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Role of the C-Terminal Extension in the Interaction of S100A1 with GFAP, Tubulin, the S100A1- and S100B-Inhibitory Peptide, TRTK-12, and a Peptide Derived from p53, and the S100A1 Inhibitory Effect on GFAP Polymerization

Marisa Garbuglia,* Marco Verzini,* Richard R. Rustandi,† Dirk Osterloh,‡ David J. Weber,† Volker Gerke,‡ and Rosario Donato*

**Department of Experimental Medicine and Biochemical Science, University of Perugia, Perugia, Italy; †Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, Maryland; and ‡Institute for Medical Biochemistry, University of Muenster, Muenster, Germany*

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Whereas native and recombinant S100A1 inhibited GFAP assembly, a truncated S100A1 lacking the last six C-terminal residues (Phe88-Ser93) (S100A1Δ88-93) proved unable to do so. The inhibitory effects of native and recombinant S100A1 on GFAP assembly were blocked by both TRTK-12, a synthetic peptide derived from the α-subunit of the actin capping protein, CapZ, and a synthetic peptide derived from the tumor-suppressor protein, p53, in a dose-dependent manner. By fluorescent spectroscopy, TRTK-12 and the p53 peptide, like GFAP and tubulin, caused a dose- and Ca^{2+}-dependent blue-shift of the fluorescence maximum of acrylodan-S100A1. In contrast, GFAP, tubulin, TRTK-12, or the p53 peptide caused no significant changes in the fluorescence spectrum of acrylodan-S100A1Δ88-93. By chemical crosslinking, both TRTK-12 and the p53 peptide strongly reduced or blocked the formation of GFAP-S100A1 or tubulin-S100A1 complexes, respectively, and S100A1Δ88-93 was unable to complex with tubulin, whereas a remarkably reduced complexation of GFAP with the truncated protein was observed. All the above observations show that the C-terminal extension of S100A1 is an essential part of the S100A1 site implicated in the recognition of GFAP, tubulin, p53, and the α-subunit of CapZ.

Key Words: **S100A1; TRTK-12; p53 peptide; GFAP; tubulin; target-protein recognition.**

Abbreviations: GFAP, glial fibrillary acidic protein; IFs, intermediate filaments; DTT, dithiothreitol; DSS, disuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis.

S100A1 and S100B are members of a multigenic family of Ca^{2+}-binding proteins of the EF-hand type implicated in the regulation of protein phosphorylation, some enzyme activities, Ca^{2+} homeostasis, and the dynamics of microtubules and type III intermediate filaments (IFs) (for reviews, see 1-3). Tubulin, a brain aldolase, caldesmon, the type III IF subunits, glial fibrillary acidic protein (GFAP) and desmin, calponin, the α-subunit of the actin capping protein, CapZ, the giant sarcomeric kinase, twitchin, and phosphoglucomutase were identified as S100A1 and S100B target proteins (1–6). In most instances, the interaction of S100A1 and/or S100B with target proteins proved Ca^{2+}-dependent.

Recently, the solution structure of both apo and Ca^{2+}-loaded S100B was determined by NMR spectroscopy and X-ray crystallography (7–11). S100B, either apo or Ca^{2+}-loaded, was shown to exist as a homodimer in which the two monomers are held together by noncovalent bonds and are related by a two-fold symmetry axis. The main change in the S100B structure in the presence of Ca^{2+} is a rotation of helix III relative to helix IV (which flank the canonical C-terminal EF-hand Ca^{2+}-binding site), and a consequent reorientation of the hinge region (7, 10, 11). These conformational changes expose a cleft, defined by residues in helices III and IV, the hinge region (that connects the N-terminal half to the C-terminal half of the monomer) and the C-terminal extension, which is absent in the apo structure and may constitute a binding surface for S100B target proteins. Given the reciprocal arrangement of the two monomers in the S100B dimer, two binding surfaces become exposed on opposite sides of the dimer in the presence of Ca^{2+}. A similar X-type

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four-helix bundle arrangement of monomers was observed in dimeric S100A6 (12–14), and thus, it was suggested that many of the S100 proteins that dimerize do so with the same structural motif.

S100A1 exibits a ≈56% sequence identity to S100B, with the least amount of identity found in the hinge region and the C-terminal extension (15, 16). However, several residues in the hinge region and the C-terminal extension, as well as in helices III and IV, that are potentially implicated in the recognition of target proteins (6, 10, 11), are conserved in S100A1 and S100B. Thus, Ser42, Phe44, Leu45 and Lys49 in the S100A1 hinge region, and Phe88 and Phe89 in the S100A1 C-terminal extension correspond to Ser41, Phe43, Leu44 and Lys48, and Phe87 and Phe88, respectively, in S100B (15, 16). Also, Val78, Thr82, Ala84 and Cys85 in the S100A1 helix IV correspond to Val77, Thr81, Ala83 and Cys84 in the S100B helix IV (15, 16). These residues might be implicated in the binding of S100B and S100A1 to similar target proteins.

S100B and S100A1 interact with TRTK-12, a synthetic peptide derived from a sequence contained in the α subunit of CapZ (17, 18). Binding of TRTK-12 to S100A1/B blocks the ability of both S100 proteins to inhibit the assembly of GFAP (19). S100B also interacts with the tumor suppressor protein, p53, and inhibits its phosphorylation and oligomerization (20). Recently, S100B was shown to interact with a peptide derived from the putative phosphorylation site and S100B binding domain of p53, and it was shown to inhibit the phosphorylation of the p53 peptide (21, 22). Binding of the p53 peptide was proposed to occur at a binding surface of S100B that is exposed in the presence of Ca^{2+} (21).

We report here that: (i) the p53 peptide strongly reduces the ability of S100A1 to inhibit the assembly of the IF subunit GFAP, similarly to the S100B- and S100A1-inhibitory peptide TRTK-12; (ii) a recombinant S100A1 lacking the C-terminal extension (Phe88-Ser93) (S100A1Δ88-93), neither inhibits GFAP assembly nor interacts with GFAP, tubulin, the p53 peptide, or TRTK-12, as investigated by fluorescent spectroscopy; and (iii) the p53 peptide reduces or abolishes the interaction of S100A1 with GFAP and tubulin similarly to TRTK-12, and no complex formation between S100A1Δ88-93 and tubulin occurs whereas S100A1Δ88-93 exhibits some interaction with GFAP, as investigated by chemical crosslinking. Together, these data suggest that the C-terminal extension is a fundamental part of the binding surface on S100A1 that is implicated in the binding of GFAP, tubulin, and, likely, p53 and CapZα, and that additional parts of the S100A1 monomer might have a role in the recognition of GFAP.

MATERIALS AND METHODS

Materials. Native S100A1 was purified from porcine heart by either of two described procedures (23, 24). Recombinant S100A1 or a truncated form of S100A1 lacking the last six residues (Phe88-Ser93) (S100A1Δ88-93) was expressed in the *E. coli* strain BL 21 (DE 3) pLysS (Novagen) transformed with an expression plasmid containing the human S100A1 gene (25) and purified from bacteria extracts as described (23). GFAP and tubulin were purified from bovine spinal cord (26). TRTK-12 was synthetized by GENOSYS. The p53 peptide (acetyl-SHLKSKKGQSTSRHKKLMFKTE-am), derived from human p53 (residues 367-388) was prepared as described (21). A polyclonal anti-S100A1/B antiserum was from Dakopatts, and the fluorescent probe, acrylodan, from Molecular Probes. All other reagents were analytical grade reagents from Sigma, BDH, Fluka, Bio-Rad, or Carlo Erba.

Sedimentation assay. GFAP was assembled in the absence or presence of each of the above proteins plus or minus the p53 peptide or TRTK-12 (27). Samples were centrifuged to separate supernatants from pellets. Equal volumes of supernatants and pellets were subjected to SDS-PAGE (10% acrylamide) (28). Gels were stained with Coomassie blue and the relative proportions of unassembled and assembled GFAP were calculated by densitometry.

Fluorescent spectroscopy. S100A1 or S100A1Δ88-93 was labeled with acrylodan and characterized as reported (4). Samples of acrylodan-S100A1 or acrylodan-S100A1Δ88-93 in 10 mM imidazole-HCl, pH 7.0, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 M NaCl, 0.2 mM EGTA plus ≈100 μM free Ca^{2+} were incubated at 25°C in the absence or presence of various proteins or peptide as described in the legend to Figs. 3 and 4. Fluorescent emission spectra (excitation: 380 nm; emission: 400-650 nm) were recorded at 25°C using a Perkin-Elmer LS 50 B spectrofluorometer. Emission spectra were corrected for dilution which never exceeded 5%.

Chemical crosslinking. For chemical crosslinking experiments, S100A1 or S100A1Δ88-93 was incubated at 22°C for 30 min in 10 mM imidazole-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM EGTA plus ≈ 100 μM free Ca^{2+} (final volume 0.1 ml) in the presence of various proteins or peptide as described in the legend to Fig. 6 prior to addition of the bifunctional crosslinker disuccinimidyl suberate (DSS) (final concentration 0.5 mM). After incubation for 15 min at room temperature, reactions were terminated by addition of 2% SDS (w/v) and 2% 2-mercaptoethanol and samples analyzed by SDS-PAGE (10% acrylamide). Separated peptides were stained with Coomassie blue or transblotted onto nitrocellulose (29) for Western blot analyses with a polyclonal anti-S100A1/B antiserum.

Other procedures. The following molecular masses were applied: 21 kDa for the S100A1 dimer and the S100A1Δ88-93 dimer; 50 kDa for GFAP; 50 kDa for tubulin; 2800 Da for the p53 peptide; and 1470 Da for TRTK-12. Free Ca^{2+} concentration was measured as described (30).

RESULTS AND DISCUSSION

By a sedimentation assay, the p53 peptide reduced the inhibitory effect of S100A1 (Fig. 1A) on GFAP assembly into IFs, in a dose-dependent manner (Fig. 1B). The p53 peptide proved less inhibitory than TRTK-12. Nearly identical results were obtained with S100B (not shown). The p53 peptide and TRTK-12 proved synergistic when tested together at subsaturating concentrations (not shown). The lower potency of the p53 peptide, as compared with TRTK-12, as an S100A1- and S100B-inhibitory peptide likely depends on its lower hydrophobicity: a Phe385Trp mutation in

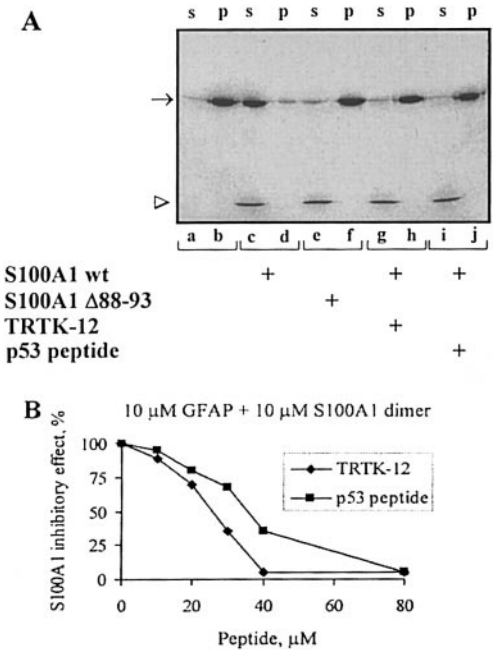

FIG. 1. Assembly of GFAP in the absence or presence of S100A1 plus or minus the p53 peptide or TRTK-12, or S100A1Δ88-93. (A) GFAP (10 μM) was assembled into IFs (0.1 M imidazole-HCl, pH 6.8, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM EGTA, ~ 100 μM free Ca^{2+}) for 30 min at 30°C in the absence of other proteins or peptides (lanes *a,b*) or presence of wild-type S100A1 (lanes *c,d*), S100A1Δ88-93 (lanes *e,f*), wild-type S100A1 plus TRTK-12 (lanes *g,h*), or wild-type S100A1 plus the p53 peptide (lanes *i,j*). Samples were centrifuged to separate supernatants (*s*) from pellets (*p*), identical volumes of which were analyzed by SDS-PAGE. Gels were stained by Coomassie blue. S100A1 dimer and S100A1Δ88-93 dimer were used each at 10 μM, TRTK-12 at 40 μM and the p53 peptide at 80 μM. The position of GFAP is indicated by an arrow and that of monomeric S100A1 or S100A1Δ88-93 by an arrowhead. (B) GFAP (10 μM) was assembled as above in the absence or presence of S100A1 dimer (10 μM) plus increasing concentrations or either TRTK-12 or the p53 peptide as indicated. Samples were then processed as described above. Results are expressed as the percent inhibitory effect of S100A1 on GFAP assembly vs. the concentration of individual peptides.

the p53 peptide was shown to remarkably increase the affinity of the peptide binding to S100B (22). In contrast, S100A1Δ88-93 was unable to inhibit the GFAP assembly (Fig. 1A). Together, these data suggest that the C-terminal extension is an essential part of the binding surface on S100A that is implicated in the recognition of GFAP, and that GFAP, TRTK-12 and the p53 peptide may share a common binding site on S100A1 that includes the S100A1 C-terminal extension.

To test this possibility, acrylodan-S100A1 or acrylodan-S100A1Δ88-93 was used in binding studies with various combinations of GFAP, TRTK-12, and the p53 peptide. Tubulin, another S100A1 (and S100B) target protein (23,31), was also used in these analyses. GFAP caused a ≈30-nm blue-shift of the fluorescence maximum and a ≈50% increase in the fluorescence intensity of acrylodan-S100A1 (Fig. 2A), and virtually no changes in the emission spectrum of acrylodan-S100A1Δ88-93 (Fig. 2B). Qualitatively similar changes in the emission spectrum of acrylodan-S100A1 were registered with TRTK-12, the p53 peptide, or tubulin, and, as with GFAP, virtually no changes in the emission spectrum of acrylodan-S100A1Δ88-93 were observed in the presence of each of the above proteins or peptides (not shown, but see Fig. 3B). GFAP, the p53 peptide, TRTK-12 and tubulin each caused a dose-dependent and saturable blue-shift of the fluorescence maximum of acrylodan-S100A1 (Fig. 3A). The different extents of changes caused by individual ligands likely depend on differences in their respective structures, although we cannot exclude subtle differences in the structural requirements of S100A1 binding to each of these molecules. Fig. 3B shows the lack of significant changes in the fluorescence maximum of acrylodan-S100A1Δ88-93 in the presence of increasing concentra-

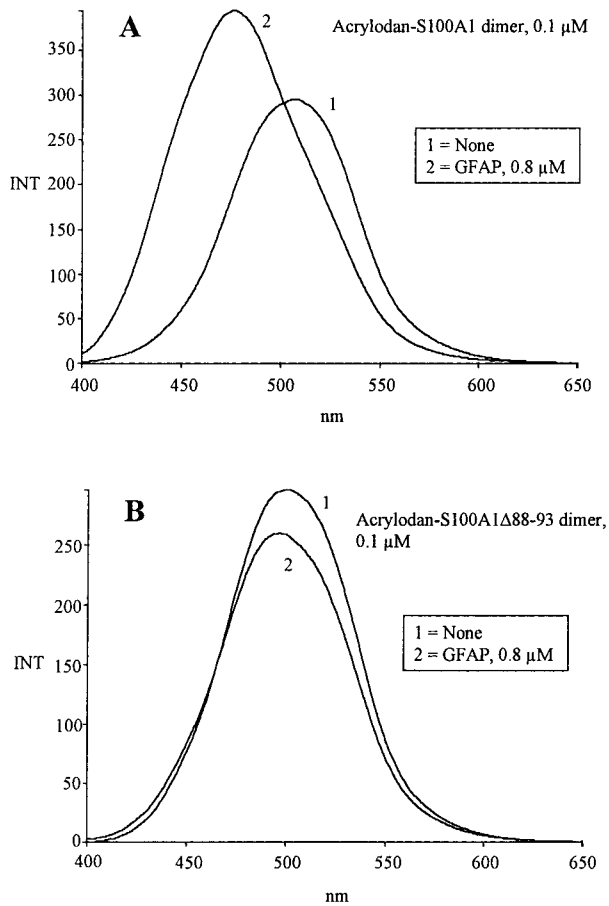

FIG. 2. Binding of GFAP to S100A1 or S100A1Δ88-93 as investigated by fluorescent spectroscopy. (A) Fluorescence emission spectrum of acrylodan-S100A1 dimer (0.1 μM) incubated as described under Materials and Methods in the absence (trace 1) or presence of 0.8 μM GFAP (trace 2). Note the GFAP-induced blue shift of the fluorescence maximum and increase in fluorescence intensity. (B) The same experiment as above, except that acrylodan-S100A1Δ88-93 was used instead of acrylodan-S100A1. Note the lack of significant changes in the fluorescence spectrum upon addition of GFAP.

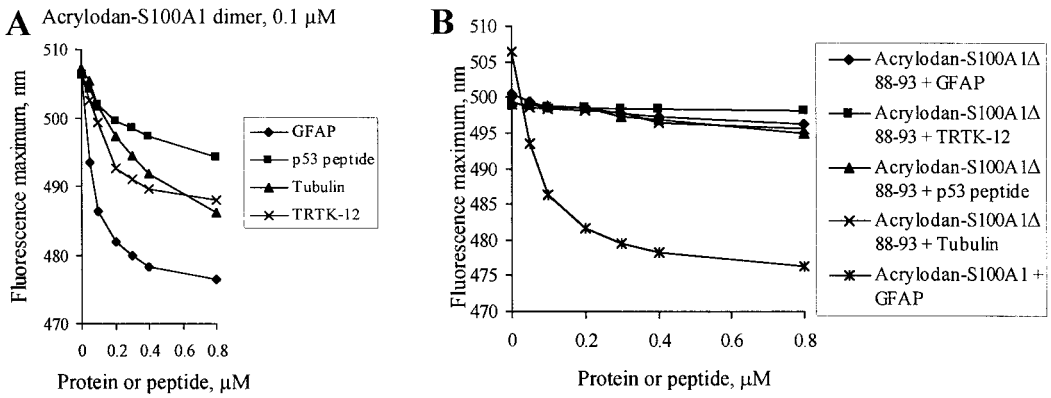

FIG. 3. Binding of GFAP, tubulin, the p53 peptide and TRTK-12 to acrylodan-S100A1 as investigated by fluorescent spectroscopy. (A) Conditions were as described in Fig. 2A, except that GFAP, tubulin, the p53 peptide or TRTK-12 was added to acrylodan-S100A1 at increasing concentrations. Results are expressed as the fluorescence maximum of the emission spectrum (nm) registered after individual additions vs. the concentration of each added substance as indicated. Note that: (i) each of the proteins or peptides tested causes a dose-dependent, albeit quantitatively different blue-shift of the fluorescence maximum; and (ii) in each case an inflection point in the binding curve can be observed at about a molar ratio of 2 mol of ligand/mol S100A1 dimer, pointing to a binding stoichiometry of 1 mol of ligand/mol of S100A1 monomer. (B) The same experiment as above was performed with acrylodan-S100A1Δ88-93. Note the absence of significant ligand-induced changes in the fluorescence maximum. The binding curve obtained with GFAP and acrylodan-S100A1 (see A) was included for comparison purposes.

tions of GFAP, the p53 peptide, TRTK-12, or tubulin, as compared to the dose-dependent change caused by GFAP in the fluorescence maximum of acrylodan-S100A1. As lack of changes in the emission spectrum of S100A1Δ88-93 in the presence of each of the above ligands could be due to altered or no Ca^{2+}-binding properties of S100A1Δ88-93, we analyzed the effects of increasing Ca^{2+} concentrations on the emission spectrum of acrylodan-S100A1 and acrylodan-S100A1Δ88-93. Ca^{2+} caused a dose-dependent red-shift of the fluorescence maximum (Fig. 4) and a decrease in fluorescence intensity (not shown) in both cases, with half-maximal effect at ≈1 μM and maximal effect at ≈3-5 μM free Ca^{2+} in the case of S100A1, and at ≈3-5 and ≈50 μM free Ca^{2+}, respectively, in the case of S100A1Δ88-93. Thus, deletion of the C-terminal extension did not appear to compromise the ability of S100A1Δ88-93 to undergo Ca^{2+}-dependent conformational changes, as investigated by the present experimental approach, differences concerning the extent and apparent affinity rather than the occurrence or non-occurrence of changes in the two proteins. However, above ≈50 μM free Ca^{2+} similar Ca^{2+}-dependent changes in the conformation of the two proteins were registered.

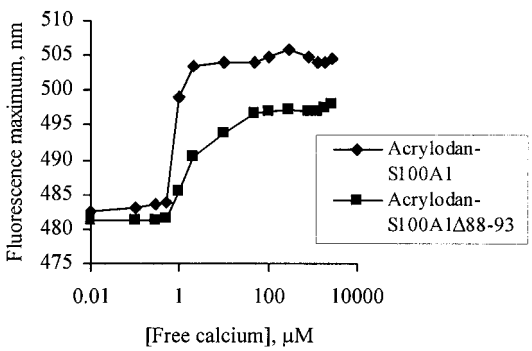

FIG. 4. Ca^{2+}-induced changes in the fluorescence maximum of acrylodan-S100A1 or S100A1Δ88-93. Conditions were as in Fig. 2A, except that Ca^{2+} was added to 0.1 μM acrylodan-S100A1 dimer or S100A1Δ88-93 dimer to the final free concentrations indicated.

Both the p53 peptide and TRTK-12 strongly reduced or abolished the formation of GFAP-S100A1 and tubulin-S100A1 complexes, as investigated by chemical crosslinking (Fig. 5). Under the present experimental conditions, the p53 peptide and TRTK-12 proved more potent as inhibitors of the tubulin-S100A1 than the GFAP-S100A1 interaction. The higher affinity of S100A1 binding to GFAP (≈0.5 μM) (27) than to tubulin (≈6 μM) (31) might explain these differences. No complex formation between S100A1Δ88-93 and tubulin could be detected by this approach (Fig. 5A and B). In contrast, some complex formation occurred between S100A1Δ88-93 and GFAP (Fig. 5B). These data suggest the following: (i) TRTK-12 and the p53 peptide likely interact with a similar, if not identical site on S100A1, thereby interfering with the S100A1-GFAP interaction: differences in the potency of individual peptides might depend on their different structures; (ii) the C-terminal extension appears indispensable for S100A1 to bind tubulin; and (iii) the C-terminal extension plays a major role in the S100A1-GFAP interactions, but other parts of the S100A1 molecule likely contribute to the generation of the GFAP binding site on S100A1. We would exclude that the lack of the

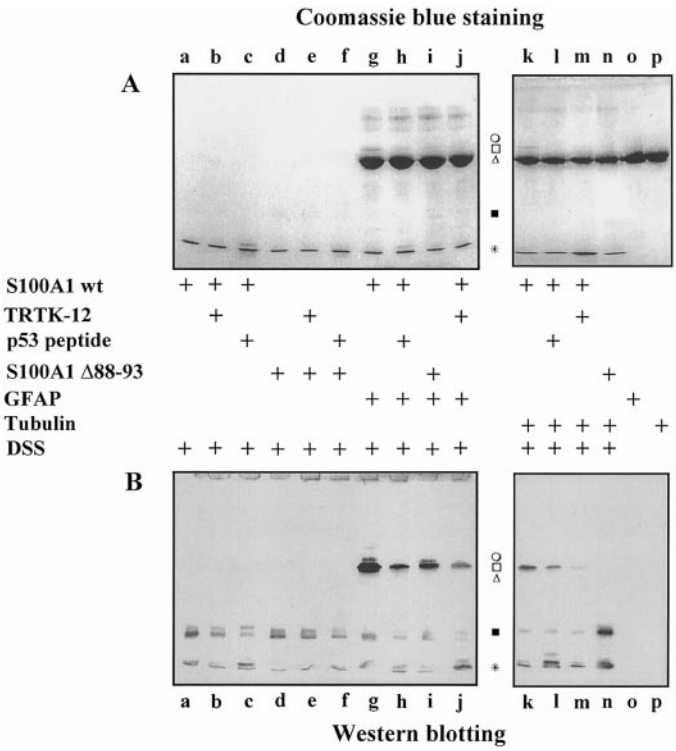

FIG. 5. Interaction of wild-type S100A1 or S100A1Δ88-93 with GFAP, tubulin, TRTK12, or the p53 peptide, or various combinations of these, as investigated by chemical crosslinking. GFAP (16 μM) or tubulin (16 μM) was incubated as described under Materials and Methods with 8 μM wild-type (wt) S100A1 dimer or S100A1Δ88-93 dimer plus or minus 20 μM TRTK-12 or p53 peptide, as indicated, before the addition of DSS to 0.5 mM. After 15 min, the reaction was stopped and samples were subjected to SDS-PAGE. Separated polypeptides were either stained with Coomassie blue or transblotted onto nitrocellulose for Western blot analyses with a polyclonal anti-S100A1/B antiserum. Coomassie-blue stained gels (A) show a DSS-induced 60-kDa GFAP-S100A1 complex (lane *g*) and tubulin-S100A1 complex (lane *k*) (□). These complexes comigrate in the SDS gel because of the similar molecular masses of GFAP and tubulin (see lanes *g-p*). This (60-kDa) small band (lanes *g* and *k*) is no longer visible if the reaction mixture contained either TRTK-12 or the p53 peptide. No DSS-induced complex formation occurs if S100A1Δ88-93 had been used. The positions of monomeric S100A1 or S100A1Δ88-93, and monomeric, uncomplexed GFAP or tubulin are indicated by (*) and (△), respectively. Western blot analyses (B) permit the visualization of the 60-kDa GFAP-S100A1 and tubulin-S100A1 complexes (□), a 70-kDa GFAP-S100A1$_2$ complex (○), and DSS-crosslinked S100A1 and S100A1Δ88-93 dimers (■). Note that: (i) the p53 peptide or TRTK-12 does not completely abolish the formation of the GFAP-S100A1 complexes under the present experimental conditions (lanes *h* and *j*, respectively) (however, higher concentrations of individual peptides did; not shown); (ii) TRTK-12 nearly completely abolishes the formation of the 60-kDa tubulin-S100A1 complex (lane *m*), whereas the p53 peptide is less able to do so (lane *l*); and (iii) a much reduced, but significant complex formation occurs between S100A1Δ88-93 and GFAP (lane *i*), whereas no complexation of S100A1Δ88-93 with tubulin can be seen (lane *n*).

C-terminal extension (which takes part in the dimerization of S100B (7, 8)) may determine the inability of S100A1Δ88-93 to interact with the ligands tested here and to inhibit the assembly of GFAP because of gross disturbances in the dimerization of the protein; in fact, chemical crosslinking shows that S100A1Δ88-93 dimers form in the presence of DSS as is the case with wild-type S100A1 (Fig. 5B), and wild-type S100A1 and S100A1Δ88-93 can be purified from bacteria by exactly the same protocol, which points to similar physiochemical properties (see Materials and methods). Also, recent work has shown that hydrophobic residues in the C-terminal extension of S100A1 are not essential for dimerization (25).

By combining all these observations, we conclude the following: (i) the p53 peptide and, presumably, p53 interact with S100A1 thereby blocking its ability to inhibit GFAP assembly; (ii) the p53 peptide shares with TRTK-12 a common binding site on S100A1; (iii) tubulin binds to the p53 peptide/TRTK-12 site on S100A1; (iv) the same site also binds GFAP (and, likely, the IF subunit, desmin, based on previous studies (4, 32)); (v) as S100A1 binds each of the above ligands with a stoichiometry of 1 mol of ligand/mol of S100A1 monomer (4, 18, 27, 32; and the present data), the S100A1 dimer would crossbridge two homologous or heterologous ligands, assuming that the overall structural arrangement described for the S100B$_2$ dimer also applies to the S100A1$_2$ dimer; and (vi) the C-terminal extension is a major component of the binding site implicated in the S100A1 interaction with all the above ligands, and plays an essential role in the ability of S100A1 to inhibit the assembly of GFAP into IFs. Yet, other parts of the S100A1 molecule appear to take part in the interaction of the protein with at least GFAP. Future studies should identify the other parts of S100A1 that appear to intervene in the recognition of GFAP and, possibly, other S100A1 target proteins.

Previous studies have identified the S100A1 C-terminal extension as an important component of the S100A1 site implicated in the binding of TRTK-12 (25). On the other hand, binding of TRTK-12 to S100B was reported to be accompanied by marked chemical-shift changes in helix I of one S100B monomer and helix IV of the other monomer, suggesting that TRTK-12 (and, possibly, other S100B target proteins) may bind to a surface composed of residues in the two above S100B helices (33). Still another study has identified in a region composed of residues in helices III and IV, the hinge region, and the C-terminal extension the binding site for the p53 peptide (21). Our present data strongly suggest that the C-terminal extension of S100A1 plays a major role in the interaction of this protein with TRTK-12, the p53 peptide, GFAP and tubulin. These observations, together with the finding that the p53 peptide also blocks the inhibitory effects of S100B on GFAP assembly (not shown), would favor the idea that the C-terminus of S100A1 and S100B is of critical importance in the recognition and regulation of several S100A1/B target proteins.

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REFERENCES

1. Donato, R. (1991) *Cell Calcium* **12,** 713–726.
2. Zimmer, D. B., Cornwall, E. H., Landar, A., and Song, W. (1995) *Brain Res. Bull.* **37,** 417–429.
3. Schäfer, B. W., and Heizmann, C. W. (1996) *Trends Biochem. Sci.* **21,** 134–140.
4. Garbuglia, M., Verzini, M., Giambanco, I., Spreca, A., and Donato, R. (1996) *FASEB J.* **10,** 317–324.
5. Heierhorst, J., Kobe, B., Feil, S. C., Parker, M. W., Benians, G. M., Weiss, K. R., and Kemp, B. E. (1996) *Nature* **380,** 636–639.
6. Landar, A., Caddell, G., Chessher, J., and Zimmer, D. B. (1996) *Cell Calcium* **20,** 279–285.
7. Drohat, A. C., Amburgey, J. C., Abildgaard, F., Starich, M. R., Baldisseri, D., and Weber, D. (1996) *Biochemistry* **35,** 11577–11588.
8. Kilby, P. M., Van Eldik, L. J., and Roberts, G. C. K. (1996) *Structure* **4,** 1041–1052.
9. Drohat, A. C., Baldisseri, D. M., Rustandi, R. R., and Weber, D. J. (1998) *Biochemistry* **37,** 2729–2740.
10. Smith, S. P., and Shaw, G. (1998) *Structure* **6,** 211–222.
11. Matsumura, H., Shiba, T., Inoue, T., Harada, S., and Kai, Y. (1998) *Structure* **6,** 233–241.
12. Potts, B. C. M., Smith, J., Akke, M., Macke, T. J., Okazaki, K., Hidaka, H., Case, D. A., and Chazin, W. J. (1995) *Nature Struct. Biol.* **2,** 790–796.
13. Sastry, M., Ketchen, R. R., Crescenzi, O., Weber, C., Lubienski, M. J., Hidaka, H., and Chazin, W. J. (1998) *Structure* **6,** 223–231.
14. Brodersen, D. E., Etzerodt, M., Madsen, P., Celis, J. E., Thøgersen, H. C., Nyborg, J., and Kjeldgaard, M. (1998) *Structure* **6,** 477–489.
15. Isobe, T., and Okuyama, T. (1978) *Eur. J. Biochem.* **89,** 379–388.
16. Isobe, T., and Okuyama, T. (1981) *Eur. J. Biochem.* **116,** 79–86
17. Ivanenkov, V. V., Jamieson, Jr., G. A., Gruenstein, E., and Dimlich, R. V. W. (1995) *J. Biol. Chem.* **270,** 14651–14658.
18. Ivanenkov, V. V., Dimlich, R. V. W., and Jamieson, G. A., Jr. (1996) *Biochem. Biophys. Res. Commun.* **221,** 45–50.
19. Bianchi, R., Garbuglia, M., Verzini, M., Giambanco, I., Ivanenkov, V. V., Dimlich, R. V. W., Jamieson, G. A., Jr., and Donato, R. (1996) *Biochim. Biophys. Acta* **1313,** 258–267.
20. Baudier, J., Delphin, C., Grunwald, D., Khochbin, S., and Lawrence, J. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11627–11631.
21. Rustandi, R. R., Drohat, A. C., Baldisseri, D. M., Wilder, P. T., and Weber, D. J. (1998) *Biochemistry* **37,** 1951–1960.
22. Wilder, P. T., Rustandi, R. R., Drohat, A. C., and Weber, D. J. (1998) *Protein Sci.* **7**:794–798.
23. Donato, R. (1988) *J. Biol. Chem.* **263,** 106–110.
24. Donato, R., Giambanco, I., Aisa, M. C., Di Geronimo, G., Ceccarelli, P., Rambotti, M. G., and Spreca, A. (1989) *Cell Calcium* **10,** 81–92.
25. Osterloh, D., Ivanenkov, and V. V., Gerke, V. (1998) *Cell Calcium* **24,** 137–151.
26. Inagaki, M., Gonda, Y., Nishizawa, K., Kitamura, S., Sato, C., Ando, S., Tanabe, K., Kikuchi, Y., Tsuiki, S., and Nishi, Y. (1990) *J. Biol. Chem.* **265,** 4722–4729.
27. Bianch,i R., Giambanco, I., and Donato, R. (1993) *J. Biol. Chem.* **268,** 12669–12674.
28. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
29. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
30. Harafujii, H., and Ogawa, Y. (1980) *J. Biochem.* **87,** 1305–1312.
31. Donato, R., Giambanco, I., and Aisa, M. C. (1989) *J. Neurochem.* **53,** 566–571.
32. Garbuglia, M., Verzini, M., Dimlich, R. V. W., Jamieson, G. A., Jr., and Donato, R. (1996) *Biochim. Biophys. Acta* **1313,** 268–276.
33. Kilby, P. M., Van Eldik, L. J., and Roberts, G. C. K. (1997) *Protein Sci.* **6,** 2494–2503.