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Association of S100B with intermediate filaments and microtubules in glial cells

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Abstract

Previous in vitro studies have shown that the Ca^{2+}-regulated S100B protein modulates the assembly-disassembly of microtubules (MTs) and type III intermediate filaments (IFs). In the present report, by double immunofluorescence cytochemistry S100B was localized to both GFAP/vimentin IFs and MTs as well as to centrosomes in U251 glial cells. In cells treated with the MT-depolymerizing agent, colchicine, S100B remained associated with the rearranged GFAP IFs throughout the cell and, at the cell periphery, vimentin IFs. In cells treated with the MT stabilizing agent, taxol, S100B followed partly the rearrangement of MTs and partly the rearrangement of IFs. Under the latter condition, bundles of MTs with their associated S100B appeared surrounded and/or flanked by rearranged IFs with their associated S100B. Colocalization of S100B with closely arranged IFs and MTs was best evident in cells manipulated with taxol and in triton-cytoskeletons. In these cases, MTs and their associated S100B appeared surrounded and/or flanked by and/or intermingled with IFs and their associated S100B. Also, a preferential association of S100B with GFAP vs. vimentin IFs could be observed near the nucleus where colocalization of S100B with MTs was also maximal. Condensation of IFs and alteration of the MT network caused by treatment of cells with the phosphatase inhibitor, okadaic acid, resulted in a concomitant condensation/alteration of the S100B immunoreactivity. The present results lend support to the possibility that S100B may be an important factor implicated in the regulation of the dynamics of MTs and IFs.

Keywords: S100B; Intermediate filament; Microtubule

1. Introduction

S100B, formerly known as S100β, is a member of a multigenic family of Ca^{2+}-binding proteins of the EF-hand type which have been implicated in Ca^{2+} signal transduction (for reviews see [1–3]). S100B is expressed in a large variety of cell types and is abundant in glial cells where it has been localized to the cytoplasm, plasma membranes and intracellular membranes, as well as to cytoskeleton constituents [4–9]. S100B has been implicated in the regulation of several intracellular activities [1–3]. Inhibition of microtubule (MT) assembly and stimulation of MT disassembly ([10], and Refs. therein), stimulation of a brain aldolase activity [11], a phosphoglucomutase activity [12] and a membrane-bound guanylate cyclase activity [13,14], and inhibition of the phosphorylation of a number of substrate (e.g. MARCKS,

Abbreviations: BSA, bovine serum albumin; GFAP, glial fibrillary acidic protein; IF, intermediate filament; MT, microtubule; PBS, phosphate buffered saline, pH 7.4

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τ proteins, caldesmon, GAP-43, neurogranin, MyoD, p53, and annexin II) [1–3] are among the intracellular activities attributed to S100B. Also, inhibition of S100B synthesis in a glial cell line by means of antisense oligonucleotides resulted in a more organized microfilament network, suggesting the possibility that S100B might be implicated in the control of the state of assembly of microfilaments [15].

Recently, we reported that S100B and S100A1 inhibit the assembly of glial fibrillary acidic protein (GFAP), the subunit of type III intermediate filaments (IFs) specific to glial cells, and desmin, the type II IF subunit specific to muscle cells, and promotes GFAP and desmin IF disassembly in a Ca^{2+}-dependent manner by binding stoichiometrically and with a high affinity to and sequestering unassembled GFAP and desmin, respectively [16–18]. We also identified the putative S100B- and S100A1-binding site on GFAP and desmin as a stretch of residues in the GFAP and desmin N-terminal domain [19,20] that harbors the so-called RP-box motif, a sequence critical to GFAP and desmin assembly into filaments [21]. All these data suggest that S100B in glial cells and S100A1 in muscle cells might play a role in the regulation of IF dynamics.

In spite of the above biochemical evidence, little is known about the S100B-type III IF and S100B-MT interactions in glial cells. As mentioned above, circumstantial evidence indicates that S100B associates with IFs in cultured Schwann cells and MT structures [4–9]. We addressed the functional relationships between S100B and IFs and MTs in vivo by analyzing the distribution of S100B in a glial cell line under various experimental conditions. Our results indicate that: (1) S100B is distributed into at least two pools, one associated with IFs, and a second one associated with the centrosomes and MTs; (2) S100B associates with GFAP IFs throughout the cell and with vimentin IFs at the cell periphery; (3) the fraction of S100B that is associated with glial IFs follows the IF rearrangement induced by colchicine and the fraction of MT-associated S100B follows the MT rearrangement induced by taxol while maintaining its association with IFs; and (4) treatment of cells with the phosphatase inhibitor, okadaic acid, causes depolymerization of both IFs and MTs with concomitant and coincident redistribution of S100B. The present data add to the possibility that S100B may have a role in the regulation of the state of assembly of both IFs and MTs in vivo.

2. Materials and methods

2.1. Materials

U251 MG-NIH glioma cells were a generous gift from Dr. S.L. Hubbard (Toronto, Ontario, Canada). The monoclonal anti-S100B antibody (clone SH-B1), monoclonal anti-α-tubulin antibody (clone DM1A), Cy3-conjugated monoclonal anti-β-tubulin antibody (clone TUB 2.1), Cy3-conjugated monoclonal anti-vimentin antibody (clone V9), fluorescein-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG colchicine, taxol, *p*-phenylenediamine were from Sigma (Milan, Italy); monoclonal anti-vimentin antibody (clone V9) from Boehringer (Mannheim, Germany); polyclonal anti-S100B antiserum from SWant (Bellinzona, Switzerland); polyclonal anti-GFAP antiserum from Dakopatts (Denmark). MEM α-medium, fetal bovine serum, and normal mouse serum were from Gibco BRL (Life Technologies, Paisley, UK). All other reagents were analytical grade reagents.

2.2. Cell culture and immunofluorescence analyses

U251 glial cells were cultured in α-MEM with L-glutamine supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a H_2O-

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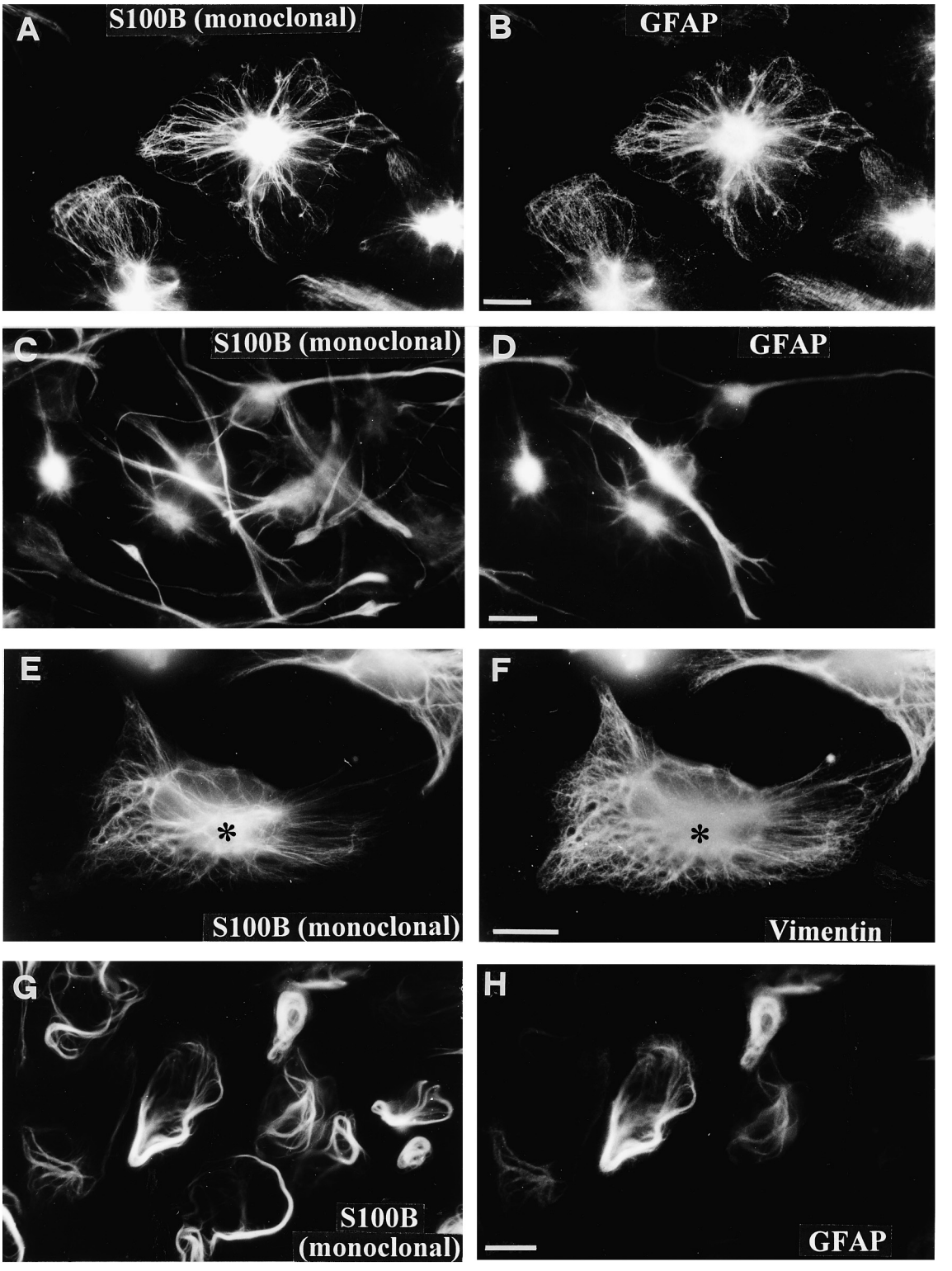
Fig. 1. S100B is colocalized with IFs in U251 MG-NIH glioma cells. By double immunofluorescence, a monoclonal anti-S100B antibody (A,C,G) decorates GFAP IFs (B,D,H) in control cells (A,B), cells cultivated for 3 days in the absence of serum (C,D), and cells treated with colchicine (G,H). Note that colchicine treatment causes bundling and collapse of IFs (H) and that S100B colocalizes with bundles and collapsed IFs (G). Cells cultivated in the absence of serum are characterized by long processes (C,D). Note the mosaic expression of GFAP (D) in contrast to the expression of S100B in nearly all cells (C). S100B (E) is also colocalized with vimentin IFs (F), but colocalization is more evident at the cell periphery. Near the nucleus (asterisk), the S100B-decorated IFs (likely GFAP IFs and/or MTs – see below) are found on a different focus plane with respect to vimentin IFs. Bar = 5 μm.


A
S100B (monoclonal)
B
GFAP
C
S100B (monoclonal)
D
GFAP
E
S100B (monoclonal)
F
Vimentin
G
S100B
(monoclonal)
H
GFAP

saturated 5% CO_2 atmosphere at 37°C. U251 glial cells were plated on glass coverslips (13 mm in diameter) in 24-multiwell plates. Cells were seeded at 3×10^4 cells/well and cultured for 24 h. In some experiments, cells were grown in the presence of serum for 24 h followed by a 3 day incubation in the absence of serum. After washings with phosphate buffered saline (PBS), cells on coverslips were fixed with methanol at −20°C for 7 min, rinsed in PBS, treated with 0.1% (v/v) Triton X-100 in PBS for 5 min at room temperature, and rinsed in PBS. Non-specific binding of antibodies or antisera was blocked by a preliminary incubation of fixed cells with 3% (w/v) bovine serum albumin (BSA) and 1% (w/v) glycine in PBS. This was followed by an incubation at room temperature for 1 h with the appropriate primary antibody diluted in 3% (w/v) BSA in PBS. Dilutions of primary antibodies were as follows: monoclonal anti-S100B, 1/20; monoclonal anti-vimentin, 1/4; polyclonal anti-S100B antiserum, 1/20; monoclonal anti-α-tubulin antibody, 1/40; polyclonal anti-GFAP antiserum, 1/200; Cy3-conjugated monoclonal anti-β-tubulin antibody, 1/100; Cy3-conjugated monoclonal anti-vimentin antibody, 1/4000. After washings for a total of 20 min with 0.1% (v/v) Tween-20 in PBS, the appropriate secondary antibody (diluted 1/50 in 3% (w/v) BSA in PBS) was applied at room temperature for 1 h. For double indirect/indirect immunostaining the two above incubations were performed in sequence. For double indirect/direct immunostaining the primary and secondary antibodies were used in sequence as described above, then cells were sequentially incubated for 1 h with 10% normal mouse serum in 3% (w/v) BSA in PBS and the appropriate Cy3-conjugated monoclonal antibody for 1 h at room temperature. The coverslips were then rinsed with 0.1% (v/v) Tween-20 in PBS for a total of 20 min, air dried and mounted on microscope slides in 80% (v/v) glycerol, 1 mg/ml *p*-phenylenediamine as an antifading agent, 0.02% (w/v) NaN_3, in PBS. In control experiments, the primary anti-S100B antiserum was either omitted or preabsorbed with the S100B before use. Cells were viewed in a Leica DM RB fluorescence microscope using an oil-immersion 100× objective and photographed on a Kodak TMax 400 or TMax 3200 film.

For drug treatment of glial cells, colchicine (final concentration 10 μg/ml) was added to the medium for 20 h; taxol in dimethylsulfoxide was added to the medium to a final concentration of 10 μM and the cells were cultivated for an additional 24 h; okadaic acid was added to the medium to a final concentration of 1 μM and the cells were cultivated for an additional 30 min. Subsequent steps were as described above.

To obtain triton-cytoskeletons containing MTs, cells were extracted at 37°C for 30–40 s with 80 mM Pipes, pH 6.8, 0.1 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10% (w/v) glycerol, 0.5% (v/v) Triton X-100, and washed twice with the same buffer without Triton X-100 before fixation as above.

3. Results

3.1. S100B is associated with GFAP/vimentin IFs in U251 glial cells

By double immunofluorescence performed with a monoclonal anti-S100B antibody (Fig. 1A) and a polyclonal anti-GFAP antiserum (Fig. 1B), the S100B immunoreactivity in U251 glial cells was found in the form of a filamentous network that was nearly superimposed to that of the GFAP IF network. Whereas most, if not all cells were S100B-

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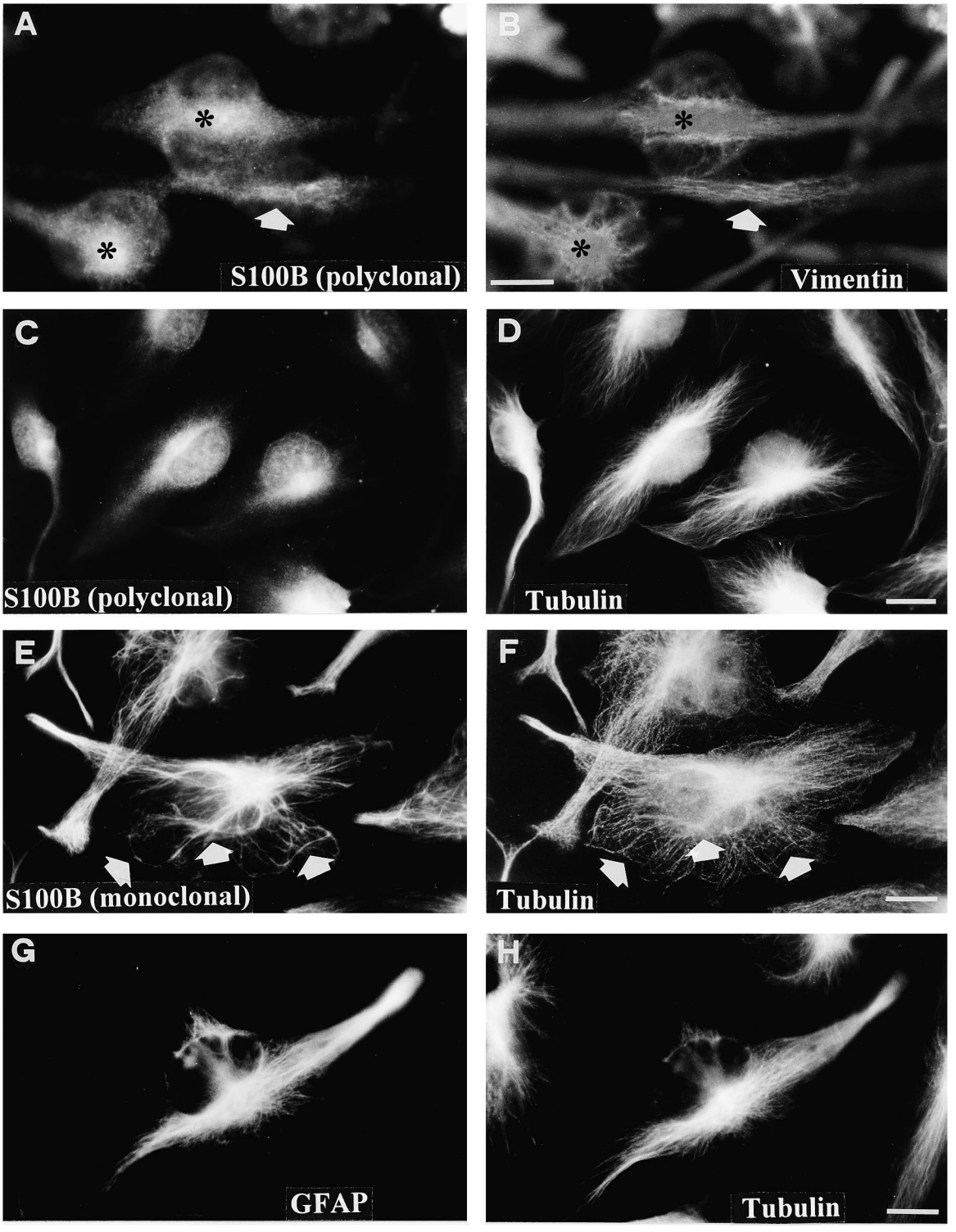
Fig. 2. S100B is colocalized with MTs in U251 MG-NIH glioma cells. By double immunofluorescence, a polyclonal anti-S100B antiserum (A) binds to a paranuclear structure (asterisks) that is found on a different focus plane with respect to vimentin IFs (B). This structure appears flanked and/or surrounded by vimentin IFs (B). This antiserum also binds to vimentin IF-associated S100B in cell processes (arrow). The above paranuclear structure is made of MTs as it can be labeled by a monoclonal anti-α-tubulin antibody (D) with which S100B is associated (C). By double, indirect/direct immunofluorescence, the monoclonal anti-S100B antibody (E) also decorates MTs (labeled by a monoclonal Cy3-conjugated anti-β-tubulin antibody) (F) near the nucleus and, to some extent, in the cell periphery (arrows). Extensive colocalization of GFAP IFs (G) and MTs (H) can be observed in a paranuclear position, as investigated by double immunofluorescence with a polyclonal anti-GFAP antiserum and a monoclonal anti-α-tubulin antibody. Note the mosaic distribution of GFAP. Bar = 5 μm.

positive, some cells did not express GFAP (not shown, but see below). In these cells the monoclonal anti-S100B antibody decorated a filamentous network as well. Mosaic expression of GFAP in glial cell lines was reported [22]. In U251 cells cultivated for 1 day in the presence of serum and for the next 2–3 days in the absence of serum (a condition in which cells proliferated at a reduced rate and were



characterized by long extensions), again the S100B immunoreactivity was in the form of a filamentous network (Fig. 1C) nearly superimposed to the GFAP IF network (Fig. 1D). Again some cells were GFAP-negative with persistence of the S100B immunoreactivity in the form of a filamentous network. These data suggest that a fraction of S100B may be associated with GFAP and, eventually, vimentin IFs (see below) in vivo. By double, indirect/direct immunofluorescence with the monoclonal anti-S100B antibody and a monoclonal anti-vimentin antibody, colocalization of S100B with vimentin IFs could also be observed at the cell periphery (Fig. 1E,F). Near the nucleus a relatively large area that was rich in GFAP IFs and their associated S100B (Fig. 1B) appeared to contain vimentin IFs on a different focus plane (compare Fig. 1E with Fig. 1F), suggesting a preferential association of S100B with GFAP rather than vimentin IFs in discrete parts of the cell.

To validate the conclusion that in U251 glial cells S100B is associated with GFAP and, at the cell periphery, vimentin IFs, cells were cultivated in the presence of colchicine (10 μg/ml) for 1 day before immunocytochemistry. It is known that MT depolymerization caused by colchicine results in a reorganization (bundling) and collapse of type III IFs around the nucleus [23,24]. Fig. 1G shows that in colchicine-treated cells the S100B immunoreactivity followed the destiny of the GFAP IF network (Fig. 1H), in agreement with the conclusion that a fraction of S100B may be associated with GFAP IFs in glial cells. In similar experiments performed with the anti-S100B monoclonal antibody and the anti-vimentin monoclonal antibody again association of S100B with vimentin IFs was best evident at the cell periphery (not shown). A polyclonal anti-S100B antiserum [25] also decorated bundles of collapsed IFs in cells treated with colchicine (not shown). In U251 glial cells treated with Triton X-100 plus a high KCl concentration, a condition in which the IF network is being preserved whereas MTs and microfilaments are being extracted [26], the S100B recognized by the monoclonal antibody was still associated with the GFAP/vimentin IF immunoreactivity (not shown).

3.2. S100B is associated with MTs in glial cells

A polyclonal anti-S100B antiserum [25] was also tested in double immunofluorescence experiments with a monoclonal anti-GFAP antibody (not shown), a monoclonal anti-vimentin antibody (Fig. 2A,B), or a monoclonal anti-tubulin antibody (Fig. 2C–F). The polyclonal anti-S100B antiserum, that decorated IFs in cell processes (Fig. 2A,B), appeared to bind mostly to an organized cell structure that was located on one side of the nucleus and/or running parallel to the long axis of the cell (Fig. 2A,B). This structure did not appear to consist of vimentin IFs since it was not decorated by the anti-vimentin antibody (Fig. 2B). Rather, this structure appeared surrounded and/or flanked by vimentin IFs, as could be observed by changing the focus plane. By contrast, the same structure appeared to contain GFAP IFs (see Fig. 2G,H). The S100B-positive, paranuclear structure decorated by the polyclonal anti-S100B antiserum (Fig. 2C,E) was also decorated by the anti-tubulin antibody (Fig. 2D,F). The paranuclear region rich in GFAP IFs, MTs, and their associated S100B also contained Golgi membranes which were detected using fluorescein-labeled wheat germ agglutinin (not shown). Golgi membranes could be decorated by the polyclonal anti-S100B antiserum (not shown, but see [6]). Experiments performed with the monoclonal anti-S100B antibody and the monoclonal anti-tubulin antibody, and with the

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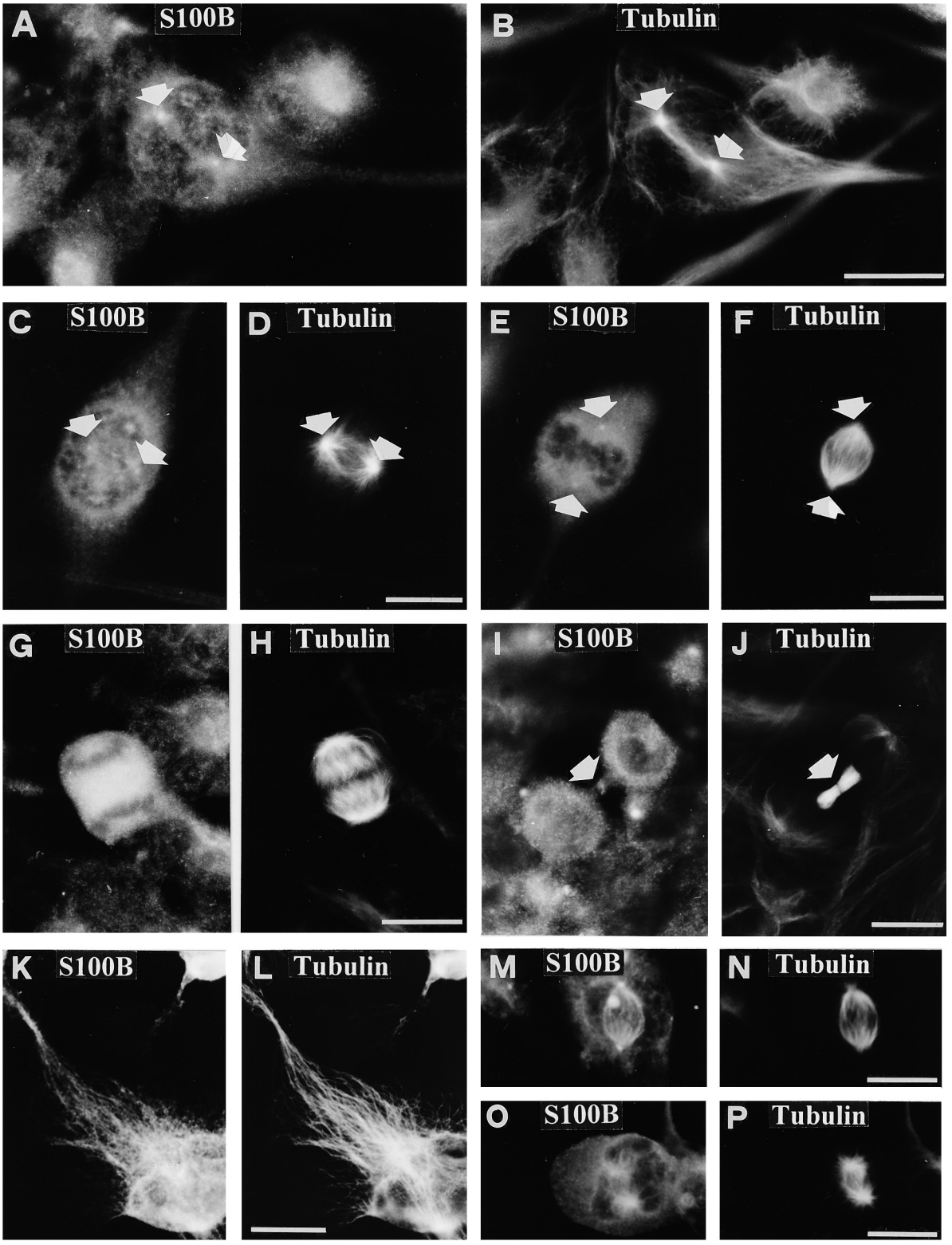
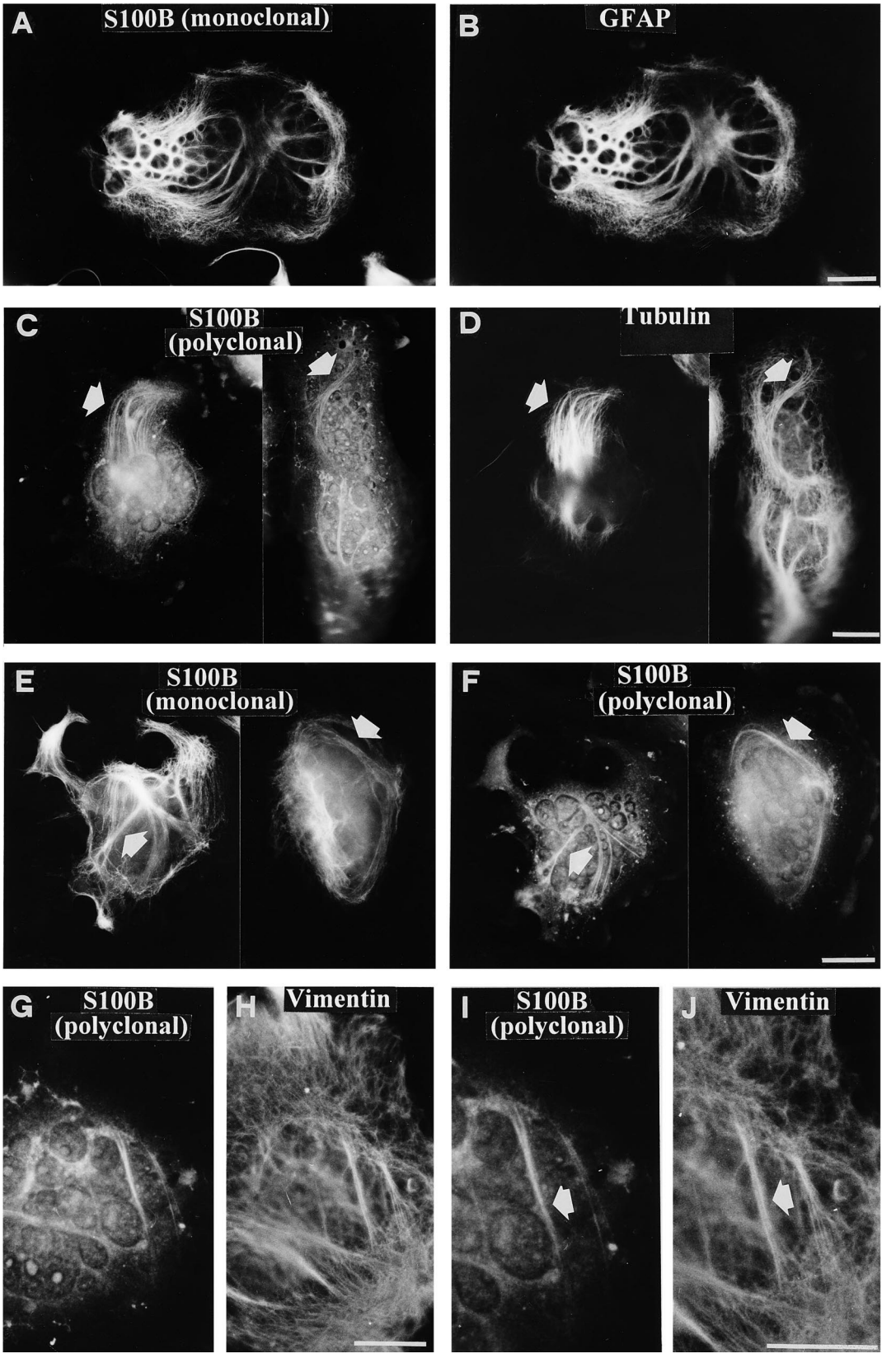
Fig. 3. Association of S100B with cytoplasmic, spindle and aster MTs, the centrosomes and the center part of the midbody in U251 MG-NIH glioma cells. Cells were double immunostained using the anti-S100B polyclonal antiserum (A,C,E,G,I,K,M,O) and the monoclonal anti-α-tubulin antibody (B,D,F,H,J,L,N,P). Intact cells (A–J) and triton-cytoskeletons (K–P) obtained under conditions that preserve MTs (see Section 2) were used in these experiments. S100B is found associated with the centrosomes (B,D,F) (arrows) in both interphase cells (A,B) and dividing cells (C–F). In prophase cells (C) S100B is also found diffusely around the nucleus. A similarly diffuse S100B staining can be seen in metaphase cells (E). In anaphase cells (G,H) S100B is found around separated chromosomes (G) where MTs are also found (H). Association of S100B with cytoplasmic MTs (K,L), spindle MTs (M,N) and aster MTs (O,P) is best evident in triton-cytoskeletons, suggesting that binding of the polyclonal anti-S100B antiserum to soluble and membrane-bound S100B in intact cells obscures detection of cytoskeleton-associated S100B. Bar = 5 μm.


A S100B
B Tubulin
C S100B
D Tubulin
E S100B
F Tubulin
G S100B
H Tubulin
I S100B
J Tubulin
K S100B
L Tubulin
M S100B
N Tubulin
O S100B
P Tubulin


A
S100B (monoclonal)
B
GFAP
C
S100B
(polyclonal)
D
Tubulin
E
S100B
(monoclonal)
F
S100B
(polyclonal)
G
S100B
(polyclonal)
H
Vimentin
I
S100B
(polyclonal)
J
Vimentin

monoclonal anti-tubulin antibody and the polyclonal anti-GFAP antiserum confirmed that near the nucleus and/or on one side of the nucleus S100B was colocalized with both, MTs and GFAP IFs (Fig. 2E–H). These experiments also showed that colocalization of S100B, as detected by the monoclonal anti-S100B antibody, with both GFAP IFs and MTs was most intense near the nucleus (Figs. 1A–D and 2). Away from this region, colocalization of MTs and S100B could also be detected at discrete cell sites (Fig. 2E,F, arrows). Fig. 2G,H shows extensive colocalization of MTs and GFAP IFs, particularly near the nucleus, i.e. in a region also rich in S100B as outlined above.

S100B, as detected using the anti-S100B polyclonal antiserum, was localized to centrosomes in both interphase (Fig. 3A,B) and dividing cells. In prophase cells (Fig. 3C,D), the polyclonal anti-S100B antiserum decorated the centrosomes and perinuclear structures. In metaphase cells, this antiserum decorated the centrosomes and a material associated with the mitotic spindle (Fig. 3E,F). In anaphase cells, S100B was detected in a pericentrosomal position and, to a larger extent, an equatorial position (Fig. 3G,H). In telophase cells, S100B was found all around the separated chromosomes and in the center part of the midbody (Fig. 3I,J).

Extraction of membranes and cytoplasmic proteins with Triton X-100 under conditions preserving MTs resulted in a clearer resolution of MT-associated S00B. The polyclonal anti-S100B antiserum distinctly decorated cytoplasmic MTs, in addition to perinuclear material containing tubulin, and spindle and aster MTs (Fig. 3K–P). By double immunofluorescence using the monoclonal anti-S100B antibody and the polyclonal anti-S100B antiserum, filamentous structures that were decorated by both antibodies were found to co-exist in the same cell along with filamentous structures preferentially decorated by individual antibodies (not shown).

To support the conclusion that S100B associates with both IFs and MTs, U251 cells were treated with the MT stabilizing drug, taxol, before immunocytochemistry. Under these conditions, the GFAP IF network appeared dramatically rearranged (Fig. 4B), as was the GFAP IF-associated S100B immunoreactivity detected by the monoclonal anti-S100B antibody (Fig. 4A). Taxol treatment caused the formation of bundles of MTs that were decorated by the polyclonal anti-S100B antiserum (Fig. 4C,D). In addition, the polyclonal anti-S100B antiserum also detected S100B between fragmented nuclear material (Fig. 4C). Here, S100B likely is associated with fragmented Golgi membranes, as suggested by coincident labeling of these structures by fluorescein-conjugated wheat germ agglutinin (not shown). As mentioned above, S100B is also found associated with the Golgi complex [6]. Also, in several instances the MTs bundles appeared surrounded and/or flanked by rearranged IFs. This was directly seen in experiments in which taxol-treated cells were reacted with the monoclonal anti-tubulin antibody and the anti-GFAP antiserum (not shown) and indirectly concluded by experiments performed with the monoclonal anti-S100B antibody and the polyclonal anti-S100B antiserum (Fig. 4E and F, respectively) as well as by experiments performed with the polyclonal anti-S100B antiserum (Fig. 4G,I) and the monoclonal anti-vimentin antibody (Fig. 4H,J). In these cases, the MT bundles and their associated S100B appeared surrounded and/or flanked by IFs and their associated S100B. An unrelated polyclonal antiserum

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Fig. 4. Double immunofluorescence analyses of S100B, tubulin, GFAP, and vimentin in U251 MG-NIH glioma cells grown in the presence of serum containing 10 μM taxol. Taxol causes a coincident rearrangement of S100B (A) and GFAP IFs (B) in cells expressing both proteins. S100B was detected with a monoclonal antibody and GFAP with a polyclonal antiserum (A,B). Taxol-induced bundles of MTs, detected with a monoclonal anti-α-tubulin antibody (arrows in D), are also S100B immunoreactive (arrows in C). The S100B immunoreactivity in C was detected with a polyclonal anti-S100B antiserum. Use of a monoclonal anti-S100B antibody (which binds to IF-associated S100B) (E) and a polyclonal anti-S100B antiserum (which binds to MT-associated S100B) (F) allows detection of partial colocalization of IFs and MTs in taxol-treated cells (arrows). Similar images can be obtained using a polyclonal anti-S100B antiserum (G) and a monoclonal anti-vimentin antibody (H). In several instances MT bundles (and their associated S100B) are flanked and/or surrounded by IFs (and their associated S100B) (arrows in I and J, that show a field in G and H, respectively, at a higher magnification). Bar = 5 μm.

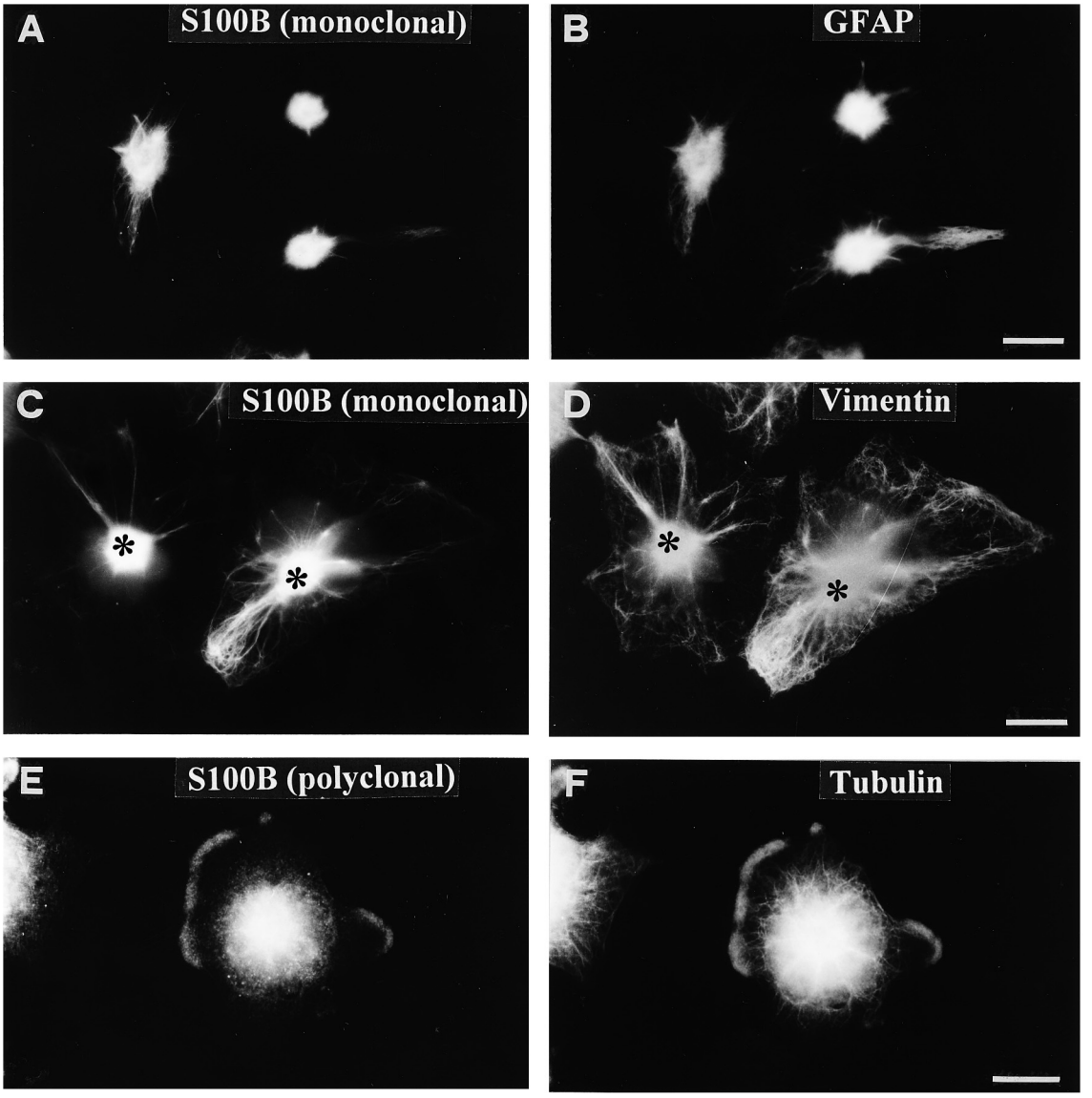

Fig. 5. Double immunofluorescence localization of S100B, tubulin, vimentin, and GFAP in okadaic acid-treated U251 MG-NIH glioma cells. Treatment of cells with the phosphatase inhibitor, okadaic acid, causes extensive disassembly of GFAP IFs (detected with a polyclonal anti-GFAP antiserum) and their condensation in a paranuclear position (B) likely due to GFAP hyperphosphorylation (see text). Under these conditions, S100B (detected with a monoclonal anti-S100B antibody) follows the GFAP IF rearrangement (A). Double, indirect/direct immunofluorescence performed with a monoclonal anti-S100B antibody (C) and a Cy3-conjugated vimentin monoclonal antibody (D) shows that vimentin IFs appear much less perturbed by okadaic acid treatment (compare D with B), whereas IF-associated S100B condensates in a paranuclear structure (asterisks in C) composed of GFAP IFs (see B) and/or MTs (see below). Again, note that the S100B-positive paranuclear structure (asterisks in C) is found on a different focus plane as compared with vimentin IFs (asterisks in D). MTs, detected with a monoclonal anti-α-tubulin antibody (F), also appear disassembled and/or condensed after okadaic acid treatment, and S100B, detected with a polyclonal anti-S100B antiserum, remains colocalized with these MTs under these conditions (E). Bar = 5 μm.

(anti-PGP 9.5) and a non-immune serum did not stain MTs or IFs in control or taxol-treated cells (not shown).

Treatment of cells with vinblastine, another MT-perturbing agent, resulted in collapsing of bundled IFs and their associated S100B (which was detected by both the monoclonal antibody and the polyclonal antiserum), and formation of typical MT paracrys-

tals which were not decorated by either anti-S100B antibody, but appeared surrounded by S100B (not shown).

3.3. Effects of okadaic acid on the distribution of S100B in U251 glial cells

The number and topographical distribution of IFs as well as their dynamics are regulated by phosphorylation of IFs and their unassembled subunits [27–29]. Accordingly, serine/threonine protein phosphatases are important in maintaining cytoskeletal integrity [30,31]. The phosphatase inhibitor, okadaic acid [32,33], caused disassembly of GFAP IFs and GFAP IF condensation in a paranuclear position in U251 glial cells (Fig. 5B) likely due to hyperphosphorylation of GFAP, with concomitant and coincident condensation of its associated S100B (Fig. 5A), whereas vimentin IFs appeared less sensitive to okadaic acid under the same conditions (Fig. 5D). Again, the perinuclear vimentin IF network was not coincident with the GFAP condensed material and its associated S100B (Fig. 5C). MTs also appeared perturbed by okadaic acid treatment, in that they appeared depolymerized to a remarkable extent (Fig. 5F). It is known that phosphorylation of MT-associated proteins results in disassembly of MTs and inhibition of MT formation [34,35]; thus, okadaic acid treatment might have resulted in hyperphosphorylation of MT-associated proteins. The paranuclear structure that was intensely decorated by the polyclonal anti-GFAP antiserum and the monoclonal anti-S100B antibody (Figs. 1–3) was also decorated by the monoclonal anti-tubulin antibody and the polyclonal anti-S100B antiserum under these conditions (Fig. 5E,F).

4. Discussion

The present data indicate that in U251 glial cells S100B is associated with at least two cytoskeleton constituents, i.e. GFAP IFs throughout the cell and/or vimentin IFs in the cell periphery, and MTs, including those found in asters and mitotic spindles, the centrosomes, and, in telophase cells, the center part of the midbody. Specifically, a fraction of S100B is found associated with GFAP/vimentin IFs and follows the rearrangements of IFs induced by colchicine, taxol, or okadaic acid treatment of glial cells. Another fraction of S100B is found associated with the centrosomes, the center part of the midbody in mitotic cells, and MTs, and follows the rearrangement of MTs induced by taxol or okadaic acid. The monoclonal anti-S100B antibody and the polyclonal anti-S100B antiserum used in the present study (which are commercially available) proved specific to S100B by Western blotting (not shown) and failed to decorate cell structures once preabsorbed with pure S100B, whereas it maintained its S100B-binding properties unaltered following preabsorption with pure S100A1 (not shown). The polyclonal anti-S100B antiserum was characterized in previous studies and found to be specific to S100B [25]. On the basis of the present findings we suppose that the monoclonal anti-S100B antibody mostly recognizes an epitope of IF-associated and, to some extent, MT-associated S100B. This epitope is less distinctly recognized by the polyclonal anti-S100B antiserum in intact cells, probably because of binding of this antiserum to a fraction of S100B that is associated with intracellular membranes [4,6,36]. In fact, the polyclonal anti-S100B antiserum decorates IFs in cells extracted with Triton X-100 plus high KCl and in cells treated with colchicine or vinblastine (not shown), decorates cytoplasmic and spindle and aster MTs in triton-cytoskeletons (Fig. 3K–P), and distinctly decorates bundles of MTs in taxol-treated cells (Fig. 4C,D). These observations suggest that in intact cells binding of the polyclonal antiserum to soluble and/or membrane-associated S100B obscures visualization of IF- and MT-associated S100B. In fact, solubilization of membranes with Triton X-100 under conditions that preserve MTs allowed detection of MT-associated S100B as investigated by means of the polyclonal anti-S100B antiserum (Fig. 3K–P).

By double immunofluorescence, the MTs decorated by the polyclonal anti-S100B antiserum appear surrounded and/or flanked by vimentin IFs and intermingled with GFAP IFs and their associated S100B, in both control and taxol-treated cells as well as in triton-cytoskeletons. Together with previous data documenting regulatory effects of S100B on the dynamics of both GFAP IFs and MTs in vitro [10,16], the present results strongly suggest that S100B may be positioned along and/or in close

proximity of IFs and MTs to take part in the regulation of their assembly state and/or to target some S100B ligands to discrete cell sites.

The presence of S100B on or close to the centrosomes, and the center part of the midbody appears interesting in light of the role of these structures as MT nucleation centers. Although S100B interferes with both the nucleation and the elongation of MTs in the presence of a few micromolar levels of free Ca^{2+} in vitro [10], its main effect consists of a retardation of the onset of MT formation, a decrease in the MT number concentration, and an increase in the mean MT length at pH 6.7, whereas an additional and marked, inhibitory effect on MT elongation can be seen at pH 7.2–7.4 [10]. The present identification of S100B on the centrosomes in both interphase and dividing cells and the center part of the midbody lends support to the possibility that S100B might play a role in the regulation of MT formation by acting at physiological sites of MT nucleation, where S100B might avoid excess tubulin polymerization. In this respect, it is important to note that S100B interacts with phosphocellulose-purified tubulin and inhibits purified tubulin polymerization in vitro [10,37]. In addition to its potential role as a regulator of MT formation, S100B might contribute to the rapid disassembly of MTs occurring at the onset of mitosis. In vitro, S100B causes a rapid and almost complete disassembly of preformed MTs in a Ca^{2+}- and pH-dependent manner, with lower Ca^{2+} concentrations needed with increasing pH values [10,38]. S100B might thus be one of the factors postulated to increase the Ca^{2+} sensitivity of polymerized tubulin at the onset of mitosis ([39], and Refs. therein).

The present data give evidence for the first time for structural relationships between S100B and two main cytoskeleton constituents, IFs and MTs, in a cell model system. Future studies should define the functional meaning of the association of S100B with IFs and MTs and the relationship between this association and transduction of the Ca^{2+} signal.

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References

[1] R. Donato, Perpectives in S100 protein biology, Cell Calcium 12 (1991) 713–726.
[2] D.B. Zimmer, E.H. Cornwall, A. Landar, W. Song, The S100 protein family: history, function, and expression, Brain Res. Bull. 37 (1995) 417–429.
[3] B.W. Schäfer, C.W. Heizmann, The S100 family of EF-hand calcium-binding proteins: functions and pathology, Trends Biochem. Sci. 21 (1996) 134–140.
[4] D. Cocchia, Immunocytochemical localization of S100 protein in the brain of adult rat. An ultrastructural study, Cell Tissue Res. 214 (1981) 529–540.
[5] D.B. Zimmer, L.J. Van Eldik, Levels and distribution of the calcium-modulated proteins S100 and calmodulin in rat C6 glioma cells, J. Neurochem. 50 (1988) 572–579.
[6] D.B. Zimmer, L.J. Van Eldik, Analysis of the calcium-modulated proteins, S100 and calmodulin, and their target proteins during C6 glioma cell differentiation, J. Cell Biol. 108 (1989) 141–151.
[7] T. Uchida, T. Endo, Immunoelectron microscopic demonstration of S100B protein-like in centriole, cilia, and basal body, J. Histochem. Cytochem. 36 (1988) 693–696.
[8] M.G. Rambotti, C. Saccardi, A. Spreca, M.C. Aisa, I. Giambanco, R. Donato, Immunocytochemical localization of S100ββ protein in olfactory and supporting cells of lamb olfactory epithelium, J. Histochem. Cytochem. 37 (1989) 1825–1833.
[9] M.G. Rambotti, A. Spreca, P. Leoncini, M. Estenoz, E. Costantino-Ceccarini, I. Giambanco, R. Donato, Detection of S100b protein in triton-cytoskeletons: an immunocytochemical study on cultured Schwann cells, J. Histochem. Cytochem. 38 (1990) 1583–1589.
[10] R. Donato, Calcium-independent, pH-regulated effects of S100 proteins on assembly-disassembly of brain microtubule proteins in vitro, J. Biol. Chem. 263 (1988) 106–110.
[11] D.B. Zimmer, L.J. Van Eldik, Identification of a molecular target for the calcium-modulated protein S100: fructose-1,6-bisphosphate aldolase, J. Biol. Chem. 261 (1986) 11424–11428.
[12] A. Landar, G. Caddell, J. Chessher, D.B. Zimmer, Identification of an S100A1/S100B target protein: phosphoglucomutase, Cell Calcium 20 (1996) 279–285.
[13] A. Margulis, N. Pazdnyakov, A. Sitaramayya, Activation of bovine photoreceptor guanylate cyclase by S100 proteins, Biochem. Biophys. Res. Commun. 218 (1996) 243–247.
[14] T. Duda, R.M. Goraczniak, R.K. Sharma, Molecular characterization of S100A1-S100B protein in retina and its activation mechanism of bovine photoreceptor guanylate cyclase, Biochemistry 35 (1996) 6263–6266.

[15] R.H. Selinfreund, S.W. Barger, M.J. Welsh, L.J. Van Eldik, Antisense inhibition of glial S100β production results in alteration in cell morphology, cytoskeletal organization, and cell proliferation, J. Cell Biol. 111 (1990) 2021–2028.
[16] R. Bianchi, I. Giambanco, R. Donato, S100 protein, but not calmodulin, binds to the glial fibrillary acidic protein and inhibits its polymerization in a Ca^{2+}-dependent manner, J. Biol. Chem. 268 (1993) 12669–12674.
[17] R. Bianchi, M. Verzini, M. Garbuglia, I. Giambanco, R. Donato, Mechanism of S100 protein-dependent inhibition of glial fibrillary acidic protein (GFAP) polymerization, Biochim. Biophys. Acta 1223 (1994) 354–360.
[18] M. Garbuglia, M. Verzini, I. Giambanco, A. Spreca, R. Donato, Effects of calcium-binding proteins ($S100a_o$, S100a, S100b) on desmin assembly in vitro, FASEB J. 10 (1996) 317–324.
[19] R. Bianchi, M. Garbuglia, M. Verzini, I. Giambanco, V.V. Ivanenkov, R.V.W. Dimlich, G.A. Jamieson Jr., R. Donato, S100 (α and β)-binding peptide (TRTK-12) blocks S100/GFAP interaction: identification of a putative S100 target epitope within the head domain of GFAP, Biochim. Biophys. Acta 1313 (1996) 258–267.
[20] M. Garbuglia, M. Verzini, R.V.W. Dimlich, G.A. Jamieson Jr., R. Donato, Characterization of type III intermediate filament regulatory protein target epitopes: S100 (β and/or α) binds the N-terminal head domain; annexin II_2-$p11_2$ binds the rod domain, Biochim. Biophys. Acta 1313 (1996) 268–276.
[21] J.E. Ralton, X. Lu, A.M. Hutcheson, R.A. Quinlan, Identification of two N-terminal non-alpha-helical domain motifs important in the assembly of glial fibrillary acidic protein, J. Cell Sci. 107 (1994) 1935–1948.
[22] C. Arcuri, V. Bocchini, P. Guerrieri, C. Fages, M. Tardy, PKA and PKC activation induces opposite glial fibrillary acidic protein (GFA) expression and morphology changes in a glioblastoma multiform cell line of clonal origin, J. Neurosci. Res. 40 (1995) 622–631.
[23] A. Ishikawa, R. Bischoff, H. Holtzer, Mitosis and intermediate-sized filaments in developing skeletal muscle, J. Cell Biol. 38 (1968) 538–555.
[24] E. Lazarides, The distribution of desmin (100 Å) filaments in primary cultures of embryonic chick cardiac cells, Exp. Cell Res. 112 (1978) 265–273.
[25] L.J. Van Eldik, W.S.T. Griffin, S100β expression in Alzheimer's disease: relation to neuropathology in brain regions, Biochim. Biophys. Acta 1223 (1994) 398–403.
[26] W.W. Franke, D. Mayer, E. Schmid, H. Denk, E. Borenfreund, Differences of expression of cytoskeletal proteins in cultured rat hepatocytes and hepatoma cells, Exp. Cell Res. 134 (1981) 345–365.
[27] E. Fuchs, K. Weber, Intermediate filaments: structure, function, and disease, Annu. Rev. Biochem. 63 (1994) 345–382.
[28] S. Heins, U. Aebi, Making heads and tails of intermediate filament assembly, dynamics and networks, Curr. Opin. Cell Biol. 6 (1994) 25–33.
[29] Y. Takai, N. Inagaki, O. Tsutsumi, M. Inagaki, Visualization and regulation of intermediate filament kinase activities, Cell Dev. Biol. 7 (1996) 741–749.
[30] J.E. Eriksson, D.L. Brautigan, R.D. Vallee, J. Olmsted, H. Fujiki, R.D. Goldman, Cytoskeletal integrity in interphase cells requires protein phosphatase activity, Proc. Natl. Scad. Sci. USA 89 (1992) 11093–11097.
[31] D.M. Toivola, R.D. Goldman, D.R. Garrod, J.E. Eriksson, Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments, J. Cell Sci. 110 (1997) 23–33.
[32] P. Cohen, The structure and regulation of protein phosphatases, Annu. Rev. Biochem. 58 (1989) 453508.
[33] S. Shenolikar, Protein serine/threonine phosphatases – new avenues for cell regulation, Annu. Rev. Cell Biol. 10 (1994) 55–86.
[34] L. Jameson, M. Caplow, Modification of microtubule steady-state dynamics by phosphorylation of the microtubule-associated proteins, Proc. Natl. Acad. Sci. USA 78 (1981) 3413–3417.
[35] G. Lindwall, D. Cole, Phosphorylation affects the ability of tau protein to promote microtubule assembly, J. Biol. Chem. 259 (1984) 5301–5305.
[36] R. Donato, B. Prestagiovanni, G. Zelano, Identity between cytoplasmic and membrane-bound S100 proteins purified from bovine brain, J. Neurochem. 46 (1986) 1333–1337.
[37] R. Donato, I. Giambanco, M.C. Aisa, Molecular interaction of S100 protein with microtubule proteins in vitro, J. Neurochem. 53 (1989) 441–446.
[38] R. Donato, Calcium-sensitivity of brain microtubule proteins in the presence of S100 proteins, Cell Calcium 6 (1985) 343–361.
[39] E.T. O'Brien, E.D. Salmon, H.P. Erickson, How calcium causes microtubule depolymerization, Cell Motil. Cytoskeleton 36 (1997) 125–135.