Cysteine Oxidation in the Mitogenic S100B Protein Leads to Changes in Phosphorylation by Catalytic CKII-α Subunit*

(Received for publication, May 20, 1997, and in revised form, November 19, 1997)

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The glial-derived calcium-binding protein S100B can be secreted to act as a neurotrophic factor or a mitogen, stimulating proliferation of glial cells. The extracellular S100B activities rely on the oxidation of the protein cysteine residues (Kligman, D., and Marshak, D. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7136–7139; Winningham-Major, F., Staeker, J. L., Barger, S. W., Coats, S., and Van Eldik, L. J. (1989) J. Cell Biol. 109, 3063–3071). Here we show that oxidation of the S100B cysteine residues, Cys-68 and Cys-84, induces a conformational change in the protein structure, unmasking a canonical CKII phosphorylation site located within the typical EF-hand calcium-binding site IIβ. Intrasubunit disulfide-bridged S100B monomer and disulfide-bonded S100B dimer are phosphorylated by the catalytic CKII-α subunit on Ser-62 with a $K_m$ of 0.5 μM and a $V_{max}$ of 10 pmol/min/100 pmol of S100B. Oxidized S100B is the best in vitro CKII-α substrate identified so far. Next we show that intrasubunit disulfide-bridged S100B monomer is the most potent S100B species to stimulate [3H]thymidine uptake by C6 glial cells in culture. In addition, the phosphorylated intrasubunit disulfide-bridged S100B monomer retains apparent mitogenic activity toward C6 glial cells, and hence, 32P-labeled S100B should be a useful probe for characterizing the mechanisms by which extracellular oxidized S100B functions. Finally, we show that formation of intrasubunit disulfide-bridged S100B monomer is stimulated by peroxynitrite anion, suggesting that production of mitogenic S100B species could be enhanced in neuropathology associated with peroxynitrite anion production.

The S100B protein belongs to the family of EF-hand calcium-binding proteins. In solution, S100B ($M_r = 10,500$) forms non-covalent dimers ($M_r = 21,000$). The S100B protein dimer binds calcium with micromolar affinity ($K_d = 10–100$ μM) and zinc ion with nanomolar affinity ($K_d = 10–100$ nM) (1–2). The S100B affinity for calcium is highly dependent on the quaternary protein structure. Zn$^{2+}$ binding or alkylation of Cys-$\beta84$ destabilizes the S100B quaternary structure to induce an increase in the protein’s affinity for calcium (1–3). The S100B is in highest concentration in the vertebrate nervous system. The gene for human S100B maps to the Down’s syndrome region of chromosome 21 (4), and increased levels of S100B proteins are found in the brain of individuals with Down’s syndrome (5) and in Alzheimer brains (6), suggesting its potential involvement in common neuropathologies associated with these diseases. In the brain, S100B is synthesized by glial cells, but a secreted form that appears to be an oxidized S100B species has neurotrophic and mitogenic activity (7–9). S100B has been detected in brain extracellular fluid and in conditioned medium from astroglial cells (10–11). Oxidized monomeric, homodimeric, and higher oligomeric disulfide-bonded forms of S100B were purified from bovine brain as a protein mixture that had neurite extension activity in chick embryonic cortical neurons (7). The reduced form of S100B has no activity in inducing neurite extension, and similarly, the activities of the recombinant protein was dependent upon the presence of the two cysteine residues $\beta68$ and $\beta84$ (12). S100B is also a glial mitogen, and that activity depends on the presence of Cys-68, suggesting that extracellular mitogenic activity of S100B also relies on the oxidation state of its cysteine residues (8, 9). Extracellular S100B stimulates both calcium flux in glial C6 cells (13) and inducible nitric oxide synthase activity in rat cortical astrocytes (14). However, the mechanism by which the extracellular form of S100B induces neurite outgrowth and/or cell proliferation is not yet known. Taking into account that in normal brain S100B accumulates in glial cells in a reduced state, an important issue will be to understand the processes and pathways that regulate formation of oxidized S100B forms in vivo and the pathways implicated in S100B secretion. The second question concerns the molecular mechanisms by which extracellular S100B elicits its effects. Is the active S100B species internalized by the target cell or are there cell-surface receptors that transduce the S100B signal? Hopefully these questions could be resolved if the biologically active oxidized S100B is clearly identified and if sufficient amounts of that species could be produced to allow more extensive structural and functional studies.

We developed a rapid and large scale method to produce mitogenic oxidized S100B, and we show that conformational changes induced by intrachain or interchain disulfide bridge formation in S100B led to unmasking a canonical CKII phosphorylation site located within the typical EF-hand calcium binding site IIβ. We also show that intrasubunit disulfide-bridged S100B monomer is the most potent S100B mitogenic species toward rat C6 glial cells.

**Experimental Procedures**

Materials—Recombinant CKII-α and holoenzyme were prepared as described previously (15). Bovine brain S100B (ββ), S100A1 (αα), and...
S100a (αβ) were purified as described previously (1–2). Intrachain disulfide-bonded S100B was prepared by the method of Mely and Gérard (16). Covalent disulfide-bonded S100B dimers were prepared by incubation of freshly purified S100B with 5 mM sodium tetrathionate (Sigma) in the presence of 1 mM calcium at room temperature. The reaction was stopped by addition of 5 mM EGTA, and the protein was dialyzed against 20 mM Tris-HCl buffer, pH 7.4. After this treatment 80–90% of S100B was disulfide-bonded S100B dimers. Oxidized S100B concentrations were determined by using the Bio-Rad protein assay reagent using purified reduced S100B as standard.

SDS-Polyacrylamide Gel Electrophoresis—11% polyacrylamide gels were run according to the method described by Schagger and Von Jagow (17) for the separation of proteins ranging from 1 to 100 kDa.

In-gel S100B Digestion and Matrix-assisted Laser Desorption/Ionization Mass Spectroscopy—S100B was run on a SDS-PAGE, and the Coomassie-stained spots were in-gel digested as described previously (20). Following this, the protein spots were excised and destained with 30% iodoacetamide prepared in 40% acetonitrile, 0.1% trifluoroacetic acid. MALDI mass spectra of peptide mixtures were obtained using a Brucker Biflex mass spectrometer (Brucker-Franzen Analytik, Bremen, Germany) equipped with a SCOUT multiblue inlet and a gridless delayed extraction source. Ion acceleration voltage was 19.5 kV, and the reflectron voltage was 20.0 kV. For delayed ion extraction, a 6.2-kV potential was applied for 167 ms before the detector was opened. A linear trap quadrupole (LTQ) equipped with a SCOUT multiprobe inlet and a gridless delayed extraction source. Ion acceleration voltage was 19.5 kV, and the reflectron voltage was 20.0 kV.

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Identification of the CKII Phosphorylation Sites on S100B—Purified intrasubunit disulfide-bonded S100B (100 μg) in standard reaction buffer was phosphorylated by CKII-α (0.5 μg) in the presence of [γ-32P]ATP. The protein was precipitated in 15% trichloroacetic acid, the pellet washed twice with ethanol and acetone, and resuspended by sonication in 200 μl of 0.1 Tris-HCl, pH 8.5, 0.05% SDS, 10 mM DTT. The protein was digested with endoproteinase Lys-C (sequencing grade from Sigma) at 35 °C for 15 h with a 1:100 protein:10 mM DTT weight ratio. The digested sample was adjusted to 6 mM guanidinium chloride plus 2% trifluoroacetic acid, incubated with 20 mM iodoacetamide for 15 min, and applied to an Octadecyl (C18), 5 μM, 4.6 × 100 mm high pressure liquid chromatography column. The peptides were separated with a gradient of 5–70% acetonitrile in 0.1% trifluoroacetic acid in 40 min at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected. The radioactivity of each fraction was determined by Cerenkov counting. The 32P-labeled peptide eluted at 30 min and was subjected to amino acid sequence analysis. The radioactivity of the eluted fractions from each sequencing cycle was determined by liquid scintillation counting.

Cell Culture—Rat C6 glioma cells were routinely maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Seromed), 20 units of penicillin per ml, and 20 μg of streptomycin per ml (Life Technologies, Inc.). For analysis of S100B mitogenic activity, C6 cells were placed into 35-mm tissue culture wells at a density of 105 cells per dish and allowed to grow for 24 h. Cells were

The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; MALDI, matrix-assisted laser desorption/ionization; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; CaM, calmodulin.

RESULTS

Oxidized S100B Dimers and Oligomers Are Phosphorylated by CKII—Fig. 1, A and B, shows an SDS-PAGE analysis and Coomassie Blue staining of two different preparations of bovine brain S100B protein stored at −20 °C for several months (lanes 1–3). Bovine brain calmodulin was run on the same gel (lane 2). Bovine brain S100B was run in the absence and presence of 1 mM calcium at room temperature. The reaction was stopped by addition of 5 mM EGTA, and the protein was dialyzed against 20 mM Tris-HCl buffer, pH 7.4. After this treatment 80–90% of S100B was disulfide-bonded S100B dimers. Oxidized S100B concentrations were determined by using the Bio-Rad protein assay reagent using purified reduced S100B as standard.

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RESULTS

Oxidized S100B Dimers and Oligomers Are Phosphorylated by CKII—Fig. 1, A and B, shows an SDS-PAGE analysis and Coomassie Blue staining of two different preparations of bovine brain S100B protein stored at −20 °C for several months (lanes 1–3). Bovine brain calmodulin was run on the same gel (lane 2). Under nonreducing conditions (Fig. 1A), S100B migrates mainly as a band with an apparent Mr of 6500 corresponding to the S100B monomer. Additional minor bands were visible at apparent Mr, 22,000 and 33,000 (lanes 1 and 2). These bands react with S100B antibodies by Western blot analysis and correspond to disulfide-bonded S100B dimers and larger oligomers (data not shown). Reduction of the proteins with 50 mM DTT prior to electrophoresis results in a shift of the lower mobility bands to the Mr, 6500 band (Fig. 1B). In contrast, the electrophoretic mobility of calmodulin, which has no cysteine
residues, was not sensitive to reducing agent (lane 2).

CaM is a recognized in vitro and in vivo substrate for CKII-α subunit (21–22). Fig. 1, A and B, also shows a comparison of the phosphorylation of CaM and S100B preparations by CKII-α. The data show that disulfide-bonded S100B dimer and oligomers are much better substrates for CKII-α than CaM and that S100B monomer is only weakly phosphorylated (Fig. 1A). As shown below, the phosphorylated S100B monomer most likely represents S100B with interchain disulfide bond. As expected, in the presence of DTT, the reduced phosphorylated S100B dimer and higher oligomers migrated at the position of S100B monomer (Fig. 1B).

To confirm the specificity of phosphorylation of the disulfide-linked form of S100B by CKII-α, we developed a method for large scale preparation of covalent disulfide-bonded S100B dimers. This was achieved by incubation of fresh purified S100B with the oxidizing agent sodium tetrathionate in the presence of calcium (Fig. 1C, lanes 3 and 4). No free SH groups remain titratable on the covalent disulfide-bond S100B dimers after denaturation in 6 M guanidinium chloride, suggesting that both Cys-β68 and Cys-β84 are involved in the formation of the disulfide-linked form of S100B (not shown, see also below). In the absence of calcium, sodium tetrathionate could not cross-link S100B as a dimer (lanes 5 and 6), and the two cysteine residues remain titratable after denaturation in 6 M guanidinium chloride (not shown). As expected, covalent disulfide-bound S100B dimers become phosphorylated by CKII-α (lanes 3 and 4), whereas the reduced protein is not (lanes 5 and 6). It is noteworthy that incubation of S100B with sodium tetrathionate in the presence of calcium (lanes 3 and 4) also resulted in phosphorylation of a population of S100B migrating as a monomer, suggesting that sodium tetrathionate might also stimulate formation of an intrachain disulfide bond between Cys-84 and Cys-68. Alkylation of the S100B preparation on both Cys-68 and Cys-84 by iodoacetamide after protein denaturation in guanidinium chloride also induced phosphorylation of the alkylated S100B monomer by CKII-α (Fig. 1C, lane 2), confirming that phosphorylation depends on the oxidation of the S100B sulfhydryl groups.

We also compared the effect of sodium tetrathionate on the Ca$^{2+}$-dependent covalent dimerization and CKII-α phosphorylation of the homodimers S100B (ββ) and S100A1 (αα) and of the heterodimer S100a (αβ) (Fig. 1D). S100B and S100A1 have a common cysteine residue, Cys-β84 and Cys-α85, which is specifically exposed to solvent in the presence of Ca$^{2+}$ (3). Only S100B has a second cysteine residue in position β68. Sodium tetraethionate catalyzed disulfide-linked forms of S100B (lane 1) but was much less efficient in catalyzing disulfide-linked forms of S100A1 and S100a (αβ) (lanes 2 and 3), confirming that both Cys-β84 and Cys-β68 are probably implicated in the formation of covalent disulfide-bound S100B dimers. Furthermore, only oxidized S100B (lane 1), and to a lower extent the heterodimer S100a (αβ) (lane 3), is phosphorylated by CKII-α indicating that phosphorylation is specific to the S100-β subunit.

Characterization of Oxidized S100B Monomer and Phosphorylation by CKII-α—A significant amount of the neurite extension factor initially purified from bovine brain was shown to migrate as a monomer on SDS-PAGE, suggesting that intrachain disulfide-bridged S100B monomer could have biological activity (7). Intrasubunit disulfide-bridged monomeric S100B species with a disulfide bond between Cys-68 and Cys-84 could be obtained by incubating the protein under denaturing conditions and gel filtration to separate intrasubunit disulfide-bridged monomeric species from intersubunit disulfide-bridged polymeric species (16).

Intrachain disulfide bond between Cys-68 and Cys-84 in the oxidized S100B monomer preparation was confirmed by MALDI mass spectroscopy analysis of the protein after digestion with endo-Asp-N (Fig. 2). After SDS-PAGE, the reduced and oxidized S100B were digested with endo-Asp-N.
protease, and the peptide mass maps were compared. Under our experimental conditions, both Asp and Glu residues are potential sites of cleavage by endo-Asp-N. Hence, the major mass peaks did not fit with the masses expected in case digestion would have been restricted to Asp residues. The mass spectroscopy spectrum obtained with the reduced S100B species revealed a series of peaks, called A, with masses of 1553.77, 1571.69, 1587.63, and 1603.66 Da that is absent in the oxidized S100B spectrum. By comparison with peptide masses expected after cleavage at Asp or Glu residues, the 1571.69-Da peak fit with the expected mass of peptide $\text{Asp}^\text{61}\text{Glu}^\text{67}\text{Asp}^\text{69}$. Oxidation of one or two methionine residues within this peptide explains mass peaks at 1587.63 and 1603.66 Da. The mass peak at 1553.77 correspond to a dehydrated form of this peptide. In the mass spectrum of the reduced S100B, the A series of peaks was immediately followed by the A’ series. A’ series have masses that correspond to A series incremented by 71 Da, corresponding to acrylamide adduct on Cys-84 generated during electrophoresis. The mass spectroscopy spectrum obtained with the oxidized S100B species revealed another series of peaks, called B, with two major peptide species having a molecular mass of 2772.27 Da and 2788.47 Da, respectively. That B series of peaks is not present in the reduced S100B spectrum. The mass difference between the B series of peaks in oxidized S100B and the A series of peaks in reduced S100B fits exactly with the mass of peptide $\text{Asp}^\text{61}\text{SDG}^\text{63}\text{Glu}^\text{67}\text{Asp}^\text{69}\text{Asp}^\text{71}$. Confirming the presence of an intramolecular disulfide bridge between Cys-68 and Cys-84 in the oxidized S100B. Note that the Asp-61–Gln-71 peptide could not be detected in the reduced S100 peptide mass map, most probably because its highly acidic properties that makes its desorption under a prototoned form very unlikely. Note also the absence of cleavage at Asp-61, Asp-63, Glu-67, and Asp-69 in the oxidized S100B, suggesting that disulfide bond stabilizes the peptide conformation, making those acidic residues not accessible to the protease.

The intrasubunit disulfide-bridged monomeric S100B species was also found to be an excellent substrate for CKII-α (Fig. 3, lane 1, and Fig. 4A, lanes 1 and 2). Incubation of the oxidized S100B monomer with 10 mM DTT prior to incubation with CKII-α results in a total inhibition of phosphorylation (Fig. 3, lanes 2 and 3), confirming that intracellular disulfide bond directly contributes to S100B phosphorylation. To determine the exact contribution of each individual Cys residue on the phosphorylation of S100B, we compared the phosphorylation of S100B alkylated with the thiol reagent Bimane on Cys-84 or on both Cys-84 and Cys-68. Specific alkylation of Cys-84 with Bimane in the presence of calcium (3) did not generate phosphorlatable S100B protein (Fig. 4A, lane 4). Alkylation of the S100B monomer on both Cys-68 and Cys-84 with Bimane after protein denaturation in guanidinium chloride was required for phosphorylation by CKII-α (Fig. 4A, lane 3). Suggesting that oxidation of Cys-68 is essential for optimum phosphorylation of S100B.

**Fig. 3.** The phosphorylation of S100B monomer by CKII-α is dependent on the oxidation state of its cysteine residues. Oxidized S100B monomer was incubated 30 min at 30 °C in the absence (lane 1) or in the presence of 5 mM DTT (lane 2) or 10 mM DTT (lane 3) prior to phosphorylation by CKII-α. The phosphorylated proteins were separated on SDS-PAGE in the absence of DTT and the gel processed for autoradiography.

**Fig. 4.** Characterization of the phosphorylation of oxidized S100B monomer by CKII-α. A, different oxidized S100B preparations were phosphorylated by CKII-α. Lane 1, oxidized S100B with an intrachain disulfide bond between Cys-68 and Cys-84. Lane 2, reduced S100B. Lane 3, alkylated S100B on Cys-68 and Cys-84 with Bimane. Lane 4, alkylated S100B on Cys-84 with Bimane. B, comparison of the phosphorylation of oxidized S100B monomer by CKII-α (lane 3) or oxidized S100B with intrachain disulfide bonds (lane 3) or oxidized S100B with iodoacetamide (lane 2), or oxidized S100B with trivalent selenide (lane 2) were phosphorylated with 70 nM CKII-α. C, the effect of Ca$^{2+}$ and Zn$^{2+}$ on S100B phosphorylation. S100B preparation stored for several months at −20 °C (lane 1) or oxidized S100B with an intrachain disulfide bond between Cys-68 and Cys-84 (lane 2) were phosphorylated by CKII-α in the absence (EGTA) or in the presence of 1 mM Ca$^{2+}$ or 5 mM Zn$^{2+}$, as indicated. A–C, the phosphorylated proteins were separated on SDS-PAGE in the absence of DTT. Gel were Coomassie Blue-stained (left panels) and processed for autoradiography (right panels). In the left margins are the positions of molecular weight standards.

**In vitro,** calmodulin is not phosphorylated by the CKII homologue $\alpha_2$β$_2$. The recombinant CKII-α subunit, however, spontaneously phosphorylates calmodulin (23). As observed with calmodulin, the $\alpha$ subunit of CKII was more efficient than the homologue ($\alpha_2$β$_2$) in phosphorylating alkylated S100B or disulfide-bridged monomeric S100B (Fig. 4B, lanes 2 and 3) or disulfide-bridged S100B dimer (not shown). The phosphorylation of monomeric and oligomeric S100B by CKII-α was proven to rely exclusively on changes induced upon oxidation of Cys residues. Neither calcium nor zinc ions, two conformational effectors of the S100B (1, 2), could modulate phosphorylation of the protein (Fig. 4C).

**Characterization of the CKII-α Phosphorylation of Oxidized S100B—** The phosphorylation of oxidized monomeric and dimeric S100B by purified CKII-α is a very rapid reaction. With 0.5 μM S100B and 80 nM CKII-α in the assay, the phosphorylation is maximal within 1–2 min (Fig. 5A). With the S100B preparation used in this experiment, the phosphorylation stoichiometry of oxidized monomeric S100B was calculated to be 0.7 mol of phosphate incorporated per mol of S100B monomer (Fig. 5A). We confirmed these kinetic and stoichiometry procedures with two different oxidized S100B monomer preparations and two different CKII-α preparations. The observed
sub-stoichiometric phosphorylation is probably due to overestimation of the oxidized S100B concentration that has been determined using the Bio-Rad protein assay reagent using purified reduced S100B as standard. To our knowledge the oxidized S100B is the best in vitro substrate for CKII-α so far identified with a $K_m$ of 0.5 μM and a $V_{max}$ of 10 pmol/min/100 pmol of S100B (Fig. 5, B and C). As expected, in a competition assay, oxidized monomeric S100B totally abolished CaM phosphorylation by CKII-α. CaM (lanes 1–3) at the concentration of 30 μM was mixed with 1 μM (lane 2) or 4 μM (lane 3) oxidized S100B monomer in buffer containing 2 mM EGTA. The proteins were phosphorylated with CKII-α (50 nM) for 1 min. Proteins were analyzed by SDS-PAGE, Coomassie Blue staining (left panel), and autoradiography (right panel).

To determine the CKII-α-phosphorylated residue on the monomeric and dimeric S100B, the 32P-labeled proteins were digested by trypsin and the resulting phosphopeptides analyzed by SDS-PAGE (Fig. 6A). In the absence of reducing agent, the phosphopeptides obtained from digestion of the monomeric and dimeric 32P-S100B migrate with apparent mass of 3.4 and 6.5 kDa, respectively. In the presence of DTT, the phosphopeptides co-migrate with the same apparent mass of 3.5 kDa. The phosphopeptides were purified by reverse phase-high pressure liquid chromatography as described under “Experimental Procedures” and sequenced. The released radioactivity coincided with the identification of Ser-62, indicating that this residue is the phosphorylated amino acid (Fig. 6B). Ser-62 is located within the calcium binding loop of site IIβ and corresponds to a canonical CKII phosphorylation site domain (Fig. 6C) (25).

Peroxynitrite Anion Stimulates S100B Oxidation and Phosphorylation—We next investigated if a more physiological oxidant than sodium tetrathionate could catalyze the formation of disulfide bridges and CKII-α phosphorylation of S100B. NO as a second messenger reacts readily with other free radicals such as superoxide anion (O$_2^-$) to form peroxynitrite (ONOO$^-$). NO and products of NO oxidation are capable of reaction with thios to give further products with biological activities (26, 27). We tested the effect of various NO donors (DEA, SIN-1), O$_2^-$ precursor (pyrogalol), and peroxynitrite anion (ONOO$^-$) on the oxidation of S100B. S100B oxidation was analyzed by phosphorylation of the S100B species with CKII-α (Fig. 7A). The most potent oxidant we found is peroxynitrite anion (ONOO$^-$) (lane 3). In contrast to sodium tetrathionate which mostly oxidized S100B as dimer, ONOO$^-$ stimulated both formation of intrachain and interchain disulfide bonds in S100B. If ONOO$^-$ is first decomposed into NO$\_2$ and ‘OH, formation of the intrachain disulfide bond within S100B monomer was inhibited (lane 4). Note that S100B oxidation into covalent disulfide bond dimer still occurred after ONOO$^-$ decomposition with an intensity comparable to that of nitric oxide (NO) precursors (DEA; SIN-1) confirming the specificity of ONOO$^-$ in generating intrachain disulfide bond within S100B-β subunit. A dose-dependent experiment showed that ONOO$^-$ at a micromolar concentration was able to mediate S100B phosphorylation (Fig. 7B). This observation, together with the fact that in our experimental condition ONOO$^-$ has a very short half-life in the second range (28), supports a physiological relevance of ONOO$^-$-mediated S100B oxidation. Incubation of the oxidized S100B protein samples with 10 mM DTT prior to incubation with CKII-α resulted in a total inhibition of phosphorylation, confirming that phosphorylation is dependent on ONOO$^-$-mediated disulfide bond formation (not shown).

Oxidized S100B Monomer Stimulates [3H]Thymidine Uptake by Rat Glial C6 Cells—It has been shown that neurotrophic S100B stimulates [3H]thymidine uptake by rat C6 glial cell (8) and also stimulates calcium fluxes and nitric oxide synthase activity in astrocytes (13, 14). All these activities have been reported to be dependent on the presence of Cys-68 or Cys-84 and on the oxidation state of these residues. However, considerable variability in specific activity between S100B prepara-
tions has been reported (8, 13–14) that could rely on the het-
erogeneity of the S100B preparation. Here we compared the
effect of the oxidized monomeric and dimeric S100B species and
of the reduced S100B on [3H]thymidine uptake by confluent rat
C6 glial cells in serum-free medium (Fig. 8A). As described
previously by Selinfreund et al. (8), the reduced S100B prepa-
ration had no effect on [3H]thymidine uptake by rat C6 cells.
The oxidized S100B dimer stimulated [3H]thymidine incorpo-
ration to a low extent, whereas the monomeric oxidized S100B
species was the most potent S100B species in stimulating
[3H]thymidine uptake by glial C6 cells. Half-maximal stimula-
tion was obtained with 0.1–0.2 nM S100B. Phosphorylation by
CKII-α had no effect on stimulation of [3H]thymidine uptake by
the monomeric oxidized S100B species (Fig. 8B).

DISCUSSION

Intracellular S100B exists mainly as a noncovalent protein
homodimer with free reduced sulfhydryl groups. However, ox-
idized S100B with a neurite extension activity has been puri-
ified from bovine brain (7). This oxidized S100B migrates with
molecular weight of 6500, 21,000, 30,000, and 40,000 in non-
reducing conditions. The neurite extension activity of the ox-
idized S100B preparation is dependent on the absence of reduc-
ing agent in the preparation, suggesting that neurite extension
activity is associated with an oxidized form of S100B. Subse-
quently it was shown that oxidized S100B is also a glial mito-
gen (8) and that S100B stimulates both calcium flux in glial C6
cells (13) and nitric oxide synthase activity in rat cortical as-
trocytes with accumulation of the NO metabolite in the condi-
tioned medium (14). Transgenic mice expressing elevated lev-
els of S100B also show abnormal astrocitosis and neurite
proliferation consistent with alterations observed in vitro on
cell culture (29). It is commonly accepted that the neurotrophic
and mitogenic activities of S100B depend on a disulfide-linked
dimeric form of the protein (for review see Ref. 9). However, we
demonstrate here that the monomeric S100B species with in-
trachain disulfide bond also has a mitogenic activity that is
even stronger than a preparation containing essentially inter-
chain disulfide-bonded S100B (Fig. 8).

The human S100B gene is found on a region of chromosome
21 that is triplicated in individuals with Down’s syndrome (4),
and it has been suggested that the dosage imbalance of this
gene is a major contributor to the abnormalities of brain devel-
opment and function that occur invariably in Down’s syndrome
individuals and Alzheimer brains (5). In Alzheimer brains a
correlation exists between S100B protein synthesis and neuro-
trophic activity (6). The S100B protein in Alzheimer brain
migrates essentially as a monomer on SDS-PAGE, strongly
supporting the notion that neurotrophic active S100B species
could be oxidized S100B monomer (6). Because only oxidized
S100B is capable of mitogenic and neurotrophic activities (7–
8), one has to envision that not only overproduction but also
alteration of the redox status of the cysteine residues in S100B
are implicated in pathogenicity. We have shown that the for-
mation of oxidized monomeric S100B can be selectively cata-
CKII-α Subunit Phosphorylates Oxidized S100B

The second major observation reported in this study is the
views of the oxidized S100B protein by peroxynitrite. A, 10 μM
S100B in 50 μl of 20 mM Tris-Cl, pH 7.5, 0.5 mM CaCl2 was incubated
30 min with 0.1 mM each of DEANO (lane 1), SIN-1 (lane 2), peroxynitrite
(lane 3), decomposed peroxynitrite (lane 4), pyrogalol (lane 5), and
a mixture of pyrogalol and DEANO (lane 6). The protein was then
phosphorylated by CKII-α. The phosphorylated proteins were sepa-
rated on SDS-PAGE in the absence of DTT, and the gel was proceeded
for autoradiography. β, oxidized S100B monomer; β2, disulfide-bonded
S100B dimer. B, 10 μM S100B was incubated for 50 min with increasing
concentrations of peroxynitrite. The proteins were phosphorylated by
CKII-α and separated on SDS-PAGE in the absence of DTT and the gel
proceeded for autoradiography.

A

B

\( \text{FIG. 7. S100B monomer oxidation by peroxynitrite.} \)

The second major observation reported in this study is the
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CKII-α and separated on SDS-PAGE in the absence of DTT and the gel
proceeded for autoradiography.

A

B

\( \text{FIG. 8. Oxidized S100B monomer stimulates glial C6 cell pro-
duction.} \)

A, subconfluent rat C6 glioma cells were kept in DMEM
containing low serum. After 48 h cells reached confluence and were
stimulated by changing media to DMEM containing 0.05% fetal bovine
serum alone (control) or plus various concentrations of reduced S100B
preparations (C), intrachain disulfide-bonded S100B monomer (●),
or interchain disulfide-bonded S100B dimer (○). 24 h, \( ^{3} \text{H} \)thymi-
dine was added, and cells were solubilized 2 h later, and incorporated
counts/min were determined. B, effect of CKII-α phosphorylation on
S100B mitogenic activity. Subconfluent C6 grown in low serum were
stimulated or not stimulated with reduced S100B (control, C) or stim-
ulated with 1 μM dephosphorylated intrachain disulfide-bonded S100B
monomer (S-S) or 1 μM phosphorylated intrachain disulfide-bonded
S100B monomer (S-S-P). After 24 h, \( ^{3} \text{H} \)thymidine was added, and
cells were solubilized 2 h later, and incorporated counts/min were
determined. A and B, data represent the mean of two experiments in
duplicate.

A

B

\( \text{FIG. 8. Oxidized S100B monomer stimulates glial C6 cell pro-
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Western blot or immunoprecipitation after metabolic labeling with $[^{35}\text{S}]$methionine. We have not yet been able to observe an in vivo phosphorylation of S100B in glial cells after metabolic labeling of cells with $^{32}\text{P}$, and we have not been able to detect any in vitro phosphorylation of immunoprecipitated S100B with purified CKII-α. It could be that in glial C6 cells, intracellular soluble S100B exists mainly in a reduced and nonphosphorylatable state and that alteration of the redox status of the cysteine residues in S100B is tightly regulated and may concern only a minor S100B population. As stated above, oxidized S100B might be produced only under certain circumstances, particularly in pathologies when S100B is overproduced and when the redox status and calcium homeostasis of the cells are perturbed, a situation that could take place in neurodegenerative disorders (31). Because the extracellular functions of the oxidized S100B species depend on its secretion, it could also be that CKII-α phosphorylation of oxidized S100B is implicated in the processes of S100B secretion, thus complicating further in vivo phosphorylation studies on S100B. A role of CKII in the regulation of intracellular trafficking and sorting mechanisms of protein substrate has recently been reported (34). We believe that the role of CKII-α phosphorylation on Ser-62 of S100B in vivo should be more easily resolved by mutagenesis studies. These studies are under way.

Finally, the specificity of CKII-α phosphorylation of oxidized S100B opens up new strategies for characterizing the mechanisms by which extracellular oxidized S100B functions. The in vitro phosphorylation of monomeric S100B by CKII-α has no effect on the S100B mitogenic activity on C6 glial cells; thus $^{32}\text{P}$-labeled S100B might serve as a useful tool to identify putative S100B receptors or target proteins. Phosphorylation of S100B by CKII-α might also be valuable to investigate the oxidation state of the S100B protein in brain pathologies where alteration of the redox status of S100B is thought to be linked with alteration of S100B functions (6).

Acknowledgment—We thank Dr. A. M. Chinn for critical reading of the manuscript.

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Cysteine Oxidation in the Mitogenic S100B Protein Leads to Changes in Phosphorylation by Catalytic CKII-α Subunit
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J. Biol. Chem. 1998, 273:3901-3908.
doi: 10.1074/jbc.273.7.3901

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