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Interaction of $S100a_0$ Protein with the Actin Capping Protein, CapZ: Characterization of a Putative $S100a_0$ Binding Site in CapZα-Subunit

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$S100a_0$, a Ca^{2+}-binding protein expressed predominantly in cardiac and skeletal muscle tissues, was demonstrated by chemical cross-linking to interact in a Ca^{2+}-dependent manner with the actin capping protein CapZ. TRTK-12, a peptide contained within the COOH-terminal region of CapZα, inhibited $S100a_0$: CapZ interaction in a dose-dependent manner. TRTK-12 was shown by cross-linking to bind $S100a_0$ in the presence of Ca^{2+}, and by fluorescence spectrophotometry to interact in a saturable manner with the anionic phospholipid and a regulator of CapZ activity, phosphatidylinositol 4-monophosphate; but not with the neutral phospholipid, phosphatidylcholine. These data suggest $S100a_0$ and polyphosphoinositides bind to the same COOH-terminal region of CapZα, thus potentially modulating CapZ activity.

S100 proteins are a family of dimeric Ca^{2+}-binding proteins consisting of $S100a_0$, S100a, S100b (subunit composition: $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$, respectively), and a variety of S100-like proteins (see reviews 1,2). Each S100 subunit contains two Ca^{2+}-binding domains which conform to the EF-hand class of Ca^{2+}-binding structures. While characterization of the biological function(s) of S100 proteins remains incomplete, it seems likely that their activities are mediated via their Ca^{2+}-dependent association with specific target proteins, as occurs with other members of the EF-hand family of Ca^{2+}-modulated proteins (e.g. calmodulin, troponin C) (3,4).

The identification of consensus S100 binding target epitopes would facilitate the analysis of S100 protein function and might permit the identification of additional S100 target proteins. Recently we completed a study in which S100b-binding peptides contained within a bacteriophage library of random sequences were identified (5). Sequence analysis of the family of S100-binding peptides obtained in those studies permitted us to identify the COOH-terminal portion of the α-subunit of the actin capping protein CapZ as a novel S100b target.

CapZ, a member of a family of heterodimeric actin capping proteins (6) that appear to be expressed in all eukaryotic cells and tissues (7–9), is comprised of a 36kDa α-subunit, and a 32kDa β-subunit which has been implicated in actin binding (10). The name CapZ (8,11) describes both its biochemical function, as an actin capping protein, and its subcellular localization at the Z-lines of skeletal muscles and Z-discs and intercalated discs of cardiac myocytes. In muscle cells, CapZ is thought to participate in regulating the length, orientation, and/or attachment of actin filaments to Z-discs (11). Interestingly, $S100a_0$, the S100 isoform expressed preferentially within skeletal muscle and cardiac tissues (in contrast to S100b which is expressed predominantly in glia), also localizes to the Z-lines and fascia adherence of the intercalated discs of cardiac myocytes (12,13). The co-expression and co-localization of $S100a_0$ and CapZ in skeletal muscle and cardiac tissues prompted us to investigate whether any structural rationale could be identified which might account for these observations.

In the present study we demonstrate that $S100a_0$, like S100b, binds to CapZ in a Ca^{2+}-dependent manner and that the synthetic peptide TRTK-12 (TRTKIDWNKILS), contained within the car-

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Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; PC, phosphatidylcholine; PIP, phosphatidylinositol 4-monophosphate.

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boxyl-terminal region of CapZα, inhibits this interaction in a dose-dependent fashion. TRTK-12 is demonstrated to bind directly to S100α-subunit in the presence of Ca^{2+} and to interact in a saturable fashion with the anionic phospholipid PIP, but not with the neutral phospholipid PC. These results suggest that S100a_0 and known regulators of CapZ, the polyphosphoinositides (14), bind to the same site in COOH-terminus of CapZα. Hence, these molecules may act in concert to modulate CapZ activity.

MATERIALS AND METHODS

Materials. Bovine brain S100a_0, bovine brain S100 rabbit antiserum (IgG fraction), PIP and PC were obtained from Sigma Chemical Co. Affinity-purified goat anti-CapZ antibody and actin capping protein, CapZ (15), were both generously provided by Dr. John Cooper, Washington University, St. Louis, MO. Alkaline phosphatase conjugated goat anti-rabbit Ig, and rabbit anti-goat IgG were obtained from Southern Biotechnology Associates. Synthetic peptides were custom synthesized by Chiron Mimotopes. All other reagents were of reagent-grade quality or better.

Chemical cross-linking. Proteins were dialyzed against buffer A (50 mM HEPES, 150 mM NaCl, pH 7.5), and preincubated for 20 min at room temperature with additives as indicated. Reactions were initiated by addition of the bifunctional cross-linking reagent BS^3 (Pierce) in buffer A. Reactions were permitted to proceed for 30 min, quenched by the addition of 200 mM 2-aminoethanol/HCl (20 mM final) in buffer A and incubated for an additional 15 min prior to preparation for SDS-PAGE by addition of an equal volume of 2X SDS-sample buffer. Protein concentration was determined by the Bradford method (16), or with a BCA kit (Pierce).

Gel electrophoresis and immunoblotting. Cross-linked complexes of S100a_0 and CapZ were analyzed using 12% (w/v) SDS-PAGE (17). S100α-subunit and TRTK-12 conjugates were analyzed using 16% (w/v) Tricine-SDS-PAGE (18). Electrophoretically separated proteins were visualized by Coomassie blue staining and/or by immunodetection with anti-S100 or anti-CapZ antibodies subsequent to transfer to nitrocellulose (19) using a commercial immunoblot kit (Bio-Rad).

Interaction of TRTK-12 peptide with phospholipids. TRTK-12 peptide, synthesized in carboxyl-terminus-amidated form, was solubilized in water, aliquoted and stored at −80°C. TRTK-12 peptide concentration was determined spectrophotometrically using the extinction coefficient for tryptophan $\epsilon^{280} = 5600 M^{-1}cm^{-1}$. Phospholipids were prepared essentially as described (14). Briefly, PIP was dissolved in water and sonicated 2 × 1 min on ice, while PC was homogenized in water and sonicated 10 × 1 min on ice, both using a Micro-Ultrasonic Cell Disrupter (Kontes). Phospholipid concentrations were determined by phosphate assay (20). The interaction of TRTK-12 with various phospholipids was evaluated by measuring changes in the fluorescence emission spectra of the peptide's tryptophan in the presence of indicated additives in buffer B (50 mM Tris, 150 mM NaCl, pH 7.5). Mixtures were excited at 295 nm and emission spectra were obtained using a Hitachi F-2000 fluorescence spectrophotometer.

RESULTS AND DISCUSSION

Ca^{2+}-dependent interaction of S100a_0 with CapZ. Chemical cross-linking experiments were performed to determine whether S100a_0 interacts with CapZ in a Ca^{2+}-dependent fashion. Optimal cross-linking conditions were determined experimentally and conjugates obtained were analyzed by SDS-PAGE and imunoblotting. Chemical cross-linking a mixture of S100a_0 and CapZ with BS^3 in the presence of $CaCl_2$ yielded multiple conjugates (M_r 75–150 kDa, and M_r > 200 kDa), which immunoblot analysis determined to contain both S100a_0 and CapZ (Fig. 1). These complexes did not form when cross-linking was performed in the absence of Ca^{2+} (2 mM EGTA). Addition of TRTK-12 peptide inhibited conjugate formation in a dose-dependent fashion (Fig. 1). These data suggest that S100a_0, like S100b (5), binds to the TRTK-12 containing, COOH-terminal domain of the CapZα in a site-specific and Ca^{2+}-dependent fashion. The Ca^{2+} requirement for this interaction was studied further. As anticipated, with decreasing concentrations of Ca^{2+} there was a corresponding reduction in the density of conjugate bands (Fig. 2).

Ca^{2+}-dependent interaction of S100a_0 with TRTK-12 peptide. While performing these experiments we noted that the migration of cross-linked S100α-dimers, formed in the presence of TRTK-12, shifted to a higher M_r (Fig. 1B: lanes 4,5 *vs.* lanes 1,6). This shift was dependent upon the presence of TRTK-12 and Ca^{2+} and consistent with S100a_0 binding to TRTK-12 and subsequent cross-linking of this multi-molecular complex. Additional experiments were performed to demonstrate directly the interaction of TRTK-12 with S100a_0. Reaction products were analyzed using Tricine-SDS-PAGE, a system which provides enhanced resolution of lower molecular weight complexes. At concentrations of TRTK-12 less than or equal to a molar equivalent of S100α-

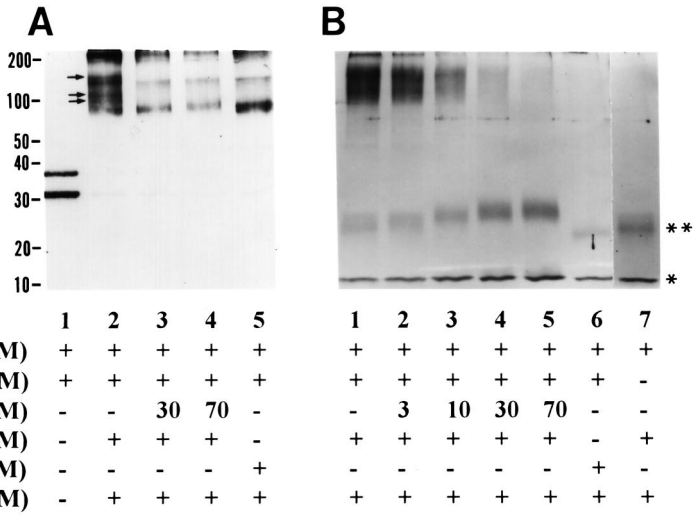


FIG. 1. Analysis of $S100a_0$ binding to CapZ. Effect of TRTK-12 peptide. Proteins were cross-linked under conditions indicated. Resultant conjugates were analyzed by immunoblotting using either (A) anti-CapZ, or (B) anti-S100 antibodies. Arrows indicate position of S100:CapZ conjugates migrating as distinct bands. Asterisks indicate the migration position of S100α-subunits (*) and cross-linked S100α-subunit dimers (**). Molecular weight standards (kDa) are indicated.

subunits, the major cross-linker dependent product detected was a single band which migrated at a M_r slightly greater than the S100α-subunit (Fig. 3: lane 5). However, as the molar ratio of TRTK-12:S100α was increased, an additional band of still higher M_r became apparent (Fig. 3: lanes 6,7). Appearance of each of these conjugates was completely dependent upon the presence of both Ca^{2+} (Fig. 3: lanes 5–7 *vs.* lanes 8–10) and TRTK-12 (Fig. 3: lanes 5–7 *vs.* lane 3). Our interpretation of these results is that the intermediate band corresponds to a 1:1 (TRTK-12: S100α) complex, while the higher M_r complex is consistent with the formation of 2:1 conjugates.

From these data we conclude the following: (i) $S100a_0$ binds both CapZ and TRTK-12, (ii) $S100a_0$ interaction with these molecules in Ca^{2+}-dependent, (iii) TRTK-12 blocks $S100a_0$:CapZ association in a site- and dose-dependent fashion, and (iv) TRTK-12 interacts directly with S100α-subunits. These results suggest strongly that the molecular interaction of $S100a_0$ and CapZ is mediated through the Ca^{2+}-dependent association of $S100a_0$ with the COOH-terminal portion of CapZα corresponding to TRTK-12 (265TRTKIDWNKILS276).

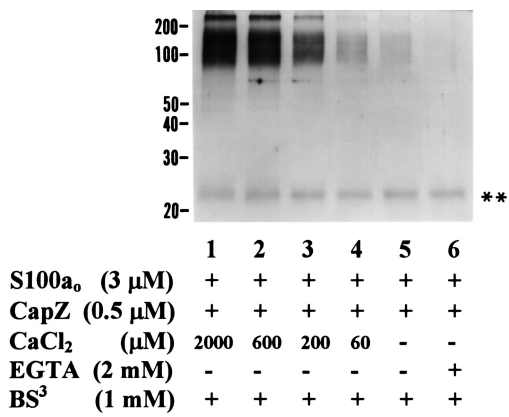
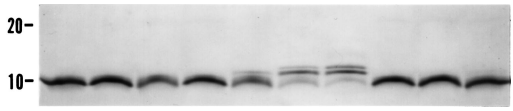


FIG. 2. Ca^{2+}-dependence of $S100a_0$ binding to CapZ. Proteins were subjected to cross-linking as indicated. Cross-linked products were analyzed by immunoblotting using anti-S100 antibodies. Asterisks indicate S100α-subunits cross-linked into dimers. S100α monomers, which migrated at the dye front, are not shown. Molecular weight standards (kDa) are indicated.



	1	2	3	4	5	6	7	8	9	10
$S100a_0$ (4 μM)	+	+	+	+	+	+	+	+	+	+
TRTK-12 (μM)	-	-	-	-	3	15	75	3	15	75
$CaCl_2$ (1 mM)	+	-	+	-	+	+	+	-	-	-
EGTA (2 mM)	-	+	-	+	-	-	-	+	+	+
BS^3 (1 mM)	-	-	+	+	+	+	+	+	+	+

FIG. 3. Analysis of TRTK-12 peptide binding to $S100a_0$. Mixtures of $S100a_0$ and TRTK-12 were subjected to cross-linking as indicated. Resultant conjugates were analyzed by electrophoresis in 16% Tricine-SDS polyacrylamide gel and Coomassie Blue staining. Molecular weight standards are indicated. S100α-subunits migrate as a 10 kDa band.

Interaction of TRTK-12 peptide with PIP. The COOH-terminal domain of CapZα-subunit and the α-subunits of related actin capping proteins are highly conserved in different eukaryotic species (7,9,21). This high level of conservation has lead others to speculate that this region mediates physiologically relevant cellular responses (9). Overlapping the S100 target epitope we have defined within the COOH-terminal region of CapZα a conserved sequence containing seven positively charged amino acids which might mediate interaction with other negatively charged molecules. In fact, Heiss and Cooper (14) demonstrated the capacity of polyphoinositides and other anionic phospholipids to bind CapZ and inhibit its actin capping activity. Moreover, the inhibitory effect of phosphatidyl-inositol 4,5 bisphosphate and other anionic phospholipids on CapZ activity appears to be mediated via their interaction with the CapZα-subunit.

To determine if the S100 target epitope contained within CapZα might mediate CapZ interaction with polyphosphoinositides, we examined the binding of TRTK-12 to PIP micelles using fluorescence spectrophotometry. Changes in the fluorescence of the peptide's single tryptophan residue in the presence and absence of PIP was used to examine TRTK-12:PIP interaction. The fluorescence spectrum of TRTK-12 alone, when excited at 295 nm, was consistent with the presence of a single tryptophan (Fig. 4: trace 1). While the addition of increasing concentrations of PIP produced little or no change in peak fluorescence, a pronounced dose-dependent, blue shift in the emission spectrum was observed (Fig. 4: traces 2–5). We interpret this spectral shift as indicative of the reorientation of the peptide's tryptophan residue into a less polar environment upon interaction with

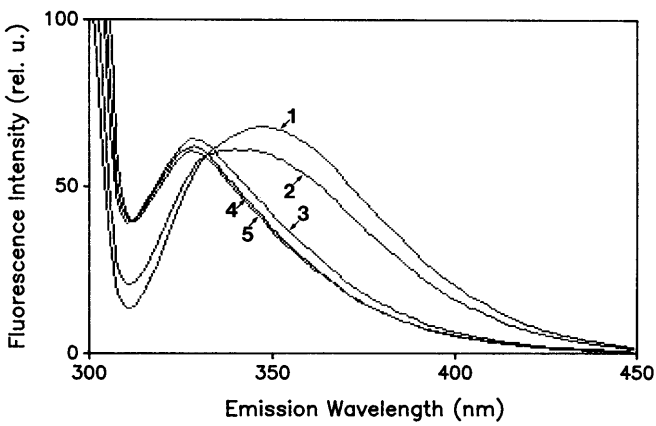


FIG. 4. Spectrophotometric analysis of the interaction of TRTK-12 peptide and PIP. TRTK-12 (1.4 μM) in Buffer B was excited at 295 nm and emission spectra recorded using a fluorescence spectrophotometer (trace 1), and after successive additions of PIP (2.3, 11, 22 and 45 μM final concentration; traces 2–5, respectively).

anionic PIP micelles. The binding of TRTK-12 to PIP was saturable and, under the conditions employed (1.4 μM TRTK-12), reached half saturation at ca. 5 μM PIP (Fig. 4). In similar experiments with liposomes prepared from the neutral phospholipid PC, no effect on TRTK-12's fluorescence spectrum was detected (data not shown).

The characteristics of the interaction of TRTK-12 with phospholipids (saturation at micromolar concentrations of PIP, and no interaction with neutral PC) correspond well to the inhibitory effects of phospholipids on CapZ activities *in vitro* (14). Thus, the COOH-terminal region of CapZα, which contains the complete TRTK-12 peptide sequence, might mediate its interaction with both $S100a_o$ and phospholipids. Moreover, since $S100a_o$ binds to the identical region of CapZα which appears to mediate its interaction with phospholipids, we hypothesize $S100a_o$ may modulate the regulatory effect of phospholipids on CapZ activity and provide a Ca^{2+}-dependent mechanism for modulating actin polymerization *in vivo*. Support for S100 proteins functioning as regulators of microfilament organization is provided by the studies of Selinfreund and co-workers (22), which demonstrated that antisense mediated inhibition of S100b expression in C6 rat glioma cells induced a flattened cell morphology and enhanced the organization of microfilament bundles.

Our results designate a specific domain within the COOH-terminus of CapZα subunit as the likely target epitope of both $S100a_o$ and anionic phospholipids. Therefore, $S100a_o$ may function to modulate the organization of actin microfilament system at local sites of high intracellular Ca^{2+} concentration (23) and at membranous surfaces within cardiac and skeletal muscle tissues.

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REFERENCES

1. Hilt, D. C., and Kligman, D. (1991) *in* Novel Calcium-Binding Proteins (C. W. Heizmann Ed.), pp. 65–103. Springer-Verlag, Berlin.
2. Zimmer, D. B., Cornwall, E. H., Landar, A., and Song, W. (1995) *Brain Res. Bull.* **37,** 417–429.
3. Means, A. R. (1988) *in* Recent Progress in Hormone Research (J. H. Clark Ed.), Vol. 44, pp. 223–262. Academic Press, San Diego.
4. Wnuk, W. (1988) *in* Calcium and Calcium Binding Proteins: Molecular and Functional Aspects (C. Gerday, L. Bolis, and R. Gilles Eds.), pp. 44–68. Springer-Verlag, Berlin.
5. Ivanenkov, V. V., Jamieson, G. A., Jr., Gruenstein, E., and Dimlich, R. V. W. (1995) *J. Biol. Chem.* **270,** 14651–14658.
6. Pollard, T. D., and Cooper, J. A. (1986) *Ann. Rev. Biochem.* **55,** 987–1035.
7. Cooper, J. A., Caldwell, J. E., Gattermeir, D. J., Torres, M. A., Amatruda, J. F., and Casella, J. F. (1991) *Cell Motil. Cytoskeleton* **18,** 204–214.
8. Schafer, D. A., Korshunova, Y. O., Schroer, T. A., and Cooper, J. A. (1994) *J. Cell Biol.* **127,** 453–465.
9. Hartmann, H., Schleicher, M., and Noegel, A. A. (1990) *Dev. Genet.* **11,** 369–376.
10. Hug, C., Miller, T. M., Torres, M. A., Casella, J. F., and Cooper, J. A. (1992) *J. Cell Biol.* **116,** 923–931.
11. Casella, J. F., Craig, S. W., Maack, D. J., and Brown, A. E. (1987) *J. Cell Biol.* **105,** 371–379.
12. Kato, K., and Kimura, S. (1985) *Biochim. Biophys. Acta* **842,** 146–150.
13. Haimoto, H., and Kato, K. (1988) *Eur. J. Biochem.* **171,** 409–415.
14. Heiss, S. G., and Cooper, J. A. (1991) *Biochemistry* **30,** 8753–8758.
15. Casella, J. F., and Cooper, J. A. (1991) *in* Methods in Enzymology (R. B. Vallee Ed.), Vol. 196, pp. 140–154. Academic Press, San Diego.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
18. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379.
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
20. Rouser, G., Siakotos, A. N., and Fleischer, S. (1966) *Lipids* **1,** 85–86.
21. Amatruda, J. F., Gattermeir, D. J., Karpova, T. S., and Cooper, J. A. (1992) *J. Cell Biol.* **119,** 1151–1162.
22. Selinfreund, R. H., Barger, S. W., Welsh, M. J., and Van Eldik, L. J. (1990) *J. Cell Biol.* **111,** 2021–2028.
23. Llinás, R., Sugimori, M., and Silver, R. B. (1992) *Science* **256,** 677–679xx.