S100B AND S100A6 DIFFERENTIALLY MODULATE CELL SURVIVAL BY INTERACTING WITH DISTINCT RAGE IMMUNOGLOBULIN DOMAINS

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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 show that S100B and S100A6, although structurally very similar, interact with different RAGE extracellular domains. Our cell assays data demonstrate that S100B and S100A6 differentially modulate cell survival. At micromolar concentration, S100B increases cellular proliferation whereas, at the same concentration, S100A6 triggers apoptosis. Although both S100 proteins induce the formation of reactive oxygen species, S100B recruits PI3K/AKT and NF-κB, whereas S100A6 activates JNK. More importantly, we show that S100B and S100A6 modulate cell survival in a RAGE-dependent manner, S100B specifically interacting with the RAGE V and C1 domains and S100A6 with the C1 and C2 RAGE domains. Altogether these results highlight the complexity of S100/RAGE cellular signaling.

The S100 proteins constitute the largest family of EF-hand calcium binding proteins. They present 25 to 65 % homology in their amino-acid sequence (1,2). All S100 proteins, except S100G, form homo- and heterodimers. Each subunit contains 2 EF-hand type calcium-binding sites. The C-terminal EF-hand presents the classical “canonical” EF-hand motif common to all EF-hand proteins and displays the highest affinity for calcium (K_D = 20-50 µM). In contrast, the N-terminal “pseudo-EF-hand” is typical for the S100 proteins and binds calcium with lower affinity (K_D = 200-500 µM) (3-5).

The S100 proteins possess various intra- and extracellular functions. Intracellular functions range from regulation of cell motility, protein phosphorylation, 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 cytokine...
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functions (4). S100B is secreted by astrocytes and increased S100B concentration has been found in the cerebrospinal fluid of patients suffering of brain trauma, ischemia or Alzheimer’s disease (AD) (6). S100A1 is released in the serum of patients suffering from acute myocardial damage and promotes neurite outgrowth at micromolar concentration (7,8). S100A4 secretion in culture medium was estimated to be about 10 µM, a concentration which induces neuritogenesis in primary rat neurons (9,10). Similarly, S100A12 is found in synovial fluid of patients suffering from rheumatoid arthritis and also triggers neuritogenesis of hippocampal neurons when added extracellularly at sub-micromolar 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, enthorinal 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 AD or Down’syndrome (6). Interestingly, overexpression of S100A6 has also been observed in patients suffering from AD or amyotrophic lateral sclerosis (ALS) (22,23).

Recently, the Receptor for Advanced Glycation Endproducts (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 (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 monocyte/macrophage, 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 paper, we have 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 show that both S100 proteins bind to RAGE and suggest that S100A6 and S100B do not recognize the same binding site. We show that, at micromolar concentration, extracellular S100B and S100A6 trigger opposite effects on cells: whereas S100B activates cell proliferation, S100A6 enhances apoptosis. More importantly, we show that binding of S100A6 and S100B induces the formation of reactive oxygen species (ROS) but also triggers different signaling pathways: whereas S100B/RAGE interaction results in the activation of PI3/AKT kinase and NF-κB transcription factor, S100A6/RAGE interaction induces the activation/phosphorylation of JNK kinase. 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 *E. coli* and purified as previously described (32). The purity of the proteins was checked by SDS-PAGE, western blot and Maldi-TOF mass spectrometry. After dialysis against phosphate-buffered saline (PBS), bacterial endotoxin was removed from the purified S100B and S100A6 by treatment with Afli-Prep Polymyxin B matrix (Bio-Rad, USA) overnight at 4°C. The absence of endotoxin contamination was assayed using the commercially available E-TOXATE kit (Sigma, Germany). S100B and S100A6 were aliquoted by 100 µl and kept at -20°C prior to use. Protein concentration was determined using the BCA protein assay kit (Pierce).

*Expression and purification of soluble RAGE and the RAGE domains.* Soluble RAGE (sRAGE), composed of the three extracellular immunoglobulin domains was expressed in *Pichia pastoris* and purified as previously...
The purified RAGE V, VC1 and C2 domains were expressed in *E. coli* and purified as described (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, Amersham Biosciences, USA) 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 was from R&D system (USA) and were resuspended in PBS. The rabbit polyclonal antibodies against S100B and S100A6 were from Dako (Denmark), the monoclonal mouse anti-β-Tubulin 1 from Sigma (Germany), the goat polyclonal anti-Flotillin 1 from AbCam (USA), the rabbit polyclonal antibodies anti-P-JNK (Thr183/Tyr185), -JNK, -P-AKT (Ser473) and anti-AKT were from Cell Signaling (USA).

**Other reagents.** SN50, SP600125 and LY294002 were from Calbiochem (USA), Ionomycin, N-Acetyl-L-Cysteine (NAC) and 4’,6-diamidino-2-phenyl-indole (DAPI) from Sigma (USA) and DCFH from Molecular Probes (USA).

**Surface Plasmon Resonance.** sRAGE was purified from HEK293 cells as previously described (26). The V, VC1 and C2 domains were purified from *E. coli* as previously described (24). sRAGE and the RAGE domains were immobilized onto CM5 Biacore sensor chips according to previously described procedures (34). Briefly, the proteins or fragments were diluted at 50 µg/ml in 20 mM NaAc pH5 and injected over the sensor chips that had been pre-activated with EDC/NHS as recommended by the manufacturer. After injection of the proteins, the surface of the sensor chip was then blocked with ethanalamine prior measurements. About 4000 RU were obtained with sRAGE and 5000 RU 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 concentration of S100B (from 0.78 to 12.6 µM) and S100A6 (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 during 1 min in contact with 0.5 M EDTA followed by 1min in contact with 50 mM Na-Borate pH 8.5 containing 1M NaCl.

**Cell culture and treatment.** The human U87-MG glioblastoma cells (ATCC, USA) were cultivated in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum (BioConceput, Switzerland), 2 mM glutamine and streptomycin/penicillin. After plating in 12-well plate, U87-MG cells were cultivated for 24 hours and were then serum-starved for additional 24 hours. Cells were then stimulated without serum for 20 minutes with DMSO, H2O, 2 mM EGTA or 1 µM ionomycin in medium supplemented with 1.5 mM CaCl2. The cell culture medium were harvested after the different treatments and centrifuged at 3’000 g for 5 minutes to remove cells. The human neuroblastoma cell line SH-SY5Y (ATCC, USA) was maintained in RPMI medium containing 10% fetal bovine serum, 2 mM glutamine and streptomycin/penicillin. After plating, cells were cultured with 10% fetal bovine serum for 24 hours and were then serum-starved for additional 24 hours before treatment with S100B or S100A6 in serum-free medium. SP600125 and LY294002 were dissolved in DMSO, 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 similar amount of DMSO or water or with the same concentration of inhibitor in 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, a S100A6 specific rabbit antibody was coated onto ELISA plate overnight at 4°C in the presence of 100mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ pH 8.1. After blocking with 10% bovine serum albumin (BSA) in PBS for 3 hours at room temperature (RT) 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 goat (19) was added in the presence of 10% BSA in PBS-T. After 3 hours incubation at RT, the wells were washed three times with PBS-T and wells were then incubated with an anti-goat antibody conjugated with HRP (Horse Radish Peroxidase) for 2 hours at RT. After a final washing procedure, the HRP substrate TMB (TMB peroxidase EIA substrate kit, Biorad) was added to the wells and the reaction was stopped by addition of H$_2$SO$_4$. The HRP products were read at 450 nm using an ELISA plate reader (TECAN). The absorbances obtained from cell culture medium were compared to 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, Switzerland) 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 equivalent time.

Cell cycle distribution analysis was determined by fluorescence-activated cell sorting (FACS). Isolation and staining of the SH-SY5Y cells were performed using the CycleTEST Plus DNA kit (Becton Dickinson, USA) and cell cycle analysis was then performed with a Becton-Dickinson FACSCalibur flow cytometer. A total of 10⁴ cells were analyzed for each sort 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, Switzerland). Detection of apoptotic cells and counterstaining with 4',6-diamidino-2-phenyl-indole (DAPI) were performed according to the instructions provided by the manufacturer. The slides were viewed and photographed by using a fluorescent Zeiss microscope and cells stained with DAPI and by TUNEL assay were then counted on random fields and by scoring at least 1000 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, USA). Cells were incubated with the caspase-Glo 3/7 substrate for 1 hour at room temperature and caspase 3/7 was measured by luminescence quantification using a microplate reader (TECAN).

Incorporation of BrdU into newly synthesized DNA was performed as cell proliferation assay. Cells were treated for 24 hours with S100B in presence of 10 µM 5-bromo-2'-deoxyuridine (BrdU). Immunofluorescent detection of BrdU incorporation was performed according to the manufacturer’s protocol (Roche, Switzerland). Nuclei were counterstained with DAPI and slides were visualized by fluorescent microscopy and photographed with a digital camera. Cells stained with DAPI and with incorporated BrdU were then counted on random fields and by scoring at least 1000 cells per experimental group.

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

Transfection and NF-κB activation assay. SH-SY5Y cells were co-transfected with the pNFκB-Luc construct (Clontech, USA) and a plasmid containing the β-galactosidase gene using the FuGENE®HD transfection reagent (Roche, Switzerland) for 24 hours in the presence of serum according to the manufacturer’s instructions. Cells were serum-starved for 24 hours before being treated with S100B or S100A6. After 24 hours in culture, cells were washed with PBS and lysed in Reporter Lysis Buffer (Promega). Luciferase activity was monitored using the Luciferase Assay System (Promega, USA) using a
microplate reader. β-galactosidase activity was quantified by mixing cell lysate with an equal volume of 2x β-galactosidase assay mix (120 mM Na$_2$HPO$_4$, 80 mM Na$_2$HPO$_4$, 2 mM MgCl$_2$, 100 mM β-mercaptoethanol and 1.33 mg/ml ONPG, incubation at 37°C and 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 nM NaF, 10 nM β-glycerophosphate, 1 mM Na$_3$VO$_4$) in presence of a protease inhibitor cocktail (Roche, Switzerland). Membrane fraction and soluble proteins were prepared as described (36). SH-SY5Y cells were washed with PBS, scraped from the plates, centrifuged and then lysis buffer (250 mM sucrose, 20 mM Hepes pH 7.4 containing a complete protease inhibitor cocktail, Roche, Switzerland) was added to the cell pellet. Cells were homogenized with a glass Dounce homogenizer and then centrifuged at 100 000 g for 1 hour at 4°C. The supernatant was further centrifuged at 100 000 g for 10 min at 4°C. The supernatant contained the cytosolic fraction. The protein concentration of both fractions was determined using the BCA protein assay kit (Pierce). For the SDS-PAGE, equal amount of protein was loaded on a 10% Bis-Tris Gel (Invitrogen, USA). 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% BSA for 1 hour 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% BSA at 1:10000 for the anti-mouse, 1:5’000 for the anti-goat and 1:1000 for the anti-rabbit secondary antibodies conjugated with HRP. Detection was performed using a chemiluminescent HRP substrate (ECL, Pierce, USA). The dilution of the primary antibodies were as follow: anti-β-Tubulin 1, 1:1’000; anti-Flotillin 1, 1:1’000, anti-P-JNK, 1:1000; 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 minutes and washed with PBS. The cells were then immunostained with the anti-C$_1$ RAGE antibody diluted at 1:500 for 1 hour, washed with PBS and incubated with the cy3-conjugated anti-rabbit secondary antibody diluted at 1:200 for 1 hour. 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 (ANOVA) and where indicated individual comparison was performed by analysis of variance with post hoc analysis using the SPSS software.

**RESULTS S100B and S100A6 bind differently to RAGE.** We have previously shown by surface plasmon resonance that S100B binds to sRAGE (26) and to the RAGE V and VC$_1$ domains (24) which had been expressed in E. coli. Consequently, the purified proteins were not glycosylated. Here, we show that both S100B and S100A6 interact 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 µM (62% species) and K$_D2$ = 2.2 nM (38% species) (Table 1). The sensorgrams of S100A6 could also be fit with a two binding sites 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 (Rmax) 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 two-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 VC$_1$ domain (Fig. 1C) and compared these with the binding of S100B to the same domains (Fig. 1B). As previously described, S100B interacted with both V and VC$_1$
domains with submicromolar affinity for the V domain (K\textsubscript{D1} = 0.5 µM; K\textsubscript{D2} = 0.6 µM) and a higher affinity for the VC\textsubscript{1} domain (K\textsubscript{D1} = 11 nM (84 %); K\textsubscript{D2} = 0.2 µM (16 %) (24). The sensorgrams obtained for S100A6 with the V domain were fitted with the two binding sites model and showed binding constant K\textsubscript{D1} = 13.5 µM (97% species) and K\textsubscript{D2} = 0.49 µM (3% species) (Table 1). Similarly, the binding data of S100A6 with the VC\textsubscript{1} domain were also best fitted with a two binding sites model and resulted in K\textsubscript{D1} = 5.6 µM (82 % species) and K\textsubscript{D2} = 0.58 µM (18% species). However, although S100B recognized 76 % and 14 % of immobilized V and VC\textsubscript{1} domains respectively, S100A6 recognized only 14 % and 0.6 % of the bound V domain and VC\textsubscript{1} domains respectively (Table 1), suggesting again differences in binding between the two S100 proteins. We also compared the binding of S100B and S100A6 to the C\textsubscript{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 RU versus 5000 RU with the V domain), a significant binding of S100A6 to the C\textsubscript{2} domain was observed, whereas the interaction of S100B to this domain was negligible. The fitting of the sensorgrams of S100A6 revealed two binding sites with K\textsubscript{D1} = 1 µM (55% species) and K\textsubscript{D2} = 28 nM (45% species) (Table 1).

**S100B and S100A6 modulate survival of human SH-SY5Y cells.** S100B and S100A6 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 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 previous studies have shown the effect of extracellular S100B on cellular functions. To determine whether S100A6 was actively released from brain-related cells, we used the human U87-MG glioblastoma cells which express endogenously 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 were found intracellularly. Interestingly, modulation of Ca\textsuperscript{2+} homeostasis influenced S100A6 extracellular levels. Increase of intracellular Ca\textsuperscript{2+} concentration following ionomycin treatment for 20 minutes enhanced S100A6 content in the extracellular medium, from 53.0±3.7 in control cells to 98.4±1.9 ng/ml in presence of ionomycin. In contrast, EGTA treatment drastically reduced S100A6 extracellular amount 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, it suggests that the observed variations of extracellular S100A6 content resulted from active secretion of the protein by glioblastoma cells.

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 resulted in the translocation of the corresponding endogenous protein (32). Such phenomenon could therefore mislead our analyses and interpretations by creating potential interferences between endogenous and extracellular added S100 proteins. We then screened several cell lines for the expression of RAGE, S100B and S100A6 and we finally choose the human neuroblastoma SH-SY5Y cell line. SH-SY5Y cells have been previously shown to express RAGE (42). We showed 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) which made 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 (matrix-assisted laser desorption/ionization-time of flight) (Fig. 2G). The MS spectra revealed a single peak for each protein with a molecular mass of 10741 Da and 10048 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 hours (Fig. 2H).

Exposure of serum-starved SH-SY5Y cells to increasing concentrations of S100B or S100A6 for 48 hours resulted in a concentration-dependent increase and decrease of cell viability, respectively, as shown by MTT assay (Fig. 3A 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 in order to compare the effect of both S100 proteins. FACS analysis of cells treated with 5 µM S100B revealed an increased amount of cells in the S and G2/M phases of the cell cycle after 24 and 48 hours, respectively, compared to the control cells (Fig. 3C and D), whereas exposure to 5 µM S100A6 enhanced cell death after 48 hours (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 BrdU into DNA. Cells were serum-starved and then stimulated with 5 µM S100B in the presence of BrdU for 24 hours. PBS-treated cells contained approximately 5% BrdU-positive cells. In contrast, S100B treatment resulted in about 13% of cells positive for BrdU 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 hours, 10.5% of TUNEL positive cells were measured in the presence of BrdU (Fig. 3G). We also investigated the role of the AKT kinase in the S100B mediated cellular signaling. As presented in Fig. 4F, an increase of the activated form of the kinase (p-AKT) was observed when the cells were treated with S100B, whereas co-treatment of the cells with both S100B and LY294002 inhibited this activation of AKT (Fig. 4H). The absence of influence of S100A6 and S100B on, respectively, AKT and JNK activation was also addressed (Fig. 4H and I). The sensitivity of the S100B- and S100A6-mediated cellular signaling to NAC suggested the involvement of reactive oxygen species (ROS). We therefore measured the time-dependent formation of ROS in the cells in the presence of 5 µM S100B or S100A6. We observed that intracellular ROS accumulated in both S100B- and S100A6-treated cells (Fig. 5A and B). However, the two S100 proteins differed by their kinetics of ROS formation. Increase in intracellular ROS level was only significant 1 hour after exposure to S100B (Fig. 5A). In contrast, stimulation with S100A6 induced a gradual and sustained ROS generation, which initiated after 30 minutes, peaked at 3 hours,
Role of RAGE in S100B-induced cellular proliferation and S100A6-mediated apoptosis. We have shown that S100B and S100A6 interact with sRAGE in vitro (Fig. 1). We therefore investigated whether RAGE was involved in the cellular responses driven by S100B and S100A6 and particularly if 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 in order 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 (αS100B or αS100A6, Fig. 7A and C), a mixture of the polyclonal antibodies directed against the V, C1 and C2 domains (αVC1C2, Fig. 7A and C) or sRAGE (Fig. 7B and D). As expected, no change in cell viability was observed when the cells were treated with non-specific mouse or rabbit immunoglobulin (rIgG or mIgG, Fig. 7A and C). 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 (αV, Fig. 7A) or the V domain itself (V, Fig. 7B) was able to reverse the increase of cell viability triggered by S100B. The VC1 domain was slightly less efficient than the V domain (VC1, Fig. 7B). Similarly, an anti-C1 polyclonal antibody (αC1, Fig. 7A) also decreased the effect mediated by S100B, but to a lesser extent. However, the purified C2 domain and the anti-C2 polyclonal antibody (C2 and αC2, Fig. 7A and B) 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 (αV and V, Fig. 7C and D). S100A6-mediated decrease of cell viability was best reversed with the anti-C1 as well as with the anti-C2 domain polyclonal antibody (αC1 and αC2, Fig. 7C). The purified C2 domain was also most efficient to reverse S100A6 mediated cellular effects (C2, Fig. 7D).

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

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, MAP kinase activation and NF-κB activity. Whereas the non-specific antibody and the anti-C2 domain antibody were without effect on 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. 8A, 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. 8B and D).

DISCUSSION

Despite their high homology, the 21 known members of the S100 EF-hand Ca\(^{2+}\)-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 receptor such as RAGE (4). This functional diversity implies the existence of regulatory mechanisms which allow RAGE to differentiate between the different S100 proteins in order 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, where 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 interacts with sRAGE in vitro. Data analysis shows 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 reveals differences in binding. Whereas S100A6 binds to sRAGE via two sites with submicromolar affinity, the purified V domain molecules recognize 2% of the immobilized molecules (Table 1) suggesting again different modes of interaction. Similar differences in binding affinity and percentage of molecule recognition between the two S100 proteins are 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 suggest that S100B and S100A6 molecules 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 (235-327). Dattilo et al. proposed a model where 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-acids flexible stretch that allows the C2 domain to move independently from the VC1 unit (24). 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 S100B effect on cell survival was mainly mediated by the RAGE V domain since both a polyclonal antibody directed against a peptide of the V domain and the purified V domain itself are the most efficient to inhibit cell survival, ROS formation as well as PI3/AKT and NF-κB activation. In addition, the C1 domain antibody could also block the increase of cell proliferation induced by S100B. These
correlate well our in vitro binding studies by SPR. The cellular effects triggered by S100A6 are 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, this suggests a central role of the three extracellular RAGE immunoglobulin domains for ligand recognition and binding. Indeed, it was shown that amphoterin and AGEs bind to the RAGE V domain, whereas S100A12 binds to the C1 and C2 domains (46,48,49). Furthermore, Aβ species, formed during the progression of AD, induce their toxicity via their binding to distinct RAGE domains (E. Sturchler, A. Galichet and C. W. Heizmann, personal communication).

S100B increases cell survival at micromolar concentration. In contrast, we found that S100A6 mediates 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 astrocystosis 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 high level of S100B could evolve S100B function from neurotrophic to pathologic. Indeed, a second S100B-overexpressing mouse model, expressing the protein at higher level, shows age-related damages 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 since RAGE expression increases following sustained exposure to its ligands (47,55). Furthermore, elevated RAGE expression sensitizes neuroblastoma cells as shown by S100B-induced increased 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 on 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 l 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 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 is released within 20 minutes following an increase of intracellular Ca2+. In addition, the S100 proteins represent about 0.2% of the soluble proteins present in the extracellular space of the rat brain, a value three- to six-fold higher than the S100 protein levels in the cytoplasmic fraction (60). Altogether, these observations make very plausible that S100B and S100A6 occur at high levels in the brain where they could thus play the role of mediators of brain cells communication like glutamate, Ca2+ 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 reactive oxygen species (ROS), the activation of PI3/AKT kinase as well as the activation of NF-κB (Fig. 9), since the increase of cell survival was significantly reverted by addition of anti-oxidant and inhibitors of PI3 kinase and NF-κB, respectively. The PI3K/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 show that S100A6 triggers apoptosis. A detailed analysis of the cellular pathway triggered by S100A6 reveals 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), since the presence of antioxidant and JNK inhibitors revert the induction of apoptosis. Surprisingly, the increase of cellular survival and apoptosis triggered, respectively, by S100B and S100A6 are both mediated through the formation of ROS. ROS elicit a wide range of cellular functions 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 Aβ- and S100B-dependent cellular toxicity or amphoterin-mediated neurite outgrowth (7,39,47). Our observations that S100B and S100A6 increase ROS formation support the hypothesis that ROS may represent a general mechanism involved in RAGE-mediated cellular activation.

In summary, our experiments clearly demonstrate that (i) S100B and S100A6 interact with different RAGE domains present on the surface of human SH-SY5Y 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 multi-protein signaling complex.

REFERENCES

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FIGURE LEGENDS

Fig. 1. Binding of S100B and S100A6 to sRAGE and the RAGE V, VC1 and C2 domains. (A) sRAGE (4500 RU) was directly immobilized onto the CM5 sensor chip as described in materials and methods. Series of S100B (0.78 to 12.6 µM, in black) or S100A6 (0.125 to 10 µM, in red) concentration were injected over the flow-cells. (B) and (C) RAGE V (black) and VC1 (red) domains (5000 RU) were immobilized and series of S100B (B) (0.78 to 12.6 µM) or S100A6 (C) (0.125 to 10 µM) concentration were injected over the flow-cells. (D) as in (A) with the immobilized RAGE C2 domain (300 RU).

Fig. 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 (2µg) produced in E. coli. (F) Western-blot analysis of purified human S100B and S100A6 with polyclonal anti-S100B or S100A6. (G) Mass spectrum of recombinant human S100B (left) and S100A6 (right). (H) S100B and S100A6 stability in RPMI medium. Conditioned medium from control cells and cells treated with 5 µM S100B or S100A6 were harvested at the time of addition (time 0) or 48 hours after addition of S100B or S100A6 and were subjected to immunoblotting for S100B (αS100B) and S100A6 (αS100A6).

Fig. 3. S100B and S100A6 affect differentially cell survival of SH-SY5Y cells. Serum-starved SH-SY5Y cells were grown without serum in the presence of increasing concentration of S100B (A) or S100A6 (B) for 48 hours and cell survival was measured by MTT assay. Cell survival was expressed as percentage of PBS-treated control cells. Data are the means ± standard deviations (SD) 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 hours before FACS 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 ± SD, *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 BrdU for 24 hours before fixation. BrdU positive cells were stained with a specific anti-BrdU antibody and the percentage of BrdU positive SH-SY5Y cells over the total number of cells was determined. A minimum of 1000 cells were counted in random fields and the presented data are the means ± SD from three separate experiments, *** p<0.0001. (G) Serum-starved SH-SY5Y were grown without serum with PBS or 5 µM S100A6 for 48 hours and the percentage of TUNEL positive cells over total number of cells was determined. A minimum of 1000 cells were counted in random fields and the presented data are the means ± SD from three separate experiments, *** p<0.0001. (H) Similar as (G) and caspase 3 and 7 activities were...
measured after the indicated length of time. Data are the means ± SD from four separate experiments, *** p<0.0001.

**Fig. 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 DMSO (A) or with 5µM S100A6 or PBS in the presence of 5 mM NAC or H2O or 10 µM SP600125 or DMSO (B) for 48 hours and cell survival was measured by MTT assay. Cell survival was expressed as percentage of control or inhibitor-treated control cells. Data are the means ± SD from eight separate experiments, *** p<0.0001. (C) Serum-starved SH-SY5Y cells were grown without serum with PBS or 5 µM S100B in the presence of BrdU for 24 hours and 5 mM NAC (or H2O) or 10 µM LY294002 (or DMSO). BrdU positive cells were counted as in Fig. 3. (D) and (E) Serum-starved SH-SY5Y were grown without serum with PBS or 5 µM S100A6 in the presence of 5 mM NAC or 10 µM SP600125 and caspase 3 and 7 activities were measured after 24 hours (D), *** p<0.0001. The percentage of TUNEL positive cells over total number of cells was determined after 48 hours (E). (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 (Ser473) and total AKT content. The numbers on top of the panel refer to the amount of activated Akt (p-Akt) after normalization to total AKT level as analyzed by densitometry, ** p<0.01. (G) Similar as in (F) except that the SH-SY5Y cells were treated with 5 µM S100A6 or PBS for 6 and 24 hours and protein subjected to immunoblotting for phosphorylated JNK (Tyr183/Tyr185) and total JNK cellular content. The numbers on top of the panel refer to the amount of activated JNK (p-JNK) after normalization to total JNK 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 hours and further immunoblotted for phosphorylated JNK (Tyr183/Tyr185) and total JNK cellular content (top panel) or the cells were treated with 10 µM LY294002 in presence or absence of 5 µM S100B and the cells were lysed after 24 hours and immunoblotted for phosphorylated AKT (Ser473) and total AKT 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. The experiments depicted in panels (F) to (I) are representative of two independent experiments performed in duplicate.

**Fig. 5.** S100B and S100A6 induce ROS formation.
Kinetics of ROS production in serum-starved SH-SY5Y cells in presence of PBS, 5 µM S100B (A) or 5 µM S100A6 (B). Cells were labeled with the fluorescent dye DCFH and analyzed as described in Material and Methods. Presented data are the average values from triplicate samples ± SD, " p<0.005. (C) SH-SY5Y PBS- (control cells) and S100B-treated cells (5 µM) were treated with 5 mM NAC. After 24 hours 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 total Akt level as analyzed by densitometry, " p<0.01. (D) Similar as in (C) in the presence of PBS or 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 total JNK level as analyzed by densitometry, " p<0.01.

**Fig. 6.** S100B modulates NF-κB transcriptional activity.
(A) SH-SY5Y cells were transiently transfected with an NF-κB-responsive cis-reporter gene construct and with a β-galactosidase expression construct. Serum-starved cells were then grown with PBS, 5 µM S100B or S100A6 and where indicated, 5 mM NAC, 10 µM LY294002 or 20 µg/ml SN50 were added in the presence of S100B. After 24 hours induction, cells were lysed and luciferase activity was quantified and normalized to β-galactosidase activity. S100B- or S100A6-modulated NF-κB transcriptional activity was expressed as percentage of PBS-treated control cells. Data are the means ±
SD from three separate experiments, *** p<0.0001. (B) Serum-starved SH-SY5Y cells were grown with PBS or 5 µM S100B in the presence of 20 µg/ml SN50 for 48 hours and cell survival was measured by MTT assay, *** p<0.0001. (C) Serum-starved SH-SY5Y cells were treated with PBS or 5 µM S100B in the presence of BrdU and 20 µg/ml SN50 for 24 hours. BrdU positive cells were counted as in Fig. 3.

**Fig. 7.** Role of RAGE in S100B-stimulated cell proliferation and S100A6-mediated apoptosis. (A) Serum-starved SH-SY5Y cells were grown with PBS (control cells) or 5 µM S100B in the presence of either an antibody directed against the three RAGE extracellular immunoglobulin domains (αVC1C2, 25 µg/ml), or one specifically made against the RAGE immunoglobulin V domain (αV, 25 µg/ml), the RAGE C1 domain (αC1, 25 µg/ml), or anti-RAGE C1 domain (αC2, 25 µg/ml), non-specific mouse (mlG, 25 µg/ml) or rabbit (rlG, 25 µg/ml) IgG or a specific anti-S100B antibody (αS100B, 25 µg/ml) for 48 hours and cell survival was measured by MTT assay, *** p<0.0001. (B) similar as (A) except that the cells were grown with 5 µM S100B in presence of either the purified sRAGE (50 µg/ml), purified RAGE V domain (V, 18.5 µg/ml), purified RAGE VC1 domain (VC1, 37 µg/ml) or the purified RAGE immunoglobulin C2 domain (C2, 18.5 µg/ml), *** p<0.0001. (C) similar as (A) except that cells were grown in presence of 5 µM S100A6 and the anti-S100B antibody was replaced by a specific anti-S100A6 antibody (αS100A6, 25 µg/ml), *** p<0.0001. (D) similar as (B) except that the cells were grown with 5 µM S100A6, *** p<0.0001. (E) Serum-starved SH-SY5Y cells were grown without serum with PBS or 5 µM S100B in the presence of 10 µM BrdU and 50 µg/ml sRAGE, 25 µg/ml anti-RAGE antibodies (αV or αC2) or 25 µg/ml non-specific IgG for 24 hours and the percentage of BrdU positive cells was counted as in Fig. 3, *** p<0.0001. (F) Serum-starved SH-SY5Y were grown without serum with PBS or 5 µM S100A6 in the presence of either 50 µg/ml sRAGE, 25 µg/ml anti-RAGE antibodies (αV or αC2) or 25 µg/ml non-specific IgG for 48 hours and the percentage of TUNEL positive cells was counted as in Fig. 3, *** p<0.0001. (G) Similar as in (F) and caspase 3 and 7 activities were measured after 24 hours. Data are the means ± SD from four separate experiments, *** p<0.0001.

**Fig. 8.** RAGE-S100 interaction is involved in ROS formation, signaling pathways activation and modulation of NF-κB transcriptional activity. (A) and (B) Serum-starved SH-SY5Y 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 αC2) or 25 µg/ml non-specific IgG. ROS formation was measured as in Fig. 5 after 1 hours, ** p<0.005. (C) Serum-starved SH-SY5Y PBS- (control) or S100B-treated cells (5 µM) were treated with 25 µg/ml of RAGE antibodies (αV or αC2) or 25 µg/ml non-specific IgGs. After 24 hours 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 total Akt level as analyzed by densitometry, ** p<0.01. (D) similar as (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 total JNK level as analyzed by densitometry, ** p<0.01. (E) similar as (C) and NF-κB transcriptional activity was measured as in Fig. 6 after 24 hours in presence of 5 µM S100B, *** p<0.0001.

**Fig. 9.** Schematic representation of signaling pathways activated by S100B and S100A6 in human SH-SY5Y neuroblastoma cells.
Table 1

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<th>% Species</th>
<th>$K_{D2}$</th>
<th>% Species</th>
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n.d. not determined
Figure 1

A

B

C

D

E. Leclerc et al. S100-RAGE interaction and influence on cell survival
Figure 3
Figure 4
Figure 5

A

B

C

D

Figure 5
Figure 6

A

NF-κB activity (No. of cultures)

Control S100B S100A6 SN50 NSC LY294002

B

Cell viability (% of control)

S100B SN50

C

Endothelial cells (%)

Control S100B SN50
Figure 7
Figure 8
Figure 9

S100B

ROS

Akt

NF-κB

ROS

JNK

Caspase 3/7

Cell proliferation

Apoptosis
S100b and S100A6 differentially modulate cell survival by interacting with distinct rage immunoglobulin domains
Estelle Leclerc, Günter Fritz, Mirjam Weibel, Claus W. Heizmann and Arnaud Galichet
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