S100B Increases Proliferation in PC12 Neuronal Cells and Reduces Their Responsiveness to Nerve Growth Factor via Akt Activation

Cataldo Arcuri, Roberta Bianchi, Flora Brozzi, and Rosario Donato‡

From the Section of Anatomy, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, 06122 Perugia, Italy

S100B is a Ca\(^{2+}\)-modulated protein of the EF-hand type expressed in high abundance in a restricted set of cell types including certain neuronal populations. S100B has been suggested to participate in cell cycle progression, and S100B levels are high in tumor cells, compared with normal parental cells. We expressed S100B in the neuronal cell line PC12, which normally does not express the protein, by the Tet-Off technique, and found the following: (i) proliferation was higher in S100B-expressing PC12 cells than in S100B-negative PC12 cells; (ii) nerve growth factor (NGF), which decreased the proliferation of S100B-negative PC12 cells, was less effective in the case of S100B-expressing PC12 cells; (iii) expression of S100B made PC12 cells resistant to the differentiating effect of NGF; and (iv) interruption of S100B expression did not result in an immediate restoration of PC12 cell sensitivity to the differentiating effect of NGF. Expression of S100B in PC12 cells resulted in activation of Akt; increased levels of p\(^{21}\)WAF1, an inhibitor of cyclin-dependent kinase (cdk) 2 and a positive regulator of cdk4; increased p\(^{21}\)WAF1-cyclin D1 complex formation; and increased phosphorylation of the retinoblastoma suppressor protein, Rb. These S100B-induced effects, as well as the reduced ability of S100B-negative PC12 cells to respond to NGF, were dependent on Akt activation because they were remarkably reduced or abrogated in the presence of LY294002, an inhibitor of the Akt upstream kinase phosphatidylinositol 3-kinase. Thus, S100B might promote cell proliferation and interfere with NGF-induced PC12 cell differentiation by stimulating a p\(^{21}\)WAF1/cyclin D1/cdk4/Rb/E2F pathway in an Akt-mediated manner.

S100B, a member of a multigenic family of Ca\(^{2+}\)-modulated proteins of the EF-hand type with both intracellular and extracellular regulatory activities, is expressed in varying abundance in astrocytes, Schwann cells, adipocytes, melanocytes, chondrocytes, skin Langerhans cells, lymphocyte subpopulations, skeletal muscle cells, and many neuronal populations (1, 2). Several intracellular target proteins have been identified for S100B (1, 2), and S100B has been shown to regulate protein phosphorylation, the dynamics of cytoskeleton components, Ca\(^{2+}\) homeostasis, some enzyme activities, and transcription factors (1, 2). Moreover, S100B has been shown to be secreted by astrocytes, thereby affecting neuronal, astrocyte, and microglia functions within the brain (1, 2). S100B likely can be released by S100B-expressing cells outside the nervous system because it is found in normal serum (1), thereby affecting functions of non-nervous cells (4).

Among the intracellular regulatory roles attributed to S100B is its participation in cell cycle progression. It has long been known that S100B levels are high in tumor cells, compared with normal parental cells (1, 2), and inhibition of S100B synthesis in an astrocyte cell line results in a decreased proliferation (5). However, the molecular mechanism underlying the potential role of S100B in cell proliferation has not been elucidated. S100B has been shown to interact with the tumor suppressor protein p53, inhibiting its phosphorylation and oligomerization (6–8), but the interpretation of this finding is controversial, ranging from participation of S100B in p53-dependent apoptosis and inhibition of cell growth (7, 8) to blockade of tumor suppressor activity of p53 (6, 9). Also, S100B was reported to stimulate Ndr, a nuclear serine/threonine protein kinase important in the regulation of cell division and cell morphology (10). These data strongly support the possibility that S100B may play a regulatory role in cell proliferation.

We studied some effects of forced expression of S100B in the neuronal cell line pheochromocytoma PC12, which was chosen because PC12 cells normally do not express S100B mRNA or protein and they cease to proliferate and differentiate into a neuronal-like cell type on exposure to the neurotrophin nerve growth factor (NGF) (11–13). We show here that overexpression of S100B results in enhanced cell survival under stress conditions, increased cell proliferation, a reduced extent of apoptosis, and decreased responsiveness to the differentiating effect of NGF. These effects are accompanied by activation of the mitogenic kinase Akt, increased levels of p\(^{21}\)WAF1, increased phosphorylation of the retinoblastoma suppressor protein Rb by cyclin-dependent kinase (cdk) 4, and increased formation of p\(^{21}\)WAF1/cyclin D1 complex. Our data support the possibility that S100B might play a role in neuronal proliferation and differentiation during development and potentially participate in neuronal tumorigenesis through an Akt/p\(^{21}\)WAF1/cyclin D1/cdk4/Rb/E2F pathway. Also, our data lend support to the notion that p\(^{21}\)WAF1 can act to promote cell proliferation under conditions that favor its association with cyclin D1/cdk4 complex (14–16).

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‡ To whom correspondence should be addressed: Section of Anatomy, Dept. of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto Casella Postale 81 Succursale 3, 06122 Perugia, Italy. Tel.: 39-075-585-7453/7448; Fax: 39-075-585-7451; E-mail: donato@unipg.it.

The abbreviations used are: NGF, nerve growth factor; cdk, cyclin-dependent kinase; FACS, fluorescence-activated cell-sorting; PI3-K, phosphatidylinositol 3-kinase; RT, reverse transcription; DOX, doxycycline.

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EXPERIMENTAL PROCEDURES

Cell Culture Conditions and Transfections—PC12 cells were transfected with pTet-Off regulator vector (Clontech) carrying neomycin resistance gene (17) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, cells were re-plated at a lower density in 1840 medium (containing 10% fetal bovine serum and 0.5% non-immune IgG) and then cultured for another 24 h in the same medium. Geneticin (0.8 mg/ml) was then added to select successfully transfected cells. Fourteen to 20 days after starting culture in the Geneticin-containing medium, Geneticin-resistant clones were transferred to separate wells. Selected clones were screened by luciferase assay (Clontech) according to the manufacturer’s instructions. The clone exhibiting the highest luciferase activity was chosen. Recombinant S100B was used for transfection with pTRE2-S100B vector. The pTRE2-S100B vector was prepared by cloning a blunt-ended fragment containing the open reading frame of the bovine S100B gene in the EcoRV site of the multicloning site of pTRE2 vector. The nucleotide sequence of the pTRE2-S100B vector was confirmed by DNA sequencing. The Tet-Off PC12 cell line, obtained as described above, was doubly transfected with pTRE2 S100B and pTK-Hyg selection vector carrying hygromycin B resistance gene (ratio, 10:1) by lipofection using Lipofectamine 2000 according to the manufacturer’s instructions. After 48 h, stably transfected clones were selected by hygromycin B (0.6 mg/ml) in 10% horse serum containing medium. 40 well-separated hygromycin B-resistant clones were transferred to separate wells. S100B-inducible clones were identified by Western blotting. S100B-transfected PC12 cells were cultivated in RPMI 1640 medium containing 10% horse serum, 5% fetal bovine serum supplemented with doxycycline (2 g/ml) for 24 h, at which the medium was renewed. NGF (100 ng/ml) was added to appropriate samples at the time of culture medium renewal to induce PC12 cell differentiation. Cells displaying processes ≥2 × longer than the cell body diameter were considered differentiated, neuronal-like cells; hence, the number of PC12 cells exhibiting processes ≥2 × the cell body diameter was taken as a measure of differentiation. Cells were cultured under these conditions for the indicated time periods (see the figure legends) without culture medium renewal unless otherwise specified, but with the addition of fresh doxycycline and NGF every other day, where required. To induce maximum S100B expression, doxycycline was omitted from culture medium. In some experiments (see Fig. 8), NGF was replaced by recombinant S100B to document extracellular S100B neurite extension activity.

RT-PCR and Western Blotting—To detect S100B mRNA, RT-PCR was performed as described in the legend of pertinent figures, washed twice with phosphate-buffered saline, and solubilized with 2.5% SDS, 10 mM Tris-HCl, pH 7.4, 0.1 mM dithiothreitol, and 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone protease inhibitor (Roche Applied Science). The following antibodies were used: polyclonal anti-S100B antibody (1:1,000; Dako); polyclonal anti-pan phospho-Ser-Thr kinase (1:100; Santa Cruz Biotechnology); polyclonal anti-Rb antibody (1:1,000; Cell Signaling Technology); polyclonal anti-phosphorylated Rb antibodies recognizing phospho-Ser780, or phospho-Ser780/781, respectively (each at 1:1,000; Cell Signaling Technology); polyclonal anti-Akt antibody (1:1,000; Cell Signaling Technology); polyclonal anti-phosphorylated (Ser^273) Akt antibody (1:1,000; Cell Signaling Technology); monoclonal anti-α-tubulin antibody (1:10,000; Sigma); monoclonal anti-cyclin D1 antibody (1:500; Santa Cruz Biotechnology); and polyclonal anti-p53 antibody (1:200; Santa Cruz Biotechnology). The primary antibody reaction was detected by enhanced chemiluminescence (SuperSignal West Pico; Pierce). Recombinant S100B was expressed, purified, and characterized as described previously (18, 19).

Fluorescence-activated Cell-sorting Analysis and Proliferation, Survival, and Apoptosis Assays—For FACS analysis, cells were seeded onto 35-mm plastic dishes (25 × 10^4 cells/dish) for 24 h, washed with Dulbecco’s modified Eagle’s medium, and cultivated for 6 days in the absence (−DOX) or presence (+DOX) of doxycycline, NGF, without renewal of culture medium. Where appropriate, doxycycline and NGF were added every other day. Cells were treated with propidium iodide (1 mg/ml) in 0.2% Triton X-100 and then processed for FACS analysis. Cells were then subjected to FACS analysis to determine the percentage of cells in the G1, G2/M, S, and G0/M phases of the cell cycle. For proliferation assay, cells were seeded onto 24-multwell plastic plates (5 × 10^3 cells/bottle/polylysine-coated well) for 24 h, washed with Dulbecco’s modified Eagle’s medium, and cultivated as described above for 6 days, after which [3H]thymidine was added. Cells were then subjected for another 24 h. Where appropriate, doxycycline and NGF were added every other day.

For survival assay, conditions were as described for the proliferation assay, except that after 6 days of culture, all sets of cells were serum-starved for 24 h, and cells cultivated in the −DOX condition were switched to the +DOX condition (to interrupt S100B expression). Cells were then treated with 0.2% trypan blue in phosphate-buffered saline. To measure apoptosis, S100B-transfected PC12 cells were seeded onto 35-mm plastic dishes (25 × 10^4 cells/dish) for 24 h, washed with Dulbecco’s modified Eagle’s medium, and cultivated for 6 days in the absence (−DOX) or presence (+DOX) of doxycycline, without renewal of culture medium. Where appropriate, doxycycline and NGF were added every other day. Cells were stained with propidium iodide and subjected to flow cytometry as described previously (20). In other experiments, PC12 cells were cultivated in the presence of doxycycline for 6 days as described above, after which H_2O_2 was added to 500 μM for 12 h to induce apoptosis, which was measured as described previously (20). To investigate possible autocrine effects of secreted S100B, PC12 cells were cultivated for 6 days in the absence or presence of doxycycline and either 50 μg/ml of non-immune IgG or 50 μg/ml of a polyclonal anti-S100B antibody (SWant), as described previously (4), and examined by phase-contrast microscopy and subjected to FACS analysis as described above. As a positive control, S100B−PC12 cells were cultivated in the presence of 500 μg/ml S100B as described previously (18) to document the neurite extension activity of S100B.

Immunofluorescence—For indirect immunofluorescence, PC12 cells were cultivated as described for the proliferation assay were fixed and processed as described previously (21). S100B and p53 were detected using a monoclonal anti-S100B antibody (1:20; Sigma) and a polyclonal anti-p53 antibody (1:50; Santa Cruz Biotechnology), respectively.

Immunoprecipitation—PC12 cells (15 × 10^5) were cultivated in the absence or presence of doxycycline for 6 days without culture medium renewal. Cells were then lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 1 μg/ml of aprotinin)) and incubated for 30 min at 4 °C under agitation. Samples were centrifuged at 14,000 rpm for 15 min at 4 °C. Supernatants were collected and used for immunoprecipitation. Extracted protein (1.5 mg) was incubated overnight at 4 °C in a final volume of 0.2 ml with 3 μg of CAM-generated anti-phospho-Ser-Thr kinase (Serine/Threonine) antibodies under vigorous agitation and centrifuged at 4 °C for 3 min at 14,000 rpm. Pellets were washed three times in radioimmune precipitation assay buffer, resuspended in SDS electrophoresis buffer, boiled for 5 min, and centrifuged as described above. Samples were then subjected to SDS electrophoresis and Western blotting with anti-cyclin D1. The immune reaction was developed by enhanced chemiluminescence (SuperSignal West Pico; Pierce).

RESULTS

Characterization of S100B-expressing PC12 Cells—PC12 cells were stably transfected with S100B cDNA, and S100B expression was induced by the Tet-Off technique (17). By RT-PCR (Fig. 1A), after 6 days of culture, S100B mRNA was found in transfected PC12 cells not exposed to doxycycline, but not in PC12 cells exposed to doxycycline or in PC12 cells carrying the gene encoding the tetracycline-controlled transactivator gene only (1T cells). By Western blotting, after 6 days of culture, the amount of expressed S100B increased with decreasing doxycycline concentration, with no S100B expression detected in the presence of 2 μg/ml doxycycline, and maximum S100B expression registered in the presence of a doxycycline concentration of ~10 ng/ml or lower (Fig. 1B, top panel). On the basis of these results and to maximize S100B expression, all experiments...
aimed at documenting effects of expression of S100B in PC12 cells were performed in the absence of doxycycline in parallel with PC12 cells cultivated in the presence of doxycycline (2 μg/ml), which served as a control. By immunofluorescence, the entire population of S100B-transfected PC12 cells cultivated for 6 days in the absence of doxycycline displayed S100B immunoreactivity (Fig. 1, C and D). By confocal laser scanning microscopy, S100B was detected diffusely in the cytoplasm of −DOX cells and was undetectable in +DOX cells (Fig. 1, E–H), and the amount of expressed S100B increased with increasing culture time in the absence of doxycycline, judging from the intensity of the immunofluorescence signal (Fig. 1, F and H) and Western blotting (Fig. 1B, bottom panel). Also, after 4 days of cultivation, the number of S100+ PC12 cells was greater than that of S100− PC12 cells (Fig. 1, F and H). This observation prompted us to investigate the effects of S100B (over)expression on cell proliferation and survival.

**S100B Increases Proliferation and Survival and Reduces Apoptosis of PC12 Cells**—To analyze the effects of S100B expression on PC12 cell proliferation, S100B-transfected PC12 cells were cultivated for 24 h, washed, and cultivated for 6 days in fresh medium in the absence or presence of doxycycline ± NGF. Fresh doxycycline and NGF were added to appropriate samples every other day without culture medium renewal. By FACs analysis, on accumulation of S100B (−DOX), a smaller fraction of cells was in G0–G1 phase, and larger fractions were in S and G2 + M phases, compared with the +DOX condition (Fig. 2, A–C). Also, at day 6, S100B+ cells incorporated a larger amount of [3H]thymidine than did S100B− cells (Fig. 2D). Thus, accumulation of S100B resulted in increased PC12 cell proliferation. Moreover, whereas NGF reduced the number of S100B− cells in S phase and strongly reduced [3H]thymidine incorporation by these cells (Fig. 2, A–D, +DOX + NGF), it was less able to do so in S100B+ cells (Fig. 2, A–D, −DOX + NGF). By cell survival assay, no significant differences were detected after 2–4 days of culture, irrespective of the expression or non-expression of S100B in the absence or presence of NGF (Fig. 2E). However, after 6 days of culture without culture medium renewal, a higher percentage of PC12 cells survived in the −DOX condition compared with the +DOX condition (Fig. 2E), indicating that expression of S100B was beneficial to these cells. Notably, whereas the percentage of surviving S100B+ cells decreased between day 4 and day 6, likely due to consumption/degradation of trophic serum factors, the percentage of surviving S100B− cells was nearly constant in the time interval investigated (Fig. 2E), pointing to a protective effect of S100B. Consistently, after 6 days of culture, S100B+ cells displayed a smaller extent of apoptosis compared with S100B− cells (Fig. 2F). Moreover, when treated with H2O2 to induce apoptosis, S100B+ PC12 cells exhibited a remarkably smaller extent of apoptosis than S100B− PC12 cells (Fig. 2F). With 1T

ng/ml doxycycline. **Bottom panel**, the S100B protein level increases in transfected PC12 cells with increasing cultivation time, at a constant tubulin level. The asterisk indicates the S100B disulfide cross-linked dimer that typically forms upon S100B storage at 20 °C. C and D, S100B-transfected PC12 cells were cultivated for 6 days in the absence of doxycycline and analyzed for S100B expression by immunofluorescence using an anti-S100B monoclonal antibody (D) and then treated with 4,6-diamidino-2-phenylindole to label the nuclei (C). E–H, immunofluorescence analysis of transfected PC12 cells. Cells were cultivated for 2 (E and F) or 4 (G and H) days in the presence (E and G) or absence (F and H) of doxycycline before fixation and immunofluorescence with an anti-S100B monoclonal antibody. S100B is found diffusely in PC12 cells cultivated in the absence (F and H) but not in the presence (E and G) of doxycycline. Also notice the stronger S100B immunofluorescence signal after 4 days of culture (H) as compared with after 2 days of culture (F). Bars in D, F, and H = 50 μm.

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**FIG. 1. Expression of S100B in transfected PC12 cells. A, RT-PCR.** S100B mRNA is expressed in PC12 cells cultivated in the absence (−DOX) but not in the presence (+DOX) of doxycycline. S100B mRNA is absent in PC12 cells carrying the gene encoding the tetracycline-controlled transactivator but not the S100B gene (IT). B, Western blot. **Top panel**, dose dependence of S100B protein expression in transfected PC12 cells exposed for 6 days to increasing concentrations of doxycycline as indicated. S100B is maximally expressed in the presence of ≤10 ng/ml doxycycline. **Bottom panel**, the S100B protein level increases in transfected PC12 cells with increasing cultivation time, at a constant tubulin level. The asterisk indicates the S100B disulfide cross-linked dimer that typically forms upon S100B storage at 20 °C.
cells, similar (if not identical) percentages of PC12 cells were in the G0-G1, S, and G2 + M phases of the cell cycle at a given time point, irrespective of the absence or presence of doxycycline (data not shown), indicating that transfection and/or doxycycline per se did not alter PC12 cell proliferation.

However, when cells were cultivated for 6 days with culture medium renewal every other day, no significant differences were detected at each time point in the cell number, irrespective of the −DOX or +DOX condition (data not shown; see Fig. 4). Thus, accumulation of S100B was necessary but not sufficient to cause increased proliferation, and consumption/degradation of the mitogens present in the culture medium likely was a prerequisite as well.

**S100B Reduces Responsiveness of PC12 Cells to NGF—**As shown in Fig. 2D, NGF was less able to cause proliferation arrest in S100B−/−PC12 cells compared with S100B+/−PC12 cells, suggesting that S100B might counteract in part the antiproliferative activity of the neurotrophin. To analyze the effects of S100B expression on NGF-induced differentiation of PC12 cells, S100B-transfected PC12 cells were cultivated for 6 days in the absence or presence of NGF + doxycycline (Fig. 3). S100B− and S100B+/−PC12 cells not exposed to NGF displayed the same morphology (the majority of the cells were round, and a few cells exhibited a few, extremely short processes), and the cell number in each field examined was much larger in the −DOX condition than the +DOX condition (Fig. 3, A−C), in agreement with data in Fig. 2. Also, whereas S100B−PC12 cells exposed to NGF displayed relatively long neurites indicative of cell differentiation (Fig. 3, D and F), S100B+/−PC12 cells exposed to NGF exhibited much shorter and fewer neurites, and the number of neurite-bearing PC12 cells was smaller in the case of S100B+/−PC12 cells than in the case of S100B−PC12 cells (Fig. 3, E and F). These data suggested that the expression of S100B in PC12 cells made these cells less responsive to the differentiating effect of NGF, raising the possibility that the reduced ability of S100B+PC12 cells to escape from the cell cycle might be the cause of the reduced responsiveness of S100B−PC12 cells to NGF. An alternative and/or additional possibility was that S100B might interfere with signaling pathways operating downstream of NGF receptors and inducing PC12 cell differentiation.

For S100B to interfere with NGF responsiveness of PC12 cells, the protein had to accumulate above a certain threshold, as indicated by the fact that the amount of S100B expressed in PC12 cells in 2 days was not sufficient for inhibition of NGF-induced differentiation (Fig. 3, H and J). In fact, a relatively
Fig. 3. NGF-induced differentiation of S100B− and S100B+ PC12 cells. PC12 cells were cultivated for 2 or 6 days as indicated in the absence (−DOX) or presence (+DOX) of doxycycline ± NGF as indicated (without renewal of culture medium). Where appropriate, doxycycline and NGF were added every other day. A–C, by phase-contrast microscopy, cells not exposed to NGF display the same morphology, irrespective of the presence (A, +DOX) or absence (B, −DOX) of doxycycline, and are round and exhibit a few, short processes. The percentage of differentiated cells under these conditions is reported in C. D–F, whereas cells exposed to NGF and doxycycline display relatively long neurites (D); those exposed to NGF in the absence of doxycycline show much shorter neurites (E). The percentage of differentiated cells under these conditions is reported in F. As mentioned under “Experimental Procedures,” PC12 cells exhibiting cell processes ≥2× longer than the cell body diameter were considered differentiated, neuronal-like cells. Collectively, these data suggest that expression of S100B in PC12 cells (−DOX condition) makes these cells less responsive to the differentiating effect of NGF. G–J, PC12 cells were cultivated for 2 days in the absence of doxycycline (G and H) to induce S100B expression, and then one sample (G) was further cultivated in the absence of doxycycline and in the presence of NGF for an additional 6 days, and the other one (H) was cultivated in the absence of doxycycline plus NGF for an additional 6 days. Parallel PC12 cells were cultivated for 2 days in the presence of doxycycline (I) and then cultivated for 6 days in the presence of doxycycline plus NGF (control sample). In all cases, where appropriate, doxycycline and NGF were added every other day, and the culture medium was renewed after the first 2-day period of culture only. The percentage of differentiated cells under these conditions is reported in J. These data suggest that S100B needs to accumulate in PC12 cells above a certain threshold for it to be able to reduce the NGF differentiating effect. K–N, PC12 cells were cultivated for 6 days in the presence of doxycycline + NGF, and then cells were switched from the +DOX condition to the −DOX condition and cultivated for another 4 days under these conditions before fixation and immunofluorescence with a monoclonal anti-S100B antibody (K). A high percentage of cells shows neurites (N), and no overt phenotypic changes can be seen, although all cells display S100B immunoreactivity. PC12 cells first were induced to express S100B for 6 days in the presence of NGF and then cultivated in the presence of doxycycline and NGF (L). All cells display S100B immunoreactivity 4 days after the switch from the −DOX to the +DOX condition, and only a minority of them show signs of differentiation (N). Thus, PC12 cells that had expressed S100B do not fully respond to the differentiating effect of NGF until S100B is present in relatively high amounts. M, PC12 cells cultivated in the presence of doxycycline + NGF throughout are S100B-negative, and the majority of them display neurites (N). *, significantly different, p < 0.0001. Bars in I and M = 50 μm.

high percentage of differentiated cells was measured in the case of PC12 cells first cultivated for 2 days in the absence of doxycycline and then cultivated for 6 days in the presence of doxycycline + NGF (Fig. 3, H and J), likely due to interruption of S100B expression after the switch from the −DOX to the +DOX condition, and the percentage of differentiated cells was similar to that measured in experiments in which doxycycline had been used throughout (Fig. 3, I and J). However, the amount of S100B accumulated in 3 days was sufficient to determine a significant inhibition of NGF-induced differentiation (see Fig. 6C). By contrast, a relatively low percentage of differentiated cells was measured in the case of PC12 cells first cultivated for 2 days in the absence of doxycycline and then cultivated for 6 days in the absence of doxycycline + NGF (Fig. 3, G and J), likely due to expression of S100B in the presence of NGF as well (see Fig. 3, K and L). On the other hand, PC12 cells that had been induced to differentiate by NGF could express S100B once switched from the +DOX condition to the −DOX condition (Fig. 3K); however, this was not accompanied by obvious phenotypic changes during the next 4–6 days of culture, with the majority of cells displaying neurites (Fig. 3, K and N). This suggested that in the time period investigated, overexpression of S100B could not reverse NGF-induced PC12 cell differentiation. When PC12 cells were first induced to express S100B for 6 days in the presence of NGF and then cultivated in the presence of doxycycline (to interrupt S100B
NGF decreased the extent of Rb phosphorylation in S100B−PC12 cells (Fig. 4B), in agreement with its ability to reduce cell proliferation, but it was less able to do so in S100B+PC12 cells (Fig. 4B), in agreement with the reduced ability of NGF to arrest proliferation of S100B+PC12 cells (see Fig. 2D). In general, the extent of Rb phosphorylation in S100B+PC12 cells cultivated in the presence of NGF was smaller than that detected in the same cells cultivated in the absence of NGF but higher than that detected in S100B−PC12 cells cultivated in the presence of NGF (Fig. 4B).

However, increased p21WAF1 levels and increased phosphorylation of Rb by cdk4 in S100B+PC12 cells were detected, provided cells had been cultivated without culture medium renewal. When cells were analyzed for p21WAF1 content and extent of Rb phosphorylation at 2, 4, and 6 days of culture with culture medium renewal every other day, no significant differences could be detected between S100B+ and S100B−PC12 cells at each time point (Fig. 4D). Under these latter conditions, although PC12 cells cultivated for 6 days in the absence of doxycycline expressed S100B irrespective of the absence or presence of NGF (see Figs. 1, C and D, and 3L) and no S100B could be detected in PC12 cells cultivated in the presence of doxycycline irrespective of the absence or presence of NGF (see Figs. 1, E and G, and 3M), no differences could be detected between S100B− and S100B+PC12 cells in terms of cell number (Fig. 4, E–J). Under these conditions, NGF stimulated differentiation of both S100B− and S100B+PC12 cells to a small extent and with similar percentages (Fig. 4, E–J), indicating that culture medium renewal strongly stimulated PC12 proliferation even in the presence of NGF. Collectively, these data suggested that, with culture medium renewal every other day, high concentrations of serum mitogens supported a similar proliferation rate in S100B− and S100B+PC12 cells and that accumulation of S100B in the presence of reduced amounts of extracellular mitogens (e.g. at 6 days of culture without medium renewal) might support mitogen-independent PC12 cell proliferation, likely due to persistent activation of the p21WAF1/cyclin D1/cdk4/Rb/E2F pathway (also see Fig. 5B). These data supported the possibility that the observed reduction of NGF sensitivity of S100B+PC12 cells at 6 days of culture without medium renewal might be due to inability of S100B+PC12 cells to escape from the cell cycle.

S100B Activates Akt—As outlined earlier, similar levels of p21WAF1 were detected in S100B−PC12 cells cultivated in the absence or presence of NGF and in S100B−PC12 cells cultivated in the presence of NGF (Fig. 4A), and this was accompanied by different extents of cell proliferation and differentiation and Rb phosphorylation (Figs. 2D, 3, A–F, and 4B, respectively). Thus, increased p21WAF1 levels per se were not sufficient to reduce or enhance PC12 cell proliferation or differentiation, and other events had to occur under the single conditions above that determined the final fate of PC12 cells. Recent work has shown that the pro-survival kinase Akt phosphorylates p21WAF1 with resulting preferential association of p21WAF1, an inhibitor of cyclin A- and E-dependent cdks (22–24), with the former cells compared with the latter ones. We found that phosphorylation of Rb at these serine residues by cdk4 cancels the Rb inhibitory effects on E2F transcription factors (14, 25). S100B+PC12 cells exhibited higher levels of phosphorylated Rb than S100B−PC12 cells (Fig. 4B), in agreement with the higher proliferation rate of the former cells compared with the latter ones. Because analyses were done with antibodies directed against Rb phosphoserine residues (i.e. phospho-Ser780, phospho-Ser795, and phospho-Ser607/611) that are phosphorylated by cdk4 (26–28), our results indicated that this kinase was being activated in S100B+PC12 cells. It is known that phosphorylation of Rb at these serine residues by cdk4 cancels the Rb inhibitory effects on E2F transcription factors (14, 25, 29). In this regard, it has been shown that p21WAF1, while inhibiting cyclin E- and A-dependent cdk2, acts as a positive regulator of cyclin D-dependent cdk4 and cdk6 (14–16). Thus, S100B might promote PC12 cell proliferation by increasing the levels of p21WAF1, which in turn activates cyclin D-dependent cdk4, with consequent phosphorylation of Rb and activation of E2F transcription factors. Consistently, a larger fraction of cyclin D1 co-immunoprecipitated with p21WAF1 in S100B−PC12 cells than in S100B−PC12 cells, in the presence of a constant amount of cyclin D1 (Fig. 4C).
of p21WAF1 (where appropriate). Cells were then solubilized and analyzed for levels except for doxycycline and NGF, which were added every other day, phosphorylation and p21WAF1-cyclin D1 complex formation.

PC12 cells were cultivated for 6 days in the absence or presence of doxycycline 

antibodies were used to detect distinct phosphoserine residues in Rb, as

FIG. 4 . S100B increases p21WAF1 levels and extents of Rb phosphorylation (A) and phosphorylated Rb (B) by Western blotting. Three

total Rb is also shown. A Western blot of tubulin is included to show equal protein loading in each lane. Relative changes in the amount of p21WAF1 and extent of Rb phosphorylation under the different conditions are reported above each panel in A and B. C, S100B (+DOX condition) and S100B (−DOX condition) PC12 cells (after 6 days of cultivation without culture medium renewal) were processed as described under “Experimental Procedures” before immunoprecipitation with agarose-conjugated anti-p21WAF1 antibody and Western blotting of immunoprecipitates with an anti-cyclin D1 antibody (IP; lanes c and d, S100B− and S100B+ PC12 cells, respectively). Lanes a and b (30 μg of protein loaded in each lane) show that lysates from S100B− (lane a, +DOX condition) and S100B+ (−DOX condition) PC12 cells contained similar amounts of cyclin D1. D, conditions were as described in A and B, except that the culture medium was renewed every other day, and cells were solubilized on days 2, 4, and 6, as indicated. Changes in the amount of p21WAF1 and extent of Rb phosphorylation relative to tubulin under the different conditions are reported above each panel. E–J, conditions were as described in A and B, except that the culture medium was renewed every other day, and cells were solubilized in the presence of NGF and analyzed by phase-contrast. Similar (if not identical) images, cell counts, and differentiated cells were registered in the presence or absence of doxycycline. Under either condition, most cells, occupying ~50% of the dish surface, were round (i.e., undifferentiated) and densely packed (E and H); a smaller percentage of cells, peripheral to the former ones, was much less densely packed and exhibited short processes (F and I), and an even smaller percentage of cells located at the extreme periphery of dishes and scattered showed overt signs of differentiation (G and J). One representative experiment of three is shown (A–J). Bar in J = 50 μm.
that S100B is capable of stimulating PC12 cell proliferation and inhibiting NGF-induced PC12 cell differentiation in an Akt-dependent manner via increase in p21\(^{WAF1}\) levels, preferential association of p21\(^{WAF1}\) with cyclin D1-cdk4, and hyperphosphorylation of Rb. Incidentally, exposure of S100B~PC12 cells to NGF for only 3 days did not result in the dramatic inhibition of Akt phosphorylation that was registered after 6 days of cultivation without culture medium renewal (compare Fig. 6F with Fig. 5A). Actually, a slightly larger extent of Akt phosphorylation was registered in these cells in the presence of NGF as compared with the absence of NGF (Fig. 6F). These differences might be explained by the known ability of NGF to cause an early and transient stimulation of PC12 cell proliferation and protect PC12 cells against apoptosis via PI3-K/Akt activation (Refs. 35–38 and the references therein). We could not extend the cultivation time in the experiments reported in Fig. 6F beyond 3 days without culture medium renewal because LY294002 caused extensive cell death.

**S100B and p53 Nuclear Translocation**—Because previous work showed that S100B blocked p53 transcription activity in non-nervous cell lines (8) and because NGF was shown to cause proliferation arrest and, in part, differentiation of PC12 cells via p53 (22–24), we explored the possibility that the expression of S100B in PC12 cells might cause increased proliferation and reduced sensitivity to NGF also by interfering with p53 activity. We found that expression of S100B did not alter the levels of total p53 in 6-day-old PC12 cell cultures (Fig. 7A). By immunofluorescence, we calculated the number of PC12 cells displaying p53 immunoreactivity within nuclei (22) under varying experimental conditions (Fig. 7B). p53 nuclear staining was detected in ~80% of S100B~PC12 cells 9 h after exposure to NGF, whereas only 4% of parallel cells not exposed to NGF showed p53 nuclear staining (Fig. 7B), in agreement with previous observations on rapid nuclear translocation of p53 in NGF-treated PC12 cells (22). These data likely accounted for the increased p21\(^{WAF1}\) levels in NGF-treated S100B~PC12 cells (Fig. 4A). Nearly the same percentages of p53-positive nuclei (i.e., ~6 and 90%) were measured in the case of S100B~PC12 cells after 9 h of culture in the absence and presence of NGF, respectively (Fig. 7B). By contrast, virtually no cells exhibited p53 nuclear staining at 24 h under any of the above-mentioned conditions (Fig. 7B), in agreement with previous observations on transient nuclear translocation of p53 in NGF-treated PC12 cells.

Western blotting. S100B expression results in Akt activation, whereas NGF inactivates Akt almost completely in S100B~PC12 cells but is less able to do so in S100B~cells. B–D, PC12 cells were cultivated for 6 days without culture medium renewal (with the exception of doxycycline, which was added every other day where required), and then cells were serum-starved for 2 days before solubilization and Western blot analysis (B) of phosphorylated and total Akt, p21\(^{WAF1}\), and phosphorylated and total Rb (left panels). Levels of phosphorylated Akt, p21\(^{WAF1}\), and phosphorylated Rb were normalized to total Akt, tubulin, and total Rb, respectively (right panels). C and D show a phase-contrast analysis of the same cells described in B. A larger extent of Akt activation can be seen in serum-starved S100B~PC12 cells, which is accompanied by increased p21\(^{WAF1}\) levels, increased Rb phosphorylation, and a larger cell number, compared with parallel S100B~cells (B–D). These data suggest that expression of S100B can support mitogen-independent PC12 cell survival and/or proliferation via Akt-induced accumulation of p21\(^{WAF1}\) and increase in Rb phosphorylation. E, conditions were the same as those described in B–D, except that cells were subjected to FACS analysis to determine the percentage of cells in the G0-G1 (A), S (B), and G2 + M (C) phases of the cell cycle. The percentage of S100B~PC12 cells in S phase was greater than that of S100B~cells, irrespective of the presence or absence of NGF, and a smaller percentage of apoptotic cells was measured in S100B~cells. Results are the averages of three experiments ± S.D. (B, right panels, and E). *, significantly different from + DOX condition, \(p < 0.01\). **, significantly different from + DOX condition, \(p < 0.001\). One representative experiment is shown (A, B, left panels of C, and D). Bar in D = 50 μm.
A representative experiment is shown (from matched H11002, the absence and presence of LY294002, whereas no p53 nuclear staining was detected under the other experimental conditions tested. Moreover, NGF induced PC12 cell differentiation after 3 days of treatment of S100B− cells (see Fig. 6A), and these cells exhibited no p53 nuclear staining (Fig. 7B). In contrast, parallel S100B− cells showed little differentiation (data not shown; also see Fig. 3G) while exhibiting p53 nuclear staining (~30% of them) (Fig. 7B). Our interpretation of these findings was that whereas no relationship appeared to exist between S100B expression and p53 nuclear translocation (in the absence of NGF) in the time interval investigated and under the experimental conditions used, S100B− PC12 cells attempted to respond to NGF anyway, albeit unsuccessfully. NGF-induced nuclear translocation of p53 in S100B− cells likely reflected the attempt of (a significant but minor percentage of) PC12 cells to cease proliferating and restart differentiating, an attempt destined to abort, as indicated by the higher proliferation rate of S100B− cells and the smaller number of differentiated S100B+ cells, compared with S100B− cells (see Figs. 2D, 3, D–F, and 6, A and C).

S100B Effects in PC12 Cells Do Not Depend on an Autocrine Activity of Released S100B−S100B− has been shown to be released by astrocytes (39) and to promote neuronal survival under stress conditions and stimulate neurite outgrowth via interaction with the receptor for advanced glycation end products (18). Thus, in principle, all or part of the effects of (over)expression of S100B in PC12 cells described above might depend on an autocrine interaction of released S100B with the receptor for advanced glycation end products or another PC12 cell surface receptor. To examine this possibility, S100B-transfected PC12 cells were cultivated for 6 days in the absence or presence of doxycycline ± an anti-S100B antibody shown to efficaciously block extracellular S100B (4) and then subjected to FACS analysis to determine the fraction of cells in the single phases of the cell cycle. For each pair of cell samples (i.e. S100B+ PC12 cells cultivated in the presence of non-immune IgG or anti-S100B antibody and S100B− PC12 cells cultivated in the

Fig. 7. S100B does not affect p53. A, conditions were the same as those described in Fig. 4, A and B, except that cells were solubilized and analyzed for levels of p53 by Western blotting. Shown is one representative experiment of three. B, conditions were the same as those described in Figs. 4, A and B, except that cells were fixed at the indicated times and analyzed by subcellular localization of p53 by indirect immunofluorescence using an anti-p53 antibody. For each experimental condition, 20 random fields were analyzed for p53 nuclear staining. Results are expressed as the percentage of cells displaying p53 nuclear staining and are the average of three independent experiments ± S.D.
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FIG. 8. S100B does not act on PC12 cells in an autocrine manner. A–D, PC12 cells were cultivated for 6 days in the absence or presence of doxycycline and either 50 μg/ml of non-immune IgG or 50 μg/ml of a polyclonal anti-S100B antibody. By phase-contrast microscopy, no differences can be seen in the density or morphology of S100B immune IgG or polyclonal anti-S100B antibody, compared with S100B of the absence or presence of non-immune IgG or anti-S100B antibody. *, significantly different from the + DOX condition, p < 0.01. E and F, PC12 cells were cultivated for 2 days in serum-free medium in the presence of doxycycline plus (F) or minus (G) 500 nM S100B and analyzed by phase-contrast microscopy. Bars in D and G = 50 μm.

In the case of S100B, no differences could be detected as investigated by both phase-contrast microscopy (Fig. 8, A–D) and FACS analysis (Fig. 8E). Also, no effects of anti-S100B antibody on NGF-induced PC12 cell differentiation could be detected (data not shown). On the other hand, administration of recombinant S100B to PC12 cells resulted in neurite extension (Fig. 8F), in agreement with previous work (18, 40–42), and administration of S100B along with an anti-S100B antibody blocked the neurite extension activity of S100B (data not shown). Thus, the effects of S100B (over)expression in PC12 cells on PC12 cell proliferation and responsiveness to NGF could not be attributed to an autocrine activity of released S100B.

DISCUSSION

It has long been known that the levels of the Ca2+-modulated protein S100B are much higher in tumor cells compared with their normal counterparts, and S100B has been implicated in the regulation of progression from the G1 phase to the S phase of the cell cycle (1, 2). A number of observations indicate that a causal relationship might exist between the levels of intracellular S100B and cell proliferation: (i) inhibition of S100B synthesis in astrocyte cell lines results in a reduced proliferation rate (5); (ii) S100B binds and stimulates Ndr, a kinase implicated in cell cycle progression (10); and (iii) S100B binds the tumor suppressor protein p53, inhibiting its phosphorylation and oligomerization (6–9). Recently, S100B was shown to block p53 transcriptional activity (8), suggesting that intracellular S100B might cause increased cell proliferation by interfering with p53 activity. However, no evidence was presented that accumulation of S100B within cells actually resulted in an enhanced proliferation and reduced ability to differentiate or maintain a differentiated state. In fact, previous work on possible blockade of p53 activity by S100B was performed on cells that do not express S100B or p53 and had been transfected with the S100B and p53 genes, and no analysis of proliferation of cells transfected with the S100B and/or p53 genes was conducted (8). We analyzed possible relationships between S100B accumulation and cell proliferation by inducing the expression of S100B in a cell type, the rat pheochromocytoma cell line PC12, that does not express the protein, differentiates into neuronal-like cells when exposed to the neurotrophin NGF, and expresses endogenous p53. We used PC12 cells stably transfected with S100B, and S100B was expressed by the Tet-Off technique (17), which allowed us to use the same cell population in analyses aimed at investigating the effects of S100B expression, in which the only varying parameter was the presence or absence of doxycycline in the culture medium. S100B-transfected PC12 cells, which did not express S100B (mRNA and protein) when cultivated in the presence of relatively high levels of doxycycline, did express it in the presence of extremely reduced amounts or in the absence of doxycycline (Fig. 1). These cells behaved identically to wild-type cells in terms of proliferation and responsiveness to NGF treatment, provided doxycycline was present in the culture medium. By contrast, accumulation of S100B resulted in an increase in PC12 cell survival and proliferation and reduction of apoptosis and responsiveness to the anti-proliferative and differentiating effect of S100B in Cell Proliferation.
of NGF (Figs. 2 and 3, A–F). On the other hand, whereas differentiated PC12 cells could be induced to express S100B, this event was not associated with overt phenotypic changes at least 4–6 days after induction of S100B expression (Fig. 3K), pointing to inability of S100B to reverse NGF-induced PC12 cell differentiation, at least during the time period investigated. Moreover, only a small percentage of PC12 cells that had been induced to express S100B for 6 days in the continuous presence of NGF underwent differentiation upon switching from the −DOX to the +DOX condition (Fig. 3, L and N), suggesting that the levels of S100B had to decrease below a certain threshold for the differentiating effect of NGF to take place. These results were obtained provided the culture medium was not renewed (with the exception of doxycycline and NGF, where appropriate); only on accumulation of relatively large amounts of S100B in PC12 cells cultivated in the presence of reduced amounts of extracellular mitogens (obtained by omitting serum renewal) did cell survival and proliferation increase and responsiveness to NGF treatment decrease. Thus, expression of S100B was not sufficient for increased PC12 cell proliferation and reduced responsiveness to NGF. Moreover, the expression of relatively small amounts of S100B, such as those attained after 2 days of culture in the −DOX condition (Fig. 1B), or expression of relatively large amounts of the protein (Fig. 1B) in cells cultivated in the presence of high levels of extracellular mitogens (obtained by renewing the culture medium every other day) did not cause obvious changes in terms of cell survival, cell proliferation, or cell responsiveness to NGF (Figs. 2E, 3, H and J, and 4, D–J). Collectively, these data suggested that (i) S100B was able to support PC12 cell proliferation, despite the decreased amount of extracellular mitogens; (ii) the inability of S100B+ PC12 cells to differentiate in response to NGF depended on the inability of these cells to escape from the cell cycle and/or interference of S100B with signaling pathways operating downstream of NGF receptors; and (iii) expression of S100B per se was not sufficient for de-differentiation of differentiated PC12 cells.

Under our experimental conditions (i.e. no culture medium renewal), S100B+ PC12 cells displayed larger amounts of p21\(^{WAF1}\) and phosphorylated Rb compared with S100B− PC12 cells (Fig. 4A). Thus, a causal relationship might exist among S100B accumulation in PC12 cells, increased levels of p21\(^{WAF1}\), increased extent of Rb phosphorylation, and increased cell proliferation. The increased levels of p21\(^{WAF1}\) in S100B+ cells expressed in the present study is in contrast to what has been observed in other cell types, in which down-regulation of p21\(^{WAF1}\) expression was detected after S100B (over)expression (8). However, our data show that S100B− PC12 cells responded normally to NGF in terms of nuclear translocation of p53 (in that NGF induced a rapid and transient nuclear translocation of p53), up-regulation of p21\(^{WAF1}\), reduction of the extent of Rb phosphorylation, proliferation arrest, and phenotypic differentiation. Also, as mentioned earlier, the levels of S100B in PC12 cells were regulated by the concentration of doxycycline in the medium, i.e. the same cell type was induced to express S100B simply by omitting doxycycline from the culture medium. Thus, at present, we attempt to explain this discrepancy by noting that our experiments were done on PC12 cells that had been cultivated for 6 days without serum renewal, whereas in the cited work, analyses were performed on H1299 human large carcinoma cells and MCF7 human breast cancer cells 48 h after transfection with the S100B and p53 genes (8). However, we cannot exclude the possibility that S100B might use different mechanisms to regulate cell proliferation in different cell types. For example, inhibitory effects of (overexpressed) S100B on the tumor suppressor activity of p53 might be restricted to neoplastic cells, as suggested by recent data showing that in primary malignant melanoma cells, p53 induces S100B expression, which in turn down-regulates p53 expression (43).

It is known that p21\(^{WAF1}\) acts as an inhibitor of cdk2, a kinase with the ability to phosphorylate Rb, thereby inhibiting cell proliferation (14, 15, 25, 29). However, p21\(^{WAF1}\) stimulates the activity of cdk4/cdk6 (14–16), i.e. kinases that phosphorylate Rb, which, in its phosphorylated form, detaches from transcription factors of the E2F family, thereby canceling its inhibitory effects on these transcription factors and allowing them to promote cell proliferation (14, 25). In addition, high levels of p21\(^{WAF1}\) have been observed in highly aggressive tumors (29), which is in contrast to the notion that p21\(^{WAF1}\) inhibits cell proliferation. Our data showing that overexpression of S100B in PC12 cells results in increased levels of p21\(^{WAF1}\), increased extent of Rb phosphorylation, and increased extent of p21\(^{WAF1}\)-cyclin D1 complex formation (Fig. 4, A–C) support the possibility that S100B might promote PC12 cell proliferation via a p21\(^{WAF1}\)/cyclin D1/ckd4/Rb/E2F pathway. A similar mechanism could be acting in at least certain neuronal tumor cells, in which accumulation of S100B above a certain threshold under certain conditions might contribute toward tumor progression. Although commonly referred to as a glial marker, S100B is normally expressed in many neuronal populations (44–48), including noradrenergic neurons (48). In addition, transient expression of S100B in neuroblasts might help to support their proliferation and confer resistance to apoptotic stimuli during development.

Our data also show that expression of S100B results in increased p21\(^{WAF1}\) levels, increased Rb phosphorylation, and stimulation of PC12 cell proliferation under conditions in which serum factors with mitogenic activity had been reduced (e.g. by avoiding serum renewal for 6 days), but not under conditions in which their levels are being kept constant (e.g. by serum renewal every other day) (Fig. 4D). This suggests that overexpression of S100B might make PC12 cells independent of extracellular mitogens, i.e. S100B, if present above a given threshold, is able to support cell proliferation in the presence of (and despite) reduced amounts of extracellular mitogens by augmenting p21\(^{WAF1}\) levels and increasing Rb phosphorylation. Data obtained with serum-starved S100B− PC12 cells (Fig. 5, B–E) support this conclusion. By contrast, in the presence of relatively high and constant levels of serum growth factors, as obtained by renewing the culture medium every other day, any S100B effects on PC12 cell proliferation might be obscured by the activity of serum mitogens. Supporting this possibility was the observation that the percentage of viable S100B− PC12 cells in a survival assay was nearly constant, irrespective of the amount of the S100B accumulated, and became significantly higher in PC12 cells allowed to accumulate S100B for 6 days before the assay, compared with matched S100B− PC12 cells (Fig. 2E). By contrast, in parallel experiments in which the culture medium was renewed every other day, no differences were registered in terms of the number of viable S100B− and S100B− PC12 cells (data not shown). Also, the role and importance of serum mitogens in PC12 cell proliferation are highlighted by the observation that very small and similar percentages of differentiated cells could be detected in both S100B− and S100B− PC12 cells exposed to NGF when the culture medium was renewed every other day and that a high cell density was seen irrespective of expression or non-expression of S100B under the same conditions (Fig. 4, E–J).

We also observed that S100B− PC12 cells exhibited a higher level of phosphorylated Akt compared with S100B− PC12 cells (Fig. 5, A and B). Because Akt was reported to phosphorylate
p21WAF1, thereby increasing p21WAF1 stability and favoring p21WAF1 association with cyclin D1-cdk4 (31–33), we reasoned that S100B might cause its effects in PC12 cells via Akt-dependent p21WAF1 phosphorylation. Consistently, the blockade of Akt phosphorylation by means of the PI3-K inhibitor LY294002 strongly reduced the S100B-induced activation of Akt, hyperphosphorylation of Rb, and stimulation of proliferation as well as S100B-dependent inhibition of NGF-induced PC12 cell differentiation (Fig. 6, A–F). Thus, we concluded that S100B might activate Akt with a resulting increase in p21WAF1 levels, which, combined with Akt-dependent p21WAF1 phosphorylation and stimulation of formation of p21WAF1-cyclin D1-cdk4 complex (30, 32), might account for both effects of S100B in PC12 cells, i.e. stimulation of proliferation and inhibition of NGF-induced differentiation. Supporting this mechanism of action was the observation that larger amounts of cyclin D1 co-immunoprecipitated with p21WAF1 in S100B+ PC12 cells compared with S100B– PC12 cells (Fig. 4C).

We could confirm that NGF dramatically reduced Akt phosphorylation in S100B– PC12 cells, and we found that it was less able to do so in S100B+ PC12 cells, at least in PC12 cells cultured for 6 days without culture medium renewal (Fig. 5A). With shorter cultivation times (e.g. 3 days), although S100B significantly increased Akt phosphorylation in both the absence and presence of NGF, NGF did not cause Akt inactivation (Fig. 5A), in agreement with the known ability of NGF to determine early and transient PC12 cell proliferation and confer resistance against apoptotic stimuli on PC12 cells via PI3-K/Akt activation (35–38). However, NGF up-regulated p21WAF1 expression (Fig. 4A), likely via p53 activation (22–24) (also see Fig. 7B). On the other hand, inhibition of Akt activation by means of the PI3-K inhibitor LY294002 strongly reduced the S100B-induced hyperphosphorylation of Rb (Fig. 6F), an event that was accompanied by a reduction of proliferation of S100B+ PC12 cells and by similar extents of NGF-induced differentiation in S100B– and S100B+ PC12 cells (Fig. 6, A–E). Thus, in S100B+ PC12 cells, a causal relationship appeared to exist between Akt activation and increased levels of Rb phosphorylation, between Rb hyperphosphorylation and cell proliferation, and between Akt activation and reduced NGF ability to induce PC12 cell proliferation arrest and differentiation. In conclusion, we propose the following: (i) S100B activates Akt; (ii) S100B-induced Akt activation results in increased levels of p21WAF1, likely due to increased stability and phosphorylation of p21WAF1 (30–33); and (iii) this process might favor the formation of cyclin D1-cdk4-p21WAF1 complex, with consequent increase in PC12 cell proliferation via hyperphosphorylation of Rb.

The same mechanism could explain the reduced sensitivity of S100B+ PC12 cells to NGF; these cells might respond poorly to NGF because of their inability to escape from the cell cycle. The possibility that this might be the case is also demonstrated by the finding that a 4–6-day treatment with NGF, which is known to cause a rapid and transient activation of p53 and produce PC12 cell proliferation arrest and differentiation (22–24) (also see Figs. 2, 3, D and F, and 5E), determined p53 nuclear translocation in ~30% of S100B+ PC12 cells, whereas no such event occurred in S100B– cells (Fig. 7B). Thus, S100B+ PC12 cells attempted to escape from the cell cycle (i.e. to re-enter G0 phase) and differentiate in coincidence with each NGF pulse, as also indicated by the reduced proliferation rate of S100B+ PC12 cells exposed to NGF compared with S100B+ PC12 cells not exposed to NGF (Fig. 2D) and the different phenotype of the former cells (which exhibit short cell processes) compared with the latter ones (which are round) (compare Fig. 3E with Fig. 3B). However, such an attempt was unsuccessful, likely due to the ability of S100B to increase Rb phosphorylation. In fact, data in Fig. 4B suggest that S100B and NGF counterbalanced each other action on Rb phosphorylation, with the result that in the presence of NGF, S100B+ PC12 cells neither proliferated (Fig. 2D) nor differentiated (Fig. 3, E and F) to the same extent as in the absence of NGF (Figs. 2, A–D, and 3, D and F).

We identified Akt as a common denominator of effects of S100B (over)expression in PC12 cells; the ability of S100B to stimulate PC12 cell proliferation and inhibit NGF-induced PC12 cell differentiation appeared to rely on S100B-induced activation of Akt. As mentioned earlier, hyperactivation of Akt in PC12 cells was shown to result in inhibition of NGF-induced differentiation (34). We could exclude a direct S100B-dependent activation of Akt because inhibition of the Akt upstream kinase, PI3-K, reduced both Akt activation and S100B-induced effects (Fig. 6). Further analyses are required to elucidate the mechanism by which S100B activates PI3-K/Akt as well as the molecular mechanism by which (over)expression of S100B protects PC12 cells against oxidant-induced apoptosis.

Lastly, we could exclude the possibility that effects of S100B (over)expression in PC12 cells might result from prior release of the protein and interaction of released S100B with the receptor for advanced glycation end products or another cell surface receptor. In fact, S100B+ PC12 cells behaved the same way irrespective of whether they had been cultivated in the presence of an anti-S100B antibody shown previously to neutralize extracellular S100B (4) or in the presence of non-immune IgG (Fig. 8). In addition, whereas extracellular S100B was shown to stimulate neurite outgrowth, i.e. neuronal differentiation (18, 40–44), and S100B+ PC12 cells extended neurites when exposed to administered S100B (Fig. 8F), in the present case (over)expression of S100B resulted in a reduced PC12 cell differentiation. Incidentally, data in Fig. 8 suggested that the simple expression or overexpression of S100B in a given cell type does not necessarily result in S100B release.

Our data provide evidence that overexpression of S100B in PC12 neuronal cells has the potential to increase their proliferation and reduce their responsiveness to NGF treatment. S100B brings about these effects by activating PI3-K/Akt via an unknown mechanism and increasing the levels of p21WAF1, the extent of Rb phosphorylation (likely by cyclin D1-cdk4), and the extent of cyclin D1-cdk4 complex formation in a PI3-K/Akt-dependent manner. This S100B activity might suggest a role of the protein in the regulation of cell proliferation and differentiation during development and, potentially, in tumor progression. Current work shows that overexpression of S100B in a myoblast cell line also results in increased proliferation and reduced differentiation, suggesting that stimulation of cell proliferation and inhibition of cell differentiation might be general properties of S100B.

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