S-100 Protein, but Not Calmodulin, Binds to the Glial Fibrillary Acidic Protein and Inhibits Its Polymerization in a Ca²⁺-dependent Manner*

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S-100 protein, a Ca²⁺-binding protein of the EF-hand type, interacts with the glial fibrillary acidic protein (GFAP) in a Ca²⁺-dependent manner. The binding of S-100 protein to GFAP was investigated by fluorescence spectroscopy using acrylodan-S-100 protein and cross-linking experiments using the bifunctional cross-linker, disuccinimidyl suberate. The binding affinity was observed to be in the nanomolar range with a stoichiometry of 2 mol of GFAP/mol of S-100 protein (dimer). S-100 protein was found to inhibit the polymerization of GFAP in a dose- and Ca²⁺-dependent manner, with a half-maximal effect at an S-100 protein/GFAP molar ratio of 0.2 and maximal effect at a molar ratio of 0.5. Identical results were obtained irrespective of whether the unfractionated bovine brain S-100 protein mixture (S-100a plus S-100b), S-100a₀, S-100a, or S-100b was used. S-100 protein was observed to be maximally effective as an inhibitor of GFAP polymerization at ~3 μM free Ca²⁺. Calmodulin neither bound to GFAP nor inhibited its polymerization. Altogether, the present results suggest that S-100 protein might be involved in the regulation of the state of assembly of glial filaments by binding to and sequestering unpolymerized GFAP.

Glial filaments (GFs)[1] represent a major cytoskeleton constituent in glial cells (1, 2). GFs are composed of a single molecular entity, the glial fibrillary acidic protein (GFAP), which self-assembles into GFs in a concentration-dependent, Mg²⁺- and pH-regulated, and nucleotide-independent manner under the ionic conditions found within cells (3). Long considered as static cytoskeleton elements, GFs have been shown to depolymerize and re-polymerize with definite kinetics *in vitro* (3–6). Relatively little is known about the factors that regulate GF formation *in vivo*. *In vitro* data indicate that, given the low critical concentration of GFAP assembly (~40 μg/ml) and the slow polymerized-unpolymerized GFAP exchange rate (3), the equilibrium is shifted toward the GF

formation. Thus, the GFAP concentration (and synthesis) appears to be a main factor in this respect. Recently, phosphorylation of GFAP by either cAMP-dependent protein kinase or protein kinase C has been reported to result in diminished or no GF formation *in vitro* (7). Also, GFs have been shown to depolymerize after phosphorylation (7). These data suggest that phosphorylation might be a way to regulate the state of GFAP assembly *in vivo*. However, cAMP accumulation in C6 glioma cells has been reported to be accompanied by increased levels of GFAP and in recovery of increased amounts of GFAP in the cytoskeleton fraction in these cells (8, 9), in agreement with data on cAMP-dependent regulation of expression of the GFAP gene (10, 11). Finally, Ca²⁺ has been shown to bind to GFAP (12) and to stimulate GFAP polymerization (13). However, Ca²⁺ concentrations >1 mM were required to obtain measurable GFAP polymerization (13), and, most important, millimolar Ca²⁺ levels were observed to change the morphology of GFs to ribbon-like structures (Ref. 3; also see Fig. 3 in Ref. 13).

S-100 is a group of dimeric, Ca²⁺-binding proteins of the EF-hand type shown to be involved in the regulation of several activities, including microtubule assembly (reviewed in Ref. 14). In the nervous system, S-100 protein is restricted to and abundant in glial cells (14). A general role of S-100 protein in the Ca²⁺-dependent regulation of the cytoskeleton has been suggested on the basis of the aforementioned effects on microtubule assembly (Ref. 15, and references therein); S-100 protein binding to caldesmon and inhibition of caldesmon-regulated activities (16–18); the observation that the selective inhibition of S-100 protein expression in a glial cell line by antisense techniques resulted in a more organized microfilament network (19); and of the S-100 protein interaction with both chains of the cytoskeleton protein complex, calpactin I (20). We have, therefore, undertaken a study aimed at verifying whether or not S-100 protein also affects GFAP polymerization. For comparison, calmodulin, another Ca²⁺-binding protein of the EF-hand type (21), was tested in parallel experiments. The results indicate that S-100 protein, but not calmodulin, binds to and inhibits the GFAP polymerization in a Ca²⁺-dependent manner.

EXPERIMENTAL PROCEDURES

Materials—Acrylodan was obtained from Molecular Probes, dithiothreitol (DTT) from Sigma, and disuccinimidyl suberate (DSS) from Pierce Chemical Co. All other products were analytical grade reagents from Sigma, Fluka AG, Bio-Rad, or Carlo Erba.

Purification of Protein—S-100 protein (mixture of S-100a (αβ) plus S-100b (ββ)) and calmodulin were purified from bovine brain as reported (22, 23), respectively. S-100a and S-100b were separated from one another by DEAE Sephacel chromatography of bovine brain S-100 protein, as reported (24). Briefly, bovine brain S-100 protein was loaded onto a column of DEAE Sephacel (1 × 10 cm) equilibrated

[1] The abbreviations used are: GFs, glial filaments; GFAP, glial fibrillary acidic protein; DTT, dithiothreitol; DSS, disuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis.

with 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM 2-mercaptoethanol (buffer A). The column was washed with 50 ml of buffer A, followed by 50 ml of buffer A containing 0.2 M NaCl. Then, the column was developed with a linear NaCl gradient (0.2–0.35 M) in a total volume of 100 ml in buffer A. By a combination of spectroscopy, *i.e.* registration of absorbtion spectra of individual chromatographic fractions, and electrophoresis in 20% acrylamide under nondenaturing conditions (25) we monitored the separation of S-100a (left tail of the chromatogram) from S-100b (right tail of the chromatogram). The absorbtion spectrum of S-100a is quite different from that of S-100b (26). Also, the two proteins are characterized by different electrophoretic mobilities under native conditions (Ref. 25; also see Fig. 1 in Ref. 15). By electrophoresis under nondenaturing conditions, the bovine brain S-100 protein mixture resulted in ~50% S-100a and ~50% S-100b. S-100a₀ (αα) was purified from porcine heart as reported (27). Individual proteins were exhaustively dialyzed against deionized double-distilled water, and lyophilized. GFAP was purified from bovine spinal cord as described (7). The purity of individual proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28). GFAP was measured by the method of Lowry *et al.* (29) against a standard solution of bovine serum albumin.

Fluorescence Experiments with Acrylodan-S-100 Protein—S-100 protein was labeled with acrylodan as described (20, 30). Briefly, S-100 protein was incubated at 4 °C for 2 h in 25 mM imidazole-HCl, pH 7.1, 0.1 M NaCl, 0.2 mM EGTA (buffer B) containing 2.5 mM CaCl₂, with a 5 M equivalent of acrylodan, a thiol-derivative of Prodan (31). The reaction was terminated by the addition of DTT to 5 mM. EGTA was then added to the mixture to 2.5 mM 30 min after the addition of DTT. Uncoupled dye was removed by gel filtration on a column of AcA 54 equilibrated with buffer B containing 1 mM DTT. The labeling ratio (dye/protein) which was determined as reported (31) was 1.8. Fluorescence emission spectra were recorded at 25 °C on a Shimadzu RF-5000 spectrofluorophotometer with excitation at 380 nm and emission collected between 400 and 650 nm. Emission data were corrected for dilution which never exceeded 5%. Experiments were done in 10 mM imidazole-HCl, pH 7.1, 1 mM MgCl₂, 1 mM DTT, 0.2 mM EGTA (buffer C) plus or minus CaCl₂ in the absence or presence of 0.1 M NaCl. The following molecular masses were considered: 21 kDa for S-100 protein (dimer), 17 kDa for calmodulin, and 50 kDa for GFAP.

Cross-linking Experiments—S-100 protein (20 μg) was incubated in a final volume of 0.1 ml of buffer C plus or minus CaCl₂ at 37 °C for 30 min in the presence of 100 μg of GFAP, after which the bifunctional cross-linker, DSS, was added to a final concentration of 0.5 mM. After varying time periods (5, 15, or 30 min) at room temperature, the reaction was terminated by the addition of SDS and 2-mercaptoethanol to 2% (w/v). Samples were then subjected to SDS-PAGE (7.5% acrylamide). Gels were stained with Coomassie Blue. In parallel experiments, electrophoretically separated polypeptides were electroblotted onto nitrocellulose paper (32) for immunochemical analyses with either a polyclonal anti-S-100 protein antiserum raised in rabbits and characterized as described (33) or a commercial polyclonal anti-GFAP antiserum. Similar cross-linking experiments were done with calmodulin instead of S-100 protein.

Polymerization of GFAP—Increasing amounts of GFAP were incubated at 30 °C for 30 min in a final volume of 0.1 ml of 0.1 M imidazole-HCl, pH 6.8, 1 mM MgCl₂, 1 mM DTT, 0.2 mM EGTA plus or minus CaCl₂, before centrifugation at 13,000 × g for 30 min (3). The supernatants were collected and the pellets resuspended in 0.1 ml of buffer C. Identical samples of supernatants and pellets were subjected to SDS-PAGE (7.5% acrylamide). Gels were stained with Coomassie Blue. To investigate the effects of S-100 protein or calmodulin on GFAP polymerization, GFAP (50 μg) was preincubated at 30 °C for 30 min in 0.1 ml of buffer C plus or minus CaCl₂ with increasing amounts of S-100 protein or calmodulin, after which imidazole-HCl, pH 6.8, was added to 0.1 M (final concentration) to trigger GFAP polymerization (1–3). After an additional 30-min incubation at 30 °C, samples were centrifuged and processed as above. The GFAP in supernatants and pellets was quantitated by densitometric scanning of Coomassie Blue-stained gels. Similar experiments were done with individual S-100a₀, S-100a, or S-100b.

RESULTS

Fluorescence Experiments with Acrylodan-S-100 Protein— Upon addition of Ca²⁺ to solutions of acrylodan-S-100 protein a slight red shift of the emission maximum (from 481 to 488

nm) and a slight reduction in the emission spectrum area (~4%) were observed (not shown), in accordance with previous data (20, 30). GFAP produced a dose-dependent blue shift of the emission maximum and increase in the emission spectrum area of acrylodan-S-100 protein in the presence of Ca²⁺ (Fig. 1, A and B), but not in its absence (not shown). Also, the final addition of EGTA to the mixture of acrylodan-S-100 protein, GFAP, and Ca²⁺ completely reverted the GFAP-induced effects on the emission spectrum (not shown). These data indicated that S-100 protein binds to GFAP in a Ca²⁺-dependent manner, that GFAP induces conformational changes in S-100 protein, and that the fluorophore in S-100 protein is located in a less polar environment upon Ca²⁺-dependent S-100 protein binding to GFAP. Since acrylodan was attached to an —SH group in the C-terminal half of individual S-100 subunits, where also functional EF-hand Ca²⁺-binding sites are located (34, 35), it was tentatively concluded that the C-terminal portions of S-100 subunits were involved in S-100 protein binding to GFAP. However, the alternative possibilities that GFAP produced a hydrophobic contact domain between the S-100 subunits and/or that the GFAP-induced changes in the emission spectrum of acrylodan-S-100 protein resulted from secondary long range conformational effects could not be excluded. The interaction of acrylodan-S-100 protein with GFAP was linearly related to the GFAP concentration up to a molar ratio of about 2 mol of GFAP/mol of S-100 protein (dimer) (Fig. 1B). At higher molar ratios only small increments in the blue shift and emission spectrum area were registered. Essentially identical results were obtained irrespective of whether or not NaCl to 0.1 M was present in the medium (not shown). Since in the experiments illustrated in Fig. 1B at the highest GFAP concentrations used GFAP could have polymerized, thereby potentially interfering with the binding to S-100 protein, experiments were also done with 0.1 μM acrylodan-S-100 protein in the presence of GFAP concentrations below the critical concentration of GFAP polymerization (Ref. 3; also see Fig. 5A). Under the latter conditions, again the GFAP-induced
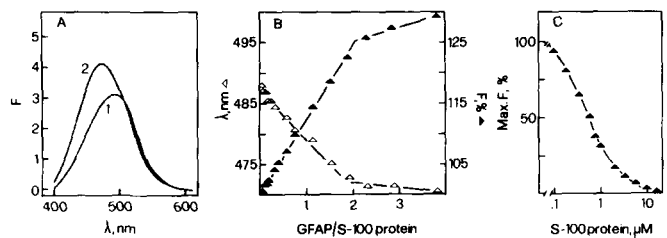
FIG. 1. **Interaction of acrylodan-S-100 protein with GFAP.** A, the emission spectrum of acrylodan-S-100 protein (0.4 μM in 10 mM imidazole-HCl, pH 7.1, 0.2 mM EGTA, 1 mM DTT, 0.3 mM CaCl₂) was registered before (*trace 1*) and after (*trace 2*) the addition of GFAP to 0.9 μM. Excitation was at 380 nm. Note the GFAP-induced blue shift of the emission maximum and increase in the emission spectrum area. The fluorescence intensity (*F*), in arbitrary units, is plotted *versus* the wavelength (λ, nm). No GFAP-induced changes in the emission spectrum were registered in the absence of Ca²⁺ (not shown). B, represented are dose-dependent changes in the emission maximum (*open symbols*) and in the emission spectrum area (*closed symbols*) upon addition of increasing concentrations of GFAP to 0.4 μM acrylodan-S-100 protein in the medium described in A, to the GFAP/S-100 protein molar ratios indicated. A distinct inflection point was observed at a GFAP/S-100 protein molar ratio of ~2. C, shown is the dose-dependent reversal of GFAP-induced changes in the emission spectrum area of acrylodan-S-100 protein by native S-100 protein. The percent change in the emission spectrum area of 0.4 μM acrylodan-S-100 protein in the presence of 1.2 μM GFAP in the above medium is plotted *versus* the concentration of native S-100 protein.

changes in the emission spectrum were linearly related to the GFAP concentration up to a GFAP/S-100 protein molar ratio of ~2 in the presence, but not in the absence of Ca^{2+}, irrespective of the absence or presence of 0.1 M NaCl (not shown). Since a distinct inflection point was detected at a GFAP/S-100 protein (dimer) molar ratio of 2, the tentative conclusion was drawn that the binding stoichiometry was 2 mol of GFAP/mol of S-100 protein. Native S-100 protein produced a dose-dependent reversal of GFAP-induced changes in the emission spectrum of 0.4 μM acrylodan-S-100 protein (Fig. 1C). Half-maximal effect was registered around 0.5 μM native S-100 protein, suggestive of a high exchange rate. In similar displacement studies, in which calmodulin was used instead of native S-100 protein, a slight (20%) reversal of GFAP-induced changes in the emission spectrum were detected at >10 μM calmodulin (not shown).

GFAP-induced changes in the emission spectrum of acrylodan-S-100 protein were monitored at increasing free Ca^{2+} concentrations (Fig. 2). A complex binding pattern was observed in that the resulting titration curve was biphasic. A first component, amounting to 30% of overall fluorescence change, was saturated at ~3 μM free Ca^{2+} and half-saturated at 1 μM free Ca^{2+}, and a second component was saturated at ~200 μM free Ca^{2+}. Irrespective of the reasons for this biphasic pattern (see "Discussion"), the conclusion could be drawn that significant binding of S-100 protein to GFAP occurred, by the present experimental approach, at free Ca^{2+} concentrations compatible with those found *in vivo* in non-resting cells.

Cross-linking Experiments—To validate the conclusion that S-100 protein interacts with GFAP in a Ca^{2+}-dependent manner and that the binding stoichiometry is 2 mol of GFAP/mol of S-100 protein, mixtures of S-100 protein and GFAP, after incubation at 30 °C for 30 min, received the bifunctional cross-linker DSS, for varying time periods, before SDS-PAGE. Irrespective of the absence or presence of Ca^{2+}, after a 5-min exposure of GFAP to DSS in the absence of S-100 protein, complexes of GFAP of apparent M_r of ~150,000 and, to a smaller extent, of ~100,000, in addition to uncomplexed
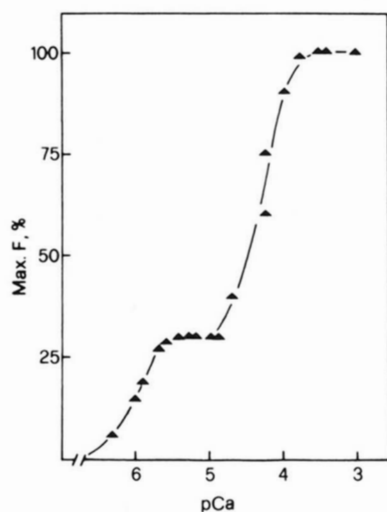
GFAP, were resolved by SDS-PAGE (not shown, but see Fig. 3A, lane 3). Under the same conditions, increasing the time of exposure of GFAP to DSS to 15 or 30 min resulted in larger proportions of the 100- and 150-kDa complexes and in the appearance of a ~200-kDa complex (not shown, but see Fig. 3A, lanes 5 and 7). Thus, DSS-induced complexes of GFAP were obtained in a time-dependent Ca^{2+}-independent manner. The calculated M_r values of GFAP complexes were compatible with the formation of dimers, trimers, and tetramers of GFAP. Trimers appeared to prevail over dimers and tetramers. When these experiments were done in the presence of S-100 protein, no changes in the electrophoretic pattern illustrated above were detected in the absence of Ca^{2+} (Fig. 3A, lanes 3, 5, and 7). In contrast, in the presence of Ca^{2+} and S-100 protein, a ~60-kDa polypeptide was resolved by SDS-PAGE concomitantly with a strong reduction in the staining intensity of the 100-, 150-, and 200-kDa DSS-induced complexes of GFAP (Fig. 3A, lanes 4, 6, and 8). Under these conditions, the GFAP trimer band appeared slightly broader than the corresponding
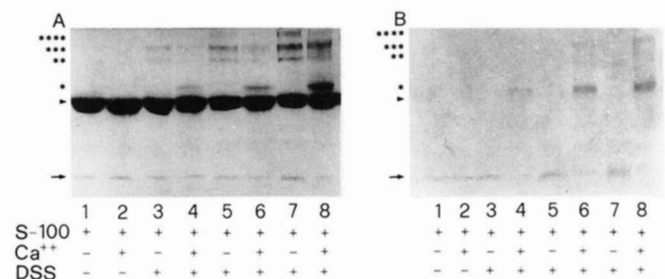

FIG. 3. **Binding of S-100 protein to GFAP as investigated by chemical cross-linking.** GFAP (1 mg/ml) was incubated in 10 mM imidazole-HCl, pH 7.1, 0.2 mM EGTA, 1 mM DTT, plus or minus 0.3 mM $CaCl_2$ as indicated, for 30 min at 30 °C with S-100 protein (0.2 mg/ml), after which the bifunctional cross-linker, DSS, was added to 0.5 mM as indicated. After 5 (A and B, lanes 3 and 4), 15 (A and B, lanes 5 and 6), or 30 min (A and B, lanes 7 and 8), SDS and 2-mercaptoethanol were added to 2%. Identical volumes of individual mixtures were boiled for 5 min and subjected to SDS-PAGE (7.5% acrylamide). Separated polypeptides were either stained with Coomassie Blue (A) or transblotted onto nitrocellulose for immunostaining with the anti-S-100 protein antiserum (B). Irrespective of the absence or presence of Ca^{2+} (A and B, lanes 1 and 2, respectively), neither GFAP complexes nor GFAP-S-100 protein complexes could be evidenced in samples from mixtures not exposed to DSS. In contrast, in the presence (A and B, lane 4), but not in the absence (A and B, lanes 3) of Ca^{2+}, after a 5-min exposure of mixtures to DSS, a ~60-kDa polypeptide, indicated by a single *asterisk* in A and B, was detected (A, lane 4), which was recognized by the anti-S-100 protein antiserum (B, lane 4). Also, after 5 min of exposure to DSS, dimers (A, double asterisk) and, to a larger extent, trimers (A, triple asterisk) of GFAP were resolved in the absence of Ca^{2+} and presence of S-100 protein (A, lane 3), which were not immunostained by the anti-S-100 protein antiserum (B, lane 3). The Coomassie Blue staining intensity of dimers and trimers of GFAP was greatly reduced in the presence of both Ca^{2+} and S-100 protein, concomitantly with the appearance of the 60-kDa polypeptide and the decrease in the Coomassie Blue staining and immunostaining intensities of S-100 subunits, when Ca^{2+} was present in the incubation mixture (arrow in A and B, lanes 4). Increasing the time of exposure of mixtures to DSS to 15 (A and B, lanes 5 and 6) or 30 min (A and B, lanes 7 and 8) resulted in the appearance of tetramers of GFAP (quadruple asterisks), best evident in the absence of Ca^{2+} (A, lanes 5 and 7, respectively), in higher Coomassie Blue staining and immunostaining intensities of the 60-kDa polypeptide in the presence of S-100 protein and Ca^{2+} (A and B, lanes 6 and 8, respectively), and in a lower Coomassie Blue staining intensity of dimers, trimers, and tetramers of GFAP in the presence rather than in the absence of Ca^{2+} (A, lanes 5 and 7, respectively). Note that after 30 min of exposure of GFAP and S-100 protein mixtures to DSS in the presence of Ca^{2+} (B, lane 8), a slight immunostaining of the GFAP trimer was detected. The *arrowhead* in A and B points to the position of uncomplexed GFAP.

FIG. 2. **Ca^{2+} dependence of the GFAP-induced changes in the emission spectrum of acrylodan-S-100 protein.** Acrylodan-S-100 protein (0.4 μM in 10 mM imidazole-HCl, pH 7.1, 0.2 mM EGTA, 1 mM DTT) was incubated with 0.9 μM GFAP. Emission spectra were registered before and after successive additions of $CaCl_2$ from a stock solution to the final free Ca^{2+} concentrations (pCa) indicated. The GFAP-induced increase in the emission spectrum area is plotted *versus* the free Ca^{2+} concentration. A similar biphasic curve was obtained when the changes in the emission maximum were plotted *versus* the free Ca^{2+} concentration (not shown).

band from experiments done in the absence of Ca^{2+} (compare *lane 7* with *lane 8* in Fig. 3A). Also, as the time of exposure to DSS of mixtures of GFAP and S-100 protein in the presence of Ca^{2+} was increased, the staining intensity of the 60-kDa polypeptide augmented, and that of the 100-, 150-, and 200-kDa GFAP complexes decreased. Concomitantly, the staining intensity of the S-100 subunit band decreased as well (Fig. 3A). The calculated M_r of the 60,000 polypeptide resolved by SDS-PAGE was consistent with the formation of a complex of 2 mol of GFAP with 1 mol of S-100 protein (dimer), in agreement with the binding stoichiometry of 2 mol of GFAP/mol of S-100 protein calculated from the fluorescence experiments. Since the appearance of the 60-kDa complex was accompanied by a decrease in the formation of dimers, trimers, and tetramers of GFAP, the conclusion was drawn that S-100 protein reduced the formation of GFAP complexes in a Ca^{2+}-dependent manner. That the 60-kDa polypeptide actually was a complex of GFAP plus one S-100 subunit was demonstrated in parallel Western blot experiments in which the polypeptides resolved by SDS-PAGE were electroblotted onto nitrocellulose paper for immunostaining with the anti-S-100 antiserum (Fig. 3B). This antiserum decorated, in addition to uncomplexed S-100 subunits, the 60-kDa polypeptide, after 5 min of exposure of GFAP/S-100 protein mixtures in the presence of Ca^{2+} to DSS (Fig. 3B, *lane 4*). Increasing the time of exposure of DSS to 15 or 30 min resulted in a greater immunostaining intensity of the 60-kDa polypeptide, as expected, and in the decoration of the ~150-kDa polypeptide (Fig. 3B, *lanes 6* and *8*, respectively). This latter finding suggested that the rather broad 150-kDa band observed in Coomassie Blue-stained gels after exposure to DSS for 30 min with GFAP/S-100 protein mixtures in the presence of Ca^{2+} was composed of two S-100-free GFAP molecules plus one GFAP-S-100 subunit complex, although the possibility that complexes of one S-100-free GFAP molecule plus two GFAP/S-100 dimer adducts could not be excluded. As observed in other work (20, 36, 37), the immunoreactivity of S-100 subunits complexed to a protein target (in this case GFAP) resulted in higher intensity than that of uncomplexed S-100 subunits. Also, no DSS cross-linked complexes of S-100 subunits were detected under the present experimental conditions, in agreement with previous work (20, 37). Similar experiments were done with calmodulin instead of S-100 protein. No DSS-induced complexes of GFAP-calmodulin could be detected, irrespective of the absence or presence of Ca^{2+} in Coomassie Blue-stained gels (not shown). Western blot experiments done with the anti-GFAP antiserum confirmed that DSS induced the formation of GFAP-S-100 complexes in a Ca^{2+}-dependent manner (Fig. 4, *lanes 4, 6,* and *8*) but not of GFAP-calmodulin complexes (not shown). Irrespective of the presence of Ca^{2+}, S-100 protein, calmodulin, or DSS, a small fraction of GFAP migrated as dimers and trimers in SDS gels. This was not appreciated in Coomassie Blue-stained gels (Fig. 4A) but was clearly evident in Western blots done with the anti-GFAP antiserum (compare Fig. 3A, *lanes 1* and *2* with Fig. 4, *lanes 1* and *2*). Recent work has shown that dimers and trimers of GFAP exist which copurify with monomers of GFAP and are resistant to SDS and boiling (38). Also, the immunostaining intensity of the 60-kDa GFAP-S-100 subunit complex in Fig. 4 was not the one expected, given the high immunoreactivity of GFAP (Fig. 4) and the high Coomassie Blue staining intensity of the 60-kDa complex (Fig. 3A, *lanes 4, 6,* and *8*). A possible explanation for this might be that complexation of S-100 protein to GFAP partially hampered the binding of the anti-GFAP antiserum to GFAP.
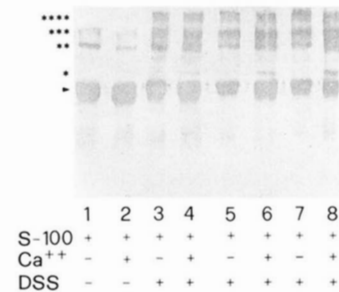

FIG. 4. **Visualization of the 60-kDa GFAP-S-100 subunit complex by immunostaining with the anti-GFAP antiserum.** Conditions were as described in the legend to Fig. 3, except that an anti-GFAP antiserum was used instead of the anti-S-100 protein antiserum. Note that dimers and trimers of GFAP, not evident in Coomassie Blue-stained gels (Fig. 3A), were decorated by the anti-GFAP antiserum in samples from mixtures of GFAP and S-100 protein incubated in the absence (*lane 1*) or presence (*lane 2*) of Ca^{2+}, but not exposed to DSS. The anti-GFAP antiserum immunostained monomers (*arrowhead*), and dimers (*double asterisk*), trimers (*triple asterisk*), and tetramers (*quadruple asterisks*) of GFAP produced by DSS (*lanes 3–8*), plus the 60-kDa GFAP-S-100 subunit complex formed in the presence of Ca^{2+} (*asterisk, lanes 4, 6,* and *8*).

GFAP Polymerization Assay—Assembly competence of GFAP and effects of S-100 protein on GFAP polymerization were studied by a sedimentation assay. The amount of polymerized GFAP recovered in pellets was linearly related to the GFAP concentration, in the GFAP concentration range tested (Fig. 5A). In contrast, the amount of GFAP remaining unpolymerized in supernatants was constant (Fig. 5A). Densitometric scanning of Coomassie Blue-stained gels revealed that the fraction of unpolymerized GFAP amounted to ~35 $\mu g/ml$, in good agreement with the calculated critical concentration of GFAP assembly (3). In the presence of Ca^{2+} (Figs. 5B and 6), but not in its absence (not shown), S-100 protein produced a dose-dependent inhibition of GFAP polymerization, whereas calmodulin proved without effect (Fig. 5B, *lanes 7* and *8*). Maximal inhibition was observed at an S-100 protein (dimer)/GFAP molar ratio of 0.5, whereas half-maximal inhibition occurred at a molar ratio of 0.2. S-100a_0 ($\alpha\alpha$), S-100a ($\alpha\beta$), and S-100b ($\beta\beta$) were equipotent to the unfractionated bovine brain S-100 protein mixture as inhibitors of GFAP polymerization (not shown). Also, at 1 μM free Ca^{2+} S-100 protein produced ~50% inhibition of GFAP polymerization, whereas a maximal inhibitory effect was observed at ~3 μM free Ca^{2+} (Fig. 7,). This observation induced us to investigate the Ca^{2+} dependence of S-100 protein binding to GFAP by DSS-induced cross-linking followed by SDS-PAGE. The results of these experiments (Fig. 7, ∇) showed that the 60-kDa GFAP-S-100 complex, as resolved by SDS-PAGE, formed with a pattern superimposable to that obtained in the experiments of GFAP polymerization inhibition.

DISCUSSION

The present data indicate that S-100 protein, but not calmodulin, binds to and inhibits the polymerization of GFAP in a Ca^{2+}-dependent manner. The binding affinity appeared to be in the nanomolar range, *i.e.* well below the estimated concentration of S-100 protein in glial cells (10–20 μM (14)). The binding stoichiometry was calculated to be 2 mol of GFAP/mol of S-100 protein (dimer), based on fluorescence spectroscopy and cross-linking experiments. A more detailed study of the kinetic parameters of S-100 protein binding to GFAP is underway. On the basis of the present data the tentative conclusion was drawn that one S-100 protein (dimer) would cross-link two GFAP molecules. This model is
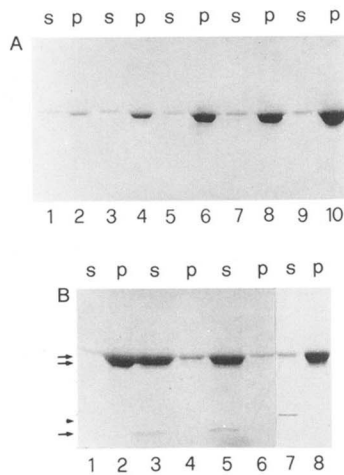
FIG. 5. **Assembly competence of GFAP (*A*) and effects of S-100 protein or calmodulin on GFAP polymerization (*B*).** *A*, increasing amounts of GFAP were incubated at 30 °C for 30 min in 0.1 ml of 0.1 M imidazole-HCl, pH 6.8, 0.2 mM EGTA, 1 mM DTT, 1 mM MgCl$_2$, 0.3 mM CaCl$_2$, and centrifuged at $13,000 \times g$ for 60 min. Supernatants were collected and pellets resuspended in 0.1 ml of buffer C. Identical volumes of supernatants (*s*) and pellets (*p*) were brought to 2% with SDS and mercaptoethanol, boiled for 5 min, and subjected to SDS-PAGE (7.5% acrylamide). Gels were stained with Coomassie Blue and densitometrically scanned to measure the relative proportions of GFAP in supernatants and pellets. Initial concentrations of GFAP were 0.1 (*lanes 1* and *2*), 0.2 (*lanes 3* and *4*), 0.4 (*lanes 5* and *6*), 0.6 (*lanes 7* and *8*), and 1.2 (*lanes 9* and *10*) mg/ml. *B*, GFAP (0.5 mg/ml) was incubated at 30 °C for 30 min in 0.1 ml of 10 mM imidazole-HCl, pH 7.1, 0.2 mM EGTA, 1 mM DTT, 1 mM MgCl$_2$, 0.3 mM CaCl$_2$ in the absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of 0.08 mg of S-100 protein/ml, 0.1 mg of S-100 protein/ml (*lanes 5* and *6*), or 0.08 mg of calmodulin/ml (*lanes 7* and *8*), before addition of imidazole-HCl, pH 6.8, to 0.1 M. After an additional 30-min incubation at 30 °C, mixtures were centrifuged as above to separate supernatants (*s*) and pellets (*p*), which were processed as described in *A* before SDS-PAGE. Gels were stained with Coomassie Blue. Note that S-100 protein nearly completely inhibited the GFAP polymerization, whereas calmodulin proved virtually without effect. Results identical to those illustrated above were obtained when no preincubation of GFAP without or with S-100 protein or calmodulin, respectively, was done before addition of imidazole-HCl, pH 6.8, to 0.1 M (not shown).
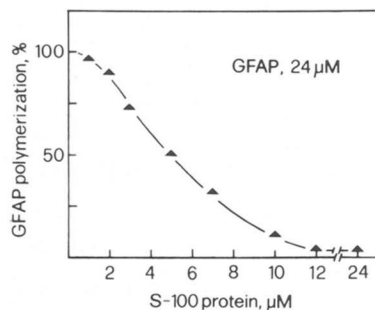
FIG. 6. **Dose dependence of the inhibitory effect of S-100 protein on GFAP polymerization.** Conditions were as described in the legend to Fig. 5, except that GFAP was used at 24 μM and S-100 protein was used at increasing concentrations (0–24 μM). The relative proportions of GFAP in supernatants and pellets were measured densitometrically after SDS-PAGE of supernatants and pellets. The GFAP recovered in pellets after polymerization in the absence of S-100 protein was taken as 100%. Identical results were obtained with pure S-100a$_0$, S-100a, or S-100b (not shown).

reminiscent of what has recently been observed when the interaction of S-100 protein with annexin II (the heavy chain of the cytoskeletal protein complex, calpactin I (39, 40)) was studied (20).
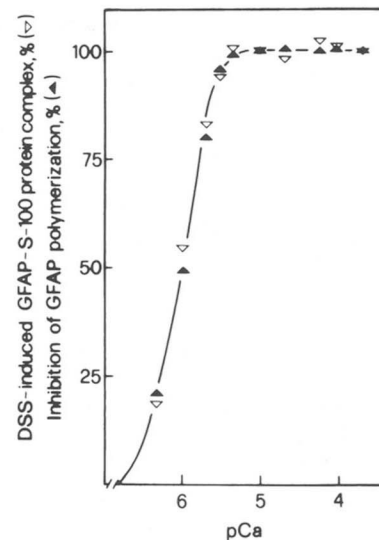
FIG. 7. **Ca²⁺ dependence of DSS-induced complex formation between GFAP and S-100 protein, and S-100 protein-dependent inhibition of GFAP polymerization.** Conditions for chemical cross-linking between GFAP and S-100 protein (∇) were as described in the legend to Fig. 3, except that incubations were done in the presence of increasing free Ca²⁺ concentrations (*p*Ca) as indicated. After 30 min of incubation at 30 °C, DSS was added to a final concentration of 0.5 mM for 5 min. The reaction was terminated and samples were subjected to SDS-PAGE as described in the legend to Fig. 3. The 60-kDa GFAP-S-100 subunit complex so resolved was quantitated by densitometry of Coomassie Blue-stained gels. Results are expressed as the percent formation of the 60-kDa complex *versus* the free Ca²⁺ concentration. Conditions for GFAP polymerization in the presence of S-100 protein were as described in the legend to Fig. 5*B*, except that the S-100 concentration was held constant and the free Ca²⁺ concentration was varied as indicated. At the end of incubations, samples were centrifuged and supernatants and pellets were subjected to SDS-PAGE. The percent inhibition of GFAP polymerization ($\blacktriangledown$) was calculated by densitometry of Coomassie Blue-stained gels as the fraction of GFAP in pellets at the different free Ca²⁺ concentrations. Parallel experiments were done in which S-100 protein was omitted. Under the latter conditions the fraction of GFAP recovered in pellets was constant, irrespective of the free Ca²⁺ concentration (not shown).

Altogether, these findings suggest the possibility that the mode of Ca²⁺-dependent interaction of S-100 protein with its protein targets in solution involves the functional cross-linking of two monomers of a given target protein by one S-100 dimer. This could also apply to the S-100 protein binding to tubulin, in which case, too, a binding stoichiometry of one tubulin dimer/S-100 dimer was calculated (37).

The cross-linking experiments illustrated in this report indicate that the DSS-induced formation of dimers, trimers, and tetramers of GFAP is not affected by Ca²⁺. These experiments also indicate that once S-100 protein had bound to GFAP, interactions between GFAP molecules are greatly reduced. This mechanism could be at the basis of the observed S-100 protein-dependent inhibition of GFAP polymerization in the presence of Ca²⁺. S-100 protein appears to be a potent inhibitor of GFAP polymerization, as the half-maximal inhibitory effect is obtained at an S-100 protein/GFAP molar ratio of 0.2. Although no information is available at present on the kinetics of GFAP polymerization in the presence of S-100 protein plus Ca²⁺ (this will be matter of a future study), we expect that S-100 protein inhibits the GFAP polymerization by sequestering unpolymerized GFAP, anticipating that S-100 protein interferes with the nucleation step of GF formation, as is the case with the S-100 protein inhibitory effect on microtubule assembly (22). However, we cannot exclude the

possibility that S-100 protein interferes with the unpolymerized-polymerized GFAP exchange.

Individual S-100 protein isoforms (S-100a$_0$, S-100a, and S-100b) proved equipotent to the unfractionated mixture of S-100a plus S-100b as inhibitors of GFAP polymerization. Also, by two different experimental approaches, S-100 protein appeared to bind to GFAP and to affect GFAP polymerization at free Ca^{2+} concentrations compatible with those found *in vivo* in non-resting cells. These observations lend support to the view that the Ca^{2+}-binding affinity of S-100 protein depends on its conformation (see Ref. 14). Remarkably, half-maximal S-100 protein binding to GFAP as investigated by chemical cross-linking and half-maximal inhibitory effects on GFAP polymerization occurred at a free Ca^{2+} concentration (1 μM) at which GFAP produced only 15% of maximal changes in the fluorescence of acrylodan-S-100 protein. This indicates that at free Ca^{2+} concentrations >1–2 μM, in the presence (or due to the presence) of GFAP, Ca^{2+} produces larger changes in the S-100 protein conformation, possibly owing to binding to the unconventional EF-hand site that is located in the N-terminal half of individual S-100 subunits (34, 35). Alternatively, fluorescence changes at relatively high Ca^{2+} concentrations might depend on GFAP-induced reinforcement of hydrophobic interactions between S-100 subunits.

It has been proposed that GFAP polymerization requires that the net positive charge of the C-terminal end piece of GFAP not be reduced (41). Accordingly, the inhibitory effect on GF formation of phosphorylation of residues in that GFAP end piece has been ascribed to reduction in basicity of a C-terminal stretch of residues that appears to be involved in GFAP polymerization (7). Since S-100 protein is an acidic protein (pI ~ 4.2), we cannot exclude that its inhibitory effect on GFAP polymerization depends on interaction between negatively charged residues in S-100 subunits with basic residues in GFAP. However, we note the following: 1) both the binding of S-100 protein to GFAP and the S-100 protein inhibitory effect on GFAP polymerization are Ca^{2+}-dependent. 2) Calmodulin, another very acidic EF-hand Ca^{2+}-binding protein, and bovine serum albumin (not shown) neither bind to GFAP nor affect its polymerization. 3) The GFAP-induced changes in the emission spectrum of acrylodan-S-100 protein in the presence of Ca^{2+} are consistent with the chromophore being located in a more hydrophobic environment. Whether this is a hydrophobic stretch in GFAP or is the hydrophobic domain in the C-terminal half of individual S-100 subunits (34, 35) is not known at present. Actually, Ca^{2+} binding to acrylodan-S-100 protein in the absence of other protein species results in a slight red shift of the emission maximum together with a slight reduction in the emissiom spectrum area (20, 30), indicating that under these conditions the chromophore is located in a more polar environment. In turn, this is in agreement with the notion that Ca^{2+} induces —SH groups in S-100 protein to be more exposed to the solvent (42). Also, we can exclude that S-100 protein inhibits the GFAP polymerization by sequestering Ca^{2+} since identical inhibitory effects of S-100 protein were observed at 3–1,000 μM free Ca^{2+}, and no Ca^{2+}-dependent stimulation of GFAP polymerization was detected within this range of Ca^{2+} concentrations in the absence of S-100 protein.

Interestingly, the genes encoding GFAP and the β subunit of S-100 protein are both regulated by cAMP (Ref. 10, and references therein). Whether there is any functional relationship between the data illustrated in the present report and the common regulation by cAMP of GFAP and S-100b genes is an attractive possibility that deserves investigation.

Also, whether or not S-100 protein regulates the state of assembly of GFs *in vivo* remains to be established. Preliminary immunocytochemical observations at the electron microscope level indicate that in Triton-cytoskeletons of cultured glial cells S-100 protein is found associated with intermediate filaments (43).

REFERENCES

1. Bignami, A., Eng, L. F., Dahl, D., and Uyeda, C. T. (1972) *Brain Res.* **43**, 429–435
2. Dahl, D., and Bignami, A. (1983) in *Handbook of Neurochemistry* (Lajtha, A., ed) Vol. 5, pp. 127–151, Plenum Press, New York
3. Nakamura, Y., Takeda, M., Angelidis, K. J., Tada, K., Hariguchi, S., and Nishinura, T. (1991) *Glia* **4**, 101–110
4. Rueger, D. C., Huston, J. S., Dahl, D., and Bignami, A. (1979) *J. Mol. Biol.* **135**, 53–68
5. Lucas, C. V., Beusch, K. J., and Eng, L. F. (1980) *Neurochem. Res.* **5**, 247–255
6. Lucas, C. V., Reaven, E. P., Beusch, K. J., and Eng, L. F. (1980) *Neurochem. Res.* **5**, 1199–1209
7. Inagaki, M., Gonda, Y., Nishizawa, K., Kitamura, S., Sato, C., Ando, S., Tanabe, K., Kikuchi, K., Tsuiki, S., and Nishi, Y. (1990) *J. Biol. Chem.* **265**, 4722–4729
8. Backhovens, H., Gheuens, J., and Slegers, H. (1987) *J. Neurochem.* **49**, 348–354
9. Messens, J., and Slegers, H. (1992) *J. Neurochem.* **58**, 2071–2080
10. Freeman, M. R., Beckman, S. L., and Sueoka, N. (1989) *Exp. Cell Res.* **182**, 370–383
11. Minura, M., Tamura, T.-A., and Mikoshiba, K. (1990) *J. Neurochem.* **55**, 1180–1188
12. Yang, Z. W., and Babitch, J. A. (1988) *Biochemistry* **27**, 7038–7045
13. Yang, Z. W., Kong, C. F., and Babitch, J. A. (1988) *Biochemistry* **27**, 7045–7050
14. Donato, R. (1991) *Cell Calcium* **12**, 713–726
15. Donato, R. (1988) *J. Biol. Chem.* **263**, 106–110
16. Skripnikova, E. V., and Gusev, N. B. (1989) *FEBS Lett.* **257**, 380–382
17. Fujii, T., Machino, K., Andoh, H., Satoh, T., and Kondo, Y. (1990) *J. Biochem. (Tokyo)* **107**, 133–137
18. Pritchard, K., and Martson, S. B. (1991) *Biochem. J.* **277**, 819–824
19. Selinfreund, R. H., Barger, S. W., Welsh, M. J., and Van Eldik, L. J. (1990) *J. Cell Biol.* **111**, 2021–2028
20. Bianchi, R., Pula, G., Ceccarelli, P., Giambanco, I., and Donato, R. (1992) *Biochim. Biophys. Acta* **1160**, 67–75
21. Persechini, A., Moncrief, N. D., and Kretsinger, R. H. (1989) *Trends Neurosci.* **12**, 462–467
22. Donato, R. (1984) *Biochem. Biophys. Res. Commun.* **124**, 850–856
23. Donato, R., Giambanco, I., Aisa, M. C., Ceccarelli, P., and Di Geronimo, G. (1988) *Cell Biol. Int. Rep.* **12**, 565–566
24. Zolese, G., Giambanco, I., Curatola, G., De Stasio G., and Donato, R. (1993) *Biochim. Biophys. Acta* **1162**, 47–53
25. Isobe, T., Nakajima, T., and Okuyama, T. (1977) *Biochim. Biophys. Acta* **494**, 222–232
26. Baudier, J., and Gerard, D. (1983) *Biochemistry* **22**, 3360–3369
27. Donato, R., Giambanco, I., Aisa, M. C., di Geronimo, G., Ceccarelli, P., Rambotti, M. G., and Spreca, A. (1989) *Cell Calcium* **10**, 81–92
28. Laemmli, U. K. (1970) *Nature* **227**, 680–685
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
30. Baudier, J., Glasser, N., and Duportail, G. (1986) *Biochemistry* **25**, 6934–6941
31. Pendergast, F. G., Meyer, M., Carlson, G. L., Iida, S., and Potter, J. D. (1983) *J. Biol. Chem.* **258**, 7541–7544
32. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
33. Donato, R., Prestagiovanni, B., and Zelano, G. (1986) *J. Neurochem.* **46**, 1333–1337
34. Isobe, T., and Okuyama, T. (1978) *Eur. J. Biochem.* **89**, 379–388
35. Isobe, T., and Okuyama, T. (1981) *Eur. J. Biochem.* **116**, 79–86
36. Baudier, J., and Cole, R. D. (1988) *Biochemistry* **27**, 2728–2736
37. Donato, R., Giambanco, I., and Aisa, M. C. (1989) *J. Neurochem.* **53**, 566–571
38. Shaw, G., and Hawkins, J. (1992) *NeuroReport* **3**, 461–464
39. Gerke, V., and Weber, K. (1984) *EMBO J.* **3**, 227–233
40. Glenney, J. R. (1985) *FEBS Lett.* **192**, 79–82
41. Inagaki, M., Takahara, H., Nishi, Y., Sugawara, K., and Sato, C. (1989) *J. Biol. Chem.* **264**, 18119–18127
42. Calissano, P., Moore, B. W., and Friesen, A. (1969) *Biochemistry* **8**, 4318–4326
43. Rambotti, M. G., Spreca, A., Leoncini, P., Estenoz, M., Costantino-Ceccarini, E., Giambanco, I., and Donato, R. (1990) *J. Histochem. Cytochem.* **38**, 1583–1589