BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 18–22 (1997)
ARTICLE NO. RC977416

Purification and Partial Amino Acid Sequence of a Novel Protein of the Reticulocalbin Family

Ming Jhy Hseu,* Chon-Yo Yen,* Chiu-Chen Tseng,* and Mu-Chin Tzeng*,†,[1]

**Institute of Biological Chemistry, Academia Sinica, and †Institute of Biochemical Sciences, National Taiwan University, P.O. Box 23-106, Taipei, Taiwan, Republic of China*

Received August 25, 1997

Binding proteins in neuronal membranes for a phospholipase A_2 with presynaptic neurotoxicity have been purified. Three polypeptides of 87, 65, and 50 K Da were obtained from the synaptic membrane fraction of guinea pig brain utilizing an immobilized crotoxin (a phospholipase A_2) column. For large scale purification, porcine brain was used instead, and two polypeptides of 50 and 18 K Da were found. The 65 and 18 K polypeptides may represent hitherto unidentified components of the crotoxin-binding proteins. Partial N-terminal amino acid sequence and a partial sequence for an internal peptide fragment have been determined for the 50 K polypeptide. Search of protein data bank reveals that this polypeptide or protein is a novel member of the reticulocalbin family of calcium-binding proteins.

Neurotoxins of protein nature have been found in many venoms and a few bacterial species. Some of these toxins act at the postsynaptic level, but the more lethal ones tend to act at the presynaptic level. Some of the presynaptically acting toxins exhibit phospholipase A_2 (PLA_2, E.C. 3.1.1.4) activity. These neurotoxic PLA_2s (or PLA_2 neurotoxins) are members of a group of extracellular (or secreted) PLA_2 proteins found in most animals, which may also produce intracellular type of PLA_2. PLA_2s play important roles in phospholipid metabolism, signal transduction, and host defense. Some of them also exhibit neurotoxicity, myotoxicity, coagulation action and other biological effects, which may or may not be related to hydrolysis of phospholipids. Although the biological actions may vary, the PLA_2 chains of the extracellular type from different sources show high degrees of homology in protein structures. A small number of the secreted PLA_2s, including crotoxin from the South American rattlesnake *Crotalus durissus terrificus*, at primarily at the presynaptic level to cause synaptic blockade by inhibiting the release of neurotransmitters, though most of them also manifest postsynaptic toxicity and other effects. Very recently, it has been shown that these neurotoxic PLA_2s bind to the neuronal membranes strongly, whereas the non-neurotoxic PLA_2s do not, though they may bind to non-neuronal membranes (ref. 1-9 for reviews). In a few cases, identification of toxin-binding proteins selectively present on synaptic membranes with the use of photoaffinity labeling or simple chemical cross-linking techniques has been reported (10-20). Purification and cloning of receptor (or acceptor) proteins for non-neurotoxic PLA_2s and for a neurotoxic PLA_2 have been achieved (21-27). In this communication we have purified and partially sequenced a crotoxin-binding protein, which is a novel member of the reticulocalbin family of calcium-binding proteins.

MATERIALS AND METHODS

Solubilization of the crotoxin-binding proteins. The fraction enriched in synaptic membranes prepared according to published procedures (28,29) was suspended at a concentration of 10 mg protein/ml in solution A (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM $SrCl_2$, 0.5 mM EGTA), and specified concentration of a detergent. After agitation at 4°C for 2 h, the mixture was centrifuged at 15,000 x g for 20 min and then at 105,000 x g for 1 h at 4°C. The resulting supernatant was referred to as the detergent extract.

Purification of the crotoxin-binding proteins. The detergent extract was fractionated by gel filtration with a Sepharose 6B column equilibrated with solution A containing 0.1% Triton X-100. Fractions of 2 ml eluate were collected and assayed for ^{125}I-crotoxin-binding activity. The active fractions were pooled and further purified by affinity chromatography with an immobilized crotoxin column, in which crotoxin was coupled to CNBr-activated Sepharose 4B (5 mg protein per ml of gel) according to the manufacturer's instructions. The Sepharose-crotoxin gel was equilibrated with solution A and then added to the pooled active fractions above. After gentle rocking at 4°C overnight, the gel was packed into a plastic column and then washed with 25 ml of the above solution containing 0.2% Triton X-100 at a flowrate of 50 ml/hr. The absorbed proteins were then

[1] To whom correspondence should be addressed. Fax: 886-2-3635038.

0006-291X/97 $25.00

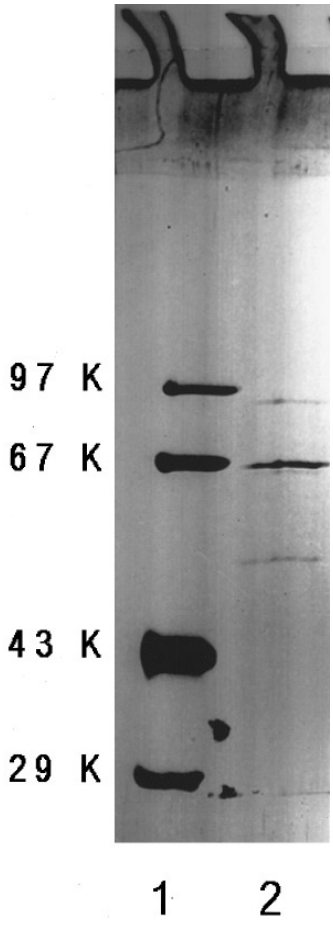

FIG. 1. SDS-PAGE of crotoxin-binding proteins from guinea pigs after purification by affinity chromatography using immobilized crotoxin column. Samples were solubilized in 0.1 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, and 5% β-mercaptoethanol and analyzed by SDS-PAGE using 10% acrylamide. Protein bands were detected by silver staining. Lane 1, protein standards (phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase, from top down); lane 2, purified fraction (1 μg) from affinity column.

dissociated from the affinity gel by eluting at 10 ml/hr with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% Triton X-100 and 50 mM NaCl. Fractions of 5 ml were collected for binding and cross-linking assays. All solutions in the protocol were ice-cooled and all of the purification steps were performed at 4°C.

Binding and cross-linking assays. To measure the binding activity of the solubilized preparations, each sample was mixed with ^{125}I-crotoxin in 0.2 ml solution A with specified concentration of a detergent. Parallel incubation containing 1 μM unlabeled crotoxin in addition was carried out in order to determine nonspecific binding. The mixtures were incubated for 2.5 h at 30°C. A rapid gel filtration method was used for separating the bound ^{125}I-crotoxin from the unbound. The Sephadex G-75 gel in a 3 ml syringe was washed with 2 bed volumes of column wash buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl and 0.2% Triton X-100) followed by centrifuging at 100 x g for 90 sec. The incubation mixture was carefully applied onto the column followed by centrifugation again. The complex consisting of crotoxin and its binding proteins was recovered in the void volume and quantified by γ-counting.

Cross-linking of ^{125}I-crotoxin with solubilized binding proteins was performed by treating the resulting binding complex above with 0.25 mM DSS for 15 min at 10°C. Then the reaction was stopped with 0.3 M glycine, pH 7.4. After the addition of 100 μg BSA, the proteins were precipitated by cold trichloroacetic acid at a concentration of 10% (w/v) with 1 h standing at 0°C. The precipitate was recovered by centrifugation, washed twice with acetone, and analyzed by SDS-PAGE (13).

CNBr cleavage. For cleavage by cyanogen bromide, protein samples were dissolved in 70% formic acid, and CNBr was added to give a final concentration of 120 mg/ml. The reaction was allowed to proceed at room temperature for 16 hr in the dark (30). At the completion of the reaction, CNBr and formic acid in the reaction mixture were removed by centrifugation in vacuum, and after addition of deionized water the operation was repeated thrice.

Electrophoretic techniques. Protein samples were separated according to their sizes by SDS-PAGE with resolving gel of 10% acrylamide using the discontinuous buffer system of Neville (31). After electrophoresis, the gel was stained by Commassie blue R-250 (0.25 W/V Commassie blue in 2-propanol/acetic acid/water, 30:10:60) and then destained with several changes of methanol/acetic/water (30:7:63).

Peptide fragments generated by CNBr cleavage were resolved by Tricine-SDS-PAGE according to the procedure of Schagger and von Jagow (32). The protein samples were dissolved in 4% SDS, 12% glycerol (W/V), 50 mM Tris, 2% mercaptoethanol (V/V), 0.01% Serva blue G, pH 6.8, and then incubated for 30 min at 40°C. The composition of the gel are stacking gel, 4% T, 3% C; spacer gel, 10% T, 3% C; and separating gel, 16.5% T, 6% C, with 2, 1.5, and 7.5 cm in length, respectively. Electrophoresis was carried out at 30 V for 1 hr followed by 110 V for 16 hr at room temperature.

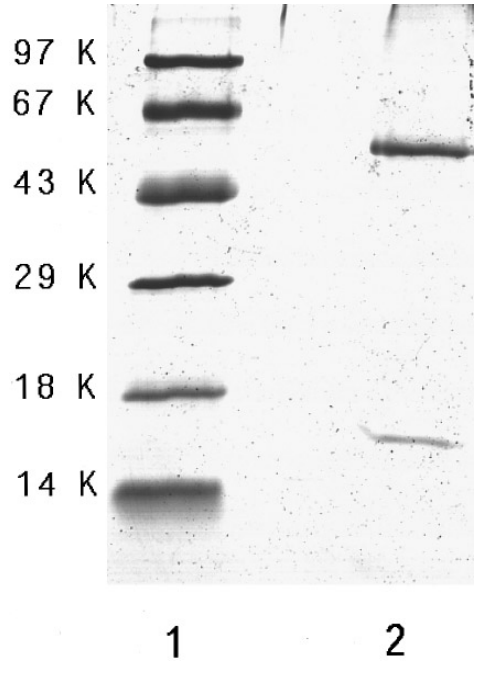

FIG. 2. SDS-PAGE of crotoxin-binding proteins from porcine brain after purification by affinity chromatography. Protein bands were stained by Coomassie Blue. Other procedures are as in Fig. 1. Lane 1, protein standards; lane 2, purified fraction (1 μg) from the affinity column.

CBP-50	KPTEKKDRVHHEPQLL
Reticulocalbin	LRAKPTVRKERVVRPDSEL

FIG. 3. Comparison of the N-terminal sequences of CBP-50 and reticulocalbin.

Electroblotting and visualization of proteins on PVDF membranes. Following electrophoresis, gels were equilibriated with electroblotting buffer (10 mM 3-(cyclohexylamino)-1-propane sulfonic acid, and 10% methanol, pH 11). Electroblotting onto PVDF membrane was performed at constant current of 2.5 A per cm^2 of gel area at room temperature for 30 min using a semi-dry blotter from Labconco. Proteins on PVDF membranes were visualized by staining with 0.1% Commassie blue R-250 in 4% methanol, and 1% acetic acid followed by destaining with 50% methanol. The destained membranes were extensively washed with deionized water, and the stained bands were cut out and subjected to automatic amino acid sequencing. For the above procedures, the manuals of both Applied Biosystems and Micro Separations Inc. were followed.

Protein quantitation. For the quantitation of protein samples, BCA protein assay reagents from Pierce were used for the sake of preventing interferences from salts and nonionic detergent.

RESULTS AND DISCUSSION

In order to solubilize the crotoxin-binding proteins from guinea pig brain, the synaptic membrane fraction was extracted with detergents. The extracts were then incubated with ^{125}I-crotoxin in the absence or the presence of unlabeled crotoxin and assayed for binding of ^{125}I-crotoxin using a gel filtration procedure. When the extraction was made with 0.5% digitonin or 1.5% CHAPS, specific binding activity was not found in the supernatants obtained after the detergent extracts were centrifuged at 105,000 x g for 1 h. When a buffer containing 4% Triton X-100 was used, about 90% of the proteins were in supernatant, and the crotoxin-binding activity was found to be solubilized. The crotoxin-binding proteins in solubilized form showed binding characteristics (data not shown) the same as the untreated membrane (10, 13).

Affinity chromatography with crotoxin immobilized onto Sepharose 4B was carried out to purify the crotoxin-binding proteins from the detergent extract. Binding activity for ^{125}I-crotoxin was desorbed by eluting with 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 50 mM NaCl, and 0.2% Triton X-100. EDTA was used for elution because the binding of crotoxin to its binding proteins requires divalent ion. When this eluate was analyzed by SDS-PAGE, only three major bands, presumably proteins, were found under reducing condition, the molecular weights being ca. 87 K, 65 K and 50 K (Fig. 1). The 87 K and the 50 K bands are consistent with previous affinity labeling experiments (12, 13), while the 65 K band was not identified before, which may be a new (subunit of the) binding protein for crotoxin.

For large scale purification of crotoxin-binding proteins, membrane preparation from porcine brain was used. Membrane fractions from the porcine brain behaved like those from the guinea pig brain towards crotoxin, both in equilibrium binding assay and in chemical cross-linking experiments (data not shown). The synaptosome fraction from porcine brain was lysed in 6 mM Tris-HCl, pH 8.1, and the resulting membrane pellets were extracted with 4% Triton X-100. Again, the crotoxin-binding proteins were found to be solubilized in biologically active form. After purification by affinity chromatography with crotoxin coupled to Sepharose 4B, only two major bands, presumably proteins, were found in SDS-PAGE under reducing condition, the molecular weights being ca. 50 and 18 K (Fig. 2). The 87 K and the 65 K bands were missing in the purified materials from porcine brain, perhaps due to species differences. Whereas the 18 K band was also not identified before, and may be a new crotoxin-binding protein or a new subunit of it, at least for the pig.

The two bands purified from porcine brain were cut out from the gel and extracted with an ISCO electroelutor. These two proteins will be referred to as CBP-50 and CBP-18. From 1.0 kg of porcine brain 50-60 μg of CBP-50 and 20-25 μg of CBP-18 were obtained. When CBP-50 was subjected to amino acid sequence determination, the first 16 residues from N-terminal was unambiguiously determined to be KPTEKKDRVHHEPQLL. A survey of the SWISS-PROT Database shows that this sequence is novel and the only protein with significant similarity to the above sequence is reticulocalbin (from the mouse) (33), which is about 43% identical (Fig. 3). Examination of the sequence revealed that the first five residue KPTEK is a potential phosphorylation site for both protein kinase C and cGMP-dependent protein kinase, since it fits the general pattern (K/R)X(T/S)X(K/R) for such site (34).

CBP-50	IVDKIDADKDGFVTEGELKSRIKHAQKXYI
Reticulocalbin(81-110)	IVDRIDSDGDGLVTTEELKLWIKRVQKRYI
TCBP-49(70-99)	IIKKIDSDSDGFLTENELSQWTQMSFKHYA
Troponin C(25-54)	AFDMFDADGGGDISTKELGTVMRMLGQNPT
Calmodulin(15-44)	AFSLFDKDGDGTITTKELGTVMRSLGQNPT
Parvalbumin(46-75)	AFAIIDQDKSGFIEEDELKLFLQNFKADAR

FIG. 4. Alignment of CBP-50 and some calcium-binding proteins. The numbers in parentheses denote the positions of the partial sequences in the polypeptide chains. X, unidentified amino acid residue.

	Helix	Loop	Helix
EF-hand motif	EL LL L	O O OG LO O	L LL L
CBP-50	IVDKI	DADKDGFVTEGE	LKSRIKHA
Reticulocalbin (81-105)	IVDRI	DSDGDGLVTTEE	LKLWIKRV

FIG. 5. Comparison of a potential calcium-binding domain in CBP-50 with the calcium-binding EF-hand motif. L and O reprensent hydrophobic and oxygen-containing residues, respectively.

CBP-50 was chemically cleaved by CNBr into peptide fragments. When the fragments were subjected to SDS-PAGE, three bands with apparent molecular weights of about 14,000, 8,000, and 4,000, were observed. Other peptide fragments escaped detection for some reason. The peptide bands in the gel were electroblotted onto PVDF membrane and then cut out for sequence determination. The following sequence of 30 residues was unambiguously identified except for one residue for the 8000 Da band: IVDKIDADKDGFVTEGELKSRIKH-AQKXYI. Search for similar sequences in the SWISS-PROT database revealed that this internal sequence of CBP-50 showed homology to only 5 proteins, i.e., reticulocalbin, TCBP-49, calmodulin, troponin C, and parvalbumin (27, 33-35), which all bind calcium (Fig. 4). For this region of 30 residues, CBP-50 has 63% of residues identical to reticulocalbin, 50% to TCBP-49, 33% to parvalbumin, and 23% to troponin C and calmodulin. Sequence information could not be obtained for the other two fragments for unknown reasons.

Similar to these calcium-binding proteins, the above partial sequence of CBP-50 also contains a calcium-binding domain. When compared with the defined calcium-binding domain (35), it is evident that the segment DADKDGFVTEGE in the above sequence fulfils the requirement for a loop of 12 residues in the helix-loop-helix (HLH) motif that is an important structural feature of the sites for calcium binding. Moreover the sequence also accords with the general feature of a high affinity Ca^{2+}-binding EF-hand domain according to Kretsinger's rule (36). To wit, 5 oxygen-containing residues in the loop region important for the coordination of Ca^{2+} are present, as well as a conserved glycine residue in the center responsible for bending the loop (Fig. 5). Other segments of the partial sequence match well with the test sequence in the helical regions. These similarities further implicated CBP-50 as a calcium-binding protein. Taken together, CBP-50 is most likely a new member of the reticulocalbin family of calcium-binding proteins.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Science Council, R.O.C., to M.-C. Tzeng (NSC-85-2311-B001-051 and NSC-86-2311-B001-080).

REFERENCES

1. Tzeng, M.-C. (1993) *J. Toxicol.-Toxin Rev.* **12,** 1–62.
2. Harris, J. B. (1991) *in* Snake Toxins (Harvey, A. L., Ed.), pp. 91–129, Pergamon Press, Tarrytown, NY.
3. Hawgood, B., and Bon, C. (1991) *in* Handbook of Natural Toxins, Vol. 5: Reptile Venoms and Toxins (Tu, A. T., Ed.), pp. 3–52, Dekker, New York.
4. Davidson, F. F., and Dennis, E. A. (1991) *in* Handbook of Natural Toxins, Vol. 5: Reptile Venoms and Toxins (Tu, A. T., Ed.), pp. 107–145, Dekker, New York.
5. Harvey, A. L. (1990) *Int. Rev. Neurobiol.* **32,** 201–239.
6. Rosenberg, P. (1990) *in* Handbook of Toxinology (Shier, W. T., and Mebs, D., Eds.), pp. 67–277, Dekker, New York.
7. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13060.
8. Mayer, R. J., and Marshall, L. A. (1993) *FASEB J.* **7,** 339–348.
9. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) *Biochim. Biophys. Acta* **1170,** 217–231.
10. Tzeng, M.-C., Hseu, M. J., Yang, J. H., and Guillory, R. J. (1986) *J. Protein Chem.* **5,** 221–231.
11. Tzeng, M.-C., Hseu, M. J., and Yen, C.-H. (1989) *Biochem. Biophys. Res. Commun.* **165,** 689–694.
12. Hseu, M. J., Guillory, R. J., and Tzeng, M.-C. (1990) *J. Bioenerg. Biomember.* **22,** 39–50.
13. Yen, C.-H. and Tzeng, M.-C. (1991) *Biochemistry* **30,** 11473–11477.
14. Othman, I. B., Spokes, J. W., and Dolly, J. O. (1982) *Eur. J. Biochem.* **128,** 267–276.
15. Rehm, H., and Betz, H. (1982) *J. Biol. Chem.* **257,** 10015–10022.
16. Rehm, H., and Betz, H.(1983) *EMBO J.* **2,** 1119–1122.
17. Rehm, H., and Lazdunski, M. (1988) *Biochem. Biophys. Res. Commun.* **153,** 231–240.
18. Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., and Lazdunski, M. (1989) *J. Biol. Chem.* **264,** 11503–11510.
19. Lambeau, G., Schimd-Alliana, A.,Lazdunski, M., and Barhanin, J. (1990) *J. Biol. Chem.* **265,** 9526–9532.
20. Scott, V. E. S., Percej, D. N., Keen, J. N., Findlay, J. B. C., and Dolly, J. O. (1990) *J. Biol. Chem.* **265,** 20094–20097.
21. Krizaj, I., Dolly, J. O., and Gubensek (1994) *Biochemistry* **33,** 13938–13945.
22. Krizaj, I., Faure, G., Gubensek, F., and Bon, C. (1997) *Biochemistry* **37,** 2779–2787.
23. Ishizaki, J., Hanasaki, K., Higashino, K., Kishino, J., Kikuchi, N., Osama, O., and Arita, H. (1994) *J. Biol. Chem.* **269,** 5897–5904.
24. Lambean, G., Ancian, P., Barhanin, J., and Lazdunski, M. (1994) *J. Biol. Chem.* **269,** 1575–1578.
25. Ancian, P., Lambeau, G., Mattei, M.-G., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 8963–8970.

26. Schlimgen, A. K., Helms, J. A., Vogel, H., and Perin, M. S. (1995) *Neuron* **14,** 519–526.
27. Dodds, D., Schlimgen, A. K., Lu, S. Y., and Perin, M. S. (1995) *J. Neurochem.* **64,** 2339–2344.
28. Whittaker, V. P. (1995) *Biochem. J.* **72,** 694–706.
29. de Robertis, E., de Iraldi, A. P., de Lores Arnaiz, G.R., and Salganicoff, L. (1962) *J. Neurochem.* **9,** 23.
30. Jahnen, W., Ward, L. D., Reid, G. E., Moritz, R. L., and Simpson, R. J. (1990) *Biochem. Biophys. Res. Commun.* **166,** 139–145.
31. Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246,** 6328–6334.
32. Schagger, H., and Jagow, G. V. (1987) *Anal. Biochem.* **166,** 368–379.
33. Ozawa, M., and Muramatsu, T. (1993) *J. Biol. Chem.* **268,** 699–705.
34. Pearson, R. B., and Kemp, B. E. (1991) *Methods Enzymol.* **200,** 63–81.
35. Strynadka, N. C. J., and James, M. N. G. (1989) *Annu. Rev. Biochem.* **58,** 951–998.
36. Kretsinger, R. H. (1980) *Ann. N.Y. Acad. Sci. USA* **83,** 7578–7582.