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Identification of S-100 proteins and S-100-binding proteins in a detergent-resistant EDTA/KCl-extractable fraction from bovine brain membranes

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The Triton X-100-resistant residue of brain membranes contains appreciable amounts of S-100 proteins. This fraction of S-100 can be solubilized by high concentrations of EDTA plus or minus high concentrations of KCl. Whereas KCl (0.6 M) extracts the detergent-resistant S-100, NaCl (1 M) does not. Endogenous Ca^{2+} is required and is sufficient for S-100 to remain associated with the detergent-resistant residue. However, 0.6 M KCl extracts a further fraction of Triton X-100-resistant S-100. In contrast, the Triton X-100-extractable fraction of S-100 resists the action of EDTA. These data suggest that Ca^{2+} regulates the extent of association of S-100 with Triton X-100-resistant components in brain membranes, whereas the association of S-100 with the lipid bilayer of brain membranes and/or with some intrinsic membrane proteins is less Ca^{2+}-regulated. Several S-100-binding proteins are identified in the detergent-resistant residue of brain membranes by an overlay procedure.

Protein, S-100; Membrane protein; Cytoskeleton; Ca^{2+}

1. INTRODUCTION

Although mostly cytoplasmic, brain S-100 proteins, a family of Ca^{2+}-binding proteins of the EF-hand type (reviews [1,2]), also exist in a membrane-associated form which can be solubilized by detergents [1,2]. Membrane-bound (Triton X-100-extractable) S-100 proteins from bovine and rat brain are identical to their cytoplasmic counterparts according to a number of criteria [3].

Several cell types, but not mature brain cells, express a cytoskeletal protein complex, calpactin I, whose heavy chain (p36) is the substrate of virus-induced protein kinase activity and binds to phospholipids, actin, and fodrin, and whose light chain (p11) behaves as the regulatory subunit and has a high sequence homology with both α- and β-subunits of S-100 [4–6].

In view of the reported ability of S-100 proteins to control the assembly-disassembly of microtubule protein [7–9] and to interact with microtubule proteins [10–12] and with steady-state microtubules [13], we sought to determine whether a fraction of the membrane-associated S-100 exists which resists extraction of brain particulate matter with detergents, and whether S-100 is capable of interacting with cytoskeletal components other than microtubules. The recent observation that S-100 modulates the phosphorylation of p36 in vitro by binding to it [14] supports the possibility that S-100 may be involved in the regulation of membrane skeleton constituents in vivo.

We show here that the Triton X-100 residue of brain membranes contains relatively large amounts of S-100 proteins, and that S-100 proteins bind in vitro to a number of Triton X-100-resistant,

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EDTA-KCl-extractable proteins from brain membranes.

2. MATERIALS AND METHODS

2.1. *Preparation of bovine brain Triton X-100-resistant proteins*

All operations were performed at 4°C. Bovine brain was homogenized in 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM NaN_3, 1 mM phenylmethylsulphonyl fluoride (buffer I) (1:5, w/v), and centrifuged at 900 × *g* to remove nuclei and cell debris. The supernatant was saved and the pellet re-extracted as above. The two supernatants were combined and centrifuged at 150000 × *g* in a Kontron (Centrikon T1055) centrifuge. The pellet (P_2) was washed twice with buffer I. The washed P_2 pellet was resuspended in buffer I containing 1% (v/v) Triton X-100, 0.1 M KCl, 2 mM $CaCl_2$ (buffer II) (1:15, w/v), kept at 4°C for 30 min under agitation, and centrifuged at 150000 × *g* for 2 h. The pellet was resuspended in buffer II minus Triton X-100 and centrifuged again. This operation was repeated once more. The final pellet was resuspended in 20 vols of 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM NaN_3, 10 mM EDTA (buffer III), kept at 4°C for 30 min under agitation, and centrifuged as above. The supernatant was concentrated 10 times, centrifuged to remove insoluble material, and used for subsequent analyses. This protocol (protocol I) was a slightly modified version of that used in [15] to purify calpactin I. Alternatively, the washed P_2 pellet was processed as in [16] with some modifications (protocol II). Briefly, it was resuspended in 10 mM Tris-HCl, pH 8.3, 5 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 10 mM EGTA, 1% Triton X-100 (1:15, w/v), and kept at 4°C for 30 min before centrifugation at 150000 × *g* for 2 h. The supernatant was then adjusted to ~1 mM free Ca^{2+} (11 mM $CaCl_2$) while maintaining the pH at 7.5 with Tris base, kept at 4°C for 30 min under agitation, and centrifuged as above. The pellet was resuspended in 20 vols buffer III, kept at 4°C for 60 min under agitation, and centrifuged. The supernatant was used for subsequent analyses.

2.2. *Processing of Triton X-100-resistant protein preparations*

Triton X-100-resistant proteins obtained by protocols I and II were subjected to SDS-PAGE [17]. Gels were either stained with Coomassie blue or electroblotted onto nitrocellulose paper [18]. In the latter case, the membrane was either stained with amido black or subjected to further processing with an anti-S-100 antiserum specific to the S-100β subunit [19] or with biotinylated S-100b (see below). In addition, Triton X-100-resistant proteins were subjected to the procedure used to purify cytoplasmic S-100 proteins [20].

2.3. *Biotinylation of S-100b protein and related binding studies*

S-100b protein purified from rat brain [20] was biotinylated as in [21]. Biotinylation does not change the electrophoretic and immunological properties of S-100b (not shown). Bovine brain cytosol, Triton X-100-extractable proteins from brain membranes, Triton X-100-resistant proteins obtained by protocols I and II, and brain membrane proteins extractable with 0.6 M KCl (see the text) were separated by SDS-PAGE and transferred onto nitrocellulose paper. The membrane was incubated at room temperature in 20 mM Tris-HCl, pH 7.5, 0.9% NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (buffer IV) containing 5% (w/v) skimmed dry milk and 0.02% (v/v) antifoam A, for 60 min, washed with buffer IV, and incubated in buffer IV containing skimmed dry milk and antifoam A plus 6 μg biotinylated S-100b/ml for 4 h at room temperature. The membrane was washed with buffer IV, and incubated in buffer IV containing skimmed dry milk and antifoam A plus 0.5 μg avidin peroxidase/ml for 60 min at room temperature. The membrane was washed with buffer IV and treated with diaminobenzidine (0.3 mg/ml) plus H_2O_2 (0.05%, v/v) in 20 mM Tris-HCl, pH 7.5, 0.9% NaCl.

3. RESULTS AND DISCUSSION

EDTA extracts obtained by protocols I and II contained a large number of proteins among which was a low-M_r polypeptide which comigrated with an analogous low-M_r polypeptide present in the brain cytosol and with pure brain S-100 (fig.1). This polypeptide was recognized by the anti-S100β antiserum (fig.2). By enzyme-linked immunosorbent assay, S-100 represented ~0.4% of proteins in the brain cytosol, ~1.2% of proteins in the EDTA-extract (protocol I) and ~0.8% of proteins in the EDTA-extract (protocol II). On a weight basis, these values corresponded to 148, 8 and 5 mg/kg bovine brain, respectively. Subjecting individual EDTA extracts to the procedure used to purify cytoplasmic S-100 [20] resulted in purification of the low-M_r polypeptide (fig.1). The behavior of

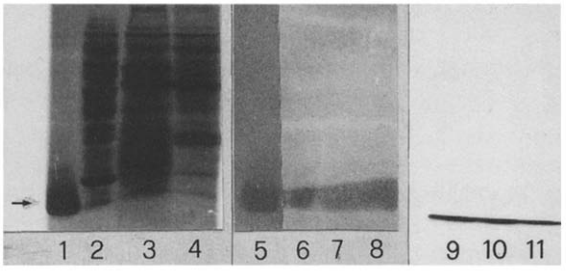

Fig.1. Identification of S-100 proteins in Triton X-100-resistant, EDTA-extractable protein fractions from bovine brain membranes by SDS-PAGE and immunoblotting. SDS-PAGE (12.5% acrylamide) of (1) bovine brain S-100 (80 μg), (2) bovine brain cytosol, (3) EDTA extract (protocol I), (4) EDTA extract (protocol II). (5–8) Same material as in (1–4) was subjected to immunoblotting using the anti-S-100β antiserum. (9–11) SDS-PAGE (12.5% acrylamide) of (9) S-100 purified from bovine brain cytosol, (10) S-100 purified from the EDTA-extract (protocol I) and (11) S-100 purified from the EDTA extract (protocol II). Arrow indicates position of S-100. Proteins were stained with amido black after transfer from SDS gels to nitrocellulose paper (1–4) or with Coomassie blue (9–11).

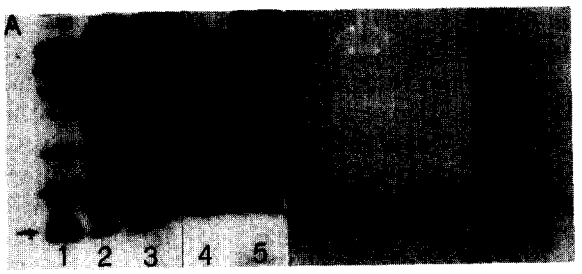

Fig.2. Further characterization of Triton X-100-resistant, EDTA-extractable S-100 and of Triton X-100-extractable S-100. (A) SDS-PAGE (12.5% acrylamide) of (1) M_r standards (from top to bottom: bovine serum albumin, tubulin, actin, trypsin, myoglobin, and bovine brain S-100), (2) bovine brain cytosol, (3) EDTA extract (protocol I), (4) EDTA extract (protocol I) after treatment of brain membranes with 1 M NaCl before the Triton X-100 step, and (5) Triton X-100-extractable material from bovine brain membranes. Arrow indicates position of S-100. (B) Same material as in A subjected to immunoblotting using anti-S-100β antiserum.

Triton X-100-resistant, EDTA-extractable S-100 on DEAE-Sephadex A-50 and on Sephadex G-200 chromatography was identical to that of cytoplasmic S-100 (not shown). The same holds true for its absorption spectrum and amino acid composition (not shown). Thus the purified polypeptide was authentic S-100.

Including EDTA in buffer I (protocol I) resulted in a very low yield of EDTA-extractable proteins from the Triton X-100 residue, as well as in the disappearance of S-100 from the EDTA-extractable material (not shown). Omitting exogenous $CaCl_2$ during the Triton X-100 step (protocol I) resulted in a smaller decrease in the content of EDTA-extractable proteins (not shown). No S-100 was detected in these preparations by Coomassie blue staining of SDS gels, although faint staining was detected by immunoblotting (not shown). On the other hand, irrespective of including EDTA in buffer I or of omitting $CaCl_2$ during the Triton X-100 step, S-100 was recovered in the Triton X-100-extractable material (fig.2), confirming previous observations [3]. Prior treatment of the washed P_2 pellet with 1 M NaCl in buffer I (protocol I) before the Triton X-100 step extracted a large number of proteins and little, if any, S-100 (not shown). The final EDTA extract, on the other hand, did contain S-100 (fig.2). Similar results were obtained after 1 M NaCl treatment of the washed P_2 pellet and further processing through protocol II (not shown).

Since S-100 binds to brain membranes with high-affinity Ca^{2+} dependence [22], experiments were performed to exclude (or verify) the possibility that Triton X-100-resistant S-100 obtained by protocol I actually results from binding of Triton X-100-extractable S-100 to the Triton X-100 residue during the Triton X-100 step owing to the presence of $CaCl_2$. For this purpose, the washed P_2 pellet was directly treated with 10 mM EDTA in buffer I. A large number of membrane polypeptides were extracted by this procedure (not shown). S-100 was among these polypeptides (not shown). We also examined the effect of high concentrations of KCl on both the Triton X-100 residue (protocol I) and the washed P_2 pellet. Similar polypeptide profiles were obtained by SDS-PAGE, in either case S-100 being recovered in the extracts (not shown). Moreover, not only was KCl (0.6 M) capable of extracting S-100 when the washed P_2 pellet had been obtained by processing brain tissue without EDTA (protocol I), but also KCl extracted S-100 from membranes prepared in the presence of

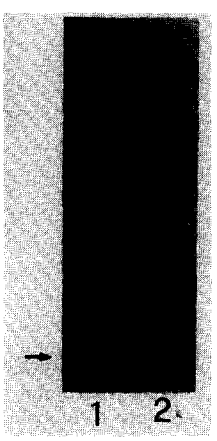

Fig.3. Detection of S-100 proteins in the 0.6 M KCl-extractable protein fraction from brain membranes. The Triton X-100 residue from brain membranes obtained by protocol I, but in the presence of 1 mM EDTA, was washed twice with buffer I plus 1 mM EDTA, and finally extracted with buffer I containing 0.6 M KCl at 4°C for 60 min. After centrifugation, the supernatant (0.6 M KCl extract) was subjected to SDS-PAGE (12.5% acrylamide). Gels were either stained with Coomassie blue (lane 1) or electroblotted onto nitrocellulose paper for immunoblotting with the anti-S-100β antiserum (lane 2). Arrow indicates position of S-100.

EDTA (fig.3). Collectively, these data indicate that a substantial fraction of S-100 is associated in vivo with the Triton X-100-resistant residue in the brain, and that exogenous $CaCl_2$ is required during the detergent step (protocol I) exclusively to stabilize this association, as is the case with calpactin I [15].

S-100b interacted with a number of polypeptides present in the brain cytosol, the Triton X-100-extractable material, EDTA extracts obtained by protocols I and II, and 0.6 M KCl-extractable material (fig.4). The M_r values of S-100b-binding polypeptides ranged between 15000 and 130000. Some of the S-100b-binding species appeared to be common to individual extracts. Strong S-100b binding was observed at the level of at least six polypeptides characterized by M_r values of 15000–18000 and 25000–30000 in the 0.6 M KCl extract (fig.4). The S-100b binding

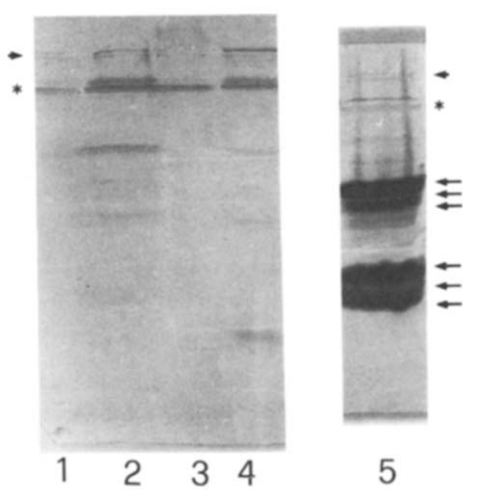

Fig.4. Identification of S-100b-binding polypeptides in bovine brain cytosol and in extracts from brain membranes. Bovine brain cytosol (1), EDTA extract (protocol I) (2), EDTA extract (protocol II) (3), Triton X-100 extract (4), and EDTA/KCl extract from brain membranes (5) were separated by SDS-PAGE (12.5% acrylamide) and electroblotted onto nitrocellulose paper. After blocking the free sites on the nitrocellulose paper with skimmed dry milk, the membranes were incubated with biotinylated S-100b and then with avidin-peroxidase. Note that S-100b binds to a number of polypeptides present in the materials loaded onto the SDS gels. Among these polypeptides the most reactive species are characterized by apparent M_r values of 70000 (asterisk) and 130000 (arrowhead) (lanes 1–5), and of 15000–18000 and 25000–30000 (arrows) (lane 5).

to all these polypeptides was Ca^{2+}-independent (not shown).

Altogether, these data indicate that membrane-bound S-100 protein in the brain is distributed in two pools, one resistant to EDTA plus 1 M NaCl and extractable with Triton X-100 [3], the other resistant to Triton X-100 and extractable with EDTA/KCl. Proteins that are present in the detergent-resistant material from any tissue usually are operationally defined as cytoskeletal or cytoskeleton-associated. Association of S-100 with axonemes, centrioles, basal bodies, mitotic spindle poles, and glial filaments in vivo, and with steady-state microtubules in vitro has been reported [13,23–27]. Also, work in progress in our laboratory indicates that a significant fraction of membrane-associated S-100b in cultured Schwann cells resists extraction with detergents (in preparation). Thus, the present data are strongly suggestive of the existence of a membrane skeleton-associated fraction of S-100 proteins in bovine brain.

The association of S-100 with membranes could be due to binding to either membrane proteins or membrane lipids. The ability of S-100 to interact with artificial membranes of different phospholipid composition is well established [28–30]. Electrostatic interactions have been suggested to drive the binding of S-100 to liposomes whereas Ca^{2+} would strengthen the binding by inducing the exposure of hydrophobic domains on S-100 [28], a suggestion recently confirmed by our observations [30]. Here, we have identified a number of membrane-associated proteins that bind S-100b. Further characterization of these proteins as well as a quantitative analysis of S-100b binding to them will be the subject of future work.

On the basis of the above considerations and in view of the dimeric nature of S-100, we suggest that S-100 binds to membrane lipids and proteins, with Ca^{2+} regulating the nature and extent of association. We speculate that, given the substantial Ca^{2+} independence of S-100b binding to Triton X-100-resistant membrane proteins in vitro (fig.4), S-100b binding to these proteins also occurs in vivo in the presence of extremely low free Ca^{2+} concentrations, that Ca^{2+} might regulate the proportion of the S-100 bound to them, and that Ca^{2+} must decrease to very low levels before dissociation of the bound S-100. For instance, S-100 binds to

tubulin and microtubule-associated tau proteins, and inhibits the microtubule protein and pure tubulin assemblies Ca^{2+}-independently at pH 7.5, yet Ca^{2+} enhances these effects of S-100 [11,12,31–33]. Also, p36 binds to artificial membranes at submicromolar free Ca^{2+} concentrations in the presence of p11 [34], yet $CaCl_2$ is required to stabilize the association of calpactin I with natural membranes [15]. On the other hand, since S-100 is dimeric, it is also possible that the whole S-100 molecule may cross-link peripheral (cytoskeletal?) proteins to membranes. p11, which has a high sequence homology with S-100 subunits [4–6], regulates the extent of association of p36 with natural and artificial membranes [16,34], and the effect of p36 on aggregation of chromaffin granules at micromolar levels of Ca^{2+} [35]. Also, S-100 binds to and regulates the phosphorylation of p36 Ca^{2+}-independently [14]. It is possible that S-100 binding to and S-100 effects on phosphorylation of p36 are but a reflection of the sequence homology between S-100 subunits and p11. However, in view of all these observations, it is reasonable to hypothesize a role of S-100 in regulating the association of definite cytoskeletal components with membranes.

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REFERENCES

[1] Donato, R. (1986) Cell Calcium 7, 123–146.
[2] Van Eldik, L.J. and Zimmer, D.B. (1988) in: Calcium and Calcium Binding Proteins (Gerday, C. et al. eds) pp.114–127, Springer, Berlin.
[3] Donato, R., Prestagiovanni, B. and Zelano, G. (1986) J. Neurochem. 46, 1333–1337.
[4] Gerke, V. and Weber, K. (1985) EMBO J. 4, 2917–2920.
[5] Glenney, J.R. jr and Tack, B.F. (1985) Proc. Natl. Acad. Sci. USA 82, 7884–7888.
[6] Hexham, J.M., Totty, N.F., Waterfield, M.D. and Crumpton, M.J. (1986) Biochem. Biophys. Res. Commun. 134, 248–254.
[7] Baudier, J., Briving, C., Deinum, J., Haglid, K., Sorskog, L. and Wallin, M. (1982) FEBS Lett. 147, 265–267.
[8] Endo, T. and Hidaka, H. (1983) FEBS Lett. 161, 235–238.
[9] Donato, R. (1983) FEBS Lett. 162, 310–313.
[10] Fujii, T., Gocho, N., Akabane, Y., Kondo, Y., Suzuki, T. and Ohki, K. (1986) Chem. Pharm. Bull. (Tokyo) 34, 2261–2264.
[11] Donato, R. (1987) Cell Calcium 8, 283–297.
[12] Baudier, J., Mochly-Rosen, D., Newton, A., Lee, S.-H., Koshland, D.E. jr and Cole, R.D. (1987) Biochemistry 26, 2886–2893.
[13] Donato, R. and Giambanco, I. (1989) J. Neurochem., in press.
[14] Hagiwara, M., Ochiai, M., Owada, K., Tanaka, T. and Hidaka, H. (1988) J. Biol. Chem. 263, 6438–6441.
[15] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[16] Glenney, J.R. jr, Tack, B.F. and Powell, M.A. (1987) J. Cell Biol. 104, 503–511.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Spreca, A., Rambotti, M.G., Rende, M., Saccardi, C., Aisa, M.C., Giambanco, I. and Donato, R. (1989) J. Histochem. Cytochem., in press.
[20] Donato, R. (1984) Biochem. Biophys. Res. Commun. 124, 850–856.
[21] Billingsley, M.L., Pennypacker, K.R., Hoover, C.G. and Kinkaid, R.L. (1987) Biotechniques 5, 680–685.
[22] Donato, R. (1976) J. Neurochem. 27, 439–447.
[23] Cocchia, D. (1981) Cell Tissue Res. 214, 529–540.
[24] Michetti, F. and Cocchia, D. (1982) Cell Tissue Res. 223, 575–582.
[25] Uchida, T. and Endo, T. (1988) J. Histochem. Cytochem. 36, 693–696.
[26] Zimmer, D.B. and Van Eldik, L.J. (1988) J. Neurochem. 50, 572–579.
[27] Rambotti, M.G., Saccardi, C., Spreca, A., Aisa, M.C., Giambanco, I. and Donato, R. (1988) J. Cell Biol. 107, 733a.
[28] Calissano, P., Alemà, S. and Fasella, P. (1974) Biochemistry 13, 4553–4560.
[29] Morero, R.D. and Weber, G. (1982) Biochim. Biophys. Acta 703, 241–246.
[30] Zolese, G., Tangorra, A., Curatola, G., Giambanco, I. and Donato, R. (1988) Cell Calcium 9, 149–157.
[31] Donato, R. (1988) J. Biol. Chem. 263, 106–110.
[32] Baudier, J. and Cole, R.D. (1988) Biochemistry 27, 2728–2736.
[33] Baudier, J. and Cole, R.D. (1988) J. Biol. Chem. 263, 5876–5883.
[34] Powell, M.A. and Glenney, J.R. jr (1987) Biochem. J. 247, 321–328.
[35] Drust, D.S. and Creutz, C.A. (1988) Nature 331, 88–91.