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CHROMOBINDIN A, A Ca^{2+}- AND ATP-DEPENDENT CHROMAFFIN GRANULE-BINDING PROTEIN, IS FOUND IN A VARIETY OF TISSUES AND IN YEAST*

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Summary: Chromobindin A is a ring shaped, multisubunit protein which exhibits Ca^{2+} and ATP-dependent binding to chromaffin granule membranes. Here we report biochemical and immunochemical evidence for the presence of chromobindin A in a surprisingly broad range of tissues: The protein is abundant in bovine skeletal muscle, pancreas, adrenal cortex and in brain gray and white matter; it is present in low quantities in the parotid, intestine and spleen and is undetectable in lung. Interestingly, chromobindin A was also detected in extracts of yeast. Electron micrographs of the yeast protein reveal a morphology virtually identical to the mammalian protein. These results suggest that chromobindin A is an important protein in a wide variety of cell types and that it has been highly conserved through evolution.

Chromobindin A is an 800 kDa protein that is composed of 2 copies of 6 subunits plus one copy of a seventh subunit joined together to form a ring-shaped molecule 17.5 nm in diameter (1). Previous studies have shown that the protein binds to a protease sensitive receptor on chromaffin granule (secretory vesicle) membranes (1-3). This binding is both Ca^{2+}- and ATP-sensitive; in the presence of ATP, the binding is Ca^{2+}-dependent: Ca^{2+} is

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required for binding. In the absence of Ca^{2+}, binding is ATP-sensitive: The protein binds to the receptor in the absence of ATP, but in the presence of ATP, binding is prevented. Therefore, it is possible to isolate mg quantities of highly purified chromobindin A by Ca^{2+} and ATP-dependent chromatography over chromaffin granule membranes linked to CNBr-activated Sepharose 4B (1).

At present, the function of chromobindin A is unclear. Recently we have found that chromobindin A has a small amount of RNA bound to the complex and that the protein has ATPase activity (2). The observation that the protein binds to chromaffin granule membranes in a Ca^{2+} and ATP-dependent manner could suggest that the protein is involved in exocytosis since exocytosis requires ATP and is triggered by an increase in cytoplasmic Ca^{2+}; however, this observation does not define a function for the protein. Possible roles for the complex include binding the granules to the cytoskeleton, movement of the granules along the cytoskeleton, modulation of granule binding to the cell membrane, membrane recycling or perhaps some other aspect of exocytosis or membrane-cytoskeletal interactions. By examining the tissue distribution of chromobindin A it may be possible to rule out some of these functions.

Materials and Methods

The preparation of chromaffin granule membranes and the coupling of granule membranes to CNBr-activated Sepharose 4B was as described previously (3). Postmicrosomal supernatants from bovine tissues were prepared by homogenizing 10g of tissue in 25 ml of 300 mM sucrose, 2mM EGTA, 5 mM HEPES-NaOH, pH 7.3. The homogenate was centrifuged at 500 x g for 10 min and the supernatant was then centrifuged at 20,000 x g for 30 min and finally at 100,000 x g for 1 hr. Yeast postmicrosomal supernatants were prepared similarly from 100gm of late log phase cultures of Saccharomyces cerevisiae strain YP3 (kindly provided by Dr. Mitch Smith, University of Virginia).

Affinity Chromatography: Chromaffin granule membranes bound to CNBr activated Sepharose 4B were packed into a 1.6 x 20 cm water jacketed column maintained at 37°C. The column then was washed with one bed volume of column buffer (240 mM sucrose, 30 mM KCl, 1 mM $MgCl_2$, 2.5 mM EGTA, 25 mM HEPES/NaOH, pH 7.3 at 37°C), followed by one bed volume of column buffer plus 4.5 mM $CaCl_2$ (2mM free), one bed volume of column buffer plus 1 mM ATP and finally the column was equilibrated with column buffer plus 2.0 mM free Ca^{2+}. The Ca^{2+}- and ATP-dependent granule-binding-proteins were isolated by adding 4.0 mM $CaCl_2$ (about 2 mM free Ca^{2+}) and 1 mM $MgCl_2$ to the postmicrosomal supernatants and passing the supernatants over the column. The column was washed with 2 bed volumes of column buffer plus 2.0 mM free $CaCl_2$, and then with one bed volume of column buffer without Ca^{2+}. The Ca^{2+}

and ATP-dependent granule-binding-proteins were finally eluted with column buffer plus 1 mM ATP.

Analytical Procedures: Protein was measured as described by Bradford (4) using bovine serum albumen as a standard. SDS-gels were run as described by Laemmli (5) and were stained with Coomassie Blue. The standards were phosphorylase b (97 kDa); bovine serum albumin (67 kDa); ovalbumin (45 kDa); carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa); and lysozyme (14 kDa). Antisera production and immunoblotting were performed as described previously (6). Electron microscopy of samples negatively stained with 1% uranyl acetate was performed as described (1).

Results and Discussion

Postmicrosomal supernatants were prepared from a variety of bovine tissues and run, in the presence of Ca^{2+}, over an affinity column prepared from chromaffin granule membranes. The column was washed with a Ca^{2+} containing buffer followed by a buffer containing 2.5 mM EGTA. The column was then washed with a buffer containing 2.5 mM EGTA and 1 mM ATP to elute Ca^{2+}- and ATP-dependent membrane-binding proteins, such as chromobindin A. Fig. 1A shows an SDS gel of the proteins eluted by the EGTA/ATP buffer using postmicrosomal supernatants from a variety of bovine tissues. Lane 1 of this figure contains authentic chromobindin A, isolated from the adrenal medulla. A similar group of proteins are isolated from the adrenal cortex (lane 2), liver (lane 3), brain white matter (lane 4) brain gray matter (lane 5), pancreas (lane 6), and skeletal muscle (lane 7). These proteins are not observed if postmicrosomal supernatant from the parotid (lane 8), intestine (lane 9), spleen (lane 10) or the lung (lane 11) are passed over the column. Since the samples shown in lanes 2-7 contain proteins of the same molecular weight and similar granule binding properties as adrenal medullary chromobindin A it is likely that these proteins are very similar or identical to chromobindin A. Further support for this idea comes from an examination of western blots prepared with an antibody to adrenal medullary chromobindin A. This antibody reacts with three of the seven subunits in the chromobindin A complex and in western blots of one dimensional gels, it reveals a doublet at 56 kDa and 59 kDa. Fig 1B is a western blot of a gel identical to the one shown in Fig 1A. In all of the lanes, except the one with proteins isolated from the lung, there is a doublet at 56 kDa and 59 kDa, suggesting that chromobindin A, or a very similar protein is present in all of these tissues, although in very small quantities in the parotid, intestine and spleen.

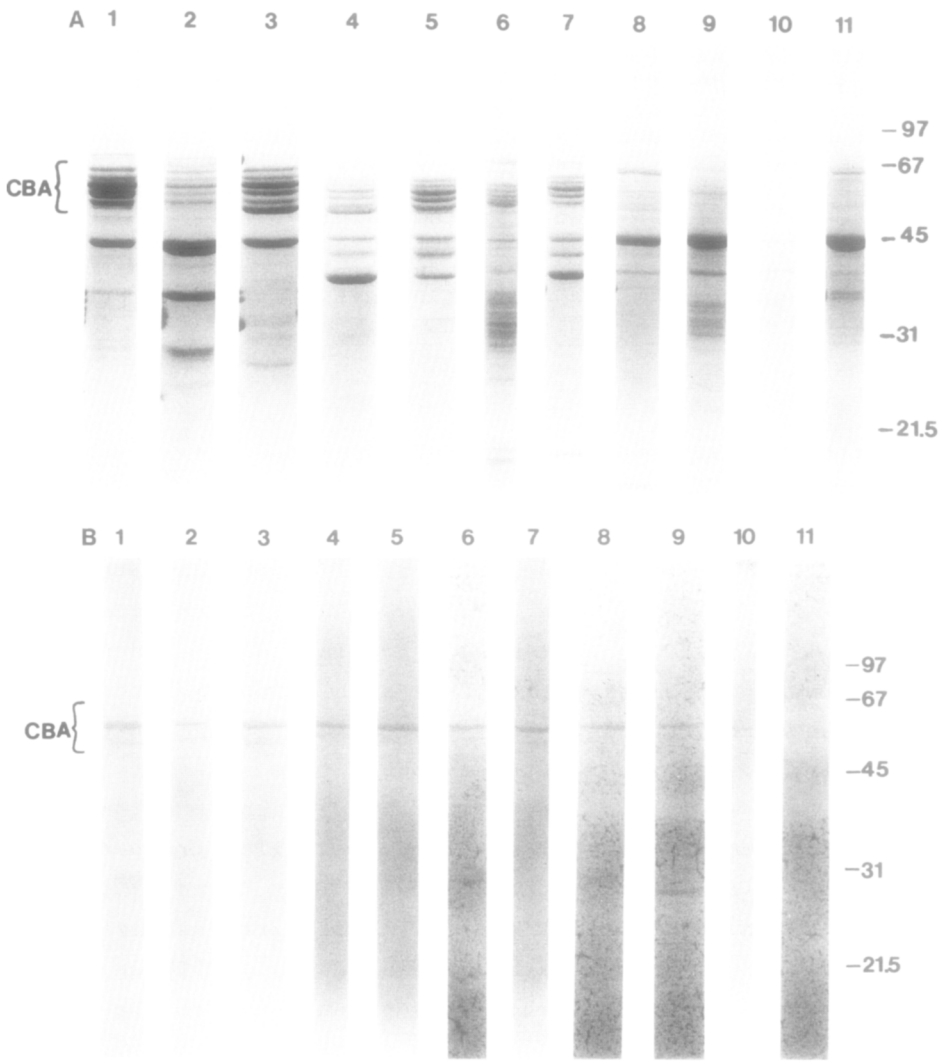

Fig. 1. Tissue Distribution of Chromobindin A: A. Proteins were isolated from various bovine tissues by Ca^{2+}-and ATP-dependent chromatography over chromaffin granule membranes attached CNBr activated Sepharose 4B and then submitted to SDS-gel electrophoresis and stained with Coomassie blue. Lane 1, Adrenal Medulla. Lane 2, Adrenal cortex. Lane 3, Liver. Lane 4, Brain white matter. Lane 5, Brain gray matter. Lane 6, Pancreas. Lane 7, Muscle. Lane 8, Parotid. Lane 9, Intestine. Lane 10, Spleen. Lane 11, Lung. B. A western blot of the gel shown in Fig. 1A was prepared, using an antibody to chromobindin A.

Although, from these experiments, it is difficult to absolutely quantitate the amount of chromobindin A per gram of tissue, it appears that chromobindin A is present in the following order of abundance: Adrenal medulla, liver>muscle, pancreas> adrenal cortex, brain gray matter> brain white matter>>parotid, intestine>spleen>>lung. Since chromobindin A is present in secretory tissue as well as in tissues that do not

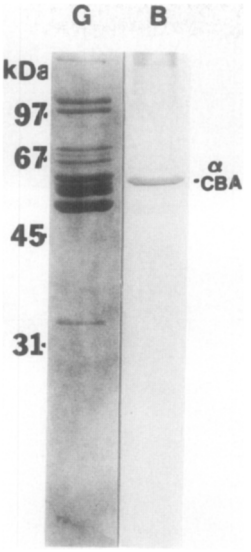

Fig. 2. Biochemical and Immunochemical Identification of Yeast Chromobindin A: Lane "G", SDS gel of yeast proteins eluted from a chromaffin granule membrane column by EGTA and ATP; Lane "B", Western blot of proteins in lane G probed with antiserum to bovine chromobindin A. "αCBA" marks the position of a yeast protein(s) that cross-reacts with bovine chromobindin A.

have high secretory capacity, such as muscle, and since it is present in very small quantities in some other tissues that have secretory activity, these results suggest that chromobindin A is not involved exclusively in exocytosis. The protein may be important in certain exocytotic pathways, but it is also likely to be important in other aspects of cellular function.

The data presented above indicates that chromobindin A has a wide tissue distribution. We were then interested in knowing if chromobindin A also has a wide species distribution: Has the protein been conserved through evolution? We therefore prepared Ca^{2+}- and ATP-dependent chromaffin granule-binding proteins from yeast postmicrosomal supernatant. This experiment resulted in the isolation of proteins of similar molecular weight and antibody crossreactivity as obtained from bovine adrenal medullary tissue (Fig. 2). We also compared the structure of the yeast protein to the structure of bovine adrenal medullary chromobindin A by electron microscopy of the negatively stained protein (Fig. 3). The structure of the yeast chromobindin A is nearly identical to the structure of the bovine protein, appearing as a circular complex 18nm in diameter. Thus, the yeast protein is similar to mammalian chromobindin A in terms of

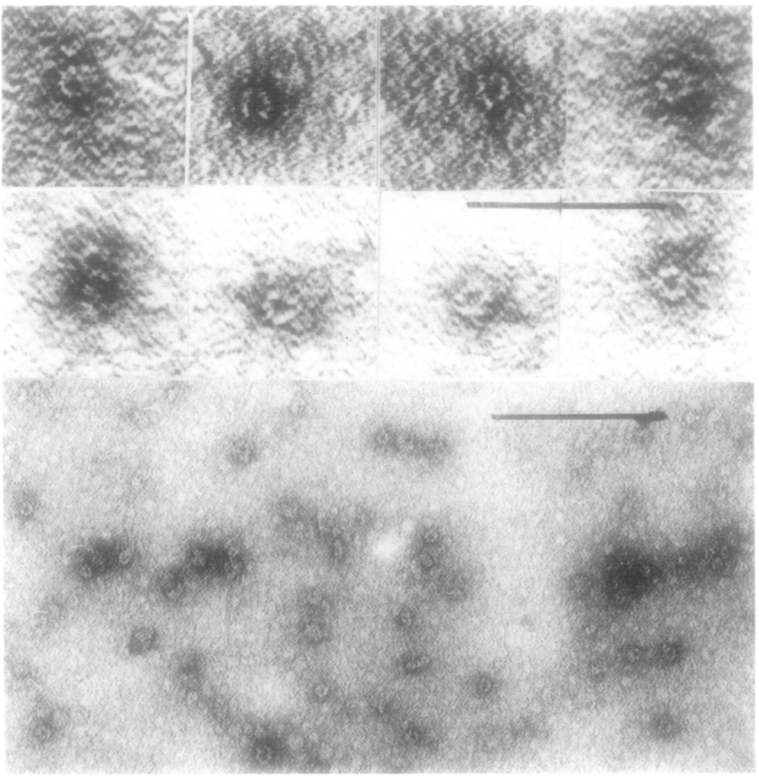

Fig. 3. Electron Micrographs of Yeast Chromobindin A: The top half of the figure shows 8 selected examples of individual chromobindin A molecules. The length of the bar is 100nm. The bottom half of the figure shows a survey view at lower magnification. The length of the bar is 250nm.

granule binding characteristics, molecular weights of the subunits, antibody cross reactivity and morphology under the electron microscope. These results imply chromobindin A has been preserved through evolution. In addition, this finding may lead to the determination of the physiological role of chromobindin A through manipulation of the genes for its subunits in yeast.

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