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INTRACELLULAR VITAMIN A–BINDING PROTEINS

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INTRODUCTION

The description of physiologic functions of retinol (vitamin A alcohol) in most biologic, biochemical, nutritional, or physiologic textbooks is limited to this lipid-soluble micronutrient's involvement in vision. Here, retinal (vitamin A aldehyde) serves as the chromophore in the photoreceptor system (10). Consequently, it is often not appreciated that vitamin A is essential for normal cellular differentiation and for maintenance of the proper differentiated state

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of many, if not all, epithelia (83). The function of the vitamin in differentiation is more important for the survival of the animal than is its involvement in vision. When animals are fed a retinol-deficient diet, some cells of epithelial tissues undergo squamous keratinizing metaplasia, thereby rendering the animal less resistant to lethal infections. On the other hand, the testes of animals fed a retinol-deficient diet show extensive loss of the germinal epithelium with consequent sterility (83). The sterility is also due to alterations in cellular differentiation due to lack of vitamin A. The mechanism by which retinol and/or its important metabolite, retinoic acid, exert their molecular effects in cellular differentiation is presently under intensive study. Although direct evidence on whether the involvement of retinol in differentiation is different from that of retinoic acid is still not available, it has become clear that the action of these compounds (collectively called retinoids) results in the simultaneous activation and repression of many specific genes (13).

In recent years, many efforts to elucidate how retinoids direct proper differentiation and maintain epithelia, as well as to understand their effects on malignant growth, have been centered on intracellular retinoid-binding proteins in tissues other than the eye. The first intracellular binding protein for retinoids discovered was that binding retinol (4). We now know of two other quite specific intracellular retinoid transport proteins that solubilize, protect, and deliver vitamin A–like compounds. These proteins, listed in Table 1, are the subject of this review. Several recent reviews on this subject are also available (14, 15, 17–19, 49, 71, 77, 79). As the literature in this area is rather extensive, we limit ourselves to the recent progress achieved by the many laboratories interested in the nonvisual functions of vitamin A. Such a decision leaves out two novel intracellular binding proteins whose presence is limited to visual tissue. These are the cellular retinal-binding protein, abbreviated CRALBP (27, 75) and the interphotoreceptor or interstitial retinol-binding protein IRBP (2, 11, 40, 42), which are reviewed elsewhere (10, 12).

CELLULAR RETINOL-BINDING PROTEIN

Detection and Quantitation

Cellular retinol-binding protein (CRBP) was first detected by sucrose gradient centrifugation (4). Gel filtration has been used subsequently (4, 28). Recently, miniature molecular-sieving columns (44) and high-performance liquid chromatography were utilized to detect and estimate the amount of CRBP (3, 8, 60, 74). These methods require preincubation of tissue extract or cytosols with radioactive retinol. The free ligand is then separated from that bound to the protein by each of the methods mentioned above. The amount of specifically bound retinol is determined by the difference between the amount of protein-bound radioactivity recovered compared to a sample that was in-

Table 1 Properties of intracellular vitamin A–binding proteins

Name	Abbreviations	Molecular weight	Endogenous ligand	Primary location
Cellular retinol-binding protein	CRBP	15,700	all-*trans*-retinol	cells of vitamin A–sensitive tissues
Cellular retinoic acid–binding protein	CRABP	15,500	all-*trans*-retinoic acid	cells of vitamin A–sensitive tissues
Cellular retinol-binding protein, type two	CRBPII	15,600	all-*trans*-retinol	absorptive cells of the small intestine

cubated with the radioactive retinol and an excess (usually 100–200-fold) of nonradioactive retinol.

Specific antibodies to both rat and human CRBP have permitted the development of sensitive radioimmunoassays that appear to be more sensitive than the nonimmunochemical methods (1, 25, 55).

Physiochemical and Spectral Properties

CRBP has been purified to homogeneity and characterized from a number of tissues and species, including rat (53, 54, 76), human (26, 47), and dog liver (43); rat testis (54, 63); and bovine retina (65). Less well characterized are preparations from transplantable mouse colon (67), oviduct magnum of laying hens (22, 59, 61), and rat testis cytosol, nucleus, and chromatin (73).

The molecular weight of purified preparations from rat and human liver as well as testis has been determined by various methods to be about 15,000 in a single polypeptide chain. This is confirmed by sequencing (20, 76). The preparations from other sources were reported to have molecular weight between 13,000 to 16,600; thus the molecular weight in all species is similar if not the same. CRBP, unlike the well-characterized blood transport protein for retinol called RBP, does not bind to transthyretin (63). Isoelectric focusing of the CRBP purified from rat testis separated that preparation into two bands with pI 24.8 and 4.9 (63). Preparations from various sources have different mobilities when electrophoresed in acrylamide gels (30). A preliminary report has announced the crystallization of rat liver CRBP and presented early x-ray data on these crystals (46).

The spectrum of CRBP complexed with retinol is dominated by the absorbance of bound retinol with a maximum at 350 nm. The spectrum is considerably altered from its spectrum in organic solvents: its maximum is red-shifted 25 nm and a fine structure, represented by a shoulder at 330 nm, is introduced; a second peak has a maximum at 368 nm (53). The fluorescence of retinol when it binds CRBP is considerably enhanced compared to its

fluorescence in a solvent as ethanol. This increase of intensity is about 7- to 8-fold. The fluorescence excitation spectrum has a major peak at 350 nm, a second peak at 368 nm, and a slight shoulder at about 330 nm. There is a small peak at 290 nm, which suggests there is energy transferred from tryptophan residues to the retinol. The emission spectrum peaks at 470 nm. Under the same conditions of buffer and pH, the spectra of rat CRBP are identical to those for human CRBP (47).

Tissue and Cellular Distribution

Production of antibody and introduction of sensitive radioimmunoassays has allowed the detection and quantitation of CRBP in cytosols of many organs (1, 55). The following organs were found to contain CRBP: brain (whole, cerebellum, cerebral cortex), hypothalamus, epididymis, eye, kidney, liver, lung, ovary, pituitary, prostate, skin, spinal cord, spleen, testis, thymus and uterus. In the intestine CRBP has been detected in jejunal and colorectal mucosa, jejunal and ileal muscle, but not in ileal mucosa. Serum and skeletal muscle appear to be devoid of CRBP.

The protein has been detected in cytosols of various types of tumors and cells in culture (as reviewed previously) (19). CRBP has been positively detected by radioimmunoassay in both isolated liver parenchymal and stellate cells (5, 6). Its presence in the endothelial cells and Kupfer cells remains to be ascertained because reports from the same laboratory are contradictory (5, 6).

The above results on the cell distribution of CRBP have been to a certain extent corroborated by localization of the protein by immunohistochemistry. Using affinity-purified anti-CRBP antibody and adult liver, the protein was localized not only in the hepatocytes but to a greater extent in the stellate (fat-storing) cells (36). In the fetal liver the protein was found in perisinusoidal cells that resemble stellate cells (37).

In spite of the agreement on the localization of CRBP in the rat liver by two laboratories (25, 36), there is a considerable difference in the quantitation of the protein by radioimmunoassay in the liver and other organs. One problem arises because CRBP is a poor immunogen. This may be due not only to its relatively small molecular weight but also more probably to its conformation. Anti-rat CRBP antibody has been successfully produced by different means; specifically by immunization of rabbits with CRBP polymerized by glutaraldehyde (55), by injection of the protein into a turkey (1, 36), or by administration of CRBP into a rabbit intranodally (25). Some radioimmunoassays require the presence of Triton X-100 (1, 34) or sodium dodecyl sulfate (25). It is, therefore, not surprising that the quantitation of CRBP varies, depending on the laboratory. These discrepancies may be compounded by 4- to 5-fold differences in the amounts of CRBP required

in the displacement curves used for the quantitation in different laboratories (1, 55).

These problems underline the striking differences in findings from experiments that address the question of whether vitamin A status influences the amount of CRBP in the liver or other organs (5, 25, 34). If vitamin A indeed increases the level of CRBP in the liver (36), the failure to detect the protein immunohistochemically in adult liver stellate cells, as previously reported (57), might be explained by the vitamin A status of the experimental animals. More importantly, detergents used in radioimmunoassays may reveal epitopes present in another newly discovered retinol-binding protein CRBPII (48). This may be the case when radioimmunoassay is used to determine CRBP in rat neonatal liver and adult intestine, where CRBPII is much more abundant than CRBP (48). These two proteins have very considerable sequence homology. See Figure 1.

By immunohistochemistry, CRBP was not detected in the gut epithelium, but only in connective tissue cells in the lamina propria and in the cells localized within the gut-associated lymphoid tissue (21). In contrast, another immunohistochemical study indicated CRBP was present in the gut epithelium (25). As rat small intestine contains CRBP, type II (48) it is possible that the antiserum against CRBP was cross-reacting with CRBPII, which has been definitely localized in the absorptive cells of the small intestine (21). Consequently some care must be taken in determining antibody specificity. Other reports essentially agree that CRBP is present in the cytoplasm of the Sertoli cells of rat testis (38, 58) and in the cytoplasm of the principal cells of the epididymis (38, 58). Localization of CRBP in the islets of Langerhans has been reported (35).

Putative Functions

The major aspects of retinol metabolism can be clearly defined and to a certain degree logistically separated: first, the absorption, storage, and mobilization of retinol from the stores; second, its catabolism; and third, its action in cellular differentiation.

Does CRBP mediate retinol action in cellular differentiation? Does this protein play dual or even multiple roles in vitamin A metabolism? CRBP was discovered more than 14 years ago by an approach similar to, if not identical with, that which previously led to the discovery of steroid hormone receptors (4). Subsequent progress in the mechanism of steroid hormone action, the present status of which was discussed quite recently (31), has been impressive. The proposed mechanism involves a translocation of the ligand, mediated by a cytoplasmic-specific binding protein (receptor), into the nucleus. The direct interaction of the steroid receptor complex with chromosomal material presumably alters gene expression. Recently, immunochemical

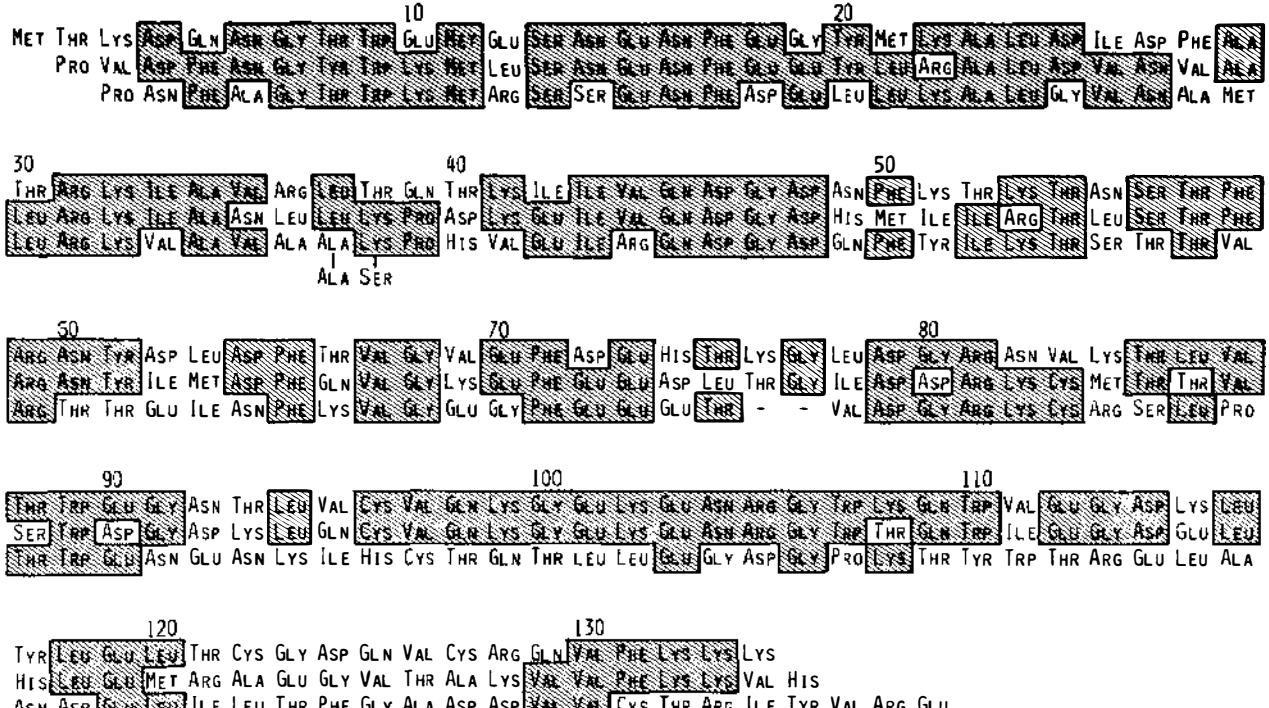

Figure 1 **Amino acid sequences of cellular vitamin A–binding proteins: *Top*, CRBPII; *Middle*, CRBP; and *Bottom*, CRABP.**

localization of the steroid receptor revealed that most, if not all, estrogen receptors are present in the nucleus and not in the cytoplasm, regardless of prior exposure to estrogen (31). These observations may require some revision of the proposed mechanism by which steroid hormones exert their action at the molecular level.

In contrast to the localization of steroid receptors, all published immunohistochemical studies indicate that the majority of CRBP is found in the cytoplasm (7, 36, 37, 57, 58). In addition, indirect immunoferritin electron microscopy demonstrated the presence of CRBP in the nucleoplasm, but in smaller quantities than in the cytoplasm (7). Thus, the distribution of CRBP within the cell appears to be different from that of estrogen receptor (31). The intracellular localization of the protein supports the contention that CRBP may be a vehicle that allows transfer of the ligand into the nucleus, as originally envisioned in the proposed mechanism of steroid hormone action. Some observations supporting this contention have already been reported. Pure CRBP complexed with radioactive retinol is apparently able to transfer the vitamin specifically into the nucleus to specific binding sites on the chromatin, without itself remaining bound to the chromosomal material (41, 80). Transport of the ligand by CRBP into the nucleus apparently does not involve an "activation" step as is required for steroid receptors (29). Presently, there is no direct evidence on whether or not the deposition of retinol by CRBP into the nucleus is connected with alterations in the genomic expression known to be caused by the vitamin in vivo.

Other immunolocalization studies have led authors to suggest that CRBP in the stellate cells may be involved in the uptake of retinol into these cells, in facilitation of its storage, or in the mobilization of retinol (back to parenchymal cells) (37). Similarly, CRBP in parenchymal cells may play analogous roles and may also be involved in retinyl ester hydrolysis, in retinol esterification, and in the transport of retinol to the place of synthesis of serum RBP in the liver. This putative function of CRBP may not be limited to the liver. CRBP has been localized in rat pigment epithelium. All vitamin A involved in vision must cross this cell layer and these cells can also store retinol. The presence of high levels of CRBP in these cells led to the conclusion that CRBP may be involved in the transcytoplasmic transport of retinol.

It is quite possible that CRBP functions not only as a transcytoplasmic and intercellular vehicle but also as a protein that prevents the catabolism of retinol, another aspect of vitamin A metabolism. In some organs like epididymis, the protein is not equally expressed in cells of the same morphologic appearance (38, 58). It is certainly of interest that the epididymis and the liver contain large amounts of CRBP. One may ask whether CRBP might be involved in the very high secretory activity of the two tissues.

CELLULAR RETINOIC ACID–BINDING PROTEIN

Detection and Quantitation

Sucrose gradient centrifugation was used for successful detection of cellular retinoic acid–binding protein (CRABP) (50, 68, 69). Agarose gel electrophoresis (30, 39), batch assay by removing the free ligand via charcoal-coated dextran (82), and miniature molecular-sieving assays have been described (44). Recently, high-performance liquid chromatography was used to measure CRABP (3, 8, 60, 74). Here again the methods require preincubation with radioactive retinoic acid. The free ligand is then separated from that bound to the protein by the above-mentioned procedures. The amount of specifically bound retinoic acid is calculated as described above for determination of CRBP.

Specific antibodies against CRABP have been produced, and sensitive radioimmunoassays are now available (1, 25, 55).

Physiochemical and Spectral Properties

CRABP has been purified to homogeneity from rat testes (52, 54, 62) and from bovine retina (64) and adrenals (78). Reports on purification of the protein from chick embryonic skin (66) and from mouse colon tumor (67) have also been published.

The protein from rat testis is a single polypeptide chain with a molecular weight of about 15,000 (50). Human breast tissue CRABP was reported to have a molecular weight of 12,000 (30), whereas the proteins from chick embryo skin and from transplantable tumors have molecular weights of 17,800 and 14,600 respectively (67, 68). Sequencing of bovine CRABP gives a molecular weight of 15,600 (78). The binding protein from embryonic chick skin has a pI value of 4.6 (69), rat testes a pI of 4.7 (62). Like CRBP, CRABP does not bind to plasma transthyretin (63, 64).

The absorption spectrum of CRABP with bound retinoic acid shows typical protein absorbance at 280 nm but is dominated by the peak at 350 nm from the bound retinoic acid (52). The spectrum of retinoic acid bound to CRABP is very similar to free retinoic acid in organic solution. It is of interest that the absorption spectrum of CRABP–retinoic acid complex is similar to that of the CRBP-retinol complex; both spectra differ substantially from that of the serum retinol-binding protein. The fluorescence of retinoic acid bound to CRABP is about an order of magnitude greater in intensity than for retinoic acid in ethanol. In addition, the fluorescence excitation spectrum of bound retinoic acid shows a small peak of 290 nm, consistent with energy transfer from tryptophan residues to retinoic acid. The main excitation peak is a doublet, with maxima at 350 and 368 nm, in contrast to the smooth peak observed for retinoic acid in ethanol. A definite shift in the emission spectrum

is at maximum 475 nm for bound retinoic acid, in contrast to maximum 490 nm for retinoic acid in ethanol (52).

Tissue and Cellular Distribution

CRABP has now been detected by radioimmunoassay and other assays in the following normal tissues: adrenal, brain (whole), cerebellum, cerebral cortex, hypothalamus, epididymis, eye, kidney, lung, ovary, pituitary, prostate, skin, spinal cord, spleen, testis, thymus, and uterus. Colorectal mucosa and jejunal and ileal muscle contain CRABP, but it cannot be detected in the jejunal and ileal mucosa (1, 25, 55).

The protein has been detected in many fetal tissues including the skeletal muscle (51), which appears to be devoid of CRABP in adult animals. The presence of CRABP in adult liver is still unresolved (e.g. 5, 6).

Two reports using immunohistochemistry or radioimmunassay unit agree that germinal cells of the testis contain CRABP (38, 58). Spermatozoa and the stereocilia of the principal cells throughout the epididymis and epithelia of the distal vas deferens also contain CRABP (58), which was also localized in the islets of Langerhans (35).

Putative Functions

Although the question of whether CRABP is necessary for retinoic acid action has yet not been resolved, some evidence for its involvement in the effects of retinoic acid on differentiation comes from work in cell culture (32, 71). Frequently, cell lines are tested for responsiveness to retinoic acid by using the parameter of differentiation that is accompanied by repression or activation of the expression of specific proteins. Here the question of whether CRABP is necessary for a cell to be responsive to retinoic acid has been examined. Striking correlations between the presence of the binding protein in the cell and its responsiveness to retinoic acid have been reported (70, 72). Teratocarcinoma cells, in which CRABP was detected, differentiated when retinoic acid was added to the medium. However, mutant cells selected for failure to differentiate in response to the retinoic acid did not contain detectable CRABP. Furthermore, it is also of interest that the binding affinity of CRABP to various derivatives of retinoic acid has been shown in various systems sensitive to retinoic acid to reflect at least partially the biological activity of these compounds (16, 33, 82).

The above is contrasted by the human myelocytic leukemia cell line HL-60, which is prompted to differentiate to mature granulocytes in the presence of retinoic acid, but in which CRABP could not be detected (9, 24). Furthermore induction of tissue transglutaminase in mouse peritoneal macrophages by retinoic acid occurs in the absence of CRABP (45). Alternate mechanism(s) not involving CRABP should be taken into consideration to explain the effects

of retinoic acid in these systems. It is quite possible that the role of CRABP is limited to differentiation of epithelia only.

The means by which retinoic acid or genomic expression might exert their influence was investigated in the studies of purified CRABP, retinoic acid, and isolated testicular nuclei and chromatin (81). Cellular retinoic acid–binding protein (CRABP), a potential mediator of retinoic acid action, enables retinoic acid to bind in a specific manner to nuclei and chromatin isolated from testes of control and vitamin A–deficient rats. The binding of retinoic acid was examined after complexing [^{3}H]retinoic acid with CRABP purified from rat testes. The binding was specific, saturable, and temperature dependent. If CRABP charged with nonlabeled retinoic acid was included in the incubation, binding of radioactivity was diminished, whereas inclusion of free retinoic acid, or the complex of retinol with cellular retinol-binding protein (CRBP) or serum retinol-binding protein had no effect. Approximately 4.0×10^4 specific binding sites for retinoic acid were detected per nucleus from deficient animals. The number of binding sites observed was influenced by vitamin A status. Refeeding vitamin A–deficient rats (4 hr) with retinoic acid lowered the amount of detectable binding sites in the nucleus. CRABP itself did not remain bound to these sites, which means that retinoic acid was transferred from its complex with CRABP to the nuclear sites.

Further work is necessary to understand the metabolic consequences of retinoic acid transfer to the genome.

CELLULAR RETINOL-BINDING PROTEIN, TYPE II

Detection and Quantitation

Cellular retinol-binding protein, type II (CRBPII), was first detected as a retinol-binding activity in extracts of whole rat pups less than one day old (48). It eluted from ion exchange columns well separated from other known vitamin A–binding proteins. It has been named cellular retinol-binding protein, type two (CRBPII), because it is of the same size as CRBP and also binds retinol. Most assay systems that separate bound from free ligand are not useful as they cannot distinguish between CRBP and CRBPII. Detection and quantitation have been accomplished by radioimmunoassay with antiserum that does not cross-react with CRBP, CRABP, or RBP.

Physiochemical and Spectral Properties

CRBPII has been purified to homogeniety from rat and partially characterized. It is a single polypeptide chain with a molecular weight of about 16,000, confirmed by its sequence derived from a cloned cDNA (23). The protein exists in two forms, one apparently the N-blocked analogue of the other.

The spectrum of CRBPII complexed with retinol is quite similar to that observed for CRBP-retinol except the wavelengths of maximum absorption of the bound retinol are shifted about 2 nm to 348 and 366 nm (48).

As for CRBP, CRBPII-bound retinol has considerably enhanced fluorescence compared to retinol in organic solution. The degree of energy transfer from tryptophan residues is also quite similar for the two proteins. Such energy transfer is quite dependent on both distance and orientation. It is interesting that both proteins have four tryptophan residues in identical positions (see below).

Tissue and Cellular Distribution

In contrast to CRBP, CRBPII is much more limited in distribution, as determined by radioimmunoassay (48). In the adult, CRBPII is essentially restricted to the small intestine where it is quite abundant, comprising about 1% of the total soluble protein of the jejunal mucosa. The protein first appears in the intestine several days before birth. It is also transiently present in liver, appearing several days before birth but disappearing prior to weaning.

Immunohistochemistry has localized the protein to the villus-associated enterocyte, the mature absorptive cell (21). The protein was not detected in the proliferative cells and immature absorptive cells found in the crypts. The level of protein was highest in absorptive cells at the tips of the villi. CRBPII was not observed in goblet cells, nor was it detectable in the epithelium of the stomach or the colon. In contrast, as discussed in this review and elsewhere, CRBP was not detected in the absorptive cells of the small intestine when antiserum demonstrated to be specific for CRBP was used for immunohistochemical studies.

Putative Functions

The precise cellular location of CRBPII in the small intestine virtually dictates a role for it in the absorption and necessary intestinal metabolism of vitamin A. The high levels of the protein may indicate it serves as a passive reservoir for absorbed retinol, or it may function more actively. As retinol is esterified in the absorptive cell prior to its incorporation into chylomicrons, a potential CRBPII function would be to present retinol to the appropriate esterifying enzyme.

When CRBPII was investigated as a carrier of retinol for esterification by microsomes from rat small intestine, it was observed that retinol was esterified utilizing an endogenous acyldonor but was not esterified with exogenous acyl CoA (56). Most importantly, the esters produced from the endogenous acyldonor were palmitate, stearate, oleate, and linoleate, the same esters known to be produced in vivo also, and the proportions were similar to those observed in vivo.

RELATEDNESS OF INTRACELLULAR VITAMIN A–BINDING PROTEINS

Recent work suggests that the proteins discussed in this review are members of a single family. Rat CRBP and bovine CRABP have been sequenced by conventional methods. The sequence of rat CRBPII has been derived from a cloned cDNA. There is very considerable similarity, as shown in Figure 1 where identical residues are boxed. The primary sequences of CRBP and CRABP can be aligned with no gaps; 75 of 133 comparable residues are identical. Bovine CRABP, with the introduction of the two-residue disjunction and two-residue gap shown, has 47 residues identical with CRBP, 44 with CRABP. Further evidence of the close relationship is shown by the fact that the genes encoding CRBP and CRBPII are closely linked on mouse chromosome 9 (within 3.0 centimorgans) (23). It will be interesting to see if other retinoid-binding proteins are discovered in this family.

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