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# The Cellular Retinoic Acid Binding Proteins

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**The two cellular retinoic acid binding proteins, CRABP I and CRABP II, belong to a family of small cytosolic lipid binding proteins and are highly conserved during evolution. Both proteins are expressed during embryogenesis, particularly in the developing nervous system, craniofacial region and limb bud. CRABP I is also expressed in several adult tissues, however, in contrast, CRABP II expression appears to be limited to the skin. It is likely that these proteins serve as regulators in the transport and metabolism of retinoic acid in the developing embryo and throughout adult life. It has been proposed that CRABP I sequesters retinoic acid in the cytoplasm and prevents nuclear uptake of retinoic acid. A role in catabolism of retinoic acid has also been proposed. Recent gene targeting experiments have shown that neither of the two CRABPs are essential for normal embryonic development or adult life. Examination of CRABP I expression at subcellular resolution reveals a differential cytoplasmic and/or nuclear localization of the protein. A regulated nuclear uptake of CRABP I implies a role for this protein in the intracellular transport of retinoic acid. A protein mediated mechanism which controls the nuclear uptake of retinoic acid may play an important role in the transactivation of the nuclear retinoic acid receptors.**

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## INTRODUCTION

Vitamin A and its biological derivatives, retinol, retinaldehyde and retinoic acid (RA), known collectively as retinoids, serve important roles in embryogenesis, differentiation and homeostasis. Retinol, the major form of vitamin A, is mainly stored in the liver. It is recruited from the liver bound to the plasma retinol-binding protein (RBP) and taken up by cells via a membrane receptor for RBP. Inside cells retinol is oxidized to retinaldehyde and subsequently to RA. This is the active form of the vitamin in non-ocular tissues and it functions as a ligand for a family of ligand-controlled nuclear receptors [1-4]. In the eye, the 11-*cis* stereoisomer of retinaldehyde acts as the chromophore of visual pigments [5].

Retinoids are hydrophobic in nature and are therefore poorly soluble in an aqueous environment. To ensure proper storage, transport and cellular acquisition of retinoids, a number of retinoid-binding proteins have evolved. As mentioned earlier the plasma transport of retinol is carried out by RBP. In non-ocular tissues, two classes of intracellular retinoid-binding proteins have evolved, the cellular retinol-binding proteins type I and II (CRBPI/II) and the cellular retinoic acid-binding proteins type I and II (CRABPI/II).

Once retinol enters a target cell it is probably bound by CRBP I. This protein is abundant in several tissues which are involved in storage, mobilization and transcellular transport of retinol [6, 7]. CRBP I is also involved as a substrate carrier in the conversion of retinol into RA via a retinaldehyde intermediate [8]. Thus, with the exception of cells involved in storage and transcellular transport of retinol, CRBP I is probably a good indicator of cells which are able to synthesize RA [9].

The effects of RA are mediated via two classes of RA receptors, the RARs ($\alpha$, $\beta$, $\gamma$) and the RXRs ($\alpha$, $\beta$, $\gamma$). These proteins form heterodimeric complexes and act as ligand-controlled transcription factors. In the presence of RA, these heterodimers can activate or suppress the transcription of genes which contain a RA response element (RARE) [1-4]. The relationships between the processes which govern cellular acquisition of retinol and synthesis of RA and the nuclear retinoic acid receptors are unknown. However, since both CRABP I and II can bind RA they are good candidates to regulate the effects of RA by controlling the availability

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of RA to the nuclear receptors. The precise roles of the binding proteins in relation to the nuclear receptors is unknown but we do have some clues as to their function (see [7] for a comprehensive review). In this article we will examine the structure and biochemistry of the CRABPs, summarise their expression patterns and postulate on how they might function.

## STRUCTURE AND BIOCHEMISTRY OF CRABP I AND II

CRABP I and II belong to a family of cellular lipid binding proteins which includes CRBP type I and II, the myelin protein P2, adipocyte P2 and several other fatty acid binding proteins. The CRABPs appear to be as equally related to myelin protein P2 and adipocyte P2 as they are to the CRBPs. Their relationship to the fatty acid binding proteins is more distant. These sequence similarities suggest that members of the cellular lipid binding protein family have evolved from a common ancestral gene. Both CRABPs are small proteins, each consisting of 136 amino acids with a molecular weight of approx. 15–16 kDa. They are highly conserved proteins during evolution. The amino acid sequence of mouse CRABP I is identical to the bovine sequence [10, 11]. It is also essentially identical to the human CRABP I sequence with only one replacement out of 136 residues [12] (Fig. 1). CRABP II is also evolutionarily conserved, with the human sequence being 94% identical to that of the mouse [12, 13]. The amino acid replacements between the two mammalian CRABP IIs are often found in residues with hydrophobic side chains, probably facing into the interior of the proteins while the hydrophilic residues, which are exposed on the surface, are conserved. The identity between the two different mammalian CRABPs is 73%. A remarkable feature is that the amino acid replacements between the two CRABPs are mainly found in hydrophilic residues whose side chains contribute to the surface of the proteins. The distinct but yet highly evolutionary conserved surfaces of the two CRABPs implies that both proteins are under strong selective pressure and that the two CRABPs interact with different cellular proteins. A third RA-binding protein xCRABP has been identified in *Xenopus laevis* [14]. Even though this 15 kDa protein displays a similar identity to CRABP I and CRABP II, it has not been classified as either type I or type II.

Both CRABP I and II bind the *all trans* form of RA *in vitro* but only CRABP I has been shown to bind the ligand *in vivo* [15]. This is probably a reflection of the different equilibrium dissociated constants ($K_d$), as the $K_d$ of binding of *all trans* RA to CRABP I is in the low nanomolar range [16] while that of CRABP II is significantly higher [17]. This difference in affinity for *all trans* RA suggests that CRABP I and CRABP II

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             1
CRABP I b    MPNFAGTWKM RSSENFDELL KALGVNAMLR KVAVAAASKP HVEIRQDGDQ FYIKTSTTVR TTEINFKVGE

CRABP I m    .......... .......... .......... .......... .......... .......... ..........

CRABP I h    .......... .......... .......... .......... .......... .......... ..........

xCRABP       ....S.H... KQ....E.M. ......L... .I........ A...K.E.ET .......... .......L.G

CRABP II h   ....S.N..I IR....E... .V....V... .I........ A...K.E..T .......... ..........

CRABP II m   ....S.N..I IR....E.M. ......M.M. .I........ A...K.EN.T .......... .......I..

             71
CRABP I b    GFEEETVDGR KCRSLPTWEN ENKIHCTQTL LEGDGPKTYW TRELAND-E- LILTFGADDV VCTRIYVRE

CRABP I m    .......... .......... .......... .......... .......-.- .......... .........

CRABP I h    .......... .....A.... .......... .......... .......-.- .......... .........

xCRABP       ..D.Q..... N.....E... .........V ...E....S. .......-.A ....MT.... ........L NFWITPSL

CRABP II h   E...Q..... P.K..VK..S ...MV.E.K. .K.E....S. ....T..G.- ....MT.... ....V....

CRABP II m   E...Q..... P.K..VK..S G..MV.E.R. .K.E....S. S...T..G.- ....MT.... ....V....
```

**Fig. 1. Sequence alignment of the cellular retinoic acid binding proteins. The amino acid sequence of bovine CRABP I is used as a paradigm. Identical residues are indicated by dots (. . .). Gaps (—) were introduced in the sequences to aid alignment and amino acid differences are shown. Abbreviations: b, bovine; m, mouse; h, human; x, *Xenopus*.**

have different functions in the cell and perhaps more importantly, under conditions of molar excess of the binding proteins relative to RA, CRABP I would be the major RA-binding protein (see below). Whether or not CRABP I and CRABP II have other physiological ligands other than *all trans* RA is not known but neither of them can bind 9-*cis* RA, the preferred ligand for the RXR receptors, with high affinity [18].

Considering the close similarity in the primary structure of the cellular retinoid-binding proteins and the fatty acid-binding proteins it would not be surprising to find that their tertiary structures are rather similar as well. CRABP I and II have recently been crystallized and their tertiary structures determined (Jones T. A., personal communication). Both proteins are remarkably similar to each other and to the other members of this protein family ([19–21] and references therein). The proteins consist of a flattened globular domain built up of two orthogonal $\beta$-sheets. The bound ligand, *all trans* RA, is sandwiched between the two $\beta$-sheets with the cyclohexenyl ring of RA exposed to the solvent and the carboxyl group is deeply buried in the binding pocket. The fact that the cyclohexenyl ring is exposed is interesting in light of recent studies demonstrating that several metabolites of RA have modifications in the ring [22–24].

## EXPRESSION AND FUNCTION OF CRABP I AND II

To elucidate the possible roles of the CRABPs it is important to establish the expression patterns of the proteins and to compare these with the expression patterns of CRBP I, the nuclear RA-receptors and different cellular processes, during embryonic development, or growth and differentiation in adult tissues. Thus an analysis of the expression patterns of both proteins could provide insight into how synthesis, transport, localization and action of RA is regulated and how this is linked to cellular processes. At present, the expression patterns of the CRABPs are well documented during embryogenesis, especially in the mouse [25–33]. In adult tissues, however, more information is needed.

If either of the two CRABPs govern the transport and regulation of action of RA, then an important criteria for such a role would be expression of the proteins in cells where RA is active. Administration of a radiolabelled synthetic derivative of RA, [$^{14}$C]TTNPB, to pregnant mice, identified sites where RA accumulated in the embryo [26]. These areas, which expressed CRABP I, were the craniofacial structures, the visceral arches, the dorsal root ganglia, cells along the gut and the major vessels of the trunk region. In the CNS, localization of CRABP I and the RA analogue was restricted to the hindbrain, the roof of the midbrain and to cells in the mantle layer of the neural tube. The preferential binding of RA to CRABP I, and not to CRABP II as judged by the lack of accumulation of RA in CRABP II-expressing tissues, might just be a reflection of the different affinities of the two proteins for RA. It seems likely that endogenous RA would localize similarly to the exogenously added TTNPB. Thus, CRABP I-expressing cells might be target cells for RA. In CRABP I-expressing embryonic tissues, [$^{14}$C]TTNPB accumulated up to 25 times more than background levels and the use of [$^{14}$C]TTNPB of known specific activity has allowed an estimation of available RA-binding sites [34]. In CRABP I-expressing tissues, mainly the neuronal and craniofacial structures and, the limb buds, the concentration of specific RA-binding sites is approx. 1–10 $\mu$M. Calculations have shown that these levels correspond to approx. $10^7$–$10^8$ CRABP I molecules per cell. Furthermore, the tissue concentration of RA is in the order of 40 nM [35]. This means that RA saturation of CRABP I is low and probably below 5%. If limiting amounts of endogenous RA was freely distributed between the two CRABPs then the difference in their affinity for the ligand suggests that CRABP II would be largely devoid of endogenous RA (compare to accumulation of TTNPB, see above). However, since limited information is available on the formation and transport routes of RA in the developing embryo it may be too early to draw such a conclusion.

CRABP II is also widely expressed during mouse embryogenesis [29, 31]. The levels of expression of both CRABP I and II varies in embryonic tissues in a spatiotemporal manner and this is clearly illustrated in the overlapping and sometimes complimentary patterns observed. In adults CRABP II is predominantly expressed in the skin [13]. Why CRABP II is expressed in some of the same tissues as CRABP I is difficult to understand when one considers the higher affinity of CRABP I for RA and the low ligand saturation for this binding protein. Of course, caution should be taken when interpreting such data for the resolution of the *in situ* hybridization technique makes it difficult to discriminate between individual cells. So, for example, what appears to be an overlapping expression pattern for CRABP I and CRABP II in the mouse embryo could very well be, instead, an differential one in neighbouring cells. If CRABP II and CRABP I are co-expressed, the type II protein may act as a binding protein for an unidentified ligand or it may indeed play a role in RA transport and metabolism distinct from that of CRABP I. There are tissues in the developing embryo where CRABP II is expressed and CRABP I is not. These are derived from the endoderm and include the pharyngeal pouches, oesophagus, tracheal epithelium and liver [29].

In contrast to CRABP I the expression of CRABP II is regulated by RA [13, 36]. This is intriguing since CRABP II has been found to be co-expressed with CRBP I in a number of cells, however, expression of CRABP II can also be found in cells with no detectable expression of CRBP I [30]. However, as pointed out

earlier CRBP I is involved in the synthesis of RA and it is appealing to speculate that the expression of CRABP II is upregulated in such cells as a consequence of the synthesis of RA. It is not certain if these observations have any implications as to the role of CRABP II.

The mechanism underlying the regulation of CRABP II expression by RA has been extensively studied. In F9 teratocarcinoma cells treated with RA, CRABP II mRNA levels are increased approx. 50-fold by post-transcriptional regulation [37]. In contrast, a later study in P19 embryonal carcinoma cells showed that RA induced CRABP II mRNA transcription was mediated by RXR–RAR heterodimers operating through RAREs [36]. In humans, topical application of RA to the skin significantly induced the levels of CRABP II mRNA and protein in this tissue [12]. The induction of CRABP II expression in the skin by RA is interesting in light of the observation that in several hyperproliferative conditions of the skin CRABP II expression is highly induced [38]. Concomitantly, it was observed that CRABP I expression was drastically reduced [39]. It is not clear whether there is a causal relationship between these observations but the down regulation of CRABP I expression might result in increased levels of RA in the skin and consequently induction of CRABP II expression.

A striking observation during mouse embryogenesis is the differential spatiotemporal restricted expression patterns of the CRABPs and CRBP I. As previously mentioned, CRABP II and CRBP I are coexpressed in some tissues but coexpression of CRABP I and CRBP I is rarely seen [9, 25, 28–30]. A nonoverlapping distribution of the latter two binding proteins was seen in the CNS and in neighbouring tissues during the formation of limbs and craniofacial structures [9]. Here, the expression of CRBP I was limited to the endoderm and surface ectoderm while that of CRABP I was found in the underlying mesenchyme. Since retinol accumulated in the ectoderm it was proposed that this tissue is a site of RA synthesis and that the role of CRABP I in the mesenchyme is to accumulate RA from the ectoderm [9]. This suggested that retinol/RA pathways may play a role in epithelial–mesenchymal interactions which are vital for pattern formation during embryonic development.

The relationship between expression of the CRABPs and the two families of nuclear RA-receptors is clearly complex. Expression of RAR$\alpha$ and RXR$\beta$ are seen in most embryonic tissues while expression of RAR$\beta$, RAR$\gamma$, RXR$\alpha$ and RXR$\gamma$ is spatially and temporally restricted [4, 40]. A direct comparison with the expression patterns of the CRABPs reveal that the different subtypes of RA-receptors may be both exclusive and overlapping with the CRABPs. This demonstrates that there is no apparent correlation between the expression of CRABPs and cells which are able to respond to RA in the early embryo. Instead, it seems that most, if not all, cells are able to respond to RA and that the CRABPs are involved in the temporal and spatial regulation of the RA response in individual cells.

The tissue localization of the CRABPs have provided some ideas as to potential roles of these proteins in RA-metabolism and action but few functional studies have emerged so far. The high level of expression of CRABP I in cells that are sensitive to the teratogenic effects of excess RA has led to the suggestion that CRABP I acts as a buffer in the cytoplasm by limiting the availability of RA to its nuclear receptors [25, 27, 29, 30, 41]. This notion was supported by overexpression studies of CRABP I in F9 teratocarcinoma cells [42]. It was shown that elevated levels of CRABP I reduced the expression of several but not all of the RA responsive genes tested. However, it may be difficult to fully interpret the role of CRABP I in such cells because the levels of endogenous CRABP I change with time [43] and CRABP II expression is induced following addition of RA [13]. A role of the CRABPs in catabolism of RA has also been suggested. It was found that the CRABP I–RA complex is a substrate for RA-metabolizing enzymes [44]. Similar results were obtained with F9 cells expressing CRABP I [24]. Here increased expression levels of CRABP I were found to increase metabolism of RA into polar metabolites. While such *in vitro* experiments point to a role of CRABP I in catabolism of RA, other roles of the proteins cannot be excluded. To our knowledge the role of CRABP II in RA metabolism and catabolism has not been tested. A recent report demonstrated that ectopic expression of xCRABP in *Xenopus* embryos greatly enhanced the mRNA levels of HoxB9 and HoxB4 and that malformations similar to those observed following RA exposure were seen in the embryos [14]. It is well documented that some Hox genes are upregulated in the presence of RA [45]. The apparent potentiation of the effects of RA seen following the ectopic expression of xCRABP is thus contradictory to the proposed model in F9 cells in which CRABP I sequesters RA in the cytoplasm and limits its access to the nuclear receptors.

Using gene targeting techniques it was recently established that neither of the two CRABPs are necessary for normal murine embryonic development or adult life [46, 47]. Even deletion of both CRABPs resulted in mice which were apparently normal [47]. In light of the strong evolutionary conservation of the CRABPs these results were rather unexpected. It is possible that cellular mechanisms may compensate for the loss of the two CRABPs and that under conditions of mild environmental stress, these mice exhibit a normal phenotype. In mice lacking CRABP I the teratogenic dose of RA was similar to that of normal mice. The higher sensitivity of the CRABP I-expressing cells for the teratogenic action of excess RA is thus independent of CRABP I-expression. This result is not consistent with the previously suggested protective role of the protein

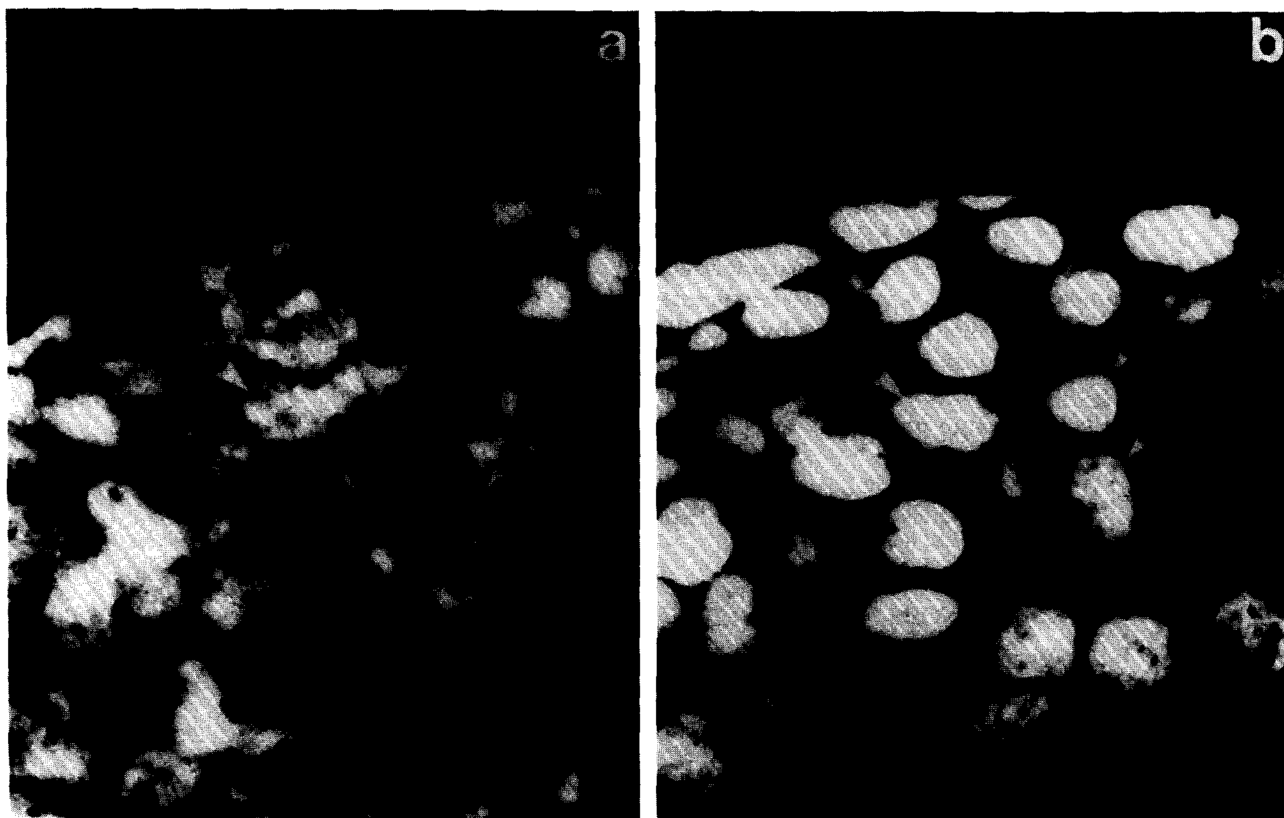


**Fig. 2. Differential subcellular localization of CRABP I in neural crest-derived frontonasal mesenchymal cells revealed by confocal microscopy. (a) Optical section showing an overview of a coronally cut medial nasal process with CRABP I-expressing neural crest-derived mesenchymal cells (day 10.5 postcoitum). Cells with a preferential cytoplasmic localization of CRABP I (small arrowheads) and cells with a preferential nuclear localization of CRABP I (large arrowheads) are shown. (b) The same section showing the location of propidium iodide stained nuclei. Bars 10 μm.**

and how it acts like a sink thereby limiting inappropriate activation of nuclear receptors. Instead, the sensitivity of the CRABP I-expressing cells may be due to the fact that these cells are target cells for RA during embryonic development and that nuclear uptake of RA and subsequent receptor activation has to be temporally regulated.

To explore the possibility that CRABP I might be involved in intracellular transport and nuclear uptake of RA we have used laser scanning confocal microscopy to examine the subcellular distribution of CRABP I in mouse embryonic cells. The results showed a remarkable nuclear localization of CRABP I in some cells while neighbouring cells had an apparent exclusion of the binding protein from the nucleus (Fig. 2) [34]. The differential subcellular distribution of CRABP I was observed in most if not all embryonic cell types expressing the protein, e.g. various neural crest-derived cells, mesenchymal cells of the limb bud and several neuronal cell types. Densitometric analyses of these cells showed that for cells accumulating CRABP I in the nucleus, the protein was found to be localized within the boundaries of the organelle with little or no expression in the cytoplasm. In the nucleus, CRABP I often displayed a speckled distribution consistent with the possibility that it is part of subnuclear structures. The reciprocal pattern was observed for those cells with a nuclear exclusion of the protein. Since CRABP I is the major binding protein for RA and the ligand saturation of CRABP I is low in the embryo (see above) it is quite likely that the subcellular localization of CRABP I would determine the location of RA and consequently its availability for the nuclear RA-receptors. It has been suggested previously that CRABP I may be involved in the transport of RA into the nucleus [48]. The primary structure of CRABP I lacks the basic stretch of amino acids that comprises a nuclear localization signal but the low molecular weight of CRABP I would facilitate its passive entry through the nuclear pores. However, the present data is not consistent with such a model since the protein is found to be excluded from the nucleus in many cells. Instead, we propose that the subcellular compartmentalization of CRABP I is controlled by other proteins which may exclude or allow nuclear uptake of the protein.

Regulated nuclear import of several transcription factors is an important feature of regulated gene activation during cellular growth and differentiation. Latent forms of glucocorticoid receptor, proteins belonging to the Rel/NFκB family and other proteins are

stored in the cytoplasm and can be rapidly activated upon nuclear import (see [49] for a review, [50] and [51]). It is possible that RA controlled gene expression involves a similar mechanism in some cells where RA is stored as a latent compound complexed with CRABP I in the cytoplasm and this complex is rapidly imported into the nucleus when appropriate. In the future, proteins that interact with CRABP I either in the cytoplasm or the nucleus need to be characterized. Such information would give us a much clearer picture on how RA is transported into the nucleus by CRABP I and gain further insight into the regulatory mechanism behind transcriptional activation of RA responsive genes in such cells. The same may apply for CRABP II.

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