM. Therefore, we used this concentration of 5 μM for all further experiments to compare the effect of both S100 proteins. Fluorescence-activated cell sorting analysis was performed to score the fraction of cells in S or G2/M phase of the cell cycle and dead cells (E). Each measurement (n = 2) was repeated in duplicate, and presented data are the means ± S.D. *, p < 0.05. F, S100B induces cell proliferation. Serum-starved SH-SY5Y cells were grown without serum with PBS or 5 μM S100B for 24 h before fixation. BrdUrd-positive cells were stained with a specific anti-BrdUrd antibody, and the percentage of BrdUrd-positive SH-SY5Y cells over the total number of cells was determined. A minimum of 1,000 cells were counted in random fields, and the presented data are the means ± S.D. from three separate experiments. ***, p < 0.0001. G, serum-starved SH-SY5Y cells were grown without serum with PBS or 5 μM S100A6 for 48 h, and the percentage of TUNEL-positive cells over the total number of cells was determined. A minimum of 1,000 cells were counted in random fields, and the presented data are the means ± S.D. from three separate experiments. ***, p < 0.0001. H, similar to G and caspase 3 and 7 activities were measured after the indicated length of time. Data are the means ± S.D. from four separate experiments. ***, p < 0.0001.

FIGURE 3. S100B and S100A6 differentially affect cell survival of SH-SY5Y cells. Serum-starved SH-SY5Y cells were grown without serum in the presence of increasing concentrations of S100B (A) or S100A6 (B) for 48 h, and cell survival was measured by MTT assay. Cell survival was expressed as percentage of PBS-treated control cells. Data are the means ± S.D. from eight separate experiments. ***, p < 0.0001. C, D, and E, same as A and B except that SH-SY5Y cells were treated with PBS, 5 μM S100B, or 5 μM S100A6 for 24 and 48 h before fluorescence-activated cell sorting analysis to score the fraction of cells in S (C) or G2/M (D) phase of the cell cycle and dead cells (E). Each measurement (n = 2) was realized in duplicate, and presented data are the means ± S.D. **, p < 0.05. F, S100B induces cell proliferation. Serum-starved SH-SY5Y cells were grown without serum with PBS or 5 μM S100B in the presence of 10 μM BrdUrd for 24 h before fixation. BrdUrd-positive cells were stained with a specific anti-BrdUrd antibody, and the percentage of BrdUrd-positive SH-SY5Y cells over the total number of cells was determined. A minimum of 1,000 cells were counted in random fields, and the presented data are the means ± S.D. from three separate experiments. ***, p < 0.0001. G, serum-starved SH-SY5Y cells were grown without serum with PBS or 5 μM S100A6 for 48 h, and the percentage of TUNEL-positive cells over the total number of cells was determined. A minimum of 1,000 cells were counted in random fields, and the presented data are the means ± S.D. from three separate experiments. ***, p < 0.0001. H, similar to G and caspase 3 and 7 activities were measured after the indicated length of time. Data are the means ± S.D. from four separate experiments. ***, p < 0.0001.

FIGURE 2. Expression of S100A6 in U87-MG glioblastoma cells and of RAGE in human SH-SY5Y neuroblastoma cells: production of recombinant human S100B and S100A6. A, cell lysate was prepared from human U87-MG glioblastoma cells and immunoblotted for S100A6, and β-tubulin 1 was used as loading control. B, immunofluorescence of RAGE antigen (red) in SH-SY5Y cells. Cells were counterstained with DAPI for DNA visualization (blue). C, soluble and membrane proteins were prepared from SH-SY5Y cells and subjected to immunoblotting for RAGE and flotillin 1, a protein marker for membrane localization. D, cell lysates were prepared from human SH-SY5Y and immunoblotted for S100B or S100A6, and β-tubulin 1 was used as loading control. E, SDS-PAGE of purified human S100B and S100A6 (2 μg) produced in E. coli. F, Western blot analysis of purified human S100B and S100A6 with polyclonal anti-S100B (αS100B) or -S100A6 (αS100A6). G, mass spectrum of recombinant human S100B (left) and S100A6 (right). H, S100B and S100A6 stability in RPMI 1640 medium. Conditioned media from control cells and cells treated with 5 μM S100B or S100A6 were harvested at the time of addition (time 0) or 48 h after addition of S100B or S100A6 and were subjected to immunoblotting for S100B and S100A6.
S100-RAGE Interaction and Influence on Cell Survival

FIGURE 4. Signaling pathways in S100B-mediated cell proliferation and S100A6-induced apoptosis. Serum-starved SH-SY5Y cells were grown without serum with 5 μM S100B or PBS in the presence of 5 mM NAC or H₂O or 10 μM LY294002 or Me₂SO (A) or with 5 μM S100A6 or PBS in the presence of 5 mM NAC or H₂O or 10 μM SP600125 or Me₂SO (B) for 48 h, and cell survival was measured by MTT assay. Cell survival was expressed as percentage of control (as analyzed by densitometry). **, p < 0.01. (F), SH-SY5Y cells were cultivated without serum with 5 μM S100B or with PBS, and the cells were lysed after the indicated length of time and further immunoblotted for phosphorylated AKT (Ser-473) and total AKT cellular content. The numbers on top of the panel refer to the amount of activated AKT (p-AKT) after normalization to the total AKT level as analyzed by densitometry. **, p < 0.01. H, SH-SY5Y cells were cultivated without serum with 5 μM S100B or with PBS, and the cells were lysed after 24 h and further immunoblotted for phosphorylated JNK (Tyr-183/Tyr-185) and total JNK cellular content (top panel), or the cells were treated with 10 μM LY294002 in the presence of 5 μM S100B, and the cells were lysed after 24 h and further immunoblotted for phosphorylated JNK (Ser-473) and total JNK cellular content (bottom panel). The numbers on top of the panel refer to the amount of activated AKT (p-AKT) or JNK (p-JNK) after normalization to the total levels of AKT or JNK as analyzed by densitometry. **, p < 0.01. J, SH-SY5Y cells were treated with 5 μM S100B or PBS for 4 h and 24 h, and the proteins were subjected to immunoblotting for phosphorylated JNK (Tyr-183/Tyr-185) and total JNK cellular content. The numbers on top of the panel refer to the amount of activated JNK (p-JNK) after normalization to the total JNK level as analyzed by densitometry. **, p < 0.01. Opposite to the above results, 3.5% of PBS-treated cells were TUNEL-positive as measured in the presence of S100A6. In contrast, 13% of cells positive for BrdUrd incorporation in the presence of 5 μM S100B revealed an increased number of cells in the S and G₂/M phases of the cell cycle after 24 and 48 h, respectively, compared with the control cells (Fig. 3, C and D), whereas exposure to 5 μM S100A6 enhanced cell death after 48 h (Fig. 3E).

To investigate more closely the increased number of SH-SY5Y cells in the S phase of the cell cycle induced by S100B, we monitored DNA synthesis by incorporation of BrdUrd into DNA. Cells were serum-starved and then stimulated with 5 μM S100B in the presence of BrdUrd for 24 h. PBS-treated cells contained ~5% BrdUrd-positive cells. In contrast, S100B treatment resulted in about 13% of cells positive for BrdUrd incorporation (Fig. 3F). Similarly to confirm that S100A6-induced cell death was apoptosis, SH-SY5Y cells treated with either 5 μM S100A6 or PBS were examined by the TUNEL assay. After 48 h, 10.5% of cells were TUNEL-positive as measured in the presence of S100A6. In contrast, 3.5% of PBS-treated cells were TUNEL-positive (Fig. 3G). Moreover the presence of S100A6 also resulted in the alteration of caspase 3/7 activities that increased after 24 and 48 h treatment with S100A6 (Fig. 3H). Altogether these results suggest that 5 μM extracellular S100B and S100A6 differentially modulated cell viability of the SH-SY5Y cells; S100B promoted DNA replication and cell proliferation, whereas S100A6 induced apoptosis.

Modulation of Signaling Transduction Pathways by S100B and S100A6—To identify key intermediates of the signaling pathways triggered by S100B and S100A6, SH-SY5Y cells were exposed to S100B and S100A6 in the presence of specific inhibitors of the various pathways. Treatment of the cells with a 5 μM concentration of the antioxidant NAC efficiently prevented enhanced cell viability and abolished the increase of BrdUrd incorporation in the presence of 5 μM S100B (Fig. 4, A and C). Similar effects were observed with the specific inhibitor of PI 3-kinase, LY294002 (Fig. 4, A and C). Interestingly the treat-
Members of the S100 protein family have been shown to activate the transcription factor NF-κB (25, 43). We used a luciferase reporter plasmid system to investigate the influence of S100B and S100A6 on NF-κB transcriptional activity. Treatment of the cells with 5 μM S100B increased NF-κB activity, and the presence of either the antioxidant NAC or the PI-3 kinase inhibitor LY294002 blocked S100B-mediated NF-κB activity (Fig. 6A). In contrast, NF-κB activity was not significantly altered when the cells were exposed to 5 μM S100A6 (Fig. 6A). SN50 is a cell-permeable peptide that inhibits NF-κB by blocking its translocation to the nucleus (44), and S100B-induced activation of NF-κB activity was inhibited by treatment of the cells with 20 μg/ml SN50 (Fig. 6A). Similarly SN50 abolished S100B-induced cell viability and the increase of BrdUrd incorporation (Fig. 6, B and C), whereas it was inefficient against the decrease of viability induced by S100A6 (data not shown). Together these results suggest that S100B and S100A6 affect cellular functions by recruiting distinct signaling pathways through the modulation of ROS formation.

Role of RAGE in S100B-induced Cellular Proliferation and S100A6-mediated Apoptosis—We showed that S100B and S100A6 interact with sRAGE in vitro (Fig. 1). We therefore

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

![Diagram D](image4)

**FIGURE 5. S100B and S100A6 induce ROS formation.** Kinetics of ROS production in serum-starved SH-SY5Y cells in the presence of PBS, 5 μM S100B (A), or 5 μM S100A6 (B) are shown. Cells were labeled with the fluorescent dye 2,7'-dichlorofluorescein diacetate and analyzed as described under “Experimental Procedures.” Presented data are the average values from triplicate samples ± S.D. **p < 0.005.** C, SH-SY5Y PBS- (control cells) and S100B-treated cells (5 μM) were treated with 5 mM NAC. After 24 h lysates were prepared and assayed for AKT activation by Western blot analysis as done in Fig. 4. The numbers on top of the panel refer to the amount of activated AKT (p-AKT) after normalization to the total AKT level as analyzed by densitometry. ***, p < 0.01. D, similar to C in the presence of PBS or 5 μM S100A6; lysates were prepared and assayed for JNK activation by Western blot analysis as done in Fig. 4. The numbers on top of the panel refer to the amount of activated JNK (p-JNK) after normalization to the total JNK level as analyzed by densitometry. **, p < 0.01.
investigated whether RAGE was involved in the cellular responses driven by S100B and S100A6 and particularly whether a specific RAGE domain could contribute to the distinct cellular responses. We used two strategies to test this hypothesis. First, we used antibodies directed against the distinct RAGE immunoglobulin domains to block RAGE-S100 interaction (33). The second strategy was to use either purified sRAGE or the individual RAGE V, VC1, or C2 domains to sequester the S100 proteins before their interaction with cell surface RAGE (25, 45–47).

We observed a significant reversion of the cellular effects when the cells were treated with either polyclonal antibodies against S100B or S100A6 (Fig. 7A and C, αS100B or αS100A6), a mixture of the polyclonal antibodies directed against the V, C1, and C2 domains (Fig. 7, A and C, αVC1C2), or sRAGE (Fig. 7, B and D). As expected, no change in cell viability was observed when the cells were treated with nonspecific mouse or rabbit immunoglobulin (Fig. 7, A and C, mlG or rlG). We then investigated which domain of RAGE was responsible for S100B- and S100A6-mediated changes of cell viability. We observed that either the anti-V domain polyclonal antibody (Fig. 7A, αV) or the V domain itself (Fig. 7B, V) was able to reverse the increase of cell viability triggered by S100B. The VC1 domain was slightly less efficient than the V domain itself (Fig. 7B, VC1). Similarly an anti-C1 polyclonal antibody (Fig. 7A, αC1) also decreased the effect mediated by S100B but to a lesser extent. However, the purified C2 domain and the anti-C2 polyclonal antibody (Fig. 7, A and B, C2 and αC2) were without effect. In contrast, the reduction of cell viability observed when the cells were exposed to 5 μM S100A6 was not significantly suppressed by anti-V domain antibody or by the V domain itself (Fig. 7, C and D, αV and V). The S100A6-mediated decrease of cell viability was best reversed with the anti-C1 as well as with the anti-C2 domain polyclonal antibodies (Fig. 7C, αC1 and αC2). The purified C2 domain was also most efficient to reverse S100A6-mediated cellular effects (Fig. 7D, C2).

Next we investigated the effect of sRAGE and the anti-RAGE domain polyclonal antibodies on the suppression of S100B-enhanced BrdUrd incorporation. S100B-enhanced BrdUrd incorporation was attenuated in SH-SY5Y cells treated with sRAGE as well as with the anti-V or anti-C1 domain polyclonal antibody (Fig. 7E, αV and αC1). Although no significant change in S100B-enhanced BrdUrd incorporation was noted with the anti-C2 domain polyclonal antibody (Fig. 7E, αC2), because S100A6 mediated cell apoptosis in SH-SY5Y cells, we also asked whether the cells treated with S100A6 would undergo apoptosis in the presence of blocking RAGE antibodies or sRAGE. Indeed blockade of RAGE-S100A6 interaction with sRAGE or the anti-C1 or anti-C2 RAGE antibodies significantly suppressed the S100A6-induced increase of apoptosis as shown by the TUNEL assay (Fig. 7F, αC1 or αC2), and the anti-C2 also reverted the increase of caspase 3/7 activity (Fig. 7G, αC2). No significant changes were observed when the cells were treated with S100A6 and the anti-V domain polyclonal antibody (Fig. 7, F and G, αV).

Role of RAGE in Signaling Pathways Activation—To determine whether RAGE was also required to trigger the different signaling pathways recruited by the two S100 proteins, we studied the influence of sRAGE and the anti-V or anti-C2 domain polyclonal antibody on ROS formation, mitogen-activated protein kinase activation, and NF-κB activity. Whereas the nonspecific antibody and the anti-C1 domain antibody were without effect on the S100B-mediated increase of ROS formation (Fig. 8A), AKT activation (Fig. 8C), and the modulation of NF-κB activity (Fig. 8E), the anti-V domain polyclonal antibody and sRAGE efficiently reduced these three cellular events (Fig. 8, A, C, and E). On the other hand, the anti-C2 domain polyclonal antibody and sRAGE, but not the anti-V domain polyclonal antibody, inhibited both S100A6-mediated ROS accumulation and activation of JNK (Fig. 8, B and D).

DISCUSSION

Despite their high homology, the 21 known members of the S100 EF-hand Ca2+-binding proteins play distinct intracellular roles through the modulation of their subcellular localization and their interaction with various target proteins. In addition, a
unique characteristic of this protein family is that some members are actively released from cells into the extracellular space where they are involved in cell differentiation, proliferation, or apoptosis by interacting with cellular receptors such as RAGE (4). This functional diversity implies the existence of regulatory mechanisms that allow RAGE to differentiate between the different S100 proteins to elicit the appropriate cellular response. To gain insights on these regulatory mechanisms, we combined an in vitro binding experiment by SPR between RAGE and S100B or S100A6 and a cell assay using human SH-SY5Y neuroblastoma cells in which we compared the cellular responses triggered by these two S100 proteins present in the culture medium and investigated the involvement of RAGE in these processes. S100B and S100A6 were chosen because of their high expression in the brain and their involvement in human diseases (37), and the SH-SY5Y human neuroblastoma cell line was selected because of the absence of both S100B and S100A6 expression and for the endogenous expression of RAGE.

We have previously characterized the binding of S100B to sRAGE as well as to the RAGE V and VC1 domains by surface plasmon resonance (24, 26). Using the same technique, we show here that S100A6 interacted with sRAGE in vitro. Data analysis showed that the interaction is more complex than the 1:1 model suggesting that sRAGE immobilized on the surface is not homogeneous. Comparison of the S100A6 data with those of S100B revealed differences in binding. Whereas S100A6 bound to sRAGE via two sites with submicromolar affinity, binding of S100B occurred via one site with micromolar affinity and one site with nanomolar affinity. Detailed analysis of these data revealed that whereas 1% of immobilized sRAGE is recognized by S100B, S100A6 molecules recognize 2% of the immobilized molecules (Table 1), again suggesting different modes of interaction. Similar differences in binding affinity and percentage of molecule recognition between the two S100 proteins were observed with the V and VC1 domains. Finally, we observed significant binding of S100A6 to immobilized C2 domain, whereas binding of S100B to the C2 domain was negligible. In summary, the SPR experiments strongly suggested that S100B and S100A6 recognize different sites on RAGE. S100A6 appears to recognize a region included in the V domain (residues 23–119) but also a region included in the C2 domain (residues 235–327). Dattilo et al. (24) proposed a model in which the RAGE V and C1 domains form a single structural unit, whereas C2 is independent. The C1 and C2 domains are linked by a 12-amino acid flexible stretch that allows the C2 domain to move independently from the VC1 unit. This flexible stretch would then allow S100A6 simultaneous binding to the V and C2 domains through the formation of a bend or a 90° angle between these two domains.

Our cell-based assay revealed that the effect of S100B on cell survival is mainly mediated by the RAGE V domain because both a polyclonal antibody directed against a peptide of the V domain and the purified V domain itself were the most efficient to inhibit cell survival and ROS formation as well as PI3-kinase/AKT and NF-κB activation. In addition, the C1 domain antibody could also block the increase of cell proliferation induced by S100B. These results correlate well with those of our in vitro binding studies by SPR. The cellular effects triggered by S100A6 were also mediated by RAGE and were reverted by polyclonal antibodies against the C1 or C2 domain as well as by the purified C2 domain. However, and in contrast to our results by SPR, the V domain antibody was unable to block S100A6-induced increase of apoptosis. This could be explained by the lower affinity of S100A6 for this domain (13.5 μM) and by the poor recognition of the domain by S100A6 (14%). Altogether these results suggest a central role of the three extracellular RAGE immunoglobulin domains for ligand recognition and binding. Indeed it was shown that amphoterin and advanced glycation end products bind to the RAGE V domain, whereas S100A12 binds to the C1 and C2 domains (46, 48, 49). Furthermore amyloid β species, formed during the progression of Alzheimer disease, induce their toxicity via their binding to distinct RAGE domains.3

S100B increases cell survival at micromolar concentration. In contrast, we found that S100A6 mediated apoptosis of the SH-SY5Y cells at the same concentration. In vivo, overexpression of S100B in glial cells of a transgenic mouse model leads to enhanced astrocytosis and axonal proliferation in the hippocampus (50). This would be consistent with known in vitro functions of S100B inducing neurite outgrowth and glial proliferation and with the increased expression of S100B observed in the hippocampus during brain development (21, 51, 52). RAGE is expressed in developing neurites and could, in this context, serve as a cellular receptor to transduce S100B neurotrophic signal from the astrocytes to the neurons (53). However, a chronic exposure of the brain to a high level of S100B could change S100B function from neurotrophic to pathologic. Indeed a second S100B-overexpressing mouse model, expressing the protein at higher level, shows age-related damage of the hippocampus. Whereas young mice have increased dendritic branching and neurite outgrowth, older animals show cytoskeletal collapse and loss of dendrites (54). RAGE could also participate in S100B-mediated damaging effect in the brain because RAGE expression increases following sustained exposure to its ligands (47, 55). Furthermore elevated RAGE expression sensitizes neuroblastoma cells as shown by the S100B-induced increase of apoptosis in neuroblastoma cells overexpressing RAGE (7). It appears that the RAGE/S100 system is highly adaptive and enables brain cells to respond in an appropriate manner to changes in the extracellular environment. The modulation of the expression of RAGE and S100 proteins during pathological states could alter this regulatory balance and would then lead to cellular dysfunctions (23, 37, 56, 57). However, these findings raise the question of the functional relevance of micromolar concentration of S100B and S100A6 in the brain in vivo. S100B and S100A6 are highly expressed in the brain of various species. In the human brain, S100B is present at an estimated concentration of 1 μM considering a 1.6-kg human brain with an average volume of 1.6 liters and a proportion of S100B over the brain fresh weight of 0.001% (26). In addition, S100B and S100A6 represent 0.1 and 0.03% of the soluble proteins of the mouse cortex and the rat brain, respectively (19, 37, 58). S100B-overexpressing mouse models contain between 5

3 E. Sturchler, A. Galichet, and C. W. Heizmann, personal communication.
and 100 copies of the S100B gene, which is expressed under the control of its own promoter (50, 59). This implies that S100B levels in the brain of these animals might reach about 10% of the soluble proteins. In addition, the heterogeneous expression pattern of both S100B and S100A6 will also influence the content of both proteins within brain regions. Similarly modulation of secretion will also affect the content and the availability of S100 proteins in the extracellular space and subsequently their interaction with RAGE. We could show that about 66% of S100A6 content in glioblastoma cells was released within 20 min following an increase of intracellular Ca^{2+}. In addition, the S100 proteins represent about 0.2% of the soluble proteins present in the extracellular space of the rat brain, a value 3–6-fold higher than the S100 protein levels in the cytoplasmic fraction (60). Altogether these observations make it very plausible that S100B and S100A6 occur at high levels in the brain where they could thus play the role of mediators of brain cell communication like glutamate, Ca^{2+}, or acetylcholine. The secretion of S100B and S100A6 from cells in response to particular stimuli would provide micromolar concentrations of these S100 proteins at the vicinity of cells where they would then influence cellular fate through their interaction with RAGE.

The observed effect of S100B on cell survival involves the formation of ROS and the activation of PI 3-kinase/AKT as well as the activation of NF-κB (Fig. 9) because the increase of cell survival was significantly reverted by addition of antioxidant and inhibitors of PI 3-kinase and NF-κB, respectively. The PI 3-kinase/AKT pathway is a key signaling pathway involved in cell proliferation and differentiation that has been shown to be induced by ROS (61, 62). In contrast to the effect triggered by S100B on SH-SY5Y cells, we showed that S100A6 triggers apoptosis. A detailed analysis of the cellular
pathway triggered by S100A6 revealed that S100A6 induces the formation of ROS and the activation of JNK (Fig. 9), a member of the mitogen-activated kinases mostly involved in stress response (63), because the presence of antioxidant and JNK inhibitors reverted the induction of apoptosis. Surprisingly the increase of cellular survival and apoptosis triggered, respectively, by S100B and S100A6 were both mediated through the formation of ROS. ROS elicit a wide range of cellular functions

FIGURE 8. RAGE-S100 interaction is involved in ROS formation, activation of signaling pathways, and modulation of NF-κB transcriptional activity. A and B, serum-starved SH-SYSY cells treated with PBS (control cells) or with 5 μM S100B (A) or 5 μM S100A6 (B) were grown in the presence of 50 μg/ml sRAGE, 25 μg/ml RAGE antibodies (αV or αC1), or 25 μg/ml nonspecific IgG. ROS formation was measured as in Fig. 5 after 1 h. *** p < 0.005. C, serum-starved SH-SYSY PBS- (control) or S100B-treated cells (5 μM) were treated with 25 μg/ml RAGE antibodies (αV or αC1) or 25 μg/ml nonspecific IgGs. After 24 h lysates were prepared and assayed for AKT activation by Western blot analysis as done in Fig. 4. The numbers on top of the panel refer to the amount of activated AKT (p-AKT) after normalization to the total AKT level as analyzed by densitometry. ***, p < 0.01. D, similar to C but in the presence of 5 μM S100A6, and lysates were prepared and assayed for JNK activation by Western blot analysis as done in Fig. 4. The numbers on top of the panel refer to the amount of activated JNK (p-JNK) after normalization to the total JNK level as analyzed by densitometry. ***, p < 0.01. E, similar to C, and NF-κB transcriptional activity was measured as in Fig. 6 after 24 h in the presence of 5 μM S100B. *** p < 0.0001.
from proliferation to cell death, and these diametrically opposed responses rely mostly on differences in magnitude and duration of ROS production. Typically low doses of ROS favor cell proliferation, whereas severe oxidative stress causes cell death (64). ROS are known to be involved in several RAGE-mediated biological processes such as amyloid-β- and S100B-dependent cellular toxicity or amphoterin-mediated neurite outgrowth (7, 39, 47). Our observations that S100B and S100A6 increased ROS formation support the hypothesis that ROS may represent a general mechanism involved in RAGE-mediated cellular activation.

In summary, our experiments clearly demonstrated that S100B and S100A6 (i) interact with different RAGE domains present on the surface of human SH-SYSY neuroblastoma cells and (ii) trigger distinct RAGE-dependent cellular pathways. Further experiments will be needed to solve the cellular link between RAGE activation and the recruitment of signaling pathways. Indeed although RAGE has been shown to transduce cellular signaling via its C-terminal tail (7, 41, 45, 47, 65), a recent report indicated that S100B-induced nitric oxide production in microglia does not require RAGE transducing activity but depends on RAGE extracellular domains (66). In addition, in vascular smooth muscle cells, S100B-dependent RAGE signaling has been shown to occur via the non-receptor Src tyrosine kinase used by several receptors lacking intrinsic tyrosine kinase activity to transduce intracellular signals (67). This suggests that RAGE-dependent signaling could occur via different mechanisms and be part of a multiprotein signaling complex.

Acknowledgments—We thank Michael Koch for help with purification of RAGE domains, Peter Kleinert for the mass spectrometry analyses, Timo Butler for ROS measurements, Brian Dattilo and Walter Chazin for providing the plasmids for expression of RAGE domains, Bernd Weigle for sRAGE produced in HEK293 cells, and Peter M. H. Kronke for continuous support to this project.

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