1

Serum and Cellular Retinoid-Binding Proteins

Dianne Robert Soprano

Introduction

Retinoids include both the natural forms of vitamin A and a large number of synthetic analogues which may or may not display the biological activity of vitamin A. The major natural forms of vitamin A include retinol, retinal and retinoic acid (see Figure 1). All of these forms of vitamin A have a conjugated double bond system which renders them extremely hydrophobic and thus quite water insoluble.

Vitamin A is an essential nutrient in the diet for normal growth (Wolback & Howe, 1925, 1933), differentiation (Wolback & Howe, 1925, 1933), reproduction (Thompson *et al.*, 1964) and vision (Wald, 1968). Vitamin A from the diet is absorbed and transported to the liver. Once in the liver, vitamin A can either be stored or transported to individual cells of target tissues where vitamin A elicits its biological functions. The target tissues include not just the eye but virtually all tissues of the body. In addition, once within the target cell, vitamin A must be transported to its appropriate site(s) of action. Finally, vitamin A may be transported from the target tissue back to the liver or to another target tissue. In each case, an extremely hydrophobic molecule must be transported through an aqueous environment in order to perform its physiological roles.

Since the isolation of the first retinoid binding protein, retinol-binding protein (RBP) in 1968 (Kanai *et al.*, 1968), much information has been obtained related to the identification, characterization and most recently the molecular biology of a number of proteins which bind retinoids. These binding proteins include serum RBP (Goodman, 1984; Soprano & Blaner, 1993); two cellular retinol-binding proteins, CRBP-I (Chytil & Ong, 1984) and CRBP-II (Ong, 1984); two cellular retinoic acid-binding

proteins, CRABP-I (Chytil & Ong, 1984) and CRABP-II (Bailey & Siu, 1988; Giguère *et al.*, 1990*a*); four nuclear retinoic acid receptors, α-RAR (Giguère *et al.*, 1987; Petkovich *et al.*, 1987; Zelent *et al.*, 1989), β-RAR (Zelent *et al.*, 1989; Benbrook *et al.*, 1988; Brand *et al.*, 1988), γ-RAR (Zelent *et al.*, 1989; Krust *et al.*, 1989; Giguère *et al.*, 1990*b*) and RXR (Mangelsdorf *et al.*, 1990); and two unique retinoid binding proteins, cellular retinal-binding protein, CRALBP (Futterman *et al.*, 1977), and interphotoreceptor retinol-binding protein, IRBP (Bridges, 1984), found only in visual tissue. It is becoming more apparent that except for retinyl esters the important biologically active forms of vitamin A may always be bound both intracellularly and extracellularly to an appropriate binding protein in order for vitamin A to be transported through aqueous environments.

Figure 1.1. Chemical formulae for major naturally occurring retinoids. (*a*) All-*trans* retinol; (*b*) all-*trans* retinal; (*c*) all-*trans* retinoic acid.

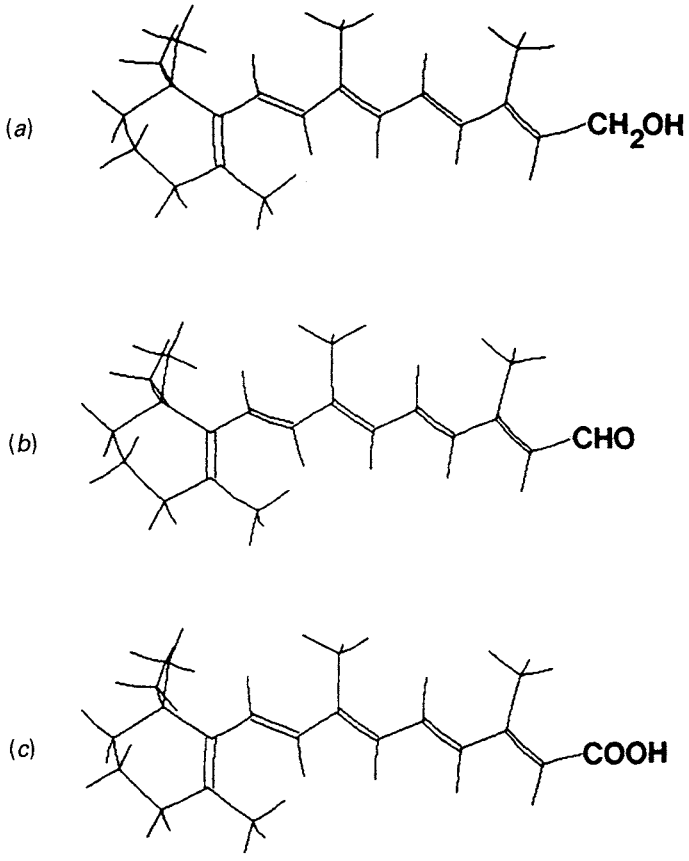

Table 1.1. *Retinoid-binding proteins*

Name	M_r	Endogenous ligand	Site of action	Function
Retinol-binding protein, RBP	21 000	all-*trans* retinol	blood	intercellular transport of retinol
Cellular retinol-binding protein, type I, CRBP-I	15 700	all-*trans* retinol	within cells of vitamin A responsive tissues	intracellular transport of retinol
Cellular retinol-binding protein, type II, CRBP-II	15 600	all-*trans* retinol, all-*trans* retinal	intestinal mucosa cells	absorption and transport of dietary vitamin A within enterocytes
Cellular retinoic acid-binding protein, type I, CRABP-I	15 500	all-*trans* retinoic acid	within cells of vitamin A responsive tissues	intracellular transport of retinoic acid
Cellular retinoic acid-binding protein, type II, CRABP-II	15 500	all-*trans* retinoic acid	developing embryo and adult skin cells	intracellular transport of retinoic acid

The focus of this chapter will be on RBP, CRBP-I, CRBP-II, CRABP-I and CRABP-II and their role in the intercellular and intracellular transport of vitamin A. Table 1 contains a summary of the general properties of each of these retinoid-binding proteins. I will discuss each of these retinoid-binding proteins in the context of their role in the normal metabolism of vitamin A within experimental animals and humans. (The RARs are believed to be involved in the transactivation of retinoic-acid-responsive genes similar to steroid or thyroid hormone receptors and are the subject of Chapter 2 in this volume.) Finally, the discussion of the specialized retinoid-binding proteins found in visual tissue is beyond the scope of this chapter; however, the topic has been reviewed by Chader (1989) and Bridges (1984).

Role of retinoid-binding proteins in the absorption of vitamin A

The natural sources of vitamin A in the diet include retinyl esters, derived from animal sources, and certain plant carotenoid pigments, such as β-carotene. Within the intestinal mucosal cells β-carotene

is converted to retinol in a two-step enzymatic process catalyzed by β-carotene, 15,15′-dioxygenase and retinaldehyde reductase. The first step involves the cleavage of β-carotene at the central double bond by a dioxygenase mechanism to yield two molecules of retinal. In the second step retinal is reduced to retinol (for review see Goodman & Blaner, 1984). On the other hand, retinyl esters are hydrolyzed in the intestinal lumen and the resulting retinol is absorbed into the mucosal cells. Therefore, retinol is the major form of dietary vitamin A in the enterocyte. This retinol is ultimately esterified with free fatty acids and incorporated into chylomicrons.

CRBP-II is a major protein in the small intestine. In fact, more than 1% of the soluble protein isolated from the rat jejunal mucosa is CRBP-II. CRBP-II was initially purified in 1984 by Ong from rat neonatal pups. This protein was found to have a relative molecular mass (M_r) of 15 600 and to bind all-*trans* retinol. More recently, two forms of CRBP-II termed CRBP-(II)A and CRBP-(II)B have been reported (Schaefer *et al.*, 1989). The primary amino acid sequence of these two forms of CRBP-II has been found to be identical. Further analysis of these two forms has demonstrated that CRBP-(II)B is acetylated at the amino terminal while CRBP-(II)A is not. Future experiments are required to determine whether these two forms of CRBP-II have different biological functions.

Both a cDNA (Li *et al.*, 1986) and a genomic clone (Demmer *et al.*, 1987) have been isolated for CRBP-II. Analysis of these clones has provided the primary amino acid sequence of CRBP-II and the structure of the CRBP-II gene. This gene contains four exons which span 0.65 kilobases and three introns with an aggregate length of 19.5 kilobases. CRBP-II has been found to be located on mouse chromosome 9 and human chromosome 3 (Demmer *et al.*, 1987). Analysis of the promoter of CRBP-II *in vitro* by transfection of the CRBP-II promoter linked to a reporter gene into CV-1 cells has demonstrated that CRBP-II gene expression is dramatically up-regulated by retinoic acid in the presence of RXR but not RAR (Mangelsdorf *et al.*, 1991). It remains to be determined whether this up-regulation of CRBP-II gene expression by retinoic acid occurs *in vivo* and is physiologically important.

Analysis of the level of CRBP-II (Ong, 1984) and CRBP-II mRNA (Li *et al.*, 1986; Levin *et al.*, 1987) has revealed an interesting developmental pattern of expression and tissue distribution. CRBP-II and CRBP-II mRNA are present in the fetal rat liver by the sixteenth day of gestation and fetal rat intestine by the nineteenth day of gestation. However, CRBP-II and CRBP-II mRNA levels in the liver abruptly decline after

parturition and remain undetectable throughout adulthood. In the intestine, however, CRBP-II and CRBP-II mRNA levels remain high, such that the level of CRBP-II in the intestine of the adult animal is 500-fold higher than any other tissue in the body which has been examined. Immunohistochemical localization studies have extended these findings by clearly demonstrating that CRBP-II is localized in the absorptive cells of the rat and human small intestine, from the duodenum to the ileum (Crow & Ong, 1985; Ong & Page, 1987). Furthermore, immunoreactive CRBP-II is not observed in the crypts of Lieburkuhn but rather appears abruptly at the crypt–villus junction. Therefore, the amount of immunoreactive CRBP-II continues to increase as the cells migrate up the villi such that the highest concentration of CRBP-II is found in the cells at the tip of the villi. Quick & Ong (1989) have extended these observations by demonstrating that the concentration of CRBP-II in the small intestine may be related to the nutritional requirement of the animal. They found that the concentration of CRBP-II rose in pregnant dams near the end of pregnancy (about day 17) and continued to rise during the lactation period. CRBP-II concentrations in the small intestine of the dam reached a peak at day 21 postpartum of approximately 3-fold greater than the concentration of CRBP-II in nulliparous, non-pregnant female control rats (Quick & Ong, 1989). This pattern of CRBP-II localization in the intestinal cells suggests that CRBP-II plays an important role in the absorption of retinol and the formation of retinyl esters ultimately to become incorporated into the chylomicron.

Two additional findings have further extended the suggestion that CRBP-II plays an important role in the absorption of dietary retinol. First, Ong and colleagues have demonstrated that CRBP-II binds not only retinol but also retinal (MacDonald & Ong, 1987). Furthermore, retinal bound to CRBP-II has been found to be available for reduction by microsomal preparations from the rat intestinal mucosa to retinol (Kakkad & Ong, 1988). This retinol remains bound to CRBP-II; in contrast to free retinol, the CRBP-II bound retinol is not oxidized to retinal. Second, retinol bound to CRBP-II can be esterified by microsomal preparations from rat small intestine mucosal cells in an acyl-CoA-independent fashion (Ong *et al.*, 1987). Taken together, these results suggest that CRBP-II may play an essential role in the accepting of retinal generated from the cleavage of β-carotene and favoring its reduction to retinol; and in the binding of retinol directly absorbed into the enterocyte. CRBP-II then appears to direct the bound retinol to the appropriate esterifying enzymes for the production of retinyl esters which are ultimately incorporated into chylomicrons. Thus, CRBP-II appears to play an active and

specific role in the intestinal metabolism of β-carotene as well as the esterification of retinol for transport to the liver via the chylomicron.

Role of retinoid-binding proteins in the transport of vitamin A between tissues

Retinyl esters incorporated into chylomicrons are ultimately taken up by the hepatocytes of the liver as part of the chylomicron remnants. A detailed discussion of these processes was reviewed by Goodman (1965). Once within the liver, retinol can either be stored as retinyl esters within the stellate cells or be transported from the liver to the target tissues of the body via the plasma.

Vitamin A is transported in plasma as retinol bound to RBP. RBP is a single polypeptide chain which is synthesized as a larger-molecular-mass precursor which is cotranslationally processed (Soprano *et al.*, 1981). This cotranslational processing results in the removal of the signal peptide and the formation of the mature approximately 21 kDa protein which is ultimately secreted. The complete primary amino acid sequence of the mature human RBP (Rask *et al.*, 1979), rat RBP (Sundelin *et al.*, 1985*c*) and rabbit RBP (Sundelin *et al.*, 1985*c*) has been reported. Human, rat and rabbit RBP each contain 182 amino acids with the human and rat displaying 86% sequence homology and the human and rabbit displaying 92% sequence homology.

RBP has one binding site for one molecule of retinol (Muto & Goodman, 1972). Normally, RBP is secreted as the RBP–retinol (holo-RBP) complex (Muto & Goodman, 1972). Three-dimensional analysis of RBP has demonstrated it to have an 8-stranded beta-barrel core that completely encapsulates the retinol molecule (Newcomer *et al.*, 1984). *In vitro*, RBP has been demonstrated to bind various retinoids including retinal, retinoic acid, *cis*-isomers of retinol and retinyl acetate. However, *in vivo* only all-*trans* retinol has been found to be bound to RBP. In plasma, holo-RBP strongly interacts with another plasma protein called transthyretin (TTR) (previously termed prealbumin) and normally circulates as a 1:1 molar RBP–TTR complex (Kopelman *et al.*, 1976; Van Jaarsveld *et al.*, 1973). It is believed that the binding of holo-RBP to TTR reduces glomerular filtration and renal catabolism of RBP. The normal level of RBP in human plasma is about 40–60 $\mu g\ ml^{-1}$.

cDNA clones have been isolated for human RBP (Colantuoni *et al.*, 1983), rat RBP (Sundelin *et al.*, 1985*c*) and rabbit RBP (Lee *et al.*, 1992). Nucleotide sequence analysis of the cDNA clones has confirmed, with slight differences, the previously reported amino acid sequence of mature RBP along with the deduction of the amino acid sequence of the signal

Table 1.2. *Relative levels of RBP mRNA in adult tissues*

Tissues	Relative RBP mRNA level
Liver	100%[a]
Epididymal fat pad	20%[a]
Kidney	5–10%[a]
Lungs	1–3%[a]
Spleen	1–3%[a]
Brain	1–3%[a]
Stomach	1–3%[a]
Heart	1–3%[a]
Skeletal muscle	1–3%[a]
Lacrimal gland	0.1–0.03%[a]
Large intestines	ND[b]
Small intestines	ND
Testes	ND
Pancreas	ND

[a] Relative levels of RBP mRNA are taken from Lee *et al.* (1992), Soprano *et al.* (1986*a*) and Makover *et al.* (1989*a*).
[b] ND, non-detectable by Northern blot assay with the limit of detection at approximately 1% of that of the liver as reported in Soprano *et al.* (1986*a*).

peptide. In addition, genomic clones have also been isolated for human RBP (D'Onofrio *et al.*, 1985) and rat RBP (Laurent *et al.*, 1985). Southern blot analysis and analysis of the genomic clones has demonstrated that the human RBP gene is a single copy gene which spans a region of approximately 10 kilobases. Both rat and human RBP genes consist of six exons and five introns and demonstrate very similar organization. Laurent *et al.* (1985) have observed that each exon of the rat RBP gene corresponds closely to distinct structural elements in the protein structure.

The hepatocytes of the liver are a major site of RBP synthesis and secretion (Soprano *et al.*, 1986*a*). However, using RBP cDNA as a probe, it has been demonstrated that along with the liver there are several extrahepatic sites of RBP mRNA and presumably synthesis of RBP in the adult rat. Table 1.2 contains a summary of the tissues and the relative level of RBP mRNA in each tissue. As can be seen the relative level of RBP mRNA ranges from undetectable (sensitivity of Northern blot assay in early studies was only to a level of RBP mRNA which was approximately 1% of that observed in the liver) to 20% of the level of RBP mRNA in the liver (Soprano *et al.*, 1986*a*; Makover *et al.*, 1989*b*;

Martone *et al.*, 1988). More recently, the lacrimal gland, using the more sensitive assay of RNase protection, has been found to contain RBP mRNA at a level of 0.01 – 0.03% of that observed in the liver (Newcomer *et al.*, 1984). It is likely that some of the tissues previously examined by Northern blot which were found to have an undetectable level of RBP mRNA may actually contain low levels of RBP mRNA. Though the amount of RBP mRNA within many of these tissues is small compared with that in the liver, the composite amount of RBP mRNA and potentially the amount of RBP secreted from extrahepatic tissues into the plasma is quite substantial.

Further analysis of kidney mRNA has demonstrated that kidney poly (A+) RNA can be translated in rabbit reticulocytes *in vitro* to yield *immunoprecipitable* RBP, indicating that kidney-derived RBP mRNA is capable of translation (Soprano *et al.*, 1986*a*). In addition, *in situ* hybridization has demonstrated that RBP mRNA is localized in the S3 segment of the proximal tubule cells of the kidney and is not distributed homogenously throughout the kidney (Makover *et al.*, 1989*a*). Therefore the amount of RBP mRNA within an individual cell of the S3 segment of the proximal tubule cells of the kidney is not small with respect to the amount observed in a liver hepatocyte. It is quite likely that RBP mRNA within other extrahepatic tissues may also be localized within individual cell type(s) of the tissue.

Several studies have examined the expression of RBP during embryonic development of rats (Soprano *et al.*, 1986*b*; Makover *et al.*, 1989*b*; Sklan & Ross, 1987). RBP mRNA can be first observed as early as seven days of gestation localized in the visceral endodermal cells. From the ninth day of gestation until the twentieth day of gestation, RBP mRNA is observed in the visceral yolk sac endoderm. Quantitation of RBP mRNA in the visceral yolk sac from the fourteenth to the twentieth day of gestation indicates that RBP mRNA levels are relatively constant throughout this period of development, averaging approximately 50% of the level observed in the adult liver. In addition, explant cultures of visceral yolk sac have been demonstrated to synthesize immunoprecipitable RBP *in vitro*. This indicates that in the visceral yolk sac RBP mRNA is translated to immunoreactive RBP. In the developing liver, RBP mRNA was first observed at the tenth day of gestation. RBP mRNA levels in the fetal liver increased steadily throughout gestation, reaching a level of 70% of that observed in the adult liver at birth. Finally, RBP mRNA and synthesis of RBP is induced in F9 teratocarcinoma cells which are differentiated to embryoid bodies (which contain visceral endoderm) (Soprano *et al.*, 1988*a*). F9 teratocarcinoma cells are believed to be very

similar to fetuses of 3–5 days' gestation. Taken together, these results suggest that RBP mRNA expression begins very early in fetal development, initially in the visceral endoderm and later in the fetal liver. It is quite likely that the very early fetal expression of RBP mRNA and synthesis of RBP reflects the essential requirement of the developing fetus for retinoids.

RBP synthesis and secretion by extrahepatic tissues has been demonstrated using *in vitro* systems including translation of kidney RNA (Soprano *et al.*, 1986*a*), synthesis and secretion of RBP by explant culture of visceral yolk sac (Soprano *et al.*, 1986*b*) and synthesis and secretion of RBP by F9 teratocarcinoma cells differentiated to embryoid bodies (Soprano *et al.*, 1988*a*,*b*). However, it has been difficult to demonstrate *in vivo* synthesis and secretion of RBP by an extrahepatic tissue which expresses RBP mRNA. This is because once RBP is synthesized and secreted by these extrahepatic tissues the RBP enters the blood and is then indistinguishable from that produced by the liver. Study of the lacrimal gland and the lacrimal gland fluid has offered a unique system to address this question. This is because the lacrimal gland fluid is isolated from the blood by an efficient blood–lacrimal barrier and the lacrimal gland contains proteins primarily synthesized in the lacrimal gland. Recent studies have demonstrated that the lacrimal gland contains RBP mRNA and that the lacrimal gland fluid contains RBP, by using a specific monoclonal antibody (Lee *et al.*, 1992). This is the first demonstration that an extrahepatic site of RBP mRNA actually synthesizes and secretes RBP *in vivo*, implying that other organs which contain RBP mRNA can also synthesize and secrete RBP.

The function of RBP synthesized in each of the extrahepatic tissues is not known. However, RBP synthesized in each of these extrahepatic tissues may play an important role(s) in the overall transport and delivery of retinol in the body. Studies of Lewis and colleagues have demonstrated that there is quantitative recycling of retinol within the body of animals (Lewis *et al.*, 1981). Therefore, we have hypothesized that when retinol leaves an extrahepatic tissue, a new molecule of RBP is synthesized locally, retinol is added to this molecule in the microsomes, and the holo-RBP is secreted into the plasma for delivery of retinol back to the liver or to other extrahepatic tissues (Soprano *et al.*, 1986*a*). Four examples are described below. First, we have proposed that RBP synthesized in the visceral yolk sac (which is a site of true placentation in the rat (Steven & Morris, 1975) may be involved in the delivery of retinol from the mother to the developing fetus (Soprano *et al.*, 1986*b*). Second, we have suggested that RBP synthesized in the kidney may be involved in the recycling of

retinol which is glomerular-filtered back to the liver and other extrahepatic tissues (Soprano *et al.*, 1986*a*). Third, we have hypothesized that RBP synthesized in adipose tissue may be involved in the transport of retinol taken up from the chylomicron or chylomicron remnant by the adipose tissue back to the liver and extrahepatic target tissues (Makover *et al.*, 1989*a*). Fourth, we have hypothesized that RBP synthesized in the lacrimal gland may be involved in the transport of retinol to the cornea of the eye via the lacrimal gland fluid (Lee *et al.*, 1992). Further research is necessary to explore these and other possible hypotheses.

As previously stated, retinyl esters from the diet are transported to the liver via the chylomicron remnant and taken up by the liver hepatocytes. Within the liver, excess retinol is stored principally in the stellate cells as retinyl esters. In cases of insufficient dietary retinol, the stored retinyl esters are released from the stellate cells of the liver (for review, see Hendriks *et al.*, 1987). Recently, it has been suggested that RBP may play a role in the transfer of retinol from the hepatocytes to the stellate cells of the liver (Blomhoff *et al.*, 1985, 1988; Gjoen *et al.*, 1987). However, stellate cells do not contain detectable amounts of RBP mRNA (Yamada *et al.*, 1987) and contain very small amounts of RBP (Blaner *et al.*, 1985). Therefore, at this time we cannot exclude other possible mechanisms for the transfer of retinol, which include: transfer of retinol mediated by direct contact of the hepatocytes and the stellate cells; and transfer between these cell types which involves CRBP. More definitive studies are required to define the mechanism of retinol transfer between the hepatocytes and the stellate cells of the liver.

The plasma level of RBP is highly regulated within individuals and remains quite constant except in extremes of vitamin A nutriture or in disease states. One factor which controls the rate of secretion of RBP and not the rate of biosynthesis of RBP is retinol. The secretion of RBP from the liver is specifically blocked in rats which have been depleted of their retinol stores, resulting in the accumulation of apo-RBP in the liver and a concomitant decline in the plasma level of RBP (Muto *et al.*, 1972). Upon repletion of retinol-depleted rats with retinol, RBP is rapidly secreted from the liver into the plasma (Smith *et al.*, 1973). On the other hand, the *in vivo* rate of RBP synthesis in the liver (Soprano *et al.*, 1982) and the level of RBP mRNA in the liver (Soprano *et al.*, 1986*a*) are not influenced by the retinol status of rats. Similar findings have also been found in the visceral yolk sac (Soprano *et al.*, 1988*b*) and the lacrimal gland (Lee *et al.*, 1992). Analysis of RBP secretion in primary hepatocytes and in Hep G2 cells demonstrates that a low level of RBP secretion occurs in the absence