Identification of the Major Chromaffin Granule-binding Protein, Chromobindin A, as the Cytosolic Chaperonin CCT (Chaperonin Containing TCP-1)*

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Chromobindin A is a multisubunit complex ATPase that binds to chromaffin granule membranes in a calcium-dependent manner and requires ATP for release from the membrane (Martin, W. H., and Creutz, C. E. (1987) J. Biol. Chem. 262, 2803–2810). Here we report that the seven previously characterized subunits of chromobindin A cross react with antisera specific to subunits of CCT, the chaperonin containing TCP-1 (Rubota, H., Hynes, G., Carne, A., Ashworth, A., and Willison, K. (1994) Curr. Biol. 4, 88–90). The chromobindin A subunits previously called chromobindins 12, 13, 14, 15, 16, 18, and 19 cross-react specifically with subunits β, δ, ε, ζ, ξ, and γ, respectively, of CCT. Additional similarities in subunit molecular weights, isoelectric points, and the morphologies of the two protein complexes as determined by electron microscopy support identification of chromobindin A as an adrenal medullary form of CCT. The chromobindin A/CCT complex was found to bind at least 7-fold more efficiently to affinity columns of chromaffin granule membranes than of adrenal medullary cytosol proteins, suggesting a specific interaction occurs between the complex and membrane components. The results indicate that the previously described characteristics of chromobindin A are likely to be relevant to the functions of CCT and suggest that the adrenal medullary form of CCT may play a role in the activities of secretory vesicle membranes.

In the search for proteins that may underlie membrane activities in exocytosis, a number of proteins have been identified that bind to chromaffin granule membranes in the presence of calcium (1–4). Using isolated chromaffin granule membranes linked to Sepharose 4B as an affinity column, such proteins, called chromobindins, can be isolated as a class (1, 3–5). Included among the chromobindins are a number of now well characterized proteins, such as annexins (6, 7), protein kinase C (3), calmodulin (3), and a phosphatidylinositol-specific phospholipase C (8), all of which may function in the process of exocytosis or other membrane-mediated events. However, in terms of yield from the membrane affinity column, the major chromobindin is chromobindin A (9–11). This protein has been characterized as a complex of seven non-identical subunits with individual molecular masses of 50–65 kDa that requires ATP to regulate its calcium-dependent interaction with the chromaffin granule membrane. The function of this protein complex, since its initial identification in 1983 (3), has remained elusive.

Recently, a protein complex with physical properties similar to chromobindin A has been identified as a mammalian chaperonin homologous to the Gro-EL chaperonin of Escherichia coli (12–16). This protein, called CCT (chaperonin containing TCP-1) has been shown in vitro to associate with and participate in the folding of newly synthesized proteins (14–16). Because of the similarity of structures and the ATP sensitivities of chromobindin A and CCT, we have sought to determine if these protein complexes are related. Our results establish that chromobindin A indeed contains subunits physically and immunologically related to subunits contained in CCT and therefore chromobindin A is related to CCT and may be a species- or tissue-specific isoform of the chaperonin complex. This result allows the information previously obtained with chromobindin A to be interpreted in a new context and raises the question of whether isoforms of CCT may play a role in protein-mediated membrane interactions underlying exocytosis.

EXPERIMENTAL PROCEDURES

The following materials were prepared as described previously: Monoclonal antibodies to CCTα (TCP-1; Ref. 12); polyclonal rabbit antisera to bovine chromobindin A (11); chromobindin A from bovine adrenal medulla by calcium/ATP affinity chromatography on isolated chromaffin granule membranes (9). "Membrane" and "Cytosol" affinity columns were prepared by coupling chromaffin granule membranes to 7.5 g of Sepharose 4B as described (3) or coupling total adrenal medullary cytosol (252 mg of a 100,000 g supernatant from a tissue homogenate prepared in 25 mM HEPES, pH 7.4, 2.5 mM EGTA, as described previously; Ref. 3) to 7.5 g of Sepharose 4B. Chromatography was performed as described (3, 9), with additional details given in the legend to Fig. 3.

One-dimensional SDS gel electrophoresis was conducted according to Laemmli (18), and two-dimensional electrophoretic acrodies- a slightly modified O'Farrell procedure (12). Western blotting was conducted using the Amersham ECL detection system as described previously (Fig. 2; Ref. 21), or according to Burnette (Fig. 4; Ref. 20) using peroxidase-conjugated second antibody and 4-chloronaphthol for detection. Protein concentrations were determined according to the Bradford procedure (22) with bovine serum albumin used as standard.

RESULTS

The first subunit of CCT to be characterized was TCP-1, tailless-complex protein 1 (12–15). In preliminary experiments using Western blots of one-dimensional gels, a highly specific monoclonal antibody (91A) to this subunit was found to react with a single subunit of bovine chromobindin A (also seen in Fig. 4, discussed below). Two-dimensional gels of chromobindin A obtained from the chromaffin granule membrane affinity column reveal a pattern of spots that is similar to that seen for CCT (Fig. 1 and Refs. 3, 9, 12, and 13) in the 50–65-kDa mass

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Fig. 1. Two-dimensional gel of the chromobindin A preparation eluted from the chromaffin granule membrane affinity column by ATP plus EGTA. Seven subunits of chromobindin A are labeled in terms of the CCT subunit antibodies with which they cross-react, as shown in Fig. 2 (α, β, γ, δ, ε, ζ, and θ subunits). Table I gives the corresponding nomenclature for the chromobindin A subunits. The position of a possible η subunit is also indicated. The lower molecular mass proteins present in the preparation do not co-purify with the chromobindin A-CCT complex through further steps of purification (9, 10).

Fig. 2. Cross-reaction of the subunits of chromobindin A with antisera specific for individual subunits of the mouse chaperonin CCT. Subunits of the chromobindin A preparation, as illustrated in Fig. 1, were separated by two-dimensional electrophoresis and transferred to nitrocellulose and probed with specific antisera. In panel A, the blot was probed with a monoclonal antibody (91a) that recognizes CCTα. In panels B, C, and D, the blot was sequentially probed with antibodies against CCTζ, CCTδ, and CCTγ respectively. In panels E, F, and G, the blot was sequentially probed with antibodies against CCTθ, CCTδ, and CCTβ. The concentrations of primary antibodies used for detection are given in Table I.

Table I
Identification of the subunits of chromobindin A by Western blotting with antibodies raised against subunits of CCT

| Chromobindin A subunit | Corresponding CCT subunit | Antibody used for detection | Dilution for Western blot |
|------------------------|---------------------------|-----------------------------|--------------------------|
| Chromobindin 12        | CCTβ                      | BC1                         | 1/2500                   |
| Chromobindin 13        | CCTδ                      | DC1                         | 1/2500                   |
| Chromobindin 14        | CCTθ                      | TC1                         | 1/2500                   |
| Chromobindin 15        | CCTα                      | 91a                         | 1/500                    |
| Chromobindin 16        | CCTζ                      | ZC1                         | 1/2500                   |
| Chromobindin 18        | CCTε                      | EC1                         | 1/2500                   |
| Chromobindin 19        | CCTγ                      | GC1                         | 1/2500                   |

* Chromobindin A subunit nomenclature is defined in Refs. 3 and 9.
* CCT subunit nomenclature is defined in Ref 13.
* Antibody characterization presented in Refs. 12, 17, and Footnote 2.

Fig. 3. Column profiles showing the protein recovered from the cytosol and chromaffin granule membrane columns. Cytosol column, the matrix was total adrenal medullary post-microsomal supernatant attached to Sepharose 4B. Membrane column, the matrix was purified chromaffin granule membranes attached to Sepharose 4B. 25 ml of adrenal medullary postmicrosomal supernatant, containing 125 mg of protein, was applied to each column. After washing the column with 50 ml of buffer (240 mM sucrose, 30 mM KCl, 2 mM MgCl₂, 2.5 mM EGTA, 5 mM CaCl₂), the column was washed with the same buffer without CaCl₂ to elute the proteins represented by the peak labeled EGTA. Subsequently the column was washed with the same buffer without CaCl₂, but including 2 mM ATP to elute the proteins represented by the peak labeled ATP. In the case of the cytosol column, the maximum protein concentrations in peak EGTA were 276 μg/ml in fraction 47 and 396 μg/ml in fraction 48. The fraction size was 6 ml.

Range. All polypeptides in this mass range co-purify in further chromatographic steps (9) and are considered to be subunits of chromobindin A. Additional spots on the gel (Fig. 1) at lower molecular weights may correspond to proteins weakly associated with chromobindin A or to impurities in the chromobindin A sample, which was obtained directly from the affinity column without further purification.

Sequential probing of Western blots of two-dimensional gels of chromobindin A with antibodies specific to subunits of CCT indicated that the seven previously identified subunits of chromobindin A specifically cross-react with characterized subunits of CCT (Fig. 2 and Table I). An additional spot at the basic end of the gel was suspected of representing the η subunit (Fig. 1), based on its position. However, this could not be confirmed since the bovine η subunit does not cross-react with antisera against the mouse η subunit. Additional biochemical and physical properties of chromobindin A and CCT, summarized under “Discussion,” support the conclusion that these complexes are highly related or identical.
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One hypothesis to explain the equivalence of CCT and chromobindin A is that the chaperonin might associate with denatured membrane proteins that have been coupled to the Sepharose column that was used to affinity purify the chromobindin fraction of adrenal medullary cytosol. Some degree of unfolding may be expected in the preparation of the column, which includes an incubation at pH 8.0 (3), and CCT may recognize these unfolded proteins in a manner analogous to its ATP-sensitive association with unfolded polypeptides (13–15, 23).

If this hypothesis were correct, then the interaction of the chaperonin with the column would probably also occur when other complex protein mixtures are coupled to the column. To test this possibility, two affinity columns were prepared in parallel. On one column, chromaffin granule membranes isolated from an adrenal medullary homogenate were attached to Sepharose 4B; on the second column, 50% of the total cytosolic proteins from the same homogenate were attached to Sepharose 4B. The remaining 50% of the cytosol was split into two equal parts and applied to the two columns. Proteins bound to the affinity columns were eluted first with EGTA, to remove annexins and other ATP-independent chromobindins, then with EGTA plus MgATP to elute chromobindin A.

As seen in Figs. 3 and 4, the chromobindin A-CCT complex is obtained in much greater yield when the chromaffin granule membranes are attached to the column rather than total cytosolic proteins. Densitometry of the gels (Fig. 4) and correction for the differences in loading of the gel lanes (see legend for Fig. 4) indicate a 7-fold greater recovery of chromobindin A with the membrane column than with the cytosol column. Since approximately 6 mg of granule membrane is attached to the membrane column (3) and 205 mg of cytosolic protein was coupled to the cytosol column in this experiment, the efficiency of the membrane column in extracting the chromobindin A-CCT is 7 × 205/6 = 239-fold greater than the cytosol column. A portion of this larger difference, however, is probably due to a geometric constraint: Many of the cytosolic proteins are coupled to interior portions of the Sepharose 4B beads, whereas the membranes are largely coupled to the outside of the beads and the chromobindin A is largely excluded from the beads.

On the other hand, the relative efficiency of the membranes at extracting chromobindin A-CCT is likely to be even greater than the 7-fold difference apparent in Figs. 3 and 4. A small amount of annexins are obtained from the cytosol affinity column (Fig. 4). This may be because some membrane contamination is present in the cytosol preparation and some of these membranes are bound to the affinity column prepared from cytosol. In addition, annexins in the cytosol, after being covalently coupled to the column, may bind small membrane fragments in the cytosol and trap more annexins in a sandwich-like reaction. If the amount of annexins obtained from the cytosol column is an indication of the amount of membrane contamination on this column, then it is possible that virtually all of the chromobindin A-CCT obtained from the cytosol column is associating with the contaminating membranes.

DISCUSSION

In addition to the immunological criteria used to identify chromobindin A as CCT, many of the previously documented properties of chromobindin A correspond to recently described characteristics of CCT. These include the following.

1) Chromobindin A has at least seven non-identical subunits of mass 50–65 kDa and PI on two-dimensional gels of 5.9–7.8 from the cytosol column and from the membrane column probed with an antiserum against chromobindin A (CBA, Ref. 11). CCT marks the migration position of the α subunit of CCT.

Fig. 4. SDS gels and Western blots of the fractions obtained from the granule membrane and cytosol affinity columns. Panel A, SDS gel of fractions from the eluates presented in Fig. 3. From left to right, the lanes are: S, molecular size standards ( phosphorylase b, 97 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 20.1 kDa; lysozyme, 14.4 kDa); C, total cytosol extract applied to the columns (100 μg loaded on gel); W1, pooled wash fractions 42–43 from the cytosol column (220 μl); E, proteins eluted from the cytosol column by removing calcium from the column buffer (fractions 64–50, 220 μl); W2, wash fractions 63–61 from the cytosol column (500 μl); A, proteins eluted from the cytosol column by addition of ATP (fractions 63–68, 500 μl); W1, wash fractions 125–126 from the membrane column (630 μl); E, proteins eluted from the membrane column by removing calcium from the column buffer (fractions 128–131, 630 μl); W2, wash fractions 139 and 140 from the membrane column (800 μl); A, proteins eluted from the membrane column by addition of ATP (fractions 143–146, 800 μl). CBA marks the position of the chromobindin A complex in lanes A. ANX marks the position of the 50–56 kDa annexins in lanes E. Panel B, Western blot of column fractions illustrated in part a, using antibodies raised against CCT subunits or against bovine chromobindin A. The standards (S) have been stained with Amido Black; molecular weights are given in the legend to panel A. Lanes C, W1, E, W2, A, W1, E, W2, and A, represent fractions as defined above, probed with rat monoclonal antibody 91a to CCTa. The two lanes on the right represent the ATP elution fractions.
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(3). In addition, the patterns of the corresponding spots on two-dimensional gels of these two proteins are very similar. Indeed, the spot profile of bovine chromobindin A is virtually identical to that of CCT purified from bovine testes except for the absence of a testis-specific subunit (S6 of mouse CCT; Ref. 13).

2) By electron microscopy chromobindin A was found to form a double ring structure with each ring composed of apparently six to seven subunits (9).

3) ATP is necessary to release chromobindin A from membranes (9), similar to the requirement for ATP to release substrate proteins from CCT.

4) Chromobindin A is not restricted to adrenal medullary tissue but is broadly distributed in mammalian tissues, and a homolog was identified in yeast (11). Recently CCT was purified from yeast and was found to consist of multiple subunits encoded by genes related to mammalian CCT genes (24). There are different subunit compositions found for chromobindin A isolated from different tissue sources (11), and diversity of CCT complexes has also been suggested (13).

5) Chromobindin A requires a protein receptor on the membrane (3, 9). This is indicated by the failure of chromobindin A to bind to pure lipids or to protease-treated membranes and may relate to the binding of CCT to substrate proteins.

In all of these features, chromobindin A is similar to CCT. Several additional properties of chromobindin A have not yet been described for CCT.

1) Chromobindin A attaches reversibly to biological membranes. This binding ability exhibits specificity for certain membrane types since chromobindin A binds to chromaffin granule membranes but not mitochondrial membranes (9).

2) Calcium regulates the attachment of chromobindin A to membranes. Although calcium is not absolutely required to attach chromobindin A to the membrane, it must be removed by chelation with EGTA if the protein is to be released from the membrane (9).

3) The ATPase activity of chromobindin A is inhibited by calcium (10).

4) Chromobindin A appears to contain a small complement of RNA which can be detected after alkaline hydrolysis as 2 mol of AMP, 2 mol of CMP, and 7 mol of GMP/mol of chromobindin A (10).

It would now be of interest to determine if these characteristics of chromobindin A relate to the functions of CCT. In particular, a role for calcium in regulating the chaperonin should be investigated, as well as the ability of the complex to interact with specific membrane types. In addition, it will be of interest to determine the nature of the specific protein target for chromobindin A-CCT on the membrane.

It has been suggested that chaperone proteins may play broad roles in cell biology in addition to catalyzing polypeptide folding during protein synthesis (18). The ability of chaperones to interact with "unfolded" regions of proteins may also be important in regulating conformational changes in functional cycles of specific proteins. Our results indicate a preference for association of the chromobindin A-CCT complex with secretory vesicle membrane proteins. A highly speculative role for chromobindin A-CCT in exocytosis could be to chaperone a hydrophobic protein domain or "spike" responsible for membrane fusion so that it does not participate in unregulated or inappropriately directed fusion events. Future work should determine whether the adrenal medullary chromobindin A-CCT complex possesses unique subunits and activities to subserve such events in the secretory pathway.

REFERENCES

1. Creutz, C. E. (1981) Biochim. Biophys. Res. Commun. 103, 1395-1400
2. Geisow, M. J., and Burgoyne, R. D. (1982) J. Neurochem. 38, 1735-1741
3. Creutz, C. E., Dowling, L. G., Sando, J. J., Villar-Palasi, C., Whipple, J. H., and Zaks, W. J. (1983) J. Biol. Chem. 258, 14664-14674
4. Creutz, C. E., Zaks, W. J., Hamsma, H. C., Crane, S., Martin, W. H., Gould, K. L., Oddie, K. M., and Parsons, S. J. (1987) J. Biol. Chem. 262, 1860-1868
5. Creutz, C. E. (1993) Methods Enzymol. 221, 190-206
6. Geisow, M. J., and Walker, J. H. (1988) Trends Biochem. Sci. 11, 420-423
7. Creutz, C. E. (1982) Science 216, 924-927
8. Creutz, C. E., Dowling, L. G., Kyger, E. M., and Frasch, R. C. (1985) J. Biol. Chem. 260, 7171-7173
9. Martin, W. H., and Creutz, C. E. (1981) J. Biol. Chem. 256, 2803-2810
10. Martin, W. H., and Creutz, C. E. (1988) J. Neurochem. 54, 613-619
11. Martin, W. H., Fromer, E., and Creutz, C. E. (1989) Biochim. Biophys. Res. Commun. 165, 37-42
12. Lewis, V. A., Hynes, G. M., Zheng, D., Sambh, H., and Willison, K. (1992) Nature 358, 249-252
13. Kubota, H., Hynes, G., Carne, A., Ashworth, A., and Willison, K. (1994) Curr. Biol. 4, 59-59
14. Frydman, J., Nimmesgern, E., Ercinlem-Bromage, H., Wall, J. S., Tempat, P., and Hartl, F. U. (1992) EMBO J. 11, 4767-4778
15. Yaffe, M. B., Farr, G. W., Miklos, D., Horwich, A. L., Sternlicht, M. L., and Sternlicht, H. (1992) Nature 358, 245-248
16. Gao, Y., Thomas, J. O., Chow, R. L., Lee, G.-H., and Cowan, N. J. (1992) Cell 69, 1043-1050
17. Kubota, H., Hynes, G., and Willison, K. (1994) Genes (Amst.), in press
18. Ellis, R. J., and Van Der Vies, S. M. (1991) Annu. Rev. Biochem. 60, 321-367
19. Laemmli, U. K. (1970) Nature 227, 680-685
20. Burnette, W. N. (1981) Anal. Biochem. 112, 195-203
21. Harrison-Lavoie, K., Lewis, V. A., Hynes, G. M., Collison, K. S., Nutland, E., and Willison, K. (1993) EMBO J. 12, 2847-2853
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
23. Roobol, A., and Carden, M. J. (1993) J. Neurochem. 60, 2237-2233
24. Miklos, D., Caplan, S., Mertens, D., Hynes, G., Pitlik, Z., Kashi, Y., Harrison-Lavoie, K., Brown, C., Horwich, A. L., and Willison, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2743-2747