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SEQUENCE HOMOLOGY OF A CANINE BRAIN CALCIUM-BINDING PROTEIN WITH CALREGULIN AND THE HUMAN Ro/SS-A ANTIGEN

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SUMMARY: A 60 kDa calcium-binding protein (CBP) was purified from canine brain and its N-terminal sequence determined to be: Glu-Pro-Ala-Ile-Tyr-Phe-Lys-Glu-Gln-Phe-Leu-Asp-Gly-Asp-Gly-X-Thr-Asp-Arg-X-Ile-Glu-Ser-Lys. This sequence is very similar to that of "calregulin", a CBP of unknown function which is similar in size and appears to be present in most animal tissues. An unexpected and even more striking similarity was found with the N-terminal sequence of the human Ro/SS-A antigen, a 60 kDa protein which has long been implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus. These findings suggest that the Ro/SS-A antigen is probably also a CBP.

It is well-established that calcium-binding proteins (CBPs) play essential roles in maintaining intracellular Ca^{2+} homeostasis (1). In recent years, however, an ever-increasing number of CBPs of unknown function, such as the suggestively-named "calregulin" (2), have been isolated from a wide variety of tissues and cells (3). Within the lumen of subcellular organelles, CBPs are involved in controlling Ca^{2+} concentration.

A particularly well-characterized example is the sarcoplasmic reticulum (SR) of striated muscle, which controls the myoplasmic Ca^{2+} concentration via membrane-bound Ca^{2+} pumps and Ca^{2+}-release channels. Within the lumen

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of the SR is a high concentration of calsequestrin, a low-affinity, high-capacity CBP whose primary function appears to be that of Ca^{2+} storage (4).

A CBP recently isolated from bovine brain was found to be associated with a membrane fraction containing a Ca^{2+}-ATPase (5). This unnamed ~58 kDa protein is similar in size to both calsequestrin and calregulin, but has a distinctly different amino acid composition. By immunogold labeling with both anti-calsequestrin and anti-Ca^{2+} pump antibodies, new cytological entities, designated "calciosomes", have recently been identified in non-muscle cells and suggested to be intracellular Ca^{2+} stores sensitive to inositol 1,4,5-trisphosphate (6,7).

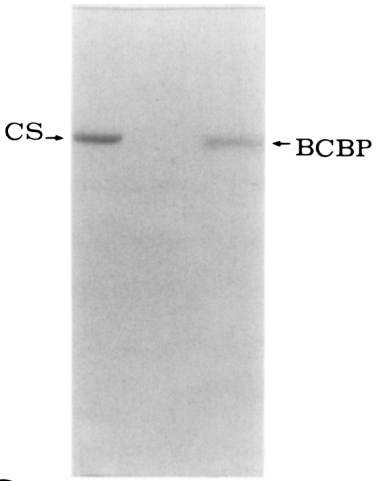
In ongoing efforts to purify and characterize the proteins associated with canine brain "calciosomes" (8), we have recently purified another ~60 kDa CBP. In this communication we present the N-terminal sequence of this protein as evidence for its homology with other CBPs and with the clinically important but little-characterized human Ro/SS-A antigen, a 60 kDa protein not previously identified as a CBP. This finding may be useful in the investigation of the molecular basis of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Canine brain CBP was isolated from whole tissue by DEAE-cellulose (9) and hydroxyapatite (4) chromatography, procedures which have proven valuable for purifying proteins of the SR. Calsequestrin was isolated from rabbit skeletal muscle by DEAE-cellulose and phenyl Sepharose chromatography (9). Following gel electrophoresis (14), the purified protein had an apparent mass of ~60 kDa, stained metachromatically blue with Stains-All, cross-reacted with anti-(rabbit liver calregulin) antibody by Western blotting, cross-reacted with anti-(rabbit muscle calsequestrin) antibody by dot blotting, and was shown to be a CBP by a calcium overlay technique (11). For N-terminal sequencing, the CBP was electroblotted onto a Millipore Immobilon membrane (12), then sequenced on an Applied Biosystems model 477A sequencer equipped with an on-line model 120 PTH amino acid analyzer, using standard protocols supplied by the manufacturer. A detailed description of the purification and ongoing characterization of canine brain CBP will be published elsewhere.

RESULTS AND DISCUSSION

Figure 1 shows the results of $^{45}Ca^{2+}$ overlays of rabbit skeletal muscle calsequestrin and canine brain CBP. Both proteins were labeled by $^{45}Ca^{2+}$, and the canine brain CBP displays a slightly lower apparent molecular



(1)

(2)

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                    5         10        15        20
CBCBP     E P A I Y F K E Q F L D G D G x T D R x I E S K
                  |                     |
RoSSA     E P A V Y F K E Q F L D G D G W T S R W I E S K
                |
RLCRG     E P V V Y F K E Q F L D G D G x x x x x x x x x
                | | |       | |
CLCRG     E P A Q F F K E E K L D G D G x x x x x x x x x
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Figure 1: Results of $^{45}Ca^{2+}$ ligand overlay of rabbit skeletal muscle calsequestrin (CS, lane a) and canine brain CBP (BCBP, lane b). The overlay was carried out as described by Zorzato and Volpe (11), using 4 ug of CS and 2 ug of BCBP.

Figure 2: Comparison of the N-terminal sequences of canine brain CBP (CBCBP, this report), human Ro/SS-A antigen (RoSSA, ref. 14), rabbit liver calregulin (RLCRG, ref. 15) and chicken liver calregulin (CLCRG, ref. 15). Sequence differences are indicated by vertical lines. Residues 1-8 of RLCRG are identical to those of rabbit uterine "high-affinity calcium- binding protein" (16). The standard single letter code for amino acids is used: A=Ala, D=Asp, E=Glu, F=Phe, G=Gly, I=Ile, K=Lys, L=Leu, P=Pro, Q=Gln, R=Arg, S=Ser, T=Thr, V=Val, W=Trp, Y=Tyr, and x is an unidentified residue.

weight. The results of duplicate N-terminal sequence analyses of the electrophoretically purified and electroblotted canine brain CBP are shown in Figure 2. A gradually increasing background during each sequencer run prevented us from identifying amino acid residues at positions 16 and 20, or any residues beyond position 24.

A GENEPRO computer search of the Protein Identification Resource database (release 20) for similar sequences yielded only one significant match: human Ro/SS-A antigen, a soluble, cytoplasmic protein first described in 1969 as an antigen reactive to antisera from a patient with systemic lupus erythematosus (13). Subsequently, the Ro/SS-A antigen has been associated with other autoimmune diseases, and found to be present in a wide variety of human tissues (14). Despite the clinical importance of this 60 kDa protein, it has only recently been purified (14). Its N-terminal

sequence has been determined, and shown to contain a major antibody-combining site (14).

It is coincidental that sequence analyses of both Ro/SS-A antigen and canine brain CBP were both successful for 24 cycles. Of the 22 positively identified residues in canine brain CBP, 20 are identical in the Ro/SS-A antigen (Figure 2). The identity betwven these two sequences may be greater than 90%, since positons 16 and 20 of canine brain CBP could easily be occupied by Trp residues, as they are in the Ro/SS-A antigen. The Val-Ile difference at position 4 is a highly conservative and frequently occuring substitution of hydrophobic residues, while the Asp-Ser difference at position 18 involves two hydrophilic residues. The great similarity of these two proteins in both size and N-terminal sequence leaves little doubt that they are homologous. It would not be surprising to find that they are in fact the same protein, with a small number of species-derived sequence differences.

We also examined available CBP sequences which were too short or too recent to be found in the database. The N-terminal sequences (residues 1-15) of rabbit liver and chicken liver calregulins have been reported (15). In view of their similar size (~60 kDa) and wide tissue disribution (15), we were not surprised to find a high degree of sequence similarity between calregulin and the canine brain CBP (Figure 2).

Recently (16), a 55 kDa rabbit uterine protein was purified which cross-reacts with antibodies raised to the previously described (17) 55 kDa "high-affinity Ca^{2+}-binding protein" (HACBP) of rabbit skeletal muscle SR. The Ca^{2+}-binding properties of the uterine protein were not determined, but its N-terminal sequence (residues 1-8 only) was found to be identical to that of rabbit liver calregulin (16). Again, this is not surprising, especially since calregulin (15) and the SR HACBP (17) each contain a single high-affinity Ca^{2+}-binding site. The HACBP also contains low-affinity Ca^{2+}-binding sites (17).

The sequence of calsequestrin (18), which binds Ca^{2+} with high capacity but low affinity, contains no segments similar to those shown in Figure 2.

It is puzzling that the canine brain CBP displays immunological cross-reactivity with both calregulin and calsequestrin, two proteins which appear to be immunologically distinct from one another (16). Further work will be needed to resolve this question.

In conclusion, the similarities in size and N-terminal sequences provide strong evidence that Ro/SS-A antigen and the CBPs are closely related, with similar structures and functional properties. While it is tempting to speculate, we do not have enough information at this point to suggest that binding of Ca^{2+} to the Ro/SS-A antigen may be involved in the pathogenesis of autoimmune diseases.

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REFERENCES

1. Collins, J.H. (1976) Soc. Exptl. Biol. Symp. 30, 303-334.
2. Khanna, N.C., and Waisman, D.M. (1986) Biochemistry 25, 1078-1092.
3. Heizmann, C.W., and Berchtold, M.W. (1987) Cell Calcium 8, 1-41.
4. MacLennan, D.H., and Wong, P.T.S. (1971) Proc. Natl. Acad. Sci. USA 68, 1231-1235.
5. Johnson Benson, R.J., and Fine, R.E. (1988) J. Cell. Biol. 107, 763a.
6. Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., and Lew, D.P. (1988) Proc. Natl. Acad. Sci. USA 85, 1091-1095.
7. Hashimoto, S., Bernardino, B., Lew, D.P., Pozzan, T., Volpe, P., and Meldolesi, J. (1988) J. Cell Biol. 107, 2523-2531.
8. Alderson, B.H., and Volpe, P. (1989) Arch. Biochem. Biophys. 272, 162-174.
9. Slupsky, J.R., Ohnishi, M., Carpenter, M.R., and Reithmeier, R.A.F. (1987) Biochemistry 26, 6539-6544.
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. Zorzato, F., and Volpe, P. (1988) Arch. Biochem. Biophys. 261, 324-329.
12. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035-10038.
13. Clark, G., Reichlin, M., and Tomais, T.B. (1969) J. Immunol. 102, 117-122.
14. Lieu, T.-S., Newkirk, M.M., Capra, J.D., and Sontheimer, R.D. (1988) J. Clin. Invest. 82, 96-101.
15. Khanna, N.C., Tokuda, M., and Waisman, D.M. (1987) Biochem. J. 242, 245-251.
16. Fliegel, L., Burns, K., Opas, M., and Michalak, M. (1989) Biochim. Biophys. Acta 982, 1-8.
17. Ostwald, T.J., and MacLennan, D.H. (1974) J. Biol. Chem. 249, 974-979.
18. Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeyer, R.A.F., and MacLennan, D.H. (1987) Proc. Natl. Acad. Sci. USA 84, 1167-1171.