S100B Protein Regulates Astrocyte Shape and Migration via Interaction with Src Kinase

**IMPLICATIONS FOR ASTROCYTE DEVELOPMENT, ACTIVATION, AND TUMOR GROWTH**

Flora Brozzi, Cataldo Arcuri, Ileana Giambanco, and Rosario Donato

From the Department of Experimental Medicine and Biochemical Sciences, University of Perugia, C.P. 81 Succ. 3, 06122 Perugia, Italy

S100B is a Ca\(^{2+}\)-binding protein of the EF-hand type that is abundantly expressed in astrocytes and has been implicated in the regulation of several intracellular activities, including proliferation and differentiation. We show here that reducing S100B levels in the astrocytoma cell line GL15 and the Müller cell line MIO-M1 by small interference RNA technique results in a rapid disassembly of stress fibers, collapse of F-actin onto the plasma membrane and reduced migration, and acquisition of a stellate shape. Also, S100B-silenced GL15 and MIO-M1 Müller cells show a higher abundance of glial fibrillary acidic protein filaments, which mark differentiated astrocytes, compared with control cells. These effects are dependent on reduced activation of the phosphatidylinositol 3-kinase (PI3K) downstream effectors, Akt and RhoA, and consequently elevated activity of GSK3\(\beta\) and Rac1 and decreased activity of the RhoA-associated kinase. Also, rat primary astrocytes transiently down-regulate S100B expression when exposed to the differentiating agent dibutyryl cyclic AMP and re-express S100B at later stages of dibutyryl cyclic AMP-induced differentiation. Moreover, reducing S100B levels results in a remarkably slow resumption of S100B expression, suggesting the S100B might regulate its own expression. Finally, we show that S100B interacts with Src kinase, thereby stimulating the PI3K/Akt and PI3K/RhoA pathways. These results suggest that S100B might contribute to reduce the differentiation potential of cells of the astrocytic lineage and participate in the astrocye activation process in the case of brain insult and in invasive properties of glioma cells.

S100B has been implicated in the regulation of the state of assembly of microtubules and type III intermediate filaments, some enzyme activities, and cell proliferation. This last issue has attracted much attention because levels of S100B are high in certain cancer cells (1, 2, 4), and S100B has been proposed to contribute to tumorigenesis by inhibiting the function of the tumor suppressor protein p53 (5, 6) and to regulate cell proliferation and differentiation by stimulating the activity of the mitogenic kinases Ndr (7) and Akt (protein kinase B) (8).

Astrocytes represent the brain cell type with the highest expression of S100B. Levels of S100B are augmented in astrogliosis, and several reports have associated the increased levels of S100B in astrocytes with the pathophysiology of degenerative and infectious/inflammatory brain disorders (1, 2, 9–11). Moreover, the human S100B gene maps to chromosome 21.q12.3 (12), with consequent high S100B levels in Down syndrome. These observations led to the hypothesis that S100B might be involved in the pathogenesis and/or pathophysiology of neurodegenerative processes (9–11). However, most of what we know about the relationships between S100B and neurodegeneration (and neuroprotection as well) comes from data obtained through the analysis of the extracellular effects of the protein. In fact, astrocytes release S100B constitutively (13, 14), and S100B release is augmented upon exposure of astrocytes to serotonin agonists (15), glutamate (16), lysophosphatidic acid (LPA) (17), or tumor necrosis factor-\(\alpha\) (18). Once released, S100B can affect neurons, astrocytes, and microglia with different effects, depending on its concentration via engagement of the receptor for advanced glycation end products in large part (19).

Little is known about the functional role(s) of S100B within astrocytes in relation to astrocyte proliferation, survival, and participation in brain development, the inflammatory response, and neoplastic transformation. In the adult normal brain, astrocytes exhibit a stellate morphology and show a slow rate of renewal (20–22). However, in case of brain insult astrocytes rapidly retract their cytoplasmic processes, proliferate, and migrate to the site(s) of damage, giving rise to the so-called reactive gliosis (20–22). These changes are largely

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7 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

8 To whom correspondence should be addressed. Tel.: 39-075-585-7453/7448; Fax: 39-075-585-7451; E-mail: donato@unipg.it.

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3 The abbreviations used are: LPA, lysophosphatidic acid; siRNA, small interfering RNA; PI3K, phosphatidylinositol 3-kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; ROCK, RhoA-associated kinase; RT, reverse transcription; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAPI, 4’,6-diamidino-2-phenylindole; TRITC, tetramethylrhodamine isothiocyanate; GST-TRBD, glutathione S-transferase-Rhotekin Rho-binding domain; PTEN, phosphatase and tensin homologue deleted on chromosome ten; Bt,cAMP, dibutyryl cyclic AMP.

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dependent on alteration of the blood-brain barrier and are mediated by serum factors and locally released cytokines. As mentioned above, levels of S100B also increase in gliomas, raising the possibility that the protein might have a role in glioma pathophysiology.

An increasing body of evidence suggests that S100B might have a role during neurogenesis, participating in astrocyte maturation (23), and in migration of granule cell precursors (24). S100B is expressed in embryonic radial glia of both cerebellum and the subventricular zone and is considered a marker of these cells, together with RC1, RC2, brain lipid-binding protein, Tenascin-C, the glutamate transporter Glast, Sox9, and others (24–27). Radial glia can act as stem cells, giving rise to both neurons and glia during development, and serve as guide wires along which the migrating neurons travel. Although radial glia were thought to disappear early after birth at the end of neurogenesis, some radial glial cells persist within the adult forebrain subventricular zone, the cerebellum (Bergmann glia), the retina (Müller cells), and the hypothalamus (periventricular tanycytes) (27, 28).

To obtain information about the role of intracellular S100B in astrocyte development, activation, and neoplastic transformation, we analyzed effects of S100B silencing in the astrocytoma cell line GL15 (29) and the retinal Müller cell line, MIO-M1 (30). We found that inhibition of S100B expression in GL15 cells by small interference RNA (siRNA) techniques resulted in stress fiber disassembly, acquisition of a stellate morphology, reduced migration and, to a lesser extent, proliferation, and GFAP filament formation and that these effects were dependent on reduced activity of the Src kinase/phosphatidylinositol 3-kinase (PI3K) module. Similarly, silencing of S100B in MIO-M1 Müller cells resulted in F-actin reorganization, stellation, and GFAP filament formation. Thus, our results suggest that S100B might participate in the regulation of cell shape, differentiation, and migration via Src-dependent activation of PI3K. Also, reduced expression of S100B protein in S100B siRNA-treated cells resulted in a slow recovery of S100B mRNA and protein production, suggesting that S100B might regulate its own expression.

EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Transfections—GL15 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 IU/ml penicillin G, 100 µg/ml streptomycin, 2 mM glutamine. The MIO-M1 cell line was grown in DMEM with high glucose and Glutamax I (Invitrogen) supplemented with 10% FBS, 100 IU/ml penicillin G, 100 µg/ml streptomycin. Both cell lines were grown in an H₂O-saturated 5% CO₂ atmosphere at 37 °C. GL15 and MIO-M1 cells were transfected with Block-IT Fluorescent Oligo, Negative Universal Control Stealth (Invitrogen), and S100B siRNA Oligo Stealth (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, the cells were switched to serum-free medium and cultivated under these conditions for the indicated time periods without culture medium renewal unless stated otherwise. Additions were made in cultivation medium as detailed in the legends to the pertinent figures: PI3K inhibitor LY294002 (Calbiochem) (10 µM), Rac1 inhibitor NSC23766 (Calbiochem) (50 µM), RhoA-associated kinase (ROCK) inhibitor Y27632 (Calbiochem) (1 µM), LPA (Sigma) (10 µM), IP2 (Calbiochem) (20 µM).

Reverse Transcription-PCR and Real Time PCR—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. S100B mRNA was detected by RT-PCR using the following primers: 5′-AGGTCTGAGCTGGAGAAGG-3′ and 5′-CTCATGTTCGAAGACTCTGT-3′. After a 10-min incubation at 95 °C, 35 cycles were performed as follows: denaturation at 94 °C for 60 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as a control. Amplification products were resolved on a 2% agarose gel (Bio-Rad) and visualized by ethidium bromide staining. To detect S100B mRNA by real time PCR, cDNA was incubated with the above primers in a reaction volume of 20 µl containing Real Master Mix (Eppendorf) and SYBR Green (Eppendorf). Reaction mixtures were incubated in a thermocycler (Stratagene) and analyzed by Multiplex Quantitative PCR system. Housekeeping glucoronidase mRNA was used as a control.

Western Blotting—Cells were cultivated as detailed in the legends of the pertinent figures, washed twice with phosphate-buffered saline (PBS), and solubilized with 2.5% SDS, 10 mM Tris-HCl, pH 7.4, 0.1 mM diethiothreitol, and 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone protease inhibitor (Roche Applied Science). The following antibodies were used: polyclonal anti-S100B (1:1000; Dako); polyclonal anti-S100B (1:1000; Epitomics); polyclonal anti-Akt (1:1000; Cell Signaling Technology); polyclonal anti-phosphorylated (Ser⁴²³) Akt (1:1000; Cell Signaling Technology); monoclonal anti-tubulin (1:10,000; Sigma); monoclonal anti-cyclin D1 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-GAP (1:1000; Dako), polyclonal anti-phosphorylated (Ser⁹) GSK3β (1:1000; Cell Signaling Technology), polyclonal anti-GSK3β (1:1000; Cell Signaling Technology), monoclonal anti-RhoA (1:1000; Santa Cruz Biotechnology); polyclonal anti-Src (1:1000; Cell Signaling); polyclonal anti-phosphorylated (Tyr⁵²⁷) Src (1:1000; Cell Signaling). The immune reaction was developed by enhanced chemiluminescence (SuperSignal West Pico or SuperSignal West Femto; Pierce). Recombinant S100B was expressed, purified, and characterized as described (31, 32).

Measurement of Cell Cycle—Cells were washed in PBS, resuspended, centrifuged (400 × g, 7 min), and processed for cell cycle analysis by propidium iodide staining and flow cytometry. Briefly, the cell pellet was resuspended in 0.5 ml of hypotonic fluorescent solution (50 µg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) in 12 × 75-mm polypropylene tubes (BD Biosciences). The tubes were kept at 4 °C for at least 30 min before flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (BD Biosciences) at 488 nm. The percentages of cells in G₀/G₁, S, and G₂/M phases were calculated using Cell FIT cell cycle analysis version 2.0.2 software.

Indirect Immunofluorescence and Detection of F-actin—For indirect immunofluorescence, cells were cultivated on glass coverslips, extensively washed with PBS, fixed with cold meth-
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The amount of TRBD-bound RhoA was normalized to the total amount of RhoA in cell lysates.

Co-immunoprecipitation Analyses—GL15 cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM CaCl₂, 10 mM NaF, and 1 mM sodium orthovanadate in the presence of a mixture of protease inhibitors (Complete; Roche Applied Science). Solubilized proteins were subjected to immunoprecipitation using either a polyclonal anti-S100B antibody (2.5 μg/ml total protein; SWant) or a polyclonal anti-Src antibody (2.5 μg/ml total protein; Cell Signaling). The S100B immunoprecipitate was probed with polyclonal anti-S100B antibody (1:1000, Epitomics), polyclonal anti-Src antibody (1:1000; Cell Signaling), polyclonal anti-p85 PI3K subunit (1:1000; Cell Signaling), and polyclonal anti-phosphatase and tensin homologue deleted on chromosome ten (PTEN) (1:1000; Cell Signaling). The Src immunoprecipitate was probed with polyclonal anti-S100B antibody (1:1000; Epitomics).

Isolation of Primary Astrocytes—Primary astrocytes were isolated from 2-day-old rat pups and cultivated as described (34).

Statistical Analysis—Each experiment was repeated at least three times. Representative experiments are depicted in the figures unless stated otherwise. The data were subjected to analysis of variance with SNK post hoc analysis using a statistical software package (GraphPad Prism version 4.00; GraphPad Software, San Diego, CA). Statistical significance was assumed when p < 0.05.

RESULTS

Subcellular Distribution of S100B in GL15 Cells—GL15 glioma cells show two phenotypes, a fusiform one and a polygonal one (29) (Fig. 1A). By immunofluorescence, S100B was found in both phenotypes and appeared associated with filamentous structures and intracellular membranes (Fig. 1A), as reported previously (35, 36).

Silencing of S100B Results in Long Term Reduction of S100B Expression in GL15 Cells—To have information about the functional role(s) of S100B in GL15 cells, we inhibited S100B expression by a specific siRNA. Control experiments in which GL15 cells were transfected with Block-IT Fluorescent Oligo showed that >95% of cells had incorporated the fluorescent oligonucleotide (data not shown). Reduced expression of S100B in S100B siRNA-treated cells was ascertained by RT-PCR (data not shown), real time PCR, Western blotting, and immunofluorescence. By real time PCR, S100B mRNA was reduced by ~98% at the end of the transfection procedure (day 0), compared with control cells (Fig. 1B). After this time point, S100B siRNA-treated GL15 cells slowly resumed expressing S100B mRNA such that at post-transfection days 5 and 12, the cells showed ~30 and 100% recovery, respectively, relative to the level detected at day 0 in control cells (Fig. 1B). However, at these same time points, control cells showed ~40 and 60 times higher levels of S100B mRNA, respectively, compared with 0 day levels (Fig. 1C). Thus, under the present experimental conditions, control cells showed an increasingly greater expression of S100B mRNA as a function of cultivation time as opposed to the relatively low expression of S100B mRNA in S100B siRNA-treated cells. By Western blotting, we detected increasing levels

anol at -20°C for 7 min, and air-dried. The cells were incubated with blocking buffer (3% bovine serum albumin (BSA), 1% glycine in PBS) overnight and incubated for 1 h at room temperature with a rabbit anti-GFAP polyclonal antibody (Dako) (1:100 in PBS containing 3% BSA) and monoclonal anti-S100B antibody (Sigma) (1:20 in PBS containing 3% BSA) and then washed in PBS. The cells were then treated with TRITC-conjugated goat anti-rabbit IgG (Sigma) (1:50 in PBS containing 3% BSA), with fluorescein-conjugated goat anti-mouse IgG (Sigma) (1:50 in PBS containing 3% BSA), washed three times with PBS containing 0.1% Tween 20 and twice with PBS alone, incubated with 4’,6-diamidino-2-phenylindole (DAPI; Sigma) (2 μg/ml) for 5 min, and air-dried. The preparations were viewed in a DMRB Leica epimicroscope equipped with a digital camera. For rhodamine-phalloidin staining, cells were extensively washed with PBS, fixed with 4% paraformaldehyde for 30 min in PBS, permeabilized for 10 min with 0.1% Triton X-100 in PBS, washed three times with PBS, and incubated with NaBH₄ (1 mg/ml in PBS). After overnight incubation with blocking buffer, the cells were incubated for 1 h at room temperature with TRITC-phalloidin (Sigma). Coverslips were mounted and viewed as described above. Where appropriate, a second transfection was performed with constitutively active RhoA (RhoAV14) cloned into a pEGFP-C1 (Clontech) vector.

Migration Assays—For migration assays, we used Boyden chambers (pore size, 8 μm) (BD Biosciences). Control and S100B siRNA-transfected cells (5 × 10⁵ cells in 0.5 ml of DMEM) were placed in the upper chamber at the end of the transfection procedure, and 0.75 ml of DMEM containing 10% FBS were placed in the lower chamber. After varying time intervals in culture, cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were fixed in methanol for 2 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the underside of the filters were viewed and counted under a microscope. For the wound healing assay, control and S100B siRNA-transfected cells were seeded onto glass coverslips at the end of the transfection procedure, grown for 24 h in DMEM containing 10% FBS, scratched with a plastic tip, and cultivated in DMEM. The cells were analyzed by phase-contrast microscopy and photographed at time intervals.

Measurement of RhoA Activity—RhoA activity was measured in a pull-down assay using the Rho-binding domain from rho-teklin cloned into pGEX-2T vector (Amersham Biosciences) (33). The vector encoding glutathione S-transferase-Rho-teklin Rho-binding domain (GST-TRBD) was amplified in bacteria, and the recombinant GST-TRBD protein was purified. Control and S100B siRNA-treated cells were washed with ice-cold Tris-buffered saline and lysed in cold buffer (50 mM Tris, pH 7.2, containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 10 mM MgCl₂, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 13,000 × g at 4 °C for 10 min, and equal amounts (190 μg) of lysates were incubated with GST-TRBD (30 μg) beads at 4 °C for 45 min. The beads were washed four times with cold buffer B (Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Bound RhoA was detected by Western blotting.

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A
CONTROL S100B siRNA

B

C

D

E

F

G

S100B/DAPI

S100B
Tubulin

S100B
Tubulin

S100B siRNA

Stellate cells, %

0 20 40 60 80 100
0 2 d 5 d 8 d 12 d

c si c si c si c si c si
of S100B as a function of cultivation time in control cells and a 
~50% decrease in S100B levels at day 0 and a ~92, 93, and 94% 
decrease at post-transfection days 2, 5, and 12, respectively, in 
small interfering RNA (siRNA) S100B-treated cells compared with their respective con- 
trols (Fig. 1D). Compared with control cells at day 0, S100B 
siRNA-treated cells showed an ~85, 80, and 70% decrease in 
S100B levels at post-transfection days 2, 5, and 12, respectively 
(Fig. 1D). Thus, although the S100B mRNA levels in S100B 
siRNA-treated cells returned to basal levels by 12 days after 
transfection, S100B protein levels remained remarkably low. By 
immunofluorescence, we confirmed the reduction of S100B 
protein content in >95% of GL15 cells at post-transfection day 
2 (Fig. 1A), and a very low expression of S100B was observed in 
S100B siRNA-treated cells at post-transfection days 5 and 8 
(data not shown, but see Fig. 4C). Residual S100B was found in 
a perinuclear position at post-transfection days 2 and 5. By con- 
trast, S100B immunofluorescence intensity in control cells 
increased as a function of cultivation time (Fig. 1E) in agree- 
ment with the Western blot data. Collectively, these results sug- 
ggested that reduction of S100B expression in GL15 cells exerted 
profound, long term effects on their ability to express the protein 
and that re-expression of S100B in S100B silenced cells underwent 
transcriptional regulation. Since an investigation of the mecha- 
nism of regulation of S100B expression was beyond the scope of 
the present work, we did not study this point any further.

Silencing of S100B Results in Disassembly of Stress Fibers and 
Acquisition of a Stellate Morphology in GL15 Cells—With 
increasing cultivation time after transfection with S100B 
siRNA, GL15 cells tended to acquire a stellate morphology. 
This characterized ~13% of cells at post-transfection day 2 and 
~40 and ~60% of cells at post-transfection days 5 and 8, respec- 	ively, with negligible stellation in the corresponding control 
cells (Fig. 1, F and G). The shape changes observed in 
S100B siRNA-transfected cells were preceded by a dramatic 
reorganization of the F-actin cytoskeleton, as analyzed by rho- 
damine-phalloidin staining (Fig. 2A). Specifically, 2 and 5 days 
after transfection with S100B siRNA, ~85 and ~87% of GL15 
cells, respectively, showed reduced or no stress fibers and col- 
lapse of F-actin onto the plasma membrane, compared with 
<20% of control cells (Fig. 2, A and E). At post-transfection day 
12, ~85% of cells showed stellation, and the vast majority of 
cells exhibited virtually no stress fibers in the body and collapse of 
F-actin onto the plasma membrane (Figs. 1G and 2A). How- 
ever, at this time point, stellate cells showed bundles of F-actin 
within extensions, the abundance of which increased with 
increasing branching. F-actin bundles were concentrated at 
sites of ramification of cell extensions (Fig. 2B). In these cells 
(re-expressed) S100B was localized to the perinuclear region 
and cell extensions at sites of reformation of F-actin bundles.

Collectively, these results suggested that reducing S100B levels 
in GL15 cells resulted in a relatively rapid, extensive, and long 
lasting disassembly of stress fibers in a high percentage of cells 
and an increasingly larger incidence of stellation as a function of 
cultivation time. However, re-expression of S100B above a cer- 
tain threshold (e.g. at post-transfection day 12; see Fig. 1D) 
coincided with reformation of F-actin bundles in cell extensions.

To investigate whether the shape changes observed in S100B 
siRNA-treated cells were dependent on reduced expression of 
the protein or reduced autocrine effects of S100B, wild-type 
GL15 cells were treated with an S100B-neutralizing antibody 
(37) or nonimmune IgG. Enzyme-linked immunosorbent assay 
measurements showed that the culture medium of control 
GL15 cells contained ~0.3 ng/ml and 10 ng/ml S100B at post-
transfection days 2 and 5, respectively (data not shown). At 
these same time points, no S100B was detected in the cultured 
medium of S100B-silenced cells (data not shown). Treatment of 
wild-type GL15 cells with an S100B-neutralizing antibody for 
up to 6 days did not induce changes in cell shape or F-actin 
organization (data not shown). Also, treatment of S100B 
silenced cells with conditioned medium from 5-day-old wild- 
type GL15 cultures did not change the pattern of F-actin reor-
ganization or stellation at days 6 and 8 (data not shown). More-
over, adding S100B to up 10 nm to the culture media of control 
and S100B-silenced cells at post-transfection day 2 did not 
cause changes in cell shape or the abundance of stress fibers in 
control cells or affect the changes in morphology or localization 
of F-actin in S100B-silenced cells (Fig. S1). By contrast, tran-
sient transfection of S100B siRNA-treated cells at postsilencing 
day 2 with either S100B-green fluorescent protein or S100B 
expression vector reversed the changes in F-actin organization 
and cell shape caused by S100B silencing (Fig. S2). Collectively, 
these results suggested that the changes in morphology and 
supramolecular organization of F-actin registered in S100B 
siRNA-treated cells were dependent on reduced intracellular 
regulatory activities of the protein.

Silencing of S100B in GL15 Cells Results in Synergistic Effects 
Submaximal Inhibition of ROCK—Stress fiber formation is 
observed in adherent cells and migrating cells in culture and is 
dependent on the activation of the small GTPase of the Rho 
family, RhoA, and its associated kinase, ROCK (38–40); inhibi-
tion of either RhoA or ROCK results in reduced formation of 
stress fibers and reduced migration. In the case of astrocytes, 
inhibition of RhoA/ROCK results in stellation and reduced 
migration (41–43). Administration of 10 μM Y27632, an inhib-
or of ROCK, for 3 h to control cells caused a massive F-actin 
reorganization and stellation (data not shown). Administration 
of a submaximal dose (i.e. 1 μM) of Y27632 for 3 h to control

![FIGURE 1. Silencing of S100B in GL15 cells results in stellation. A, control and S100B siRNA-treated cells at post-transfection day 2 were immunostained with an anti-S100B antibody (green) and counterstained with DAPI (blue). Shown are merged images only. B, quantitative PCR demonstrating the relative expression of S100B mRNA in S100B siRNA-treated cells (n) at different time points after transfection, relative to control cells (c) at the end of the transfection period (0 time) (n = 3). C, quantitative PCR demonstrating the relative expression of S100B mRNA in control and S100B siRNA-treated cells at different time points after transfection (n = 3). D, Western blot analysis of S100B in control and S100B siRNA-treated cells at different time points after transfection. Notice the time-de-
dependent accumulation of S100B in control cells and the slow and incomplete recovery of S100B protein expression in S100B siRNA-treated cells (n = 3). E, control cells were immunostained with an anti-S100B antibody and counterstained with DAPI at different time points after transfection. Shown are merged images only. Note the increase in fluorescence signal as a function of cultivation time. F, and G, S100B siRNA-treated cells show stellation (F), the incidence of which increases as a function of cultivation time (n = 3) (G), as investigated by phase-contrast microscopy. Bars, 50 μm (A, E, and F).]
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GL15 cells with S100B siRNA and 10 μM LY294002 (10 μM) for 2, 6, and 8 days resulted in ~45, 55, and 85% decrease in Akt phosphorylation, respectively (Fig. 3A). Treatment of control GL15 cells with the PI3K inhibitor, LY294002 (10 μM), for 2, 6, and 8 days resulted in ~45, 55, and 85% decrease in Akt phosphorylation, respectively (Fig. 3A), and in formation of long cell extensions (Fig. 3B). Notably, neither the reduction of S100B levels nor inhibition of PI3K by LY294002 alone resulted in the complete inhibition of Akt phosphorylation in GL15 cells, probably because of hyperactivity of PI3K in glioma cells (44). We could not use a higher concentration of LY294002 because of its cytotoxicity. However, the combined treatment of GL15 cells with S100B siRNA and 10 μM LY294002 resulted in an additive effect on both the levels of phosphorylated Akt and astrocyte stellation (Fig. 3, A and B). Collectively, these results suggested that reduction of S100B levels impacted on PI3K activity.

Given that Akt phosphorylates and inactivates the serine/threonine kinase, GSK3β (45), that levels of phosphorylated Akt were reduced in S100B siRNA-treated GL15 cells (Fig. 3A), and that GSK3β has been involved in the formation of cell extensions in several cell types via activation of Rac1 (39, 46–48), we asked whether stellation of S100B-silenced GL15 cells might translate into an enhanced activity of Rac1, a small GTPase that governs the formation of lamellipodia and cell extensions and participates in the organization of cortical F-actin (39).

Silencing of S100B Results in Reduced Phosphorylation of Akt and GSK3β and Decreased RhoA Activity in GL15 Cells—To have information about the molecular mechanism linking the reduced expression of S100B in S100B siRNA-transfected GL15 cells to stress fiber dissolution and stellation, we first analyzed the phosphorylation level of Akt, taken as an index of the activity of the Akt upstream kinase, PI3K, which was shown to activate RhoA, thereby stimulating the formation of stress fibers in astrocytes (43). At post-transfection days 2, 6, and 8, we detected a ~40, 50, and 70% decrease in the levels of Akt phosphorylation, respectively, in S100B siRNA-treated GL15 cells compared with their respective controls (Fig. 3A). Treatment of control GL15 cells with the PI3K inhibitor, LY294002 (10 μM), for 2, 6, and 8 days resulted in ~45, 55, and 85% decrease in Akt phosphorylation, respectively (Fig. 3A), and in formation of long cell extensions (Fig. 3B). Notably, neither the reduction of S100B levels nor inhibition of PI3K by LY294002 alone resulted in the complete inhibition of Akt phosphorylation in GL15 cells, probably because of hyperactivity of PI3K in glioma cells (44). We could not use a higher concentration of LY294002 because of its cytotoxicity. However, the combined treatment of GL15 cells with S100B siRNA and 10 μM LY294002 resulted in an additive effect on both the levels of phosphorylated Akt and astrocyte stellation (Fig. 3, A and B). Collectively, these results suggested that reduction of S100B levels impacted on PI3K activity.

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FIGURE 2. Silencing of S100B in GL15 cells results in stress fiber disassembly and reorganization of F-actin. A, rhodamine-phalloidin staining (red) of control and S100B siRNA-treated cells at different time points after transfection. The cells were counterstained with DAPI (blue). Shown are merged images only. Note the reduction of stress fibers at post-transfection day 2 and the time-dependent increase in cell branching as a function of cultivation time in S100B siRNA-treated cells. B, S100B siRNA-treated cells at post-transfection days 5 and 12 were subjected to rhodamine-phalloidin staining (red) and immunostained with an anti-S100B antibody (green). Shown are merged images only. A faint S100B immunofluorescence signal is found in a perinuclear position at day 5, whereas S100B is additionally found in cell extensions at day 12, in coincidence with the formation of F-actin bundles therein. C and D, effects of S100B silencing on stellation and stress fiber disassembly at post-transfection day 2 are synergistic to those exerted by the ROCK inhibitor, Y27632, as investigated by phase-contrast microscopy (C) and rhodamine-phalloidin staining (D). E, shown are the numbers of stress fiber-devoid cells in control and S100B-silenced cells in the absence or presence of treatment with Y27632 at post-transfection day 2 (n = 3). Bars, 50 μm (A–D). si, S100B siRNA-treated cells; c, control cells.
C), implying that GSK3β activity was required for S100B silencing to cause morphological changes in GL15 cells and that the reduced Akt activity detected in S100B siRNA-treated cells translated into a high GSK3β activity (actually a reduced inactivation of GSK3β). Consistently, at post-transfection days 2, 6, and 8 a ~40, 55, and 70% decrease, respectively, in the levels of...

FIGURE 3. Silencing of S100B in GL15 cells results in reduced phosphorylation of Akt and GSK3β. A, analysis of levels of phosphorylated Akt in control (c) and S100B siRNA-treated (si) cells in the absence or presence the PI3K inhibitor LY294002 (LY) (10 μM) at post-transfection days 2, 6, and 8 (n = 3). B, effects of S100B silencing on stellation at post-transfection day 6 are synergistic to those exerted by the PI3K inhibitor LY294002, as investigated by phase-contrast microscopy. C, treatment of S100B siRNA-treated cells with the GSK3β inhibitor LiCl (2 mM) at post-transfection day 4 results in reduced stellation, as investigated by rhodamine-phalloidin staining. Shown are merged images of rhodamine-phalloidin fluorescence signal (red) and DAPI (blue). D, analysis of levels of phosphorylated GSK3β in control (c) and S100B siRNA-treated (si) cells in the absence or presence of the PI3K inhibitor LY294002 (10 μM) at post-transfection days 2, 6, and 8 (n = 3). E, treatment of S100B siRNA-treated cells for 24 h with the Rac1 inhibitor NSC23766 (50 μM) at post-transfection day 8 results in reduced stellation, as investigated by rhodamine-phalloidin staining. Shown are merged images of rhodamine-phalloidin fluorescence signal (red) and DAPI (blue). Bars, 50 μm (B, C, and E). *, significantly different from control (S100B siRNA-treated cells in the absence of kinase inhibitors).
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phosphorylated GSK3β was detected in S100B siRNA-treated cells compared with their respective controls (Fig. 4D), and the combined treatment of GL15 cells with S100B siRNA and LY294002 resulted in an additive effect on levels of phosphorylated GSK3β (Fig. 3D). Thus, reduction of S100B levels impacted on the PI3K/Akt/GSK3β axis.

Also, treatment of S100B siRNA-transfected cells with the Rac1 inhibitor, NSC23766, resulted in reduced stellation of S100B-silenced cells (Fig. 3E), suggesting that reduction of S100B levels might result in an increased activity of Rac1. Because both Akt and ROCK can inactivate Rac1 (39, 45, 49), the reduced activity of PI3K/Akt in S100B siRNA-transfected GL15 cells might translate into Rac1 activation.

To have information about effects of S100B silencing on RhoA, we analyzed RhoA activity and effects of transient transfection of GL15 cells with a constitutively active mutant of RhoA (RhoAV14-EGFP). As investigated by a pull-down assay, RhoA activity was reduced in S100B siRNA-transfected cells compared with control cells, with a modest but significant effect at post-transfection day 2 and a larger effect at post-transfection day 5 (Fig. 4A). Notably, RhoA activity in control cells was higher at post-transfection day 5 in coincidence with their higher S100B levels, compared with day 2. Also, transient transfection of S100B siRNA-transfected cells with RhoAV14-EGFP resulted in negation of the effects of S100B silencing in GL15 cells, the cells that had incorporated RhoAV14 displaying a high amount of stress fibers and lacking extensions (Fig. 4B). Thus, the increased activity of RhoA under these conditions rescued the deficit of stress fiber formation and negated the cell shape changes consequent to S100B silencing. Similarly, treatment for 3 h of S100B siRNA-treated GL15 cells with LPA, a strong activator of RhoA (50), reversed the effects of S100B silencing on cell shape and the supramolecular organization of F-actin (Fig. 4C).

Collectively, these findings supported the conclusion that reduction of S100B levels in GL15 cells resulted in a reduced ability of PI3K to activate RhoA and Akt with consequent high activities of GSK3β and Rac1. Thus, S100B might participate in the formation of stress fibers and modulate the formation of cell extensions in GL15 cells by stimulation of the RhoA/ROCK axis via PI3K and inactivation of the GSK3β/Rac1 axis by PI3K/Akt.

S100B Interacts with Src Kinase in GL15 Cells—The results depicted in Fig. 3 suggested that S100B might act at the level of or upstream of PI3K. As investigated by immunoprecipitation assays, neither PI3K (85-kDa subunit) nor PTEN, a phosphatase that inactivates PI3K (51, 52), were detected in S100B immunoprecipitates, thus pointing to a lack of S100B interaction with PI3K and PTEN (data not shown). By contrast, Src, a nonreceptor tyrosine kinase with the ability to signal to PI3K (53), was detected in S100B immunoprecipitates, and, conversely, S100B was detected in Src immunoprecipitates (Fig. 5A). Furthermore, no Src was detected in S100B immunoprecipitates from S100B-silenced cells (data not shown). Also, treatment of wild-type GL15 cells with the Src inhibitor, PP2, resulted in stress fiber disassembly, F-actin collapse onto plasma membranes, and acquisition of a stellate morphology (Fig. 5B) and in reduced Akt phosphorylation levels (Fig. 5C). Thus, inhibition of Src in GL15 cells caused phenotypic and molecular changes

FIGURE 4. Silencing of S100B in GL15 cells results in reduced RhoA activity. A, control (c) and S100B siRNA-treated (si) cells were subjected to a pull-down assay to detect activated RhoA. *, significantly different from control at post-transfection day 2 (n = 3). B, control and S100B siRNA-treated GL15 cells (post-transfection day 2) were transiently transfected with RhoAV14-green fluorescent protein (EGFP) and treated with rhodamine-phalloidin (red signal) to detect F-actin. Transfected cells were recognized by GFP fluorescence (green signal). Note that cells that have incorporated RhoAV14 are elongated and exhibit a great abundance of stress fibers. C, S100B siRNA-treated cells were incubated with LPA (10 μM, 3 h) at post-transfection day 8 and subjected to rhodamine-phalloidin staining (red) and immunostained with an anti-S100B antibody (green). Cells were counterstained with DAPI (blue). Shown are merged images only. Treatment with LPA results in reformation of stress fibers and reversal of stellation in S100B-silenced cells. Bars, 50 μm (B).
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FIGURE 5. S100B interacts with and activates Src in GL15 cells. A, cell lysates were subjected to immunoprecipitation using a polyclonal anti-S100B antibody, and the immunoprecipitate was probed with a polyclonal anti-Src antibody (top) or a polyclonal anti-S100B antibody (middle). Src (top) and S100B (middle) were detected in the cell lysate (Input), the fraction of cell lysate that did not bind to the anti-S100B antibody (Unbound), the S100B immunoprecipitate (IP aS100B), and the fraction of cell lysate that did not bind to nonimmune IgG (unbound IgG). Also shown are the IgG heavy chain (IgG HC, top) and S100B marker (last lane from the right in the middle panel). Cell lysates were subjected to immunoprecipitation using a polyclonal anti-Src antibody, and the immunoprecipitate was probed with a polyclonal anti-S100B antibody (bottom). S100B was detected in the cell lysate (Input), the fraction of cell lysate that did not bind to the anti-Src antibody (Unbound), the Src immunoprecipitate (IP aSrc), and the fraction of cell lysate that did not bind to non-immune IgG (unbound IgG). Also shown is the S100B marker (last lane on the right). B, GL15 cells were treated with either vehicle (left) or 20 μM PP2 (right) for 6 h. The cells were analyzed by phase-contrast microscopy (top row) or subjected to rhodamine-phalloidin staining (red) (bottom row). Cells were counterstained with DAPI (blue) (bottom row). Inhibition of Src results in stellation and disassembly of stress fibers. C, conditions were as in D except that the cells were treated for 1 or 3 h with increasing doses of PP2 and analyzed for Akt phosphorylation levels relative to total Akt levels by Western blotting. Inhibition of Src results in reduced phosphorylation levels of Akt. D, control and S100B siRNA-treated GL15 cells at post-transfection day 1 were subjected to Western blotting using a polyclonal anti-phosphorylated (Tyr527) Src. Note the enhanced phosphorylation levels of Src in S100B-silenced cells compared with control cells, indicative of reduced Src kinase activation status in the absence of S100B.

similar to those observed in S100B-silenced cells. Moreover, as investigated by Western blotting at post-transfection day 1, levels of phosphorylated (Tyr527) Src were significantly higher in S100B-silenced cells compared with control cells (Fig. 5D), pointing to reduced Src kinase activity. These results suggested that S100B might interact with and activate Src in GL15 cells and that S100B-Src interactions might stimulate the PI3K/Akt module and the PI3K/RhoA/ROCK module.

Silencing of S100B in GL15 Cells Results in Reduced Migration—Since stress fiber contraction is required for cell movement, the dramatic changes induced in stress fiber formation following transfection with S100B siRNA suggested that migration also might be compromised in S100B-silenced cells. In a migration assay using Boyden chambers, S100B siRNA-treated GL15 cells migrated ~25 and 60% less than control cells at 6 and 24 h post-transfection, respectively (Fig. 6A). Also, control and S100B siRNA-treated cells despite the reduced levels of phosphorylated Akt and GSK3β (Fig. 3, A and D) and the significantly lower S100B levels in S100B siRNA-treated cells compared with controls (Fig. 1D). A ~20% and ~30% reduction of expression of cyclin D1, a key enzyme expressed during the G1 phase of the cell cycle and required for progression into S phase, was detected at post-transfection days 0 and 1, respectively (Fig. 6E). However, no differences in cyclin D1 expression resulted in reduced phosphorylation (i.e. reduced activation) of Akt and reduced phosphorylation (i.e. reduced inactivation) of GSK3β, we reasoned that this might result in a reduced cell proliferation. By a bromodeoxyuridine incorporation assay (data not shown) and fluorescence-activated cell sorting (Fig. 6D), 2 days after transfection, ~16% of control GL15 cells and ~7% of S100B siRNA-treated cells were in the S phase of the cell cycle (i.e. proliferating). However, at post-transfection days 5 (Fig. 6D) and 8 (data not shown), similar percentages (~5–7%) of cells were proliferating in

whereas at a submaximal dose (i.e. 1 μM) the ROCK inhibitor, Y27632, caused a ~40% reduction of migration by 6 h, a dramatically reduced migration was observed at 6 h in cells treated with both S100B siRNA and 1 μM Y27632 (Fig. 6A). Thus, the reduced extent of stress fibers in S100B siRNA-treated GL15 cells correlated positively with their reduced migration. In a wound repair assay, also reduction of S100B expression resulted in reduced migration (Fig. 6, B and C), suggesting that S100B might have a role in both random and directional migration.

Silencing of S100B in GL15 Cells Results in Moderately Reduced Proliferation—Akt is a mitogenic kinase (45, 54), and GSK3β exerts antiproliferative effects by favoring β-catenin and cyclin D1 degradation (55). Because the reduction of S100B levels in GL15 cells resulted in reduced phosphorylation (i.e. reduced activation) of Akt and reduced phosphorylation (i.e. reduced inactivation) of GSK3β, we reasoned that this might result in a reduced cell proliferation. By a bromodeoxyuridine incorporation assay (data not shown) and fluorescence-activated cell sorting (Fig. 6D), 2 days after transfection, ~16% of control GL15 cells and ~7% of S100B siRNA-treated cells were in the S phase of the cell cycle (i.e. proliferating). However, at post-transfection days 5 (Fig. 6D) and 8 (data not shown), similar percentages (~5–7%) of cells were proliferating in
Silencing S100B in GL15 cells results in reduced migration and proliferation. A, migration assay in Boyden chambers. Shown is migration of control (c) and S100B siRNA-treated (si) cells at 24 h post-transfection (left panel) and of control and S100B siRNA-treated cells at 6 h post-transfection in the absence or presence of 1 μM Y27632 (right). B and C, wound healing assay. Monolayers of control and S100B siRNA-treated cells were scratched at post-transfection day 1 and examined by phase-contrast microscopy at time intervals (C). Shown in B is a quantitative analysis of wound width in experiments in C (n = 3). D, fluorescence-activated cell sorting analysis of control (c) and S100B siRNA-treated (si) cells at post-transfection days 1 and 5. E, Western blot analysis of cyclin D1 in control (c) and S100B siRNA-treated (si) cells at the end of the transfection procedure (0 d) and at post-transfection day 1. *, significantly different from control (left column in upper and lower panels in A and left column in each pair in D and E) (n = 3). **, significantly different from all other samples (n = 3). Bars, 50 μm (C).

**Figure 6.** Silencing S100B in GL15 cells results in reduced migration and proliferation. A, migration assay in Boyden chambers. Shown is migration of control (c) and S100B siRNA-treated (si) cells at 24 h post-transfection (left panel) and of control and S100B siRNA-treated cells at 6 h post-transfection in the absence or presence of 1 μM Y27632 (right). B and C, wound healing assay. Monolayers of control and S100B siRNA-treated cells were scratched at post-transfection day 1 and examined by phase-contrast microscopy at time intervals (C). Shown in B is a quantitative analysis of wound width in experiments in C (n = 3). D, fluorescence-activated cell sorting analysis of control (c) and S100B siRNA-treated (si) cells at post-transfection days 1 and 5. E, Western blot analysis of cyclin D1 in control (c) and S100B siRNA-treated (si) cells at the end of the transfection procedure (0 d) and at post-transfection day 1. *, significantly different from control (left column in upper and lower panels in A and left column in each pair in D and E) (n = 3). **, significantly different from all other samples (n = 3). Bars, 50 μm (C).

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Conditions used in these experiments might reduce the GL15 cell proliferation rate, particularly at later time points (Fig. 6E), thus hindering potential differences between control and S100B-sienced cells.

Silencing of S100B Results in Increased Formation of GFAP Filaments in GL15 Cells—GL15 cells show a mosaic distribution of GFAP filaments in accordance with their dedifferentiated phenotype (29). Appearance of GFAP filaments is a sign of astrocyte differentiation (56–58). At post-transfection day 12, control GL15 cultures exhibited a small percentage (~13%) whereas cultures of S100B siRNA-treated GL15 cells exhibited a higher percentage (~61%) of GFAP filament-positive cells (Fig. 7A). However, levels of GFAP protein were essentially the same in control and S100B siRNA-treated GL15 cells (Fig. 7B). This suggested that the reduction of S100B levels had favored the assembly of GFAP into filaments, in accordance with the notion that S100B interferes with GFAP assembly in vitro (59).

Alternatively, the increased percentage of GFAP filament-positive cells might result from other events related to the acquisition of a more differentiated phenotype of S100B siRNA-treated GL15 cells. Whatever the case, the present data pointed reduced in differentiating primary astrocytes, which resumed expressing the protein at later differentiation stages.

Silencing of S100B Results in Stellation of MIO-M1 Müller Cells—To verify whether the changes consequent to S100B silencing were not restricted to GL15 cells, we transfected MIO-M1 cells, a cell line derived from retinal Müller cells (30), with S100B siRNA. Wild-type MIO-M1 cells expressed S100B, as investigated by RT-PCR (Fig. 8A) and immunofluorescence (Fig. 8C). Silencing of S100B resulted in reduced expression of S100B mRNA (Fig. 8A) and in formation of cell extensions (Fig. 7B), reorganization of F-actin (Fig. 8C), and formation of GFAP filaments (Fig. 8D) similar to S100B-silenced GL 15 cells. Moreover, under the serum-free conditions used here and after a long cultivation time (e.g. ≥8 days), MIO-M1 cells exhibited a tendency to form neurosphere-like aggregates in which the majority of cells were small and S100B-positive/GFAP filament-negative, whereas neighboring cells were polygonal and/or elongated in shape and S100B-negative/GFAP filament-positive (Fig. 8E). Also, MIO-M1 cells underwent spontaneous stellation after a prolonged cultivation time in the presence of serum (Fig. S3). These shape changes were accompanied by a negative correlation between the levels of S100B and the formation of GFAP filaments in GL15 cells.

Silencing S100B is transiently reduced during Bt2cAMP-induced differentiation of primary astrocytes—Given the data in Fig. 7A, we asked whether S100B expression needs to be reduced for astrocyte stellation (i.e. differentiation) to occur. To this end, we analyzed primary astrocytes for expression of S100B and cell shape changes in primary astrocytes in the absence or presence of the astrocyte-differentiating agent, Bt2cAMP. At the end of the isolation procedure, ~90% of astrocytes were S100B-positive/GFAP filament-positive, whereas a minor percentage were elongated in shape and S100B-positive/GFAP filament-negative (Fig. 7, D and F). After 3 more days of treatment with Bt2cAMP, the majority of astrocytes were ramified and/or stellate and S100B-positive/GFAP filament-positive (Fig. 7, E and F). Thus, there was a temporal window during which S100B expression was...
increasingly larger GFAP filament formation as a function of cultivation time. However, a significant decrease in S100B expression occurred between day 5 and day 10 (Fig. S3) coincident with the appearance of GFAP filaments in the majority of cells and stellation. At a later time point (i.e. day 14), differentiated cells resumed S100B expression. Thus, as in the case of primary astrocytes (Fig. 7D), there was a decrease in S100B expression in MIO-M1 cells at the beginning of their spontaneous differentiation.

**DISCUSSION**

As in the case of other cell types, migration of astrocytes requires the formation of lamellipodia at the cell front and of stress fibers made of bundles of actomyosin at the rear; contraction of these latter causes the cell to reacquire front-rear symmetry. Interference with stress fiber formation in astrocytes reduces migration and causes astrocytes to acquire a stellate shape, which is due in part to retraction of the cytoplasm (which is largely dependent on reduced activity of RhoA) and in part to the extension of cell processes (which is largely dependent on increased activity of Rac1) (42–44). The stellate shape of astrocytes in normal brain tissue reflects their reduced migratory and proliferative properties, whereas retraction of their extensions is one sign of transition from a resting to an activated state (20–22).

Astrocytes become activated in the course of brain insults, playing a dual role; they release trophic factors to protect neurons at the very beginning of insult and participate in the inflammatory response at later phases. As a part of its ability to act as an immune cell, the astrocyte has to reacquire a proliferative capacity as well as a shape suitable for migration toward the site of insult. Several blood-borne factors, such as mitogens and cytokines, as well as factors released by astrocytes themselves and microglia exert regulatory effects on astrocyte shape changes, proliferation, and migra-
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FIGURE 8. Silencing S100B in MIO-M1 Müller cells results in stellation, stress fiber disassembly, and enhanced GFAP filament formation. A, control (c) and S100B siRNA-treated (si) cells were analyzed by RT-PCR at the end of the transfection procedure (0 d) and 2 and 5 days post-transfection using S100B-specific oligonucleotides. B, control and S100B siRNA-treated cells were cultivated for 5 days and analyzed by phase-contrast microscopy. Transfection with S100B siRNA results in stellation. C, control and S100B siRNA-treated cells were cultivated for 2 days and subjected to rhodamine-phalloidin staining (red) and immunostained with an anti-S100B antibody (green). Cells were counterstained with DAPI (blue). Shown are merged images only. At post-transfection day 2, S100B silenced cells show reduced stress fibers compared with control cells. D, control and S100B siRNA-treated cells were immunostained using an anti-GFAP antibody (red) and an anti-S100B antibody (green) and counterstained with DAPI (blue). Merged images only are presented. E, MIO-M1 cells were cultivated in serum-free medium for 14 days and immunostained using an anti-GFAP antibody (red) and an anti-S100B antibody (green) and counterstained with DAPI (blue). Shown are merged images only. GADPH, glyceraldehyde-3-phosphate dehydrogenase.

Repression of S100B in astrocytes is of great interest in the case of brain insult (60). Astrocytes have an inherent capacity to respond to these factors by expressing membrane receptors that activate or deactivate signaling pathways, which in turn control gene expression and/or shape changes, and/or by regulating the expression and subcellular localization of intracellular proteins with the ability to affect metabolic and signaling processes. Neoplastic transformation also causes a reprogramming of intracellular activities in astrocytes leading to shape changes, uncontrolled proliferation, and migration. Last, during neurogenesis, astrocyte precursors proliferate and migrate to colonize the various parts of the brain and participate in the establishment of the brain cytoarchitecture and the formation of the blood–brain barrier (61–63).

The Ca\(^{2+}\)-binding protein of the EF-hand type, S100B, is a candidate to play an important role within cells by virtue of its ability to interact with several target proteins and to regulate their function. In the brain, astrocytes represent the cell type with the highest expression of the protein, and levels of S100B in astrocytes increase in astrocytomas, in the aging brain, and in the course of several brain disorders, including degenerative processes, epilepsy, and infectious/inflammatory diseases (1–3, 9, 11). On one hand, increased levels of S100B might translate into its increased secretion or release; the ability of extracellular S100B to regulate neuronal, astrocyte, and microglia functions is well documented (1, 19). On the other hand, levels of intracellular S100B might be important for regulation of astrocyte activities from inside. For example, S100B has been implicated in the regulation of cytoskeleton elements and proliferation by virtue of its ability to inhibit microtubule and type III intermediate assembly; to activate the mitogen kinases, Ndr and Akt; and to modulate the expression and activity of the tumor suppressor protein, p53 (6–8). However, whether and by which mechanisms S100B participates in astrocyte proliferation, migration, and shape changes is not fully understood.

In the present study, we analyzed the effects of repression of S100B expression in the astrocytoma cell line, GL15, and the Müller cell line, MIO-M1. Our results suggest that S100B might contribute to astrocyte migration and, to a lesser extent, proliferation, as well as to the maintenance of an undifferentiated phenotype in astrocytic cells via interaction with and activation of Src kinase and Src-dependent stimulation of PI3K activity. This is the first report documenting an interaction of S100B with Src kinase. Also, repression of S100B expression in GL15 cells interferes with their ability to re-express the protein for a relatively long time under the present experimental conditions, which translates into long lasting effects on cell shape changes and the activation status of Akt. This latter effect suggests that S100B might regulate its own expression. Notably, parallel control cells expressed increasingly larger amounts of S100B mRNA and protein as a function of cultivation time, which might contribute to maintain their undifferentiated phenotype in the present serum-free conditions.

We found that repression of S100B expression in GL15 and MIO-M1 cells resulted in disassembly of stress fibers, cortical localization of F-actin, time-dependent acquisition of a stellate morphology, and a reduced migratory capacity. Effects of reduction of S100B expression in GL15 cells on the supramolecular organization of F-actin and migration were rapid and
preceeded the acquisition of a stellate shape. Some of these effects mimicked those exerted in control GL15 cells by inhibition of either Src kinase, PI3K, or the RhoA-associated kinase, ROCK. Also, the effects of repression of S100B expression were additive or synergistic to those exerted by inhibitors of either PI3K or ROCK and were blunted by transient transfection with a constitutively active mutant of RhoA, inhibition of either GSK3β with resultant reduced activity of Rac1, or treatment with the RhoA activator, LPA. Moreover, transfection of S100B-silenced cells with S100B expression vector rescued the changes produced by S100B silencing. The activation status of the PI3K downstream kinase, Akt, and of RhoA was reduced, whereas that of GSK3β was high in S100B-silenced cells. Since PI3K activates RhoA (which, in turn, activates ROCK) and Akt (which, in turn, inactivates GSK3β), we conclude that S100B has a role in the stimulation of PI3K activity with resultant activation of Akt and the RhoA/ROCK axis and inactivation of the GSK3β/Rac1 axis (Fig. 9). Further, we suggest that S100B binds to and activates Src kinase, thereby stimulating PI3K activity (Fig. 9). Previous work has shown that S100B activates the PI3K/Akt axis in PC12 neuronal cells, thereby stimulating proliferation and countering neural growth factor-induced differentiation (7) and that S100B negatively regulates chondrocyte terminal differentiation via an as yet undetermined mechanism (64). Moreover, current data indicate that intracellular S100B also inhibits myoblast differentiation.⁴

S100B was shown to interact with the Rac1 and Cdc42 effector, IQGAP1, at the polarized leading edge and areas of membrane ruffling in astrocytoma cell lines (65). These authors proposed that S100B regulates IQGAP1 activity in relation to cell migration, without, however, providing experimental evidence for a role of S100B in cell migration. Rac1/Cdc42-regulated IQGAP1 cross-links F-actin and participates in the organization of microtubules at the cell cortical region, which leads to cell polarization and migration (39, 66, 67). In this context, S100B might act as an activator of IQGAP1, thereby favoring cell migration. Our present results showing that reduction of S100B expression in GL15 cells (and MIO-M1 cells as well; data not shown) translates into reduced migration are in line with this possibility. However, we show that S100B might also act upstream of Rac1, decreasing its activity via Src/PI3K/Akt/GSK3β; reduction of S100B levels results in more activated Rac1, as inferred from the formation of long, branched cell extensions as well as from the significant reversal of effects of S100B silencing on stellation in cells treated with the Rac1 inhibitor, NSC23766. However, we cannot exclude the possibility that the lack of regulatory effects of S100B on IQGAP1 might contribute to the reduced migration of S100B-silenced cells despite the more active Rac1.

Whereas stress fiber disassembly was observed in the vast majority of S100B siRNA-treated GL15 cells at early time points after transfection and thereafter, stellation and reduction of Akt and GSK3β phosphorylation levels and RhoA activity were increasingly larger as a function of cultivation time. These effects were unlikely to be due to the culture condition (i.e. serum-free medium), because in parallel control cells only a minority of cells exhibited F-actin reorganization, stellation was only occasionally seen, and Akt and GSK3β phosphorylation and RhoA activity were relatively high at any time point in the time interval considered. Thus, the effects detected in S100B-silenced cells were attributable to their reduced ability to express the protein, and the increasingly larger shape changes detected in S100B siRNA-treated cells as a function of cultivation time might be attributed to long term effects on the molecular machinery inactivated or activated by S100B silencing, together with the progressive reduction of nutrients and mitogens in the medium. Supporting this conclusion was the observation that a brief (3-h) treatment of S100B-silenced cells with the RhoA activator, LPA, at post-transfection day 8 resulted in an almost complete reversal of the effects of S00B silencing on F-actin organization and cell shape. Furthermore, switching S100B-silenced cells from serum-free medium to 10% FBS also resulted in a tendency of cells to resume a fusiform or polygonal shape by 24 h (data not shown). Also, we speculate that the increasingly larger amounts of S100B in control cells as a function of cultivation time in serum-free medium might contribute to maintain Akt and RhoA activities relatively high and GSK3β activity relatively low despite the reduction of nutrients and mitogens.

Our present results are at variance with data obtained by inhibiting S100B expression in astrocytoma cells by oligonucleotide antisense techniques (68). We observed an extensive disassembly of stress fibers, cortical reorganization of F-actin, and acquisition of a stellate morphology in GL15 and MIO-M1 cells upon reduction of S100B expression as opposed to the flattened morphology, increased stress fiber formation, and reduced membrane ruffling in the cited study. Moreover, we have shown that during Bt-cAMP-induced differentiation of primary astrocytes and spontaneous differentiation of MIO-M1 cells, there is a temporal window in which S100B expression decreases in coincidence with the acquisition of a

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⁴ C. Tubaro, C. Arcuri, I. Giambanco, and R. Donato, submitted for publication.
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stellate morphology, and, as mentioned earlier, effects of S100B silencing on F-actin organization and cell shape in GL15 cells can be prevented by transfection with S100B expression plasmid. We have no explanation for the discrepancies between our data and those of the cited work aside from the different techniques used to inhibit S100B expression and culture conditions.

In further support of the conclusion that S100B might interfere with differentiation cues in GL15 cells, we found that whereas a minor percentage of control cells showed GFAP filaments, a marker of astrocytic differentiation, a larger fraction of S100B-silenced cells exhibited GFAP filaments in the presence of constant levels of total GFAP. Also, S100B silencing in the Müller cell line, MIO-M1, which is not a tumor cell line and has characteristics of radial glia (30), also resulted in stellation and increased GFAP filament content. Müller cells are radial glia-like cells implicated in the development of retinal cytoarchitecture and retinal regeneration (69–71). MIO-M1 cells show characteristics of neural stem cells (72) and express S100B (Fig. 8, A and C). The observation that silencing S100B in these cells results in the formation of long extensions and assembly of GFAP filaments suggests that S100B might contribute to maintain the stem cell-like properties of MIO-M1 cells.

The present data appear interesting on several grounds. 1) Although the mechanism of enhanced expression of S100B in astrogliaoma cells is unknown, the observation that inhibition of S100B expression in GL15 cells results in the acquisition of a differentiated phenotype and reduced migration suggests that the protein might contribute to maintaining a neoplastic, invasive phenotype and that this occurs via stimulation of a Src/PI3K/RhoA/ROCK pathway and concomitant Src/PI3K/Akt-dependent attenuation of the GSK3β/Rac1 activity. Thus, accumulation of S100B in glioma cells might be mechanistically linked to tumor progression and/or invasiveness. 2) Recent evidence suggests that S100B expression in astrocytic cells is developmentally regulated with different characteristics, depending, however, on whether subventricular or cortical astrocytic cells are considered (23). These same studies have established that during the time interval between postnatal days 2 and 8, ramified, differentiating (i.e. GFAP filament-positive) astrocytes are substantially S100B-negative. This suggests that during that time interval, S100B might be down-regulated, whereas the protein becomes re-expressed during the final phase of astrocytic differentiation. Moreover, S100B is expressed in radial glial precursors (25), in the ventricular zone of embryonic mouse cerebellum (26), and in progenitors of cerebellar granule cells (24), the protein being expressed in these latter cells as long as they are migrating. Our present results are consistent with the possibility that repression of S100B expression at certain phases of development of astrocytes and certain neuronal populations might be functionally linked to their differentiation. We speculate that S100B might contribute to confer migratory capacity to undifferentiated astrocytes and neuroblasts and that S100B expression needs to be repressed for differentiation to take place. In this context, S100B might act to avoid precocious differentiation besides favoring cell migration. Interestingly, MIO-M1 cells formed neurosphere-like aggregates after a long cultivation time in serum-free medium, which were made mainly of small S100B-positive/GFAP fila-

ment-negative cells, whereas the cells found outside the neurosphere-like aggregates were polygonal and/or ramified and S100B-negative/GFAP filament-positive. Also, Bt_cAMP-induced differentiation of primary astrocytes and spontaneous differentiation of MIO-M1 cells are accompanied by a transient reduction of S100B expression. Together, these observations also support the possibility that S100B expression characterizes undifferentiated astrocytes and that astrocyte precursors remain undifferentiated as long as S100B protein levels are relatively high. However, S100B expression characterizes a terminal maturation stage of cortical astrocytes (23), and astrocytes do express S100B in the mature nervous system. Thus, mechanisms that remain to be identified should exist that are responsible for re-expression of S100B in differentiated astrocytes. Our present data suggest that re-expression of S100B in stellate GL15 cells around post-transfection day 12 does not result in reversal of the ramified morphology; however, S100B re-expression coincides with the formation of F-actin bundles within extensions. This suggests that S100B might have a role in the maintenance of the stellate shape of differentiated astrocytes by participating in the organization of the F-actin cytoskeleton in astrocytic extensions. Supporting this possibility is the observation that with increasing cultivation time under differentiation conditions, a large fraction of primary astrocytes reacquire the ability to express S100B while being ramified and GFAP filament-positive. 3) Finally, S100B might participate in migration of both activated astrocytes in the case of brain insult and neoplastic astrocytes, by virtue of its ability to regulate the Src/PI3K/RhoA/ROCK and the Src/PI3K/Akt/GSK3β/Rac1 pathways.

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