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STRUCTURE/FUNCTION OF CYTOPLASMIC VITAMIN A–BINDING PROTEINS

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ABSTRACT

Two cytoplasmic retinol–binding proteins, CRBP and CRBP II, and two cytoplasmic retinoic acid–binding proteins, CRABP-I and CRABP-II, have been well characterized. There has been significant progress in the structural analysis of these four proteins with X-ray crystallography, nuclear magnetic resonance, mutagenesis, and binding studies. In contrast, the cellular functions of these cytoplasmic vitamin A–binding proteins are less well understood. Since these proteins bind their respective ligands with high affinity, they are likely to influence retinoid signaling pathways. Analysis of retinoid metabolism in the presence or absence of these proteins provides support for the hypothesis that these proteins are involved in modulating intracellular retinoid metabolism. Molecular genetic approaches to alteration of the levels of these proteins in tissue culture cells and in whole animals have provided a powerful means toward defining the physiological roles of the cytoplasmic vitamin A–binding proteins in vivo.

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INTRODUCTION

The cytoplasmic vitamin A–binding proteins were discovered in the search for retinoid binding receptors (reviewed in 111). Sucrose gradient centrifugation, the approach that previously had identified the steroid hormone receptors, demonstrated small, ~15-kDa proteins in a variety of tissues that specifically bound labeled retinol or retinoic acid (8, 132). Thus far, two cytoplasmic retinol-binding proteins, CRBP and CRBP II, and two cytoplasmic retinoic acid–binding proteins, CRABP-I and CRABP-II, have been extensively characterized. They are the primary focus of this article.

There has been significant progress in the structural analysis of these four proteins, members of a larger family of small intracellular lipid-binding proteins (iLBPs) (reviewed in 7). The crystal structures of all four have been determined. Nuclear magnetic resonance has been used to study the structural and dynamic factors involved in molecular recognition by these proteins. The effect of modifying the protein-ligand interface has been studied by analyzing the binding interactions of mutant proteins constructed by site-directed mutagenesis and of retinoid analogues in which various portions of the molecule have been altered.

In contrast, the cellular functions of these cytoplasmic retinoid-binding proteins are less well understood. It is now clear that vitamin A signal transduction is directly mediated not by the cytoplasmic vitamin A–binding proteins, but instead by nuclear retinoid receptors homologous to the steroid hormone receptors. Because the cytoplasmic vitamin A–binding proteins bind their respective ligands with high affinity, these proteins are likely to influence retinoid-signaling pathways by modulating intracellular retinoid metabolism and by influencing ligand occupancy of the nuclear receptors. The distinct patterns of tissue distribution in the adult and the developing animal exhibited by the cytoplasmic vitamin A–binding proteins suggest that these proteins each serve different functions. Analysis of retinoid metabolism in the presence or absence of the cytoplasmic retinoid binding proteins in vitro provides support for the hypothesis that these proteins are involved in modulating intracellular retinoid metabolism. Molecular genetic approaches to modulation of the levels of the cytoplasmic vitamin A–binding proteins in tissue culture cells and, most recently, in whole animals have provided a powerful means toward defining the physiologic roles of these binding proteins in vivo.

CELLULAR RETINOL-BINDING PROTEINS

Two cytoplasmic retinol-binding proteins, CRBP and CRBP II, have been purified and extensively characterized. CRBP has been purified from rat liver (106), rat testis (107, 124), bovine retina (130), bovine testis (91), and human

liver (39, 104). The amino acid sequence of CRBP (23, 141, 144) is highly conserved across a number of vertebrate species (95–97%). CRBP II was initially purified from rat pups (105). Comparison of the primary sequences of rat CRBP II and rat CRBP reveals sequence identity of 56% (78). Comparison of the primary sequences of rat (78), chick (41), pig (115), and human CRBP II (82) indicate that the amino acid sequence of CRBP II is also highly conserved (~91%) across species. It is likely that these two proteins represent the majority of the retinol-binding activities detected in tissue extracts. However, one cannot exclude the possibility that other cytoplasmic retinol-binding proteins exist. Myelin P2 protein, a member of the iLBP family, has been reported to bind all-*trans*-retinol (147). Small cytoplasmic binding proteins that bind both retinol and retinoic acid have been detected in hepatomas (94) and in fish eye (99).

Tissue Distribution

Although CRBP and CRBP II share significant amino acid identity, they exhibit distinct tissue-specific and developmental patterns of expression. CRBP II expression is essentially confined to the small intestine after birth (76, 78). Immunohistochemical studies performed with monospecific antibodies localize CRBP II to villus-associated columnar absorptive cells (enterocytes). The diffuse staining pattern observed is consistent with localization in the cytoplasm. The initial detection of CRBP II mRNA corresponds with the time of the first appearance of the absorptive columnar epithelium in the intestine (76, 78). CRBP II is an extremely abundant intestinal protein, representing 0.4–1% of soluble intestinal protein (105). With monospecific antisera, CRBP is estimated to be 1/1000-fold less abundant in the intestine than is CRBP II; it is localized within the cells of the lamina propria and within the gut-associated lymphoid tissue (26, 158). An earlier report localizing CRBP within the intestinal epithelium (37) probably reflected cross-reaction of the antiserum with CRBP II. The localization of CRBP II within the proximal intestinal epithelium suggests that this protein is uniquely adapted for the intestinal absorption and/or esterification of dietary retinol.

During the perinatal period, CRBP II mRNA and protein is also detectable in the liver (78, 109). The mRNA levels decline abruptly shortly after birth, with protein levels becoming absent by the midsuckling period. CRBP II is localized primarily in the centrilobular region close to the central vein (110). The changes in CRBP II expression during the perinatal period may reflect changes in how retinol is delivered to the liver before and after birth.

In contrast, CRBP transcripts and protein are detected in many fetal and adult tissues. It is particularly abundant in the liver as well as other visceral organs, and in reproductive tissues. There are differences in the relative order

of tissue abundance between the rat and humans (reviewed in 111). It is likely that CRBP represents most of the retinol-binding activities detected in early surveys of tissue extracts, with the exception of the intestine (reviewed in 111). CRBP has been immunolocalized in both parenchymal cells and stellate cells (37, 67, 158) within the liver. The liver plays a central role in the storage of vitamin A as retinyl esters, and in the delivery of retinol complexed with serum retinol-binding protein to target tissues. Within the kidney, CRBP has been localized primarily in the proximal convoluted tubules of the renal cortex (67, 158). CRBP has been implicated in the translocation of retinol across blood organ barriers based on the localization of CRBP in the peritubular cells (28) and Sertoli cells (66) of the testis, the cuboidal epithelial cells of the choroid plexus in the brain, and the pigment epithelial cells of the eye (reviewed in 111).

Because teratogenicity is associated with both excess and deficiency of vitamin A, the temporal and spatial patterns of CRBP gene expression in the developing mouse embryo have been examined by in situ hybridization (125, 126) and by immunohistochemistry (50). The details of these findings are beyond the scope of this review. It appears that CRBP transcripts are detected more widely in the embryo by in situ hybridization techniques, compared with the distribution of protein detected by immunohistochemistry. Radiolabeled retinoids, administered to pregnant mice as labeled retinyl esters, accumulate in regions of the embryo in which CRBP is detected by immunostaining (50).

Gene Regulation

The levels of CRBP and CRBP II mRNA are influenced by retinoid nutritional status. Studies in whole animals have shown that CRBP protein and mRNA are reduced in retinoid-deficient rats (11, 66, 119). In contrast to CRBP, CRBP II mRNA levels are increased in the small intestine of the retinoid-deficient rat (119). A number of *cis*-acting retinoic acid response elements have been identified in both the CRBP and the CRBP II promoters (61, 92, 95, 141). CRBP mRNA and protein levels have been shown to be inducible by retinoic acid in a number of tissues and cultured cell lines (45, 51, 128, 153), presumably as a result of these response elements. The retinoic acid inducibility of transfected mouse and rat CRBP II promoters is dependent on the cell type. These transfected promoters are not retinoic acid inducible in the human intestinal Caco-2 cell line, although they are inducible in other cell lines (95). Of note, these studies used Caco-2 cells prior to the development of the differentiated intestinal phenotype. The human CRBP II gene appears to be retinoic acid inducible in polarized monolayers of differentiated Caco-2 cells (81). These different results may reflect the presence or absence of other transcriptional factors, such as HNF-4, which also bind to these elements in

various cultured cell lines (95). The mechanism by which CRBP II transcripts are induced in retinoid-deficient animals remains to be determined.

The CRBP and CRBP II genes appear to be responsive to other hormones. Glucocorticoids exert a negative effect on CRBP mRNA levels (128). This effect may be mediated by putative glucocorticoid-response elements identified in the CRBP promoter (22). 1,25-Dihydroxy vitamin D (42) reduces new synthesis of CRBP II in chick intestine in a dose-dependent manner. Intestinal CRBP II mRNA levels have been reported to increase with food intake and particularly with the intake of dietary fat (48); however, it remains to be demonstrated whether or not the peroxisomal proliferator activator receptors mediate this effect.

Ligand-Binding Studies

The endogenous ligand for both CRBP and CRBP II has been identified as all-*trans*-retinol (105, 129). Accurate determination of retinoid-binding affinities for the cytoplasmic vitamin A–binding proteins is difficult. This is due to the very high affinities involved, the low aqueous solubility of retinoids [~100–200 nM (145)], and the loss of ligand during separation of bound from free ligand (27, 91, 100, 111). These difficulties are reflected in the wide range of affinities reported by various groups. Both CRBPs show clear preference for hydrophobic ligands with terminal alcohol or aldehyde groups. CRBP and CRBP II bind all-*trans*-retinol and all-*trans*-retinal (see Figure 1 for chemical structures) tightly with a stoichiometry of 1:1 (77, 85). Replacement of the ligand alcohol/aldehyde moiety with other polar groups (acid, amide, ester) abolishes binding (77, 85, 91). Both proteins bind the 13-*cis*-isomer of retinol but not the 9-*cis*- or 11-*cis*-isomer (85). Both proteins bind 3-dehydro-retinol and trimethylmethoxyphenyl derivatives of all-*trans*-retinol (85, 122). However, removal of the methyl substituents on the ring that are ortho to the polyene chain (e.g. Ro21–7962 alcohol) abolishes binding to CRBP II but not to CRBP (122). Conformational analysis of these ligands indicates that the two methyl groups influence the planarity of the ligand. The identification of monospecific ligands may prove useful for studying the physiologic roles of these two proteins.

Although the dissociation constants for CRBP- and CRBP II-retinol in an aqueous buffer have been determined to be roughly equivalent at 10^{-8} M by fluorometric titration (77, 85), subsequent ^{19}F nuclear magnetic resonance (NMR) studies of CRBP and CRBP II isotopically labeled with 6-fluorotryptophan demonstrated that retinol complexed to CRBP II is readily transferred to CRBP, whereas retinol complexed to CRBP is not transferred to CRBP II (79). In contrast, retinal is transferred readily in both directions between CRBP and CRBP II (79). These studies suggest that under conditions of limiting

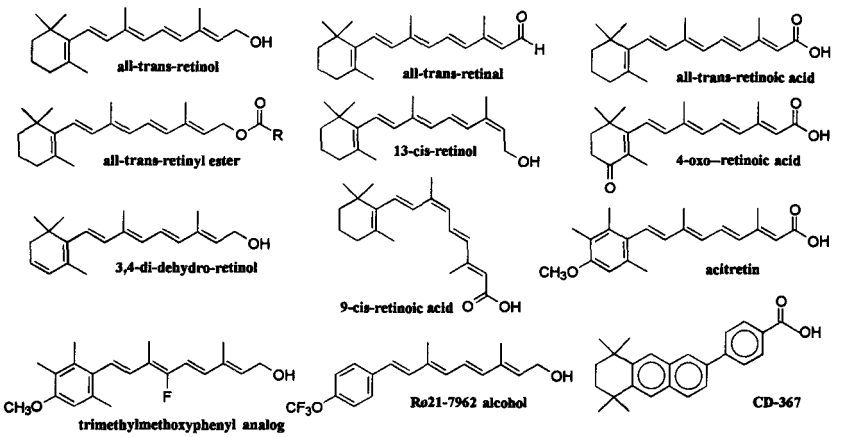

Figure 1 Chemical structures of selected retinoids.

retinol concentrations CRBP ligand-binding sites will be preferentially populated over those of CRBP II. Based on mass-action considerations, the difference in binding constants would facilitate movement of retinol from the intestinal mucosa to other tissues.

Structural Studies

X-RAY STUDIES The X-ray structures have been determined for rat holo-CRBP complexed with all-*trans*-retinol (24) and for both the apo- and holo-forms of rat CRBP II (155). These proteins share the β-barrel motif (see Figure 2) observed in the crystalline structures of other members of the iLBP family (reviewed in 7). This motif consists of a 10-stranded, anti-parallel β-barrel, with a helix-turn-helix between the first and second β-strands. In both proteins, the bound retinol is sandwiched inside the barrel with the alcohol moiety situated innermost and in close contact with the side chain of Gln-108. The conformation of all-*trans*-retinol complexed to CRBP II was derived for the four different molecules of the holo-protein present in the triclinic form (155). There were slight differences between the placement of the bound retinols in the four molecules, which had an effect on the hydrogen bond to Gln-108. These differences may account for differences in the relative binding affinities of CRBP and CRBP II for retinol and retinaldehyde.

The bound conformation of retinol complexed with CRBP has been shown to be in a 6-s-*trans* conformation (see Figure 3); its 6-s-bond torsion angle (which is defined by C5, C6, C7, and C8 and can be thought of grossly as the angle between the planes of the ring and the polyene chain) is −167° (24). The

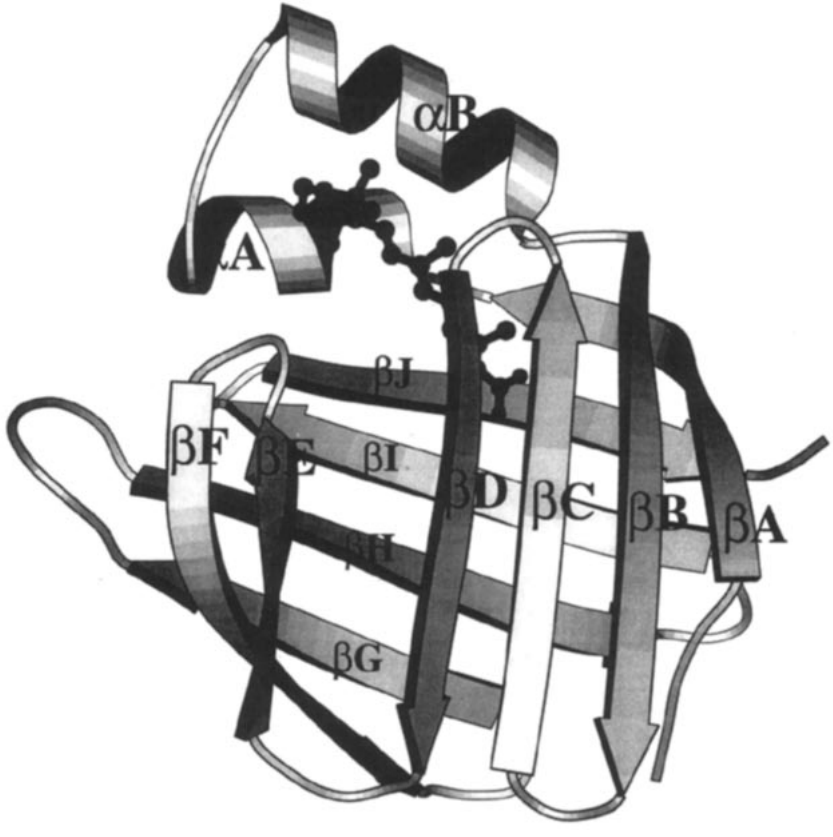

Figure 2 Representative structure of the iLBPs. The cystal structure of holo-CRABP-II (71) is shown. Drawing created with MOLSCRIPT (71a).

retinol conformations in the four different molecules of holo CRBP II appear to be more twisted from planarity −78°, −117°, −127°, and −150° (155). Inspection of the binding cavity of CRBP II does not yield an explanation why ligands predicted to adopt a more planar conformation (e.g. Ro21-7962) could not be accommodated based on steric considerations. An alternative explanation is that interactions between the methyl substituents on the ring moiety of the retinoid molecule and the protein are important in governing the binding specificity of CRBP II.

Inspection of the apo-form of CRBP II revealed no obvious portal through which the ligand could enter the binding cavity (155). Comparison of apo- and holo-CRBP II at a resolution of 2.0 Å showed no significant structural differ-

Figure 3 The 6-s-*trans*- and 6-s-*cis*-conformers of all-*trans*-retinol.

ences between the apo- and holo-forms of the protein (155). Consequently, the structural bases for a number of observations showing changes in the chromatographic properties (54) and reduced sensitivities to limited proteolysis (64) upon ligand binding remain to be explained. There is increasing evidence that in the solution state there are time-dependent fluctuations in conformation about an average structure. Some of these conformations may be more open, allowing the ligand to enter. These open states may be relatively more susceptible to proteolysis. The addition of ligand may alter the equilibrium between different conformational states rather than cause a gross change in conformation.

NMR STUDIES There is increasing evidence that ligands, while bound, can undergo significant dynamic processes. This is the case for fluororetinol bound to CRBP II. [^{19}F]fluorine NMR studies showed that fluororetinol, when bound to CRBP II, exchanges between magnetically distinct binding states (123). This exchange occurred on a much faster time scale than the exchange of the fluororetinol between the bound and the unbound states. The distinct binding states may correlate with the multiple conformations of bound retinol observed in crystalline holo-CRBP II. These studies suggested that the conformational dynamics of retinol binding are different for CRBP (123). Multidimensional heteronuclear NMR methods may prove useful in analyzing potential changes in the dynamics of these proteins that occur upon ligand binding.

EFFECTS OF MUTAGENESIS The importance of polar interactions between the ligand alcohol moiety and Gln-108 is underscored by the loss of retinol binding for both CRBP and CRBP II when Gln-108 is replaced by an arginine residue (20, 62, 143). It has been proposed that substitution of Phe-4 in CRBP by glutamine in CRBP II could account for differences in the polar interactions in these two holo-proteins. However, this was not borne out by subsequent site-directed mutagenesis experiments (63). It is important to note that site-directed mutagenesis of a single amino acid may result in global conformational changes in these proteins (20, 80).

Mutant CRBP II proteins in which Gln-108 was replaced by an arginine gained the ability to bind fatty acids with affinities comparable to the binding

affinities exhibited by the iLBP fatty acid–binding proteins (20, 62). However, mutant CRBP II did not bind retinoic acid with any higher affinity than the wild-type protein (20). Reciprocal mutagenesis experiments carried out with intestinal fatty acid–binding protein (an iLBP), in which Arg-106 was replaced with glutamine, led to weak retinol binding (62). These results, along with the observation that removal of methyl substituents on the ring moiety abolishes binding to CRBP II, indicate that interactions involving regions of the ligand other than the polar group also play a role in governing binding specificity.

Functional Roles—In Vitro Studies

The precise functional roles for CRBP and CRBP II remain to be defined. It has been proposed that these proteins play a role in cellular uptake of retinol, cytoprotection, and modulation of intracellular retinol metabolism. Furthermore, the intestinal localization of CRBP II suggests that it is uniquely adapted for the intestinal absorption and/or metabolism of retinoids.

RETINOL TRANSFER BETWEEN THE CRBPS AND MEMBRANES It is controversial whether or not a membrane-associated retinol transporter exists (9, 31, 56). Studies with artificial phospholipid bilayers have shown that retinol can spontaneously flip between the two layers (40). The transfer of retinol across a lipid bilayer can therefore occur in the absence of any specific membrane-associated binding proteins. Studies that measure transfer of radiolabeled retinol or decreases in protein-bound retinol fluorescence indicate that little dissociation (~10%) of the CRBP-retinol complex takes place when coincubated with membranes (54, 102). ^{19}F NMR studies have demonstrated that CRBP sequesters retinol from phospholipid bilayers more effectively than CRBP II does (E Li, unpublished observations).

The rate of retinol transfer from CRBP to phospholipid vesicles has been reported to be independent of vesicle concentration over a sevenfold range. This has led to the suggestion that the mechanism of transfer is by aqueous diffusion of the ligand rather than by collision between the protein and the vesicles (102). Studies of the mechanism of retinol transfer from CRBP II have not been reported.

EFFECT ON RETINOID METABOLISM One approach to examining the functional role of the CRBPs has been to examine the effect of these proteins on a number of cellular enzyme activities in vitro. The activities that have been examined are the reduction of retinaldehyde, esterification of retinol, retinyl ester hydrolysis, and synthesis of retinoic acid (reviewed in 96, 97, 111). Interpretation of these studies is complicated by the aqueous insolubility of retinoids and their affinity for membranes in which many of the retinoid metabolizing

activities are found (102, 145). Frequently, the K_m values measured for these activities exceed the solubility limits of retinol in water (0.1 μM) and are thus difficult to interpret because much of the added retinol is likely to exist in an aggregated state. Because all of the enzyme systems described below are capable of accepting unbound retinol as substrate, the binding proteins are not essential cofactors in the catalytic process. In the case of membrane-associated enzymes, differences in the rates of transfer of unbound, CRBP-, and CRBP II-retinol into the lipid bilayer could complicate analysis of the kinetics. In these studies it is difficult to distinguish effects due to mass action from those due to specific protein-enzyme interactions. The difficulties just described should not detract from the theme that has emerged from the in vitro work of a number of investigators, that the cytoplasmic retinoid-binding proteins restrict access of retinoids to some metabolic activities but not others. For CRBP, there is compelling evidence for the existence of direct protein-enzyme interactions.

Because the alcohol moiety of retinol bound to CRBP or CRBP II appears to be relatively inaccessible, the structural basis for how these cytoplasmic proteins present the bound retinol as substrate for these enzymes presents an interesting problem. Furthermore, the structural basis by which cellular enzymes discriminate between the apo- and holo-forms of CRBP and CRBP II remains to be defined.

Reduction of retinaldehyde Within the enterocyte, provitamin A carotenoids undergo enzymatic, oxidative cleavage to form retinal, which is then quickly reduced to retinol. CRBP II-retinal is poorly reduced to retinol by cytosolic retinal reductase activity but is easily reduced by microsomal retinal reductase activity prepared from intestinal mucosa (65). Free retinol is reduced by both activities.

Retinol esterification Some 90% of dietary retinol is esterified by the intestinal mucosa and is packaged into chylomicrons prior to delivery to the liver. Within the liver, retinol is reesterified and stored as retinyl esters in hepatocytes and stellate cells (reviewed in 12, 75, 156). The effect of CRBP and CRBP II on retinol esterification activities has been investigated for intestinal and hepatic microsomes (54, 108, 110, 120, 157). Two distinct activities have been described (reviewed in 111). One is acyl-coenzyme A (CoA):retinol acyltransferase (ARAT), which utilizes acylated fatty acids as the donor. The second is lecithin:retinol acyltransferase (LRAT), which uses the *sn*-1 fatty acid of phosphatidylcholine as the fatty acid donor. The two activities can be distinguished by differences in their dependence on exogenous acyl-CoA. Esterification of retinol bound to CRBP or CRBP II is acyl-CoA independent, suggesting that CRBP and CRBP II selectively direct retinol for esterification by

LRAT over esterification by ARAT. The comparable apparent K_m values of unbound retinol and retinol complexed with CRBP or CRBP II have been presented as evidence that CRBP- and CRBP II-retinol are directly accepted as substrates for LRAT. Note that no definitive conclusion can be drawn since the K_m of 0.63 μM obtained with unbound retinol as the substrate exceeds the solubility of retinol in an aqueous environment (0.1 μM). It has been observed that apo-CRBP but not apo-CRBP II inhibits liver microsomal LRAT esterification of CRBP-retinol (54). This suggests that the level of ligand occupancy for CRBP regulates esterification of retinol in the liver. This inhibition is observed even after ligand binding by the apo-CRBP is inactivated by prior treatment with *p*-(chloromercuri)benzenesulfonic acid and provides compelling evidence for direct CRBP-enzyme interactions (54).

Retinyl ester hydrolysis Stored retinyl esters within the liver are hydrolyzed for eventual release into the circulation as retinol complexed with serum retinol-binding protein. Apo-CRBP has been demonstrated to stimulate bile-salt independent hydrolysis of endogenous retinyl ester stores in the liver (13). Apo-CRBP also stimulates hydrolysis of preformed retinyl esters in retinal pigment epithelial plasma membranes (112). It has been argued that this effect is not simply due to mass action because other proteins that bind retinol, including β-lactoglobulin and bovine serum albumin, have no effect on hydrolase activity (13). It should be noted, however, that CRBP binds retinol with significantly higher affinity than either of these two proteins.

Retinoic acid synthesis Within target tissues, retinol can be metabolized to retinoic acid and polar metabolites. Because retinoic acid is the active ligand for the nuclear receptors, the cellular production of retinoic acid is likely to be a tightly regulated process. The enzymatic conversion of retinol to retinoic acid is generally thought to proceed through retinal as an intermediate (69, 97). Both cytosolic and microsomal activities have been reported for the conversion of retinol to retinal (98, 113, 117). The liver microsomal activity has been purified to two proteins of 34 and 54 kDa (14). The 34-kDa protein, termed retinol dehydrogenase I or RoDH(I), has been cloned from a mouse liver cDNA library (18). Recently, cross-linking of RoDH(I) with holo-CRBP but not apo-CRBP has been demonstrated, providing compelling evidence of a direct interaction (14, 97). This cross-linking was dependent upon NADP as a cofactor (97).

CRBP-retinal serves as a substrate for a partially purified cytosolic retinal dehydrogenase (118). Apo-CRBP inhibited the absolute rate of this reaction.

The rates of retinol oxidation by intestinal microsomes are reduced 50-fold when retinol is bound to CRBP II compared to unbound retinol (65), which suggests that CRBP II serves to sequester retinol from oxidation within the intestinal epithelium.

Functional Roles—In Vivo Studies

The effects of modulating the levels of CRBP and CRBP II on retinol uptake and esterification in the human intestinal cell line, Caco-2, were recently studied (74, 81). Differentiated Caco-2 cells express CRBP II but not CRBP and contain LRAT activity. Retinol absorption and esterification were increased up to twofold when Caco-2 cells were stably transfected to overexpress rat CRBP or CRBP II. In the case of CRBP II-transfected cells, the magnitude of retinol absorption was directly related to the total CRBP II content. When Caco-2 cells were transfected with CRBP, the levels of CRBP II decreased such that total binding protein content was not increased. However, retinol absorption was also increased approximately twofold in these cells, which suggests that CRBP is more efficient than CRBP II at promoting cellular retinol uptake. This is consistent with the increased affinity of CRBP for retinol compared with CRBP II. In these studies, synthesis of retinyl esters was directly linked to absorption regardless of the composition of the cytoplasmic retinol-binding proteins. Interestingly, the composition of the cytoplasmic retinol-binding protein may exert an effect on the polarity of retinyl ester secretion (81). These studies were carried out in the presence of micromolar concentrations of retinol in the medium, where it was likely that CRBP was exclusively in the holo-form. Under these conditions, the hypothesis that apo-CRBP inhibits retinol esterification cannot be tested. Conversion of retinol to retinoic acid in these cells has not been described. It would be interesting to compare the metabolism of retinol within these transfected cells in the presence of limiting amounts of retinol.

CYTOPLASMIC RETINOIC ACID–BINDING PROTEINS

Two cytoplasmic retinoic acid–binding proteins, CRABP-I and CRABP-II, have been extensively characterized. One or both of the CRABPs have been purified and cloned from mammals, birds, and amphibians (see Figure 4) (4, 17, 25, 30, 55, 70, 83, 86, 119, 142, 148). A protein consistent with CRABP-I based on binding specificity, molecular weight, isoelectric point, electrophoretic mobility, and amino acid composition has been isolated in fish (99). It is likely that CRABPs are conserved among all vertebrates.

The two CRABPs are highly homologous (Figure 4), sharing 72% amino acid identity in humans (4). The interspecies conservation of CRABP-I is particularly high. Mouse, bovine, and rat CRABP-I have identical amino acid sequences and share all but one amino acid with human CRABP-I (Figure 4). The amino acid substitutions between CRABP-I and CRABP-II are conserved, which suggests that these two proteins have unique functions that are conserved between species.

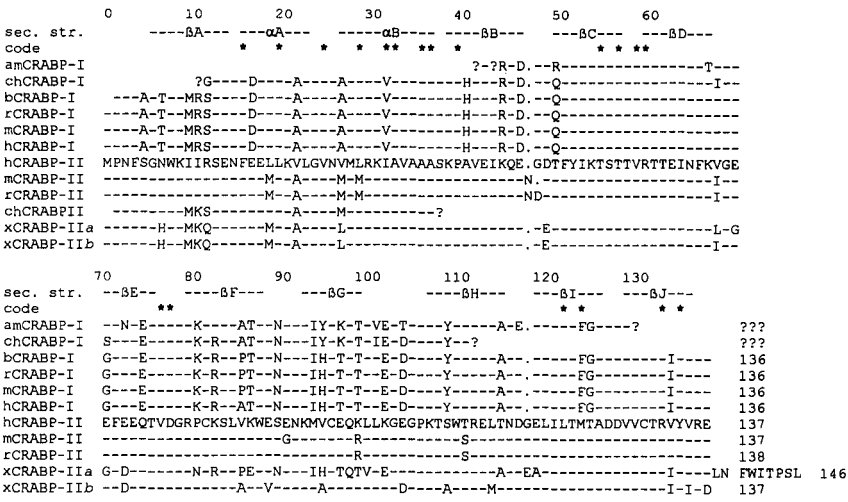

Figure 4 Sequence alignment of CRABP-I and CRABP-II from various organisms. Numbering is for mCRABP-II. Secondary structure is as reported (71). Residues within 3.5 Å of bound ligand are indicated (*). Abbreviations: am, *Ambystoma mexicanum*; ch, *Gallus gallus*; b, *Bos primigenius taurus*; m, *Mus musculus*; h, *Homo sapiens*; r, *Rattus norvegicus*; x, *Xenopus laevis*. Both xCRABPs have been tentatively assigned as CRABP-IIs, based solely on sequence comparison. Reprinted with permission from Reference 101. Copyright 1995 American Chemical Society.

Whether or not these two proteins account for all of the retinoic acid–binding activities detected in tissue extracts remains to be determined. Other cytoplasmic proteins have been identified that bind retinoic acid, including two iLBPs: adipocyte lipid-binding protein and myelin P2 protein (7). Photoaffinity experiments with tritiated all-*trans*-retinoic acid point toward the existence of several uncharacterized proteins, soluble and membrane associated, which specifically bind retinoic acid (10). The possibility of additional CRABP genes has been suggested by DNA hybridization experiments (47, 73). However, no detectable high-affinity retinoic acid–binding activity could be detected in whole-embryo cytosolic extracts in mice that are null mutants in both CRABP-I and CRABP-II (72).

Tissue Distribution

Like the CRBPs, the two CRABPs exhibit distinct tissue-specific and developmental patterns of expression. In adult animals, CRABP-I is found in many tissues (119, 158), whereas CRABP-II appears to be relatively restricted. CRABP-II expression is localized to skin in the adult animal, though not all tissues have been examined (4, 47, 133, 139). Levels of CRABP-II within the

epidermis are higher in the more differentiated suprabasal keratinocytes, as detected by in situ hybridization of RNA and immunohistochemistry (36, 136). Expression of CRABP-II is increased in some disease states of skin such as psoriasis (35, 140). Recently, it has been reported that CRABP-II transcripts were detected in rat corpus luteum at levels that exceeded other tissue examined, including the skin (17).

There have been extensive studies detailing the temporal and spatial patterns of CRABP-I and CRABP-II gene expression during embryogenesis (for recent work see 50, 55, 59, 73, 84, 88, 126). Both CRABP-I and CRABP-II are expressed strongly during embryogenesis (52). In the mouse, CRABP-I and CRABP-II transcripts are detectable in eight-day-old embryos. Transcripts levels reach a maximum near 12 days and then decline rapidly with faint signals detected in the 16.5-day-old embryos (47, 52). CRABP-I and CRABP-II expression is initially observed in the developing neuroepithelium (73, 84). At later times during morphogenesis the distribution becomes more widespread (47, 73, 84, 88, 127). In general, the pattern of CRABP-II gene expression appears less restricted than does CRABP-I (127). Furthermore, the temporal and spatial patterns of CRABP-I and CRABP-II expression are distinct from the patterns described for the nuclear retinoic acid receptors (32, 126).

Spatial patterns of expression of the CRABPs in the developing nervous system (50, 73, 84, 88, 89) and the limb bud (32, 38, 84, 90, 134) have been the subject of extensive studies, since these sites are subject to retinoid-induced teratogenesis. The apparent anterior-posterior gradient of CRABP-I levels in the chick limb bud (88, 90, 134) has been proposed to influence axis specification by retinoids. The presence of an anterior-posterior CRABP-I gradient in the mouse limb bud is unclear (32, 38, 84, 134). However, CRABP-I is expressed with a clear proximal-to-distal gradient in the anterior portion of the mouse limb bud (32, 38). Although it has been reported that the pattern of CRABP-I expression corresponds to the targets of retinoid-induced teratogenesis (125, 148), such a correlation was not found in a subsequent study (59). The rapid, embryonic localization of maternally administered retinoic acid corresponds well to the sites of CRABP-I expression (53).

Immunocytochemical studies of the CRABPs are consistent with cytoplasmic localization. There is conflicting evidence regarding whether or not the CRABPs enter the nucleus in vivo (17, 33, 158).

Gene Regulation

Levels of CRABP-I protein, mRNA, and binding activity in the rat are not significantly affected by dietary vitamin A deficiency (11, 66, 87, 119).

CRABP-II is up-regulated by retinoic acid in many cells/tissues, including whole mouse embryo, various cell lines, and human skin in vivo (3, 4, 47, 52,

93, 137). Not all cells are capable of this response, as exemplified by lack of CRABP-II induction in lung fibroblasts (4). The upstream regions of both the mouse and human CRABP-II genes contain retinoic acid response elements, which mediate the induction of CRABP-II transcription by retinoic acid (2, 34). Interestingly, in both species 9-*cis*-retinoic acid induces CRABP-II expression to a greater extent than all-*trans*-retinoic acid does (34, 93).

CRABP-I is also up-regulated by retinoic acid. This response is temporally restricted. Treatment of mouse embryos at day 7.75, but not later, leads to increased levels of CRABP-I protein and mRNA (4, 52, 73, 84). This temporal change is probably related to hypermethylation of the 5′ flanking region of the CRABP-I gene, which occurs in older mouse embyos and in adult mice but not in young embryos (150). In contrast to CRABP-II, the upstream flanking region of the mouse CRABP-I gene does not contain a retinoic acid response element with any known consensus sequence (149). Deletional analysis of the upstream region suggests that a putative AP-1 site is responsible for the retinoic acid–induced up-regulation of CRABP-I (151).

Ligand-Binding Studies

The endogenous ligand of CRABP-I purified from cattle retina and fish eye has been identified as all-*trans*-retinoic acid (99, 129). The ligand occupancy in purified CRABP-I ranges from 20–75% (99, 129, 135). This represents a minimum due to possible ligand loss during purification (27). The endogenous ligand for CRABP-II has not been characterized. One study found that no retinoid was complexed to purified CRABP-II (5). This may represent loss of ligand during purification. Levels of the CRABPs exceed that of retinoic acid in many tissues, which suggests that the level of ligand occupancy for these proteins may sometimes be low in vivo (134).

Both CRABPs bind all-*trans*-retinoic acid with very high affinity. CRABP-I binds all-*trans*-retinoic acid with stronger affinity than does CRABP-II (5, 43, 46, 100, 133, 139). This is true for both recombinant protein and endogenous protein recovered from animal tissues (46, 133, 139). The stronger affinity of CRABP-I appears to be conserved between species and has been established for rats (6), mice (43, 100), chickens (134), and humans (46). The affinity of CRABP-I for all-*trans*-retinoic acid is stronger than can be measured by existing fluorescence techniques. The apparent dissociation constant, K_d' of CRABP-I is less than 0.4 nM and the K_d' of CRABP-II is 2 nM (100). Based on Scatchard analysis of retinoic acid binding in skin extracts, it has been reported that CRABP-I and CRABP-II exist in vivo in both a high- and a low-affinity state (133). This has not been observed for purified CRABPs. The relative affinities of the CRABPs versus the retinoic acid receptors (RARs) for all-*trans*-retinoic acid is an important question. Unfortunately, though the

affinities of the RARs have been measured (1), direct comparison is not possible because of differences in methodology.

Both CRABPs prefer ligands with terminal carboxylic acids, shown by lack of binding to all-*trans*-retinol or all-*trans*-retinal (43). Failure of CRABP of unspecified type to bind retinyl esters has been reported as well (68). Both CRABPs show a strong preference for the all-*trans*-isomer of retinoic acid. Binding of 9-*cis*-retinoic acid occurs with much lower affinity. There is little if any detectable binding of 13-*cis*-retinoic acid and 9,13-di-*cis*-retinoic acid by either CRABP (43, 58, 100, 131, 138).

Both CRABPs bind many retinoic acid analogues with modified ring structures, including natural retinoic acid metabolites: 4-hydroxy-retinoic acid, 4-oxo-retinoic acid, 18-hydroxy-retinoic acid, and 3,4-didehydro-retinoic acid (43). Both CRABPs bind synthetic retinoids with altered ring structures, including the pharmacologic retinoid acitretin (100). The ring methyl groups ortho to the chain appear to be important for binding, as shown by reduced affinity for the acid form of Ro21-7962 (see Figure 1) and similar compounds (21, 60, 154).

Both CRABPs bind many synthetic retinoids with nonclassic structures. The compound CD367 is shown in Figure 1 as an example (46). One compound, tetramethyl napthyl benzoic acid, has been reported to bind CRABP-II weakly, and to bind CRABP-I not at all (116).

Structural Studies

X-RAY STUDIES The crystal structures of bovine CRABP-I and human CRABP-II complexed with all-*trans*-retinoic acid, as well as CRABP-II complexed with a synthetic retinoid, have been determined by X-ray diffraction (71). As expected, both CRABPs share the β-barrel motif exhibited by other members of the iLBP family. In both CRABPs, the second alpha-helix extends for an extra half turn, compared with the CRBPs and the fatty acid–binding proteins. This extra turn is probably the consequence of an insertion of two additional amino acids in the sequence of both CRABPs at the end of this helix as compared with other iLBPs (71).

There are several interesting points that can be made in comparing the CRABP structures with those of the CRBPs (71). Although the ligands of the CRBPs and CRABPs differ only in their polar end group, the positioning of the ligand within the binding cavity is very different (Figure 5). In both CRABPs, the carboxylic acid moiety of the ligand interacts directly with Arg-132 (CRABP-II numbering). An indirect interaction is formed with Arg-111 via bound water. This is in contrast to the polar interactions observed for both CRBPs, in which the polar end group interacts directly with the glutamine corresponding to Arg-111 in the CRABPs. Retinoic acid within the CRABPs

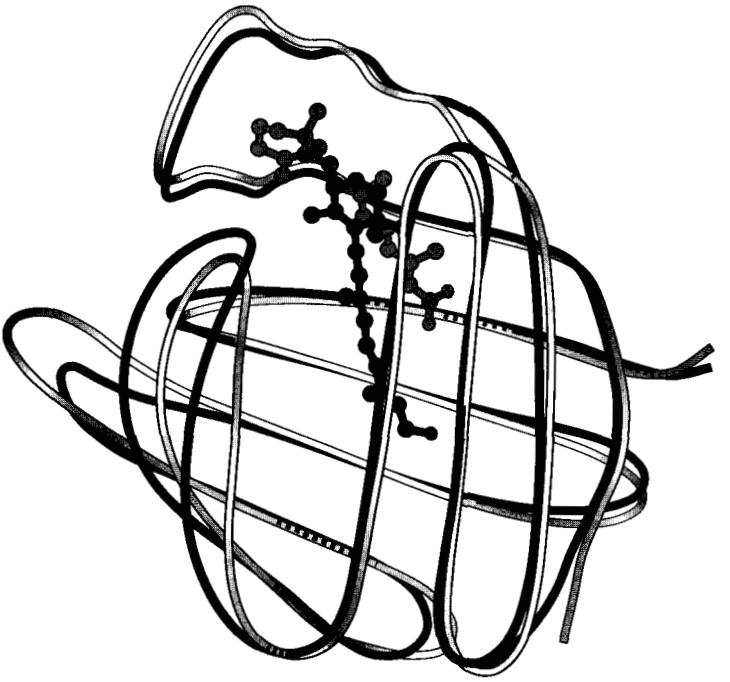

Figure 5 Comparison holo CRBP-II (155) and CRABP-II (71). CRBP-II/retinol is the darker trace. Drawing created with MOLSCRIPT (71a).

is shifted 5.9 Å (see Figure 5) compared with retinol bound to the CRBPs. This is due to the increased length of arginine relative to glutamine and to the interaction with residue 132 rather than 111.

The position of the β-ionone ring of retinoic acid bound to CRABP is displaced by ~4.5 Å within the binding cavity (see Figure 5) compared to the position of the β-ionone ring of retinol bound to CRBP. The β-ionone ring of retinoic acid bound to both CRABPs appears to be more accessible to solvent than does the ring moiety of retinol bound to the CRBPs (71). The 6-s-bond torsion angle of retinoic acid bound to CRABP-II was measured at –33°, a skewed *cis* conformation. This contrasts with the 6-s-*trans*-conformation measured for retinol bound to CRBP. In both CRABPs, the remainder of the polyene chain was planar with the exception of the carboxylate, which is twisted 55° out of the polyene plane.

As expected from their high degree of homology, the holo structures of CRABP-I and CRABP-II are very similar. One detectable difference is in the positioning of the ligand carboxylate relative to the different guanido nitrogens

of Arg-132. The difference in positioning may be due to amino acid substitutions at positions 9 and 123 and may account for the higher affinity of CRABP-I for retinoic acid (71). Amino acids within 3.5 Å of bound retinoic acid are indicated in Figure 4 (101). The majority of these amino acids are conserved between CRABP-I and CRABP-II. The amino acids at positions 31 and 123 show a conserved substitution. These may be important in determining differences in ligand-binding affinity. Most of the conserved substitutions are not within 3.5 Å of bound ligand.

Comparison of the structure of CRABP-II when complexed with all-*trans*-retinoic acid versus the synthetic ligand shows that CRABP-II can alter its conformation in response to different ligands (71). Changes as large as ~1.4 Å were observed for αA and the βC–βD loop (see Figure 2 for iLBP secondary structure elements). The bound synthetic retinoid had also undergone significant conformational changes, as compared with its unbound crystal structure.

The crystal structure of apo CRABP-I has also been solved (146). Each unit cell contains two molecules of apo CRABP-I, termed molecule A and B. The structures of these two molecules differ from each other and from the structure of holo CRABP-I. In apo CRABP-I molecule A, the turn between βC and βD is displaced outward as much as 9 Å from its position in holo CRABP-I (see Figure 6). In molecule B, much less–marked displacement of this loop is observed. The displaced βD strand forms an intermolecular β-sheet with βD of the opposing CRABP-I molecule in the unit cell. The ability to form this intermolecular β-sheet is due to the paucity of main-chain hydrogen bonds between strands βD and βE (note the gap between βD and βE in Figure 2). A shift in the conformation of the second helix, αB, apparently accompanies the movement of turn βC–βD. The marked conformational changes between crystalline apo- and holo- CRABP-I contrast with the minimal alterations found in the apo- and holo-structures of other iLBPs, including CRBP II, intestinal fatty acid–binding protein, and adipocyte lipid–binding protein (reviewed in 7).

Entrance of retinoic acid into the binding pocket may be facilitated by movement of the loop region observed in apo-CRABP-I (146). It has been suggested that CRABP-I may form intermolecular β-sheets, similar to that found in the crystal structure, with selected cellular proteins (146). This may provide a mechanism by which CRABP-I could channel retinoic acid along certain metabolic pathways.

NMR STUDIES ^{1}H and ^{15}N resonances have been assigned for the backbone and many of the sidechains for apo-CRABP-I at pH 7.5 and for holo-CRABP-I at pH 3.8 (121). The secondary structures predicted from the NMR studies are consistent with the respective crystal structures. The dynamics and folding of

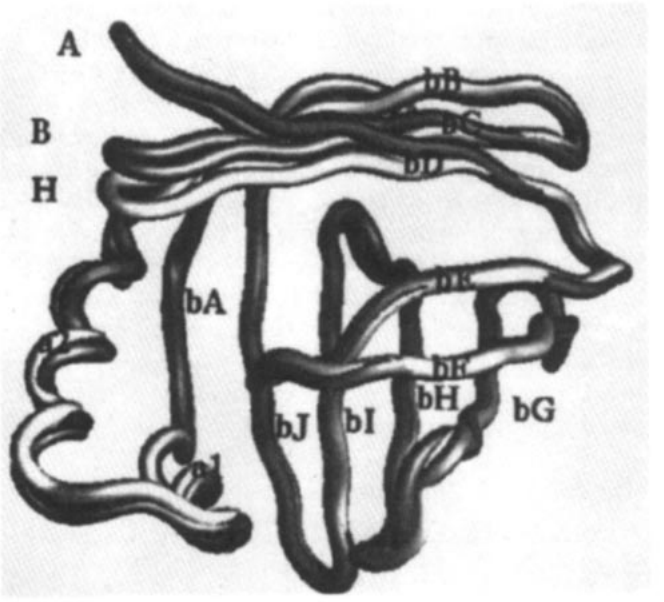

Figure 6 Comparison of apo CRABP-I molecules A and B with holo CRABP-I (H). Reprinted with permission from Reference 146. Copyright 1995 Academic Press, Inc.

CRABP-I were assessed by amide proton exchange studies. The largest differences in exchange rates between the apo and holo states occurred at αB and at the βC–βD turn, with faster exchange in the apo form. This suggests greater flexibility at these positions in the apo state, consistent with the implications of the crystal structure of apo CRABP-I. This finding is consistent with greater proteolytic susceptibility of apo versus holo CRABP-I (64), as well as with reduced thermostability of apo versus holo CRABP-I (159).

Differences in the interaction of all-*trans*-retinoic acid with CRABP-I and CRABP-II have been detected by NMR studies (101). Specifically, differences were noted in the 6-s-bond torsion angle and in protein-ligand close contacts at the ring moiety. These differences are unexpected given the high degree of homology between the two CRABPs. For CRABP-II, the 6-s-bond torsion angle measured by NMR was in good agreement with the skewed *cis* conformation found in the crystal structure (see above). In contrast, for CRABP-I, results of the NMR studies suggested that the 6-s-bond torsion angle exhibits time-dependent fluctuations, similar to that observed for retinoids in organic solution at room temperature (57). The differences in protein-ligand close contacts were tentatively assigned to residues 31 and 28 of αB (101).

EFFECTS OF MUTAGENESIS Interactions between the carboxylate group of retinoic acid and the arginines located at positions 111 and 132 have been probed by site-directed mutagenesis of both CRABPs. For CRABP-I, mutation of either of these positions to glutamine drastically reduces binding of all-*trans*-retinoic acid (159). In contrast, mutation of position 111 to alanine had minimal effects on the binding of retinoic acid by CRABP-II, whereas mutation of position 132 to alanine or glutamine greatly reduced binding (19). Loss of binding on mutating the arginine at position 132 was consistent with the crystalline structures of both holo-CRABP-I and holo-CRABP-II (71). In CRABP-I, loss of binding on mutating the arginine residue at position 111 may indicate the importance of its indirect electrostatic interaction with the carboxylate via bound water. Alternatively, it may represent local or global perturbation of the binding pocket conformation (20). None of the mutants gained affinity for retinal or retinol.

Functional Roles—In Vitro Studies

The precise functional roles of CRABP-I and CRABP-II remain to be defined. It has been proposed that these proteins influence ligand occupancy of the nuclear retinoic acid receptors, and that they modulate retinoic acid metabolism. Metabolism of retinoic acid may represent an important means by which cells regulate retinoic acid levels. It is likely that the microsomally (endoplasmically) located P450 system represents an important site of retinoic acid metabolism. The effect of CRABP-I on microsomal retinoic acid metabolism has been investigated in vitro. The metabolic half-life ($t_{1/2}$) for all-*trans*-retinoic acid incubated in the presence of microsomes is not altered by the addition of apo-CRABP-I (44). In contrast, the metabolic $t_{1/2}$ for 4-hydroxy-retinoic acid and 4-oxo-retinoic acid were significantly increased by the addition of apo-CRABP-I (44). This has been taken as putative evidence that CRABP-I directly presents selected retinoids (i.e. retinoic acid) for metabolism by P450. However, this may only reflect differential partitioning of the various retinoids between CRABP-I and the microsomal membranes.

Functional Roles—In Vivo Studies

CELL CULTURE STUDIES The effect of modulating levels of CRABP-I by genetic techniques has been investigated for two cell lines, F9 teratocarcinoma cells and primary murine embryonic palate mesenchymal cells. Both cell lines naturally express one or both CRABPs. The stable transfection of a CRABP-I expression vector into F9 cells resulted in a reduced response to exogenously added retinoic acid (15). The overexpression of CRABP-I shifted the dose-response curve such that higher concentrations were needed to stimulate retinoic acid–induced gene expression. This effect is not general and was not observed

for all of the retinoic acid–responsive genes tested. Cotransfection of reporter constructs containing retinoic acid response elements showed that higher levels of retinoic acid were needed to induce expression from the constructs. Likewise, transfection with an anti-sense CRABP-I led to a heightened response to retinoic acid. These results suggested that the presence of CRABP-I reduced nuclear retinoic acid–receptor occupancy. Increased metabolism of retinoic acid was observed in F9 cells overexpressing CRABP-I (16). Somewhat contrary results were obtained when primary cultures of murine embryonic palate cells were treated with anti-sense oligonucleotides directed at either CRABP-I or CRABP-II (103). Retinoic acid–induced expression of three endogenous genes was reduced upon treatment with the anti-sense oligonucleotides. These results suggested that the CRABPs increased nuclear retinoic acid occupancy. It is entirely possible that the function of the CRABPs is variable, depending on the particular cell, the levels of the CRABPs, and the levels of retinoic acid.

WHOLE ANIMAL STUDIES Overexpression of the CRABPs at the organismal level causes considerable pathology. Transgenic mice have been created that express CRABP-I under the influence of the lens-specific αA-crystallin promoter (114). CRABP-I is not normally expressed in the lens. These mice develop cataracts at a young age. Histologic examination of the lens from these animals suggests that the cataracts form as a result of impaired development and impaired cellular differentiation. No change was noted in expression of γF-crystallin, a retinoic acid–responsive gene, which suggests that overexpression of CRABP-I did not alter nuclear levels of retinoic acid. These animals also expressed the transgene in normal pancreatic tissues starting at 5.5 months. Starting at 4.5 months, pancreatic endocrine tumors were found that express the transgene. Mice have also been created that express CRABP-I under the influence of the human metallothioneine IIA promoter (152). Expression of the transgene is highly variable between the various founder lines. Half of the founders produced normal progeny that express the transgene. The other founders produced progeny with significant pathology. Some of the female progeny were completely sterile. The ovaries in these animals were small, did not contain follicles, and appeared to overexpress CRABP-I at high levels. Another line produced sickly progeny with abnormal livers, lungs, and spleens. The livers and lungs from these animals highly expressed CRABP-I.

Abnormally high CRABP levels have been created in *Xenopus* by injection of CRABP RNA into fertilized eggs (30). The exogenous transcripts are retained with a half-life of 20 h. The resulting embryos suffered from severe developmental abnormalities, including defects in anterior-posterior patterning. There was a severe reduction of the tail and anterior brain, as well as complete absence of the heart. The effects closely resembled the effect of treatment with retinoic acid. Two retinoic acid–responsive genes were hyper-

induced by the ectopic CRABP expression. These results are consistent with transduction or enhancement of endogenous retinoid signals by CRABP (30)

Null mutant mice of both CRABPs have been created by transgenic technology. Two groups have independently shown that CRABP-I null mutant mice have a normal phenotype (29, 49). In these mice, CRABP-II expression is unchanged and thus not up-regulated to compensate for the loss of CRABP-I. Quantitation of RNA levels for the RARs showed no changes. The lack of increased RAR-β, which is induced by retinoic acid, suggests that no increases in nuclear retinoic acid levels were present. No difference in teratogenic response to retinoic acid administered during development was observed between the null mutant and wild-type mice.

CRABP-II null mutant mice also show a predominately normal phenotype (38, 72). However, many of the homozygous CRABP-II null mutant mice have an extra, postaxial digit on the forelimb. This finding clearly establishes a role for CRABP-II in limb development. The penetrance of this effect was different between strains, ranging from 10–50%. As in the CRABP-I null mutants, no change was detected in CRABP-I expression or in RAR expression. Challenge of the CRABP-II null mutants with teratogenic doses of retinoic acid showed no differences in effects compared with wild-type littermates.

Mice that are null mutants in both CRABP-I and CRABP-II have also been produced (72). These mice exhibit a phenotype similar to that of the CRABP-II null mutants. The double knockout mice exhibited postaxial digits, but with higher penetrance (83%). Post- and preaxial digits were also occasionally found in the hind limb as well. The double knockouts also exhibit significantly ($P < 0.025$) decreased viability, with 9% mortality by 6 weeks of age. The cause of the reduced viability was not established. This increased mortality, compared to 2–3% for wild-type controls (72), could represent considerable evolutionary pressure. Like the single knockouts, no change in RAR expression was demonstrated, and no change in teratogenic susceptibility to retinoic acid was observed. No CRABP-like retinoic acid–binding activity was detected in whole-embryo cytosolic extracts, which suggests that unknown CRABPs were not compensating for the lack of CRABP-I and CRABP-II in these mice. Although the knockout mice showed no change in teratogenic susceptibility to retinoid excess, their susceptibility to retinoid deficiency remains to be determined.

CONCLUSIONS

In the past three years, significant progress has been made toward understanding the structural basis for molecular recognition by four cytoplasmic retinoid-binding proteins. Although differences in the ligand binding constants of these proteins may play a role in modulating intracellular trafficking of retinoids, recent exciting biochemical data support the hypothesis that the role

of CRBP in retinoid metabolism involves direct protein-enzyme interactions. There is compelling biochemical evidence that cellular enzymes can distinguish between the apo- and holo- forms of CRBP. Systematic comparisons of the structure and dynamics of these proteins in solution may provide further insight into the molecular basis for differential recognition of the apo- and holo-forms of these proteins. The mild phenotype observed for the null mutant mice for CRABP-I and CRABP-II has brought into serious question hypotheses regarding the role of these proteins in the retinoic acid–signaling pathway. Parallel genetic experiments may provide further insights into the physiologic roles of CRBP and CRBP II.

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