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# Cellular retinoic acid binding protein is associated with mitochondria

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**Abstract We report that immunohistochemical staining for cellular retinoic acid-binding protein (CRABP) was restricted to the cytoplasm of cortical cells in bovine adrenal. In contrast, staining for the similar protein, cellular retinol-binding protein (CRBP), was found throughout these cells. After transfections of CRABP and CRBP into cultured cells, immunofluorescence analyses again revealed cytoplasmic restriction only for CRABP, with a pronounced punctate appearance. Use of organelle-specific fluorochromes indicated that CRABP immunofluorescence overlaid exactly with the pattern of the mitochondrial-specific fluorochrome. Confirmation of this association came with subcellular fractionation of the adrenal cortex. CRABP, but not CRBP, co-sedimented with the mitochondria, a novel finding for a member of this superfamily of cellular lipid-binding proteins.** 

*Key words:* Cellular retinoic acid-binding protein; Vitamin A; Retinoid; Mitochondrion; Targeting

## 1. Introduction

Retinoic acid (RA) is an active hormonal form of the essential micronutrient vitamin A. This hormone exerts its effects on a cell by binding to its cognate RA receptors (RARs). The active receptor–ligand complex binds to RA response elements and alters the transcriptional rates of specific genes [1]. Since at least one of the three known RARs is expressed in most, if not all, mammalian cell types, mechanisms for regulating access of RA to the nucleus would appear necessary. Part of such mechanisms may involve the 15.5 kDa protein, cellular RA binding protein (CRABP or CRABP, type 1) [2]. This protein has been shown to specifically bind RA with nanomolar affinities [3,4]. There are several reports that suggest CRABP may protect the cell from the genomic action of RA by sequestering the compound and accelerating its catabolism [5,6]. RA bound to CRABP has been demonstrated to be a substrate for oxidation by members of the P450 family [7].

A potential key to attenuation of the RA signal may be found in the specific location of CRABP within the cell. Immunohistochemical experiments performed on tissue sections by our laboratory has revealed that this protein is restricted to the cytoplasmic compartment of cells, even though its size should enable free entry into the nucleus [8]. This nuclear restriction might create a barrier that prevents RA from reaching the RARs in the nucleus. However, the mechanism(s) by which this restriction is accomplished has not been established. Conversely, Gustafson et al. have reported immunohistochemical analysis of tissue sections and observed CRABP staining restricted to the cytoplasm of some cells but in the nucleus of others [9]. However, this report demonstrated that, following transfection of cells with CRABP cDNA, the expressed protein detected by immunofluorescence was nuclear excluded in all cells. One additional study reported both nuclear exclusion or inclusion, depending on their experimental conditions. They suggested that the apparent nuclear exclusion observed by immunohistochemistry or immunofluorescence may have resulted from antibody or fixation artifacts [10]. In this study, we demonstrate that CRABP is localized to the mitochondria. This association with the mitochondria effectively restricts it to the cytosolic compartment of the cell and suggests a role for mitochondria in retinoid metabolism.

## 2. Materials and methods

### 2.1. Materials

The Cos-1 and NIH 3T3 cell lines were from ATCC. ST15A cells were a gift from R. McKay. The pCMX vector was a gift from C. Tucker [11]. Polyclonal antibodies to CRABP and cellular retinol-binding protein (CRBP) were generated by this laboratory as previously described [12,13]. The antibody to cytochrome *c* was from Pharmingen (San Diego, CA, USA). Alexa Fluor 488 labeled goat anti-rabbit IgG was from Molecular Probes (Eugene, OR, USA). Organelle-specific fluorochromes were from Molecular Probes (Eugene, OR, USA). All oligonucleotides were synthesized by the Vanderbilt University Diabetes Core. Lipofectamine was from Life Technologies (Rockville, MD, USA). The alkaline phosphatase substrate used was from DAKO Corporation (Carpinteria, CA, USA). Aquamount was from Harlan. All other reagents were from Sigma.

### 2.2. Immunohistochemistry

Staining of bovine adrenal samples was performed as previously described [14].

### 2.3. DNA constructs

cDNA primers to the 5′ and 3′ ends of CRABP and CRBP that would incorporate restriction sites convenient for subcloning were used for PCR amplification. The products of these reactions were cloned into the pCMX vector [11].

### 2.4. Cellular transfections

ST15A cells in DMEM/F12 plus 10% fetal bovine serum, COS-1 cells in DMEM plus 10% fetal bovine serum or NIH 3T3 cells in DMEM plus 10% calf serum were seeded at 50% confluency onto glass cover slips in 35 mm tissue culture dishes. Plasmid (1 mg) and Lipofectamine (6 ml) were mixed with serum free media (100 ml) and incubated for 30 min at room temperature. The cells were rinsed once with serum free media and then overlaid with the plasmid/lipofectam-

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*Abbreviations:* CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol-binding protein; RA, retinoic acid

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ine mix plus additional serum free media (800 ml). The cells were placed in an incubator at 37°C for 2.5 h, rinsed once in serum free media and finally covered with serum containing media. 48 h following the transfection, cells were rinsed once with PBS and then fixed as described below.

*2.5. Immunofluorescence*

The paraformaldehyde and all detergents were prepared in PBS. Transfected cells were fixed and permeabilized with one of the following methods as indicated in Section 3: (1) immersion in ice-cold methanol:acetone (1:1) for 2 min at −20°C, (2) immersion in 3.5% paraformaldehyde for 20 min at room temperature followed by a PBS rinse and a 5 min incubation in 0.1% Triton X-100, (3) immersion in 3.5% paraformaldehyde for 20 min at room temperature followed by a PBS rinse and a 5 min incubation in 0.15% SDS or (4) immersion in 3.5% paraformaldehyde for 20 min at room temperature followed by a PBS rinse and a 5 min incubation in 40 mg/ml digitonin. Following permeabilization, the cells were rinsed with PBS, incubated for 30 min at room temperature in buffer A (3% BSA in PBS) and incubated with primary antibody (diluted in buffer A) overnight at 4°C in a humidified chamber. The cells were washed with PBS and incubated for 30 min at 37°C with Alexa 488-labeled goat anti-rabbit IgG (1 mg/ml) diluted in buffer A, washed with PBS, and mounted onto slides with Aquamount. The cells were visualized with a Zeiss Axiophot microscope (Vanderbilt University Imaging Center) equipped for fluorescence microscopy or with a Zeiss LSM 410 microscope for laser scanning confocal microscopy (Vanderbilt University Imaging Center). For the MitoTracker Red studies, transfected cells were incubated for 30 min at 37°C with MitoTracker Red (CMXRos) (50 nM). The cells were rinsed with PBS and fixed as described above.

*2.6. Mitochondrial isolation*

Bovine adrenal gland was obtained from a local slaughterhouse and transported to the laboratory in 0.25 M sucrose (solution B) on ice. The medulla was removed and the cortex was scraped from the capsule. Mitochondria were prepared from the cortex using previously described procedures [15]. All procedures were performed at 4°C. The cortical tissue was weighed and homogenized with five complete passes of a Teflon homogenizer in three volumes of solution B. The homogenate was diluted to twice the original volume with solution B, filtered through glass wool and centrifuged at $600\times g$ for 10 min. The supernatant liquid was removed and centrifuged at $6000\times g$ for 15 min. The supernatant liquid was discarded and the pellet was resuspended first in 500 ml with a 'cold finger' and finally to the original volume in solution B. This suspension was centrifuged at $9750\times g$ for 15 min. The resulting pellet was resuspended in solution B (500 ml/g of wet weight tissue). It should be noted that the addition of a buffer or salt to the 0.25 M sucrose did not permit the retention of either cytochrome *c* or CRABP with the mitochondria. Nycodenz solutions at 9 and 35% were prepared in solution B. The isolated mitochondria were further purified by centrifugation through a Nycodenz gradient as previously described [16]. Fractions (250 ml) were collected from the gradient and aliquots (25 ml) were subjected to 15% SDS–PAGE, transferred to Immobilon and analyzed by Western blotting with antibodies specific for either CRABP or cytochrome *c*.

## 3. Results and discussion

Previous immunohistochemical studies from this laboratory revealed that CRABP is restricted to the cytoplasmic compartment of cells in tissues where it is expressed [8]. In contrast, CRBP, a related protein of the same family, was shown to be present in both the cytoplasm and the nucleus, as expected for a protein of this size [17]. Bovine adrenal gland is known to be a rich source of both CRABP and CRBP [18], but the specific cellular and subcellular localizations of these proteins had not yet been examined in this organ. We, therefore, examined bovine adrenal by immunohistochemistry with antibodies specific for either CRABP or CRBP to determine sites of expression and intracellular location. Both CRABP and CRBP immunostaining localized to the parenchymal cells of the adrenal cortex but no staining was observed in the medulla (data not shown). In addition, the staining pattern of CRABP, but not CRBP, was restricted to the cytoplasmic compartment of the cortical parenchymal cells (Fig. 1A–D). In all CRABP expressing tissues examined by immunohistochemistry in this laboratory, CRABP staining has consistently been observed as exclusively cytoplasmic (unpublished results). From this experiment we conclude that CRABP is normally restricted to the cytoplasmic compartment of cells in which it is expressed.

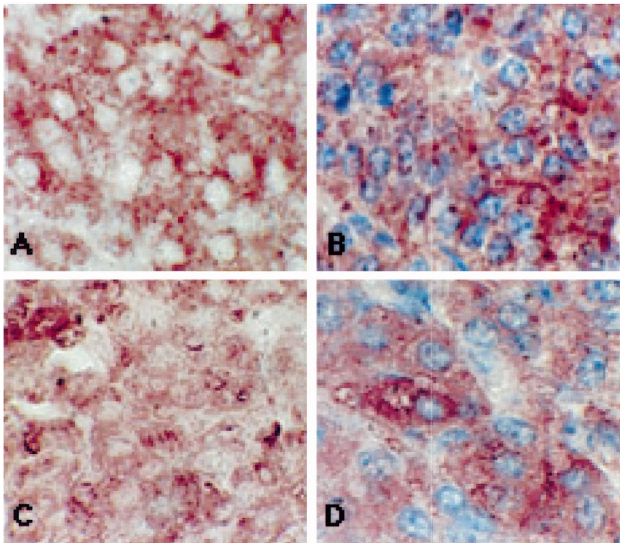


Fig. 1. Nuclear exclusion of CRABP in bovine adrenal cortical cells. Immunohistochemical localization of CRABP and CRBP was performed using an alkaline phosphatase based staining system (brown) and a hematoxylin counter-stain (blue). A: Positive staining for CRABP in adrenal cortical cells not counter-stained (1000×) and (B) same as in (A) but counter-stained with hematoxylin to reveal the nuclei. The lack of staining in the nucleus is evident. C: Positive staining for CRBP in adrenal cortical cells not counter-stained (1000×) and (D) same as in (C) but counter-stained with hematoxylin to reveal the nuclei.

To test if CRABP would be excluded from the nucleus of cells in culture that do not normally express this protein, we transiently transfected the Cos-1 cell line with a mammalian expression vector containing cDNAs encoding either CRABP or, as a control, CRBP. Following transfection, the cells were fixed with 3.5% paraformaldehyde and subsequently permeabilized with 0.1% Triton X-100. The expressed proteins were detected by immunofluorescence with specific antibodies and examined by fluorescence microscopy. CRABP immunofluorescence was observed only in the cytoplasmic compartment and exhibited a punctate pattern, suggesting a possible organelle association (Fig. 2A). CRBP immunofluorescence was observed as a diffuse staining pattern throughout the cell, including the nucleus (Fig. 2B). Interestingly, a difference in the ability to detect the two proteins by immunofluorescence was observed. Approximately 35% of the transfected cells expressing CRBP were visible by fluorescence microscopy while only 7% of the CRABP expressing cells were visible. These results could not be explained by differences in the level of protein expression since both proteins were expressed at approximately equal levels as determined by Western blotting (data not shown). In an attempt to increase the number of CRABP expressing cells visible by immunofluorescence we tested a panel of seven different polyclonal antibodies to

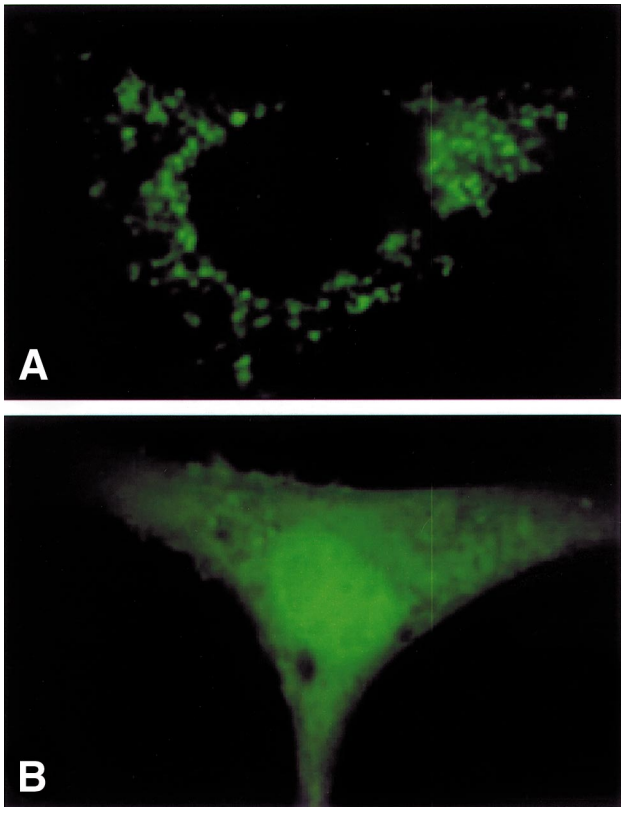


Fig. 2. Nuclear exclusion of CRABP in transfected cells. ST15A cells were transfected with the pCMX mammalian expression vector containing cDNAs encoding either: (A) CRABP or (B) CRBP. Following transfection, the cells were fixed with 3.5% paraformaldehyde, permeabilized with 0.1% Triton X-100 and protein expression was monitored by immunofluorescence as described under Section 2. Note the punctate, nuclear-excluded pattern for CRABP in contrast to the relatively homogeneous pattern for CRBP throughout the cell.

this protein. No difference in the number of CRABP expressing cells was detected following immunofluorescence with any of these antibodies. The presence of RA (1 mM) in the medium of the transfected cells had no effect on the observed fluorescence staining pattern or the inability to see the CRABP-transfected cells by immunofluorescence (data not shown). Extension of this experiment to both the ST15A and NIH 3T3 cell lines confirmed that these results were not cell line-specific (data not shown). The patterns observed for the transfected cells recapitulated the previous observations for in situ expression and indicated that the mechanism of nuclear exclusion for CRABP may be available to all cells and does not require a special cell-specific component in order to be accomplished.

The majority of the CRABP protein was undetectable by immunofluorescence microscopy following paraformaldehyde fixation and Triton X-100 permeabilization. In an attempt to increase the number of CRABP expressing cells that could be seen by immunofluorescence, different fixation and permeabilization methods were employed. Following transient transfection of ST15A cells with cDNA encoding CRABP, cells were fixed with either methanol:acetone or 3.5% paraformaldehyde. The paraformaldehyde-fixed cells were subsequently permeabilized with either 0.1% Triton X-100, 0.2% SDS or 40 mg/ml digitonin. The treated cells were then probed with CRABP specific antibodies and analyzed by immunofluorescence. As described in Fig. 1, only 7% of the cells transfected with CRABP cDNA fixed with 3.5% paraformaldehyde and permeabilized with Triton X-100 were visible by immunofluorescence microscopy and these cells exhibit a characteristic punctate staining pattern (Fig. 3A). Fixation with methanol:acetone did not significantly alter either the punctate staining pattern or the number of CRABP expressing cells that were visible by immunofluorescence (Fig. 3B). However, fixation with paraformaldehyde followed by permeabilization with SDS resulted in a three-fold increase in the number of cells that could be seen by immunofluorescence (Fig. 3C). Additionally, approximately 50% of these cells had lost their punctate staining pattern. Fixation with paraformaldehyde followed by digitonin permeabilization also resulted in a three-fold increase in the number of cells that were visible by fluorescence microscopy and a loss of the typical punctate staining pattern in all cells examined (Fig. 3D). Similar fixation and permeabilization experiments performed on cells transfected with CRBP cDNA did not result in a difference in the number of cells seen by immunofluorescence (data not shown). From these experiments we conclude that the method of fixation and permeabilization is critical for observing nuclear exclusion and punctate staining of CRABP in transfected cells. Variation in fixation methods may well explain the conflicting immunolocalization results obtained by other laboratories [9,10]. Additionally, since both SDS and digitonin permeabilization led to an increase in the number of CRABP expressing cells visible by immunofluorescence, it is possible that these detergents released the binding protein from its native position in the cell and exposed epitopes that were not previously available to our antibodies.

A punctate staining pattern can be indicative of organelle association. To determine if this was the cause of the staining pattern observed, overlay experiments with organelle-specific fluorochromes were performed. Cos-1 cells transfected with

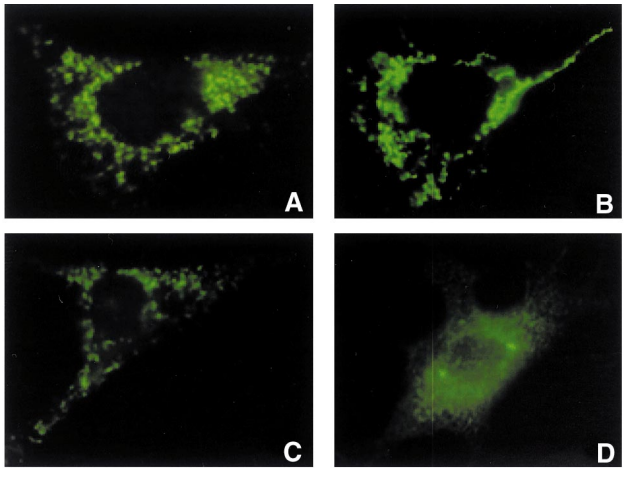


Fig. 3. Immunofluorescence pattern of CRABP varied with different fixation procedures. ST15A cells were transfected with the pCMX mammalian expression vector containing cDNA encoding CRABP. Following transfection the cells were processed with either: (A) 3.5% paraformaldehyde and 0.1% Triton X-100, (B) methanol:acetone, (C) 3.5% paraformaldehyde and 0.2% SDS or (D) 3.5% paraformaldehyde and 40 mg/ml digitonin as described under Section 2. Subsequently, protein expression was detected by immunofluorescence. Treatment with digitonin led to the loss of the punctate pattern in all fluorescing cells (D).

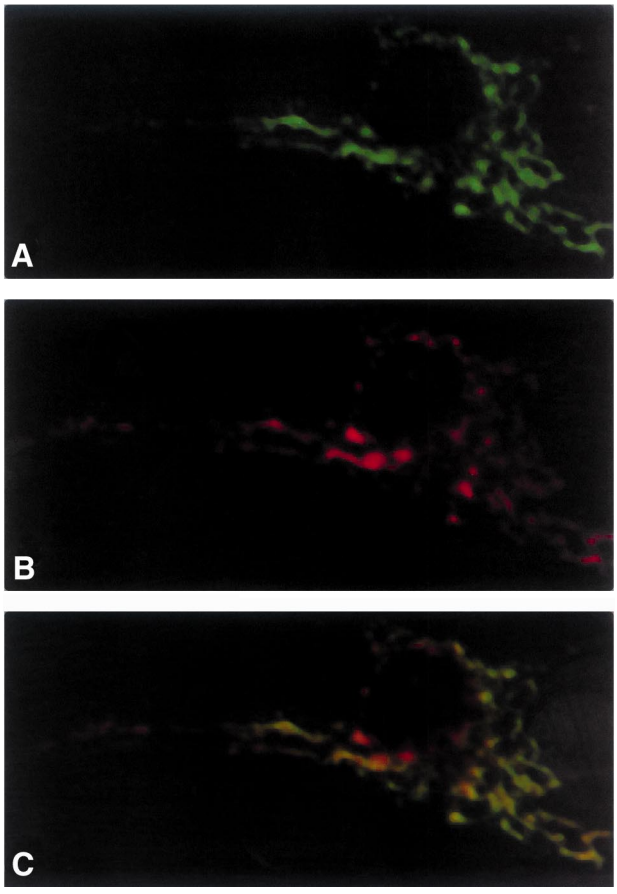


Fig. 4. MitoTracker Red-stained mitochondria are coincident with CRABP immunofluorescence. Cos-1 cells were transfected with the pCMX mammalian expression vector containing the CRABP cDNA. 48 h following transfection, the cells were incubated with MitoTracker Red, fixed with methanol:acetone probed by immunofluorescence and viewed by laser scanning confocal microscopy as described in Section 2. A: CRABP immunofluorescence. B: MitoTracker Red staining pattern. C: Overlay of CRABP immunofluorescence and MitoTracker Red. Note the similar patterns in A and B, as revealed by the overlay in C.

cDNA encoding CRABP were incubated with fluorescent dyes specific for the Golgi (BODIPY TR ceramide), endoplasmic reticulum ($DiOC_5$), lysosomes (neutral red), and mitochondria (MitroTracker Red). The cells were fixed with methanol:acetone and probed by immunofluorescence with antibodies specific for CRABP. The coincidence of binding protein immunofluorescence to organelle dye fluorescence was then compared by laser scanning confocal microscopy. Neither the Golgi, endoplasmic reticulum nor lysosome-specific fluorochrome staining patterns matched the patterns of CRABP immunofluorescence (data not shown). Only the mitochondrial-specific dye produced a staining pattern that was coincident, by confocal microscopy, with the immunofluorescence pattern of CRABP (Fig. 4A–C). From this experiment we conclude that CRABP associated with the mitochondria in these transfected cells. This is consistent with the increased but now diffuse signal seen after digitonin treatment, a detergent typically used for dissolution of the mitochondrial membrane.

As noted, bovine adrenal is a rich source of both CRABP and CRBP and demonstrated restriction of CRABP staining to the cytoplasmic compartment of cortical cells. Immunohistochemical analysis by dye deposition does not afford the resolution necessary to see an organelle-specific staining pattern. Consequently, to ensure that our cell culture results were not based on a transfection artifact, we examined the location of CRABP by subcellular fractionation of the adrenal cortex. A crude preparation of mitochondria was prepared and further purified by sedimentation through a Nycodenz gradient. The fractions from this gradient were analyzed by Western blotting with antibodies specific for either CRABP, cytochrome *c* or CRBP. Both CRABP and cytochrome *c* co-sedimented in fractions 5–8 (Fig. 5). There was no immunodetectable CRBP observed in these fractions (data not shown). As described in Section 2, it should be emphasized that any deviation from this mitochondrial preparation protocol resulted in a loss of both CRABP and cytochrome *c* from the purified mitochondrial gradient fractions. From this experiment we conclude that CRABP is normally associated with mitochondria in situ.

The discovery of the association of CRABP with mitochondria reported here was unexpected. CRABP has been studied for 25 years and has always presented as a soluble, presumably cytosolic, protein [2]. Clearly, then, the association is easily reversed and has not survived the procedures used to obtain soluble extracts for study or purification of this protein. Similarly, some other mitochondrial proteins, such as cytochrome *c*, are also easily lost to the soluble fraction during cell disruption. Mitochondria have not previously been considered to have any role in RA function or metabolism, a possibility that must now be considered.

The only demonstrated function for CRABP is to bind RA. Since this protein is associated with mitochondria, this implies that mitochondria participate in RA management. Cells expressing CRABP have an increased ability to degrade RA [6] and RA bound to CRABP is accessible for oxidation by cytochrome P450s [7]. While it has been demonstrated that a microsomal cytochrome P450, CYP26, is inducible by RA and recognizes it as a substrate [19,20], this enzyme is not present in mouse testis, a CRABP-containing organ, but is present in mouse liver, which is CRABP negative [21]. A second murine microsomal cytochrome P450 that metabolizes RA has been described but its expression is also restricted to liver in the adult [22]. Consequently, it should now be considered that the mitochondrion could also play a role in RA catabolism in those cells expressing CRABP.

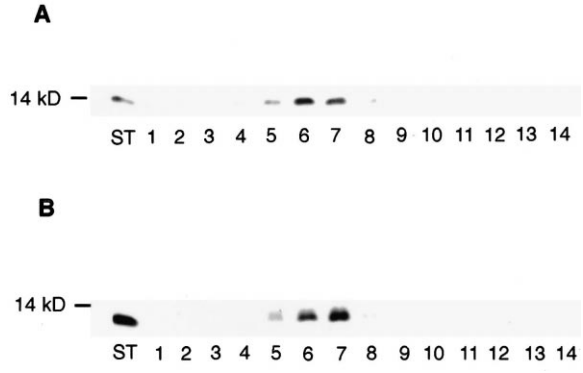


Fig. 5. CRABP co-sediments with bovine adrenal mitochondria. A crude preparation of mitochondria was prepared and centrifuged through a Nycodenz gradient as described in Section 2. Fractions from this gradient were analyzed by Western blotting with antibodies specific for either (A) CRABP or (B) cytochrome *c*.

In 1973 it was proposed that the action of RA would be similar to that of steroid hormones [23]. This was confirmed by the discovery of the RA nuclear receptor proteins. This analogy may be extended further. Since the first step in the inactivation of calcitriol (vitamin $D_3$) is a mitochondrial hydroxylation, it is possible that the RA binding protein allows the passage of RA or RA derived-compounds into the mitochondria for further processing.

Future studies will be necessary to determine the specific compartment of the mitochondria to which CRABP is targeted. Because this protein is difficult to retain within the mitochondria during cell fractionation, it is likely that it is present in the intermembrane space or attached to the outer membrane. It will be of interest to determine which amino acid residues are involved in: (1) the possible import of CRABP into mitochondria and (2) the targeting of this binding protein to its proper organelle membrane or space. In comparison with the nuclear effects of RA, little is known about the regulation of its synthesis and degradation. The results presented here open many new areas of investigation. Future studies will be aimed at identifying the role of CRABP in the mitochondrion and in RA management.

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