Acta Medica et Biologica
Vol. 45, No. 4, 171-177, 1997

Isolation and Partial Characterization of Human Placenta Retinol-binding Protein

Hiroko KOSAKA[1], Mamoru ISEMURA[2], Shoji ODANI[3], Takehiko KOIDE[4] and Teruo ONO[5]

[1]Section of Infrared Spectroscopy, Analytical Instruments Laboratory, Niigata University, Niigata; [2]Laboratory of Biochemistry, School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka; [3]Department of Biology, Faculty of Science, Niigata University, Niigata; [4]Laboratory of Life Science, Faculty of Science, Himeji Institute of Technology, Himeji; [5]Department of Biochemistry, Niigata University School of Medicine, Niigata, Japan

Received July 24 1997; accepted November 4 1997

Summary. **Retinol-binding protein (RBP) is the specific blood carrier protein for the transport of retinol (vitamin A). RBP is mainly synthesized in the liver, though several extrahepatic organs including the placenta may also synthesize RBP as high amounts of RBP mRNA have been detected in these. Here we purified human placenta RBP (pRBP) and examined its characteristics in comparison with human plasma RBP (sRBP). pRBP showed cross-immunoreactivity with sRBP, but the difference was noted in the molecular weight as determined by SDS-PAGE. Since the amino-terminal amino acid sequence of pRBP was identical with that of sRBP, the lower molecular weight of pRBP suggests that pRBP is the truncated form in which several carboxyl-terminal amino acid residues of sRBP have been removed. pRBP can also be distinguished from sRBP in the elution pattern on high performance liquid chromatography and in the time course of fluorescence quenching of retinol bound as ligands to RBPs. These data suggest that pRBP may play a specific role in the placenta in connection with reports that extrahepatic RBP may be involved in the retinol transport and recycling.**

Key words—retinol, retinol binding protein, placenta, vitamin A.

INTRODUCTION

Retinoids (vitamin A analogs) play central roles in regulation of cell growth and differentiation.[1] Retinoids are also teratogenic in humans as well as in all animal species. Therefore, transplacental transport of retinoids from the maternal plasma to embryos is believed to be highly regulated.[2] There is evidence that the placental transmission of serum retinol-binding protein (RBP) plays an important role in retinoid transfer to embryos in rats.[3] Also in the rhesus monkey, which has a close similarity to man in placental micromorphology and fetal development, significant parts of the maternal RBP and retinol are transferred to the embryo through the placenta, and RBP is considered necessary in the normal delivery of vitamin A to the fetus.[4]

On the other hand, several extrahepatic organs have been found to contain high amounts of RBP mRNA, suggesting that these tissues may also synthesize RBP, although the liver is the major RBP-synthesizing organ. The rat kidney was found to contain RBP mRNA at a level of 5-10% of that of the liver, and the lung, spleen, brain, heart and skeletal muscle were also found to contain RBP mRNA at levels of 1-3% of that of the liver.[5] In the human placenta RBP mRNA was found to be present at a significant level.[6] Moreover, SDS-PAGE-immunoblotting analysis has revealed that RBP is present in several vitamin A target tissues such as the liver, kidney, adipose tissue and skin.[7] Whether RBPs generated in these tissues are derived from plasma RBP or synthesized by these tissues themselves remains to be determined. In the present work, we purified RBP from the human placenta and examined its several properties in comparison with human plasma RBP.

MATERIALS AND METHODS

Preparation of RBPs

Human placenta RBP was isolated essentially

Correspondence: Hiroko Kosaka, Section of Infrared Spectroscopy, Analytical Instruments Laboratory, Niigata University School of Medicine, Asahimachi-dori 1, Niigata 951, Japan.

according to the method as described for rat liver cellular RBP[8] with a slight modification at the final step (HPLC gel filtration). All steps except for HPLC were carried out at 0-4℃. Human placenta tissues (total 350-450 g) were washed with 0.9% NaCl to remove blood and homogenized in 0.01 M Tris-HCl buffer, pH 7.5. The homogenate was centrifuged at 15,000 g for 15 min and the resulting supernatant liquid was then adjusted to pH 5. After the precipitated material was removed by centrifugation, the resulting supernatant liquid was treated batch-wise with CM-cellulose, CM-32 (Whatman, Maidstone, UK), pre-equilibrated in 0.01 M sodium acetate buffer (pH 5.0). The suspension was stirred for 30 min before CM-32 was removed by filtering through a glass funnel. The yellow filtrate was adjusted to pH 7.0 and concentrated by ultrafiltration. The concentrated material was subjected to gel filtration on a Sephadex G-75 (4.7×90 cm, Pharmacia Fine Chemicals, Uppsala, Sweden). Subsequently, ion-exchange chromatography was performed at pH 8.3 on a column of DEAE-cellulose DE-32(2.1×15 cm, Whatman). HPLC gel filtration was carried out using TSK gel G-2000-SW (0.75×60 cm, Tosoh Corp. Tokyo, Japan). To aid in monitoring the purification, sufficient amounts of all-trans retinol (Sigma Chemical Co. USA) were added to saturate the protein.

Human plasma RBP was isolated by almost the same procedure used for rat serum RBP[9] except for the final HPLC step. Briefly, human plasma (80 ml) was dialyzed against 0.05 M imidazole/acetate buffer, pH 6.0 and then centrifuged at 10.000 g for 20 min. The resulting supernatant was treated by a sequence of three fractionation steps including the following: a) ion-exchange chromatography on DE-32 (3.7×26 cm, Whatman) using stepwise elution with 0.05 M imidazole-acetate, pH 6.0 (buff. A), then with 0.1 M NaCl in buff. A and finally with 0.3 M NaCl in buff. A; b) the final 0.3 M NaCl eluent which contains RBP was subjected to gel filtration on Sephadex G-75, (4.6×73 cm, Pharmacia); and then to c) HPLC gel filtration on TSK gel G-2000-SW (0.75×60 cm, Tosoh).

Fluorescence detection of bound retinol

Fluorescence for the fraction eluted from columns was measured at 25℃ in a Hitachi fluorescence spectrophotometer 204-A, (excitation, 350nm; emission, 480 nm). Fluorescence measurements for the time-dependent change of protein bound retinol were performed with a Hitachi fluorescence spectrophotometer F-3010 in a 0.5-ml four-sided quartz cell at 25℃.

Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane sheets. The sheets were incubated for 15 h at 4℃ with anti-human RBP serum (Seikagaku Corp., Tokyo, Japan) at a dilution of 1 : 1000. The bound antibody was visualized using a 1 : 1000 dilution of goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate and 4-chloronaphthol.[10]

Amino-terminal protein microsequencing

Partially purified RBPs were separated by SDS-PAGE[11] and electro-blotted on to PVDF membrane. The corresponding band stained with Coomassie brilliant blue was excised and subjected to amino-terminal amino acid microsequencing by gas phase Edman degradation on a Shimadzu Inc. PSQ-1 Protein Sequencer. Cystines were not reduced nor alkylated; thus, the location was not determined.

RESULTS

Purification of placenta RBP and comparison of its elution patterns from columns with plasma RBP

When the fraction obtained by CM-32 chromatography was subjected to gel filtration on a column of Sephadex G-75 (total volume 1560 ml) equilibrated with 0.2 M NaCl-0.05 M Tris-HCl (pH 7.5), a prominent peak of fluorescence was observed at an elution volume of 1010 ml as monitored by fluorescence of bound retinol. The peak fractions were collected (total volume 170 ml) and then concentrated by ultrafiltration. In the next step, DE-32 chromatography at pH 8.3 was performed (Fig. 1). When the fluorescence was monitored, two major peaks were observed at 400 ml (Fraction 80) and at 455 ml (Fraction 91). The first peak appeared to be mainly due to retinol bound by cellular retinol-binding protein (CRBP), which was included in a family of lipid-binding proteins with a low-molecular weight of close to 15,000, and the second due to retinol bound by RBP, as judged by their molecular weight on SDS-PAGE and their fluorescence spectra.[12,13] Fractions corresponding to RBP were pooled, concentrated by ultrafiltration and then loaded on to TSK gel G-2000-SW and eluted with 50 mM sodium phosphate buffer (pH 7.1) containing 0.1 M NaCl (Fig. 2a). Partially purified plasma RBP (sRBP) was also subjected to HPLC under the same conditions (Fig. 2b). Data

indicated that placenta RBP was eluted later than sRBP. Thus, placenta RBP appeared to be different from sRBP, and therefore, was designated as pRBP.

SDS-PAGE-immunoblotting analysis

SDS-PAGE of pRBP (obtained after a DE-32 step) showed only one band near 21 kDa, indicating the essential homogeneity of the preparation (Fig. 3a and c). Its mobility was a little faster than that of sRBP, showing that pRBP has a molecular weight smaller by about 500 Da than sRBP (Fig. 3a and c). The western blotting analysis indicated that pRBP had cross-immunoreactivity with sRBP (Fig. 3b).

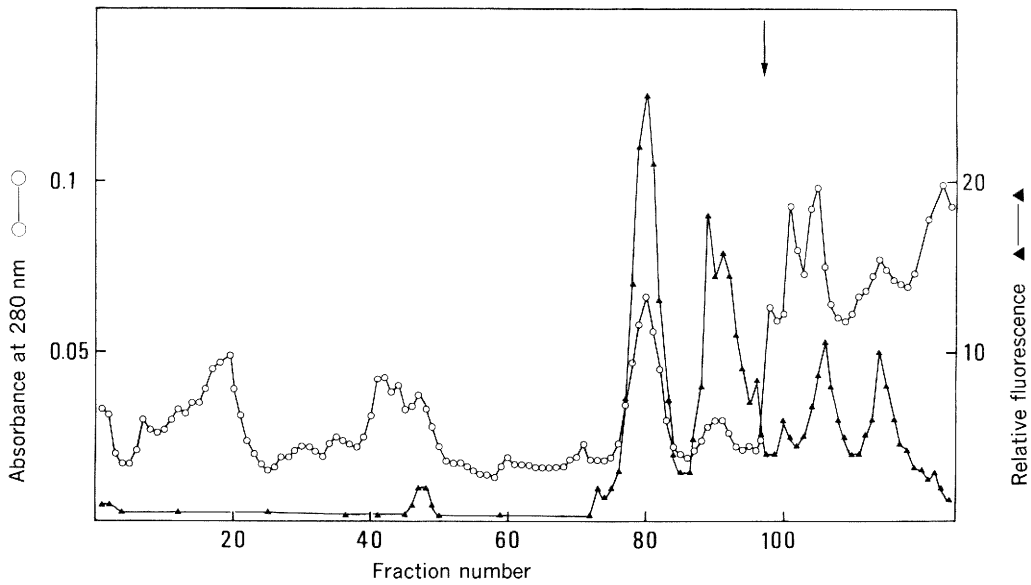

Fig. 1. DEAE-cellulose chromatography of pRBP. After application of a sample, the column (DE-32) was washed with 0.05 M Tris-acetate, pH 8.3 and then eluted with a linear gradient of NaCl from 0 to 0.1 M in 0.05 M Tris-acetate, pH 8.3 (total volume, 500 ml). The arrow shows the start of the second gradient of NaCl from 0.1 to 0.2 M in Tris-acetate, pH 8.3 (total volume, 200 ml). Fractions of 5.0 ml were collected and monitored by absorbance at 280 nm for proteins and by fluorescence with excitation at 350 nm and emission at 480 nm for retinol.

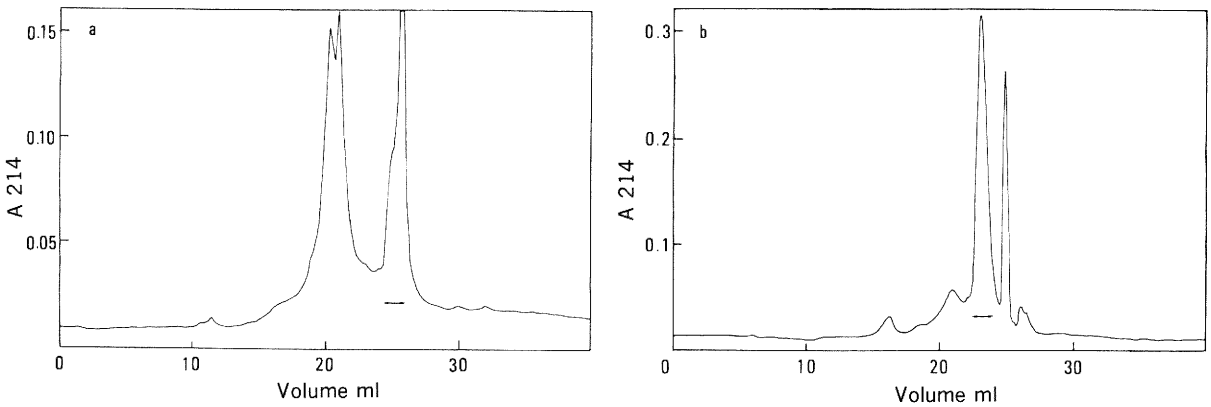

Fig. 2. HPLC of partially purified RBPs (a, pRBP; b, sRBP). Chromatography was performed on a TSK gel G-2000-SW column by using 50 mM sodium phosphate buffer (pH 7.1) containing 0.1 M NaCl. Proteins were monitored by absorbance at 214 nm. Fractions indicated by double headed arrows contained RBPs as judged by SDS-PAGE.

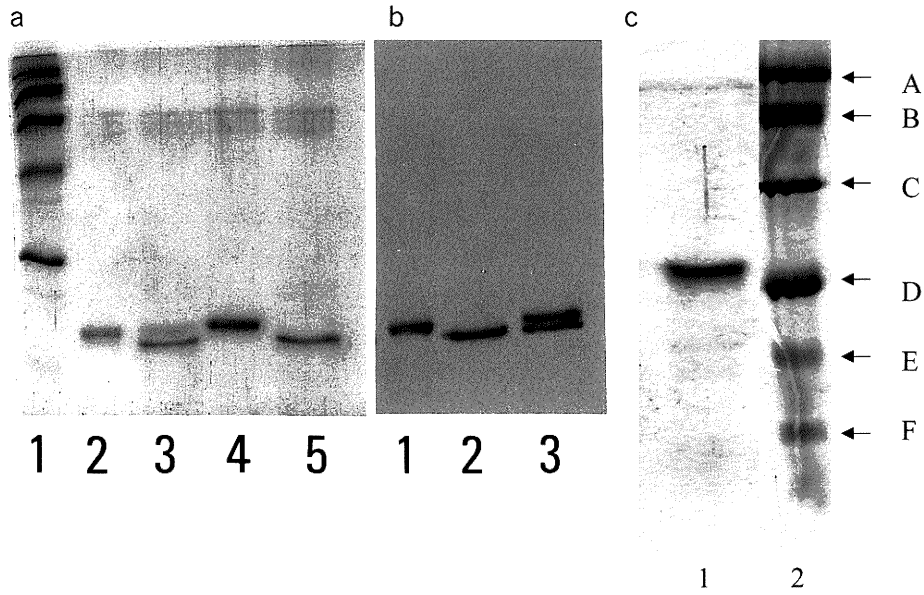

Fig. 3. SDS-PAGE and immunoblotting of RBPs. **a.** Coomassie brilliant blue staining. Lane 1, Size marker proteins (rabbit muscle myosin, 205,000; E. coli β-galactosidase, 116,000; rabbit muscle phosphorylase b, 97,000; bovine serum albumin, 66,000; ovalbumin, 45,000; bovine carbonic anhydrase, 29,000); lanes 2 and 4, sRBP; lane 3, a mixture of sRBP and pRBP; lane 5, pRBP. **b.** Immunostaining. Lane 1, sRBP; lane 2, pRBP; lane 3, a mixture of sRBP and pRBP. **c.** Coomassie brilliant blue staining. Lane 1, pRBP; lane 2, size marker proteins (A, bovine serum albumin, 66,000; B, ovalbumin, 45,000; C, bovine carbonic anhydrase, 29,000; D, soybean trypsin inhibitor, 20,100; E, bovine α-lactalbumin, 14,200; F, bovine aprotinin, 6,500)

Table 1. The amino-terminal sequence of the human placenta and plasma RBPs

	Sequence									
	1	2	3	4	5	6	7	8	9	10
Placenta RBP	Glu	Arg	Asp	–	Arg	Val	Ser	Ser	Phe	Arg
Plasma RBP	Glu	Arg	Asp	–	Arg	Val	Ser	Ser	Phe	Arg
Serum RBP*	Glu	Arg	Asp	Cys	Arg	Val	Ser	Ser	Phe	Arg

Sequence data for the human serum RBP* is cited from Refs. 19 and 26.
Hyphens (–) indicate a lack a major signal in a given cycle.

Amino-terminal protein microsequencing

The determined sequence of the N-terminal 10 amino acid residues of pRBP indicated that it was identical to that of human sRBP (Table 1).

Time-dependent change in fluorescence intensity for pRBP and sRBP

As reported previously for RBPs,[14] there was considerable enhancement of fluorescence when retinol was bound to pRBP. This increase in intensity was about 7 to 8-fold. This enhanced fluorescence intensity was gradually weakened when RBP-retinol was kept exposed to ultraviolet (UV) light in the course of fluorescence measurement as reported previously.[15] Retinol (final concentration 2 μM) was added to either pRBP or sRBP (0.93 μM), and fluorescence was monitored under UV light radiation. The initial fluorescence intensity of sRBP-retinol was about 1.7 times larger than that of pRBP-retinol (Fig. 4). The decrease in the fluorescence intensity was observed for both pRBP-retinol and sRBP-retinol by exposure to UV light. This drop in the retinol fluorescence

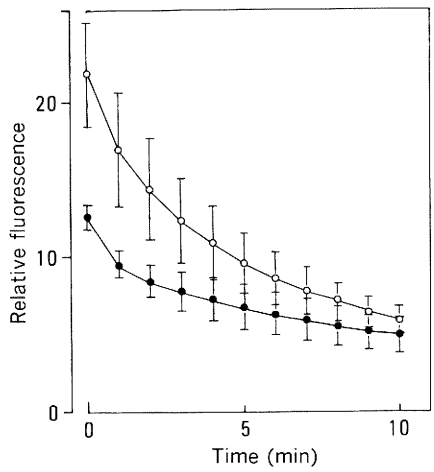

Fig. 4. Time-dependent changes in the fluorescence intensity of pRBP-retinol and sRBP-retinol. All-trans retinol (final concentration 2 μM) was added to either pRBP and sRBP (0.93 μM) in 30 mM phosphate buffer (pH 7.4) containing 70 mM KCl. The change in fluorescence (arbitary units) was measured as a function of UV light radiation time (excitation, 340 nm; emission, 470 nm). Results (pRBP-retinol, ●; sRBP-retinol, ○) represent the means±S.D. of two independent experiments.

signal has been reported by U. Cogan et al.[15] Moreover the decreasing ratio (the fluorescence intensity after 10 min radiation/initial fluorescence intensity) was about 0.40 for pRBP-retinol and about 0.27 for sRBP-retinol. Consequently, the rate of decrease in the fluorescence intensity was more gradual for pRBP-retinol than sRBP-retinol, although the initial fluorescence intensity of pRBP-retinol was smaller than that of sRBP-retinol.

DISCUSSION

sRBP is responsible for the extracellular transport of retinol. This 21 kDa-protein has been well characterized,[6] and both the primary and tertiary structures are known except that no COOH-terminus (residues 176-182) portion has yet been located by X-ray crystallographics.[16] sRBP, composed of a single polypeptide chain, is structually related to a number of extracellular proteins involved in the transport of small hydrophobic compounds, the lipocalines.[17,18] There is a discrepancy in that the amino acid sequence for mature human RBP deduced by nucleotide sequencing consists of 184 amino acids[19] rather than 182 as determined by protein sequencing of the isolated protein. Recently S. Jaconi et al. reported that mass spectrometric analysis of purified RBPs from chronic renal failure and normal sera revealed the presence not only of full-length RBP (183 residues) but also two forms of RBP differing from the native form by the loss of C-terminal Leu (RBP residuse 1-182) and the loss of C-terminal Leu-Leu (RBP residues 1-181).[7] They also reported that truncated RBP (loss of Leu and Leu-Leu) considerably increased in the serum of patients with chronic renal failure and was present at different levels in healthy vitamin A target tissues (liver, kidney, adipose tissue and skin).[7]

In the present work, we isolated RBP from the human placenta and showed that pRBP was smaller than sRBP. Consequently, pRBP appears to be truncated and some C-terminal amino acid residues were probably lost because the N-terminal sequence of ten amino acid residues of pRBP was identical with that of sRBP. Internal truncation in the sequence from Cys-4 to Cys-174 is not likely to occur as judged by the tertiary structure with three S-S linkages (Cys-4 and Cys-160, Cys-70 and Cys-174, Cys-120 and Cys-129).[16]

pRBP obtained was only in the truncated form. Since it is unlikely that the full length form was selectively lost by the purification procedures used, RBP in the normal term placenta is supposed to be entirely truncated. The origin of placental truncated RBP may lie among the following three alternatives. Provided that the origin of pRBP is maternal plasma RBP: 1) only the truncated form is selectively taken up through the placental membrane in the course maternal circulation; 2) the intake mechanism through the placental membrane involves RBP truncation; or, 3) if pRBP is an mRNA product in the placenta, pRBP is a processed form *in situ*, although the mechanism of truncation such as posttranslational cleavage is not known.

Truncated pRBP was shown to bind retinol as does sRBP. Our preliminary experiments suggest that their K_ds (apparent dissociation constants for retinol with both pRBP and sRBP) are similar. pRBP can be distinguished from sRBP in the time course of fluorescence quenching of bound retinol. pRBP is also distinct from sRBP in the elution pattern of HPLC gel filtration. The difference appears to be due to the difference in their protein conformation as well as their primary structures at the C-terminal portion.

The mechanism responsible for retinol uptake into target tissues including the placenta remains unsolved. Specific membrane receptors, which are likely to involve the retinol uptake in the retinal pigment epithelium and Sertoli cells,[20,21] were shown to exist in human placental brush-border mambranes.[22,23] Although this placental RBP receptor has not been

purified completely and its primary structure is not known,[24] it appears to be the RBP receptor in view of the fact that it is sensitive to trypsin, heat and thiol-group-specific reagents while also highly specific. Sivaprasadarao et al. have demonstrated that holo-RBP delivers retinol to the target cells by mediating the RBP receptor and that the resulting apo-RBP remains outside the cell by using isolated human placenta brush border membranes.[22,23] On the other hand, the *in vivo* experiment in the rhesus monkey proved that maternal serum holo-RBP was taken up by the placenta to deliver retinol to the fetus.[4] However, the mechanism for placental transmission of RBP and retinol remains to be established, and further examination of placental RBP is required to clarify this. Determination of its primary structure would be important from the standpoint of the interaction between the RBP receptor and RBP, since this receptor binding site seems to be located in the C-terminal region of RBP.[25] Therefore, the determination of the complete structure of pRBP, which appears to be the truncated form of sRBP, would provide a clue to understanding such a mechanism and its physiological significance.

Acknowledgments. The authors are grateful to Dr. Kuni Takahashi of Takeyama Hospital for her kindness in providing term placenta, Dr. Yoshiaki Kazama of the Department of Internal Medicine, Toyama Medical and Pharmaceutical University for his excellent technical support, and Miss Eri Takahashi for her kind assistance.

REFERENCES

1) Lorraine J. Gudas: Retinoids and vertebrate development. *J Biol Chem* **269:** 15399-15402, 1994.
2) Heinz Nau: Correlation of transplacental and maternal pharmacokinetics of retinoids during organogenesis with teratogenicity. *Methods Enzymol* **190:** 437-448, 1990.
3) Takahashi YI, Smith JE, Goodman DS: Vitamin A and retinol-binding protein metabolism during fetal development in the rat. *Am J Physiol* **233:** E263-E272, 1977.
4) Vahlquist A, Nilsson S: Vitamin A transfer to the fetus and to the amniotic fluid in rhesus monkey (Macaca mulatta). *Ann Nutr Metab* **28:** 321-333, 1984.
5) Soprano DR, Soprano KJ, Goodman DS: Retinol-binding protein messenger RNA levels in the liver and the extrahepatic tissues of the rat. *J Lipid Res* **27:** 166-171, 1986.
6) Blaner WS, Goodman DS: Purification and properties of plasma retinol-binding protein. *Methods Enzymol* **189:** 193-206, 1990.
7) Jaconi S, Rose K, Hughes GJ, Saurat JH, Siegenthaler: Characterization of post-translationally processed forms of human serum retinol-binding protein, altered ratios in chronic renal failure. *J Lipid Res* **36:** 1247-1253, 1995.
8) Ong DE, Chytil F: Cellular retinol-binding protein from rat liver. *J Biol Chem* **253:** 828-832, 1978.
9) Mcguire BW, Chytil F: Three-step purification of retinol-binding protein from rat serum. *Biochim Biophys Acta* **621:** 324-331, 1980.
10) Siegenthaler G: Gel electrophoresis of cellular retinoic acid-binding protein, cellular retinol-binding protein, and serum retinol-binding protein. *Methods Enzymol* **184:** 299-307, 1990.
11) Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:** 680-685, 1970.
12) Kanai M, Raz A, Goodman DS: Retinol-binding protein, the transport protein for vitamin A in human plasma. *J Clin Invest* **47:** 2025-2044, 1968.
13) Ong DE: Purification and partial characterization of cellular retinol-binding protein from human liver. *Cancer Res* **42:** 1033-1037, 1982.
14) Futterman S, Heller J: The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma. *J Biol Chem* **247:** 5168-5172, 1972.
15) Cogan U, Kopelman M, Mokady S, Shinitzky M: Binding affinities of retinol and related compounds to retinol binding proteins. *Eur J Biochem* **65:** 71-78, 1976.
16) Newcomer ME, Jones TA, Aqvist J, Sundelin J, Eriksson U, Rask L, Peterson PA: The three-dimensional structure of retinol-binding protein. *EMBO J* **3:** 1451-1454, 1984.
17) Pervaiz S, Brew K: Homology and structure-function correlations between alpha-1 acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J* **1:** 209-214, 1987.
18) Nagata A, Suzuki Y, Igarashi M, Eguchi N, Tou H, Urade Y, Hayaishi O: Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. *Proc Natl Acad Sci USA* **88:** 4020-4024, 1991.
19) Colantuoni V, Romano V, Bensi G, Santoro C, Costanzo F, Raugei G, Cortese R: Cloning and sequencing of a full length cDNA coding for the human retinol-binding protein. *Nucl Acids Res* **11:** 7769-7776, 1983.
20) Bavik CO, Eriksson U, Allen RA, Peterson PA: Identification and partial characterization of a retinal pigment epithelial membrane receptor for plasma retinol-binding protein. *J Biol Chem* **266:** 14978-14985, 1991.
21) Anonymous: Cell-surface receptors retinol-binding protein. *Nutr Rev* **49:** 218-221, 1991.
22) Sivaprasadarao A, Findlay JBC: The interaction of

retinol-binding protein with its plasma-membrane receptor. *Biochem J* **255:** 561-569, 1988.
23) Sivaprasadarao A, Findlay JBC: The mechanism of uptake of retinol by plasma-membrane vesicles. *Biochem J* **255:** 571-579, 1988.
24) Sivaprasadarao A, Boudjelal M, Findlay JB: Solubilization and purification of the retinol-binding protein receptor from human placental membranes. *Biochem J* **302:** 245-251, 1994.
25) Muto Y: Biological functions of specific binding proteins for vitamin A-like compounds (retinoids). *Vitamins (Japan)* **60:** 481-492, 1986.
26) Rask L, Anundi H, Peterson PA: The primary structure of the human retinol binding protein. *FEBS Letters* **104:** 55-58, 1979.