Characterization of two post-translationally processed forms of human serum retinol-binding protein: altered ratios in chronic renal failure

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Abstract Retinol-binding protein (RBP) is the specific blood carrier for the transport of retinol (vitamin A) to target tissues. As the kidney is involved in RBP metabolism, the analysis of RBP species in the serum of patients with chronic renal failure (CRF) was used as a model to study possible RBP alterations. SDS-PAGE-immunoblotting analysis of normal and CRF sera shows a doublet of RBP bands (band A and band B) near 21 kDa. Mass spectrometric analysis of purified RBPs from CRF and normal sera revealed the presence not only of full-length RBP (183 residues, migrating in band A) but also two forms of RBP differing from the native form by the loss of C-terminal Leu (i.e., RBP_1 (residues 1-182), migrating in band A also) and the loss of C-terminal Leu-Leu (i.e., RBP_2 (residues 1-181), migrating in band B). Interestingly, RBP_2 was considerably increased in the serum of CRF, whereas it was low in normal sera. In healthy retinol target-tissues and in cultured HepG2 cells, RBP_2 levels were significantly and variably present compared to RBP and RBP_1. We propose that these post-translationally modified forms of RBP occur in cells and that after their release into the blood circulation RBP_2 is cleared by the kidney in healthy individuals but accumulates in the serum of CRF patients. RBP_2 may have an important physiological role in retinol transport and/or recycling.—**Jaconi, S., K. Rose, G. J. Hughes, J-H. Saurat, and G. Siegenthaler.** Characterization of two post-translationally processed forms of human serum retinol-binding protein: altered ratios in chronic renal failure. *J. Lipid Res.* 1995. **36:** 1247-1253.

Supplementary key words kidney • retinol • retinol recycling • serum • vitamin A

Retinol (vitamin A) is the precursor of retinoic acid which regulates transcription of specific genes through nuclear retinoic acid receptors (for a review see ref. 1). Retinol is transported in serum to the vitamin A-requiring tissues as a complex with plasma retinol-binding protein (RBP) (for reviews see refs. 2, 3). RBP is mainly synthesized in the liver (2), but several extra-hepatic organs have been found to contain high amounts of RBP mRNA suggesting that these tissues may also synthesize RBP (4). RBP is a well-characterized single polypeptide chain of 21 kDa (5), coded by a single gene (6). The COOH-terminus of RBP has received particular attention because the amino acid sequence predicted from a cDNA clone encoding human RBP is one residue longer (3) than the amino acid sequence from protein analysis (7). The failure to identify the C-terminal Leu of RBP protein has received no explanation to date.

It has been reported that most plasma retinol is recycled about ten times (8) before irreversible utilization (reviewed in ref. 9). The source of RBP involved in retinol recycling remains unknown, but as RBP may also be synthesized in tissues other than the liver (3, 4, 10) this extra-hepatic source of RBP may be involved in retinol recyling (9).

The kidneys play an important role in the metabolism of low molecular weight serum proteins. Normally, small proteins are filtered in the glomeruli and are catabolized in the tubular cells. In renal diseases where the filtration function is impaired, low molecular weight proteins are no longer filtered and their levels increase in the serum (11, 12). Thus, as a consequence of kidney dysfunction, patients with chronic renal disease have elevated levels of RBP and show an increase of the molar ratio RBP:retinol (13).

There is much evidence that cellular uptake of retinol from RBP involves a specific membrane receptor-mediated mechanism (14-17). Recently, it has been suggested that RBP can be internalized by liver cells using receptor-mediated endocytosis and that, after retinol release, the

Abbreviations: RBP, serum retinol-binding protein; full length RBP, RBP (residues 1-183); RBP_1, truncated RBP (residues 1-182) or des(^{183}Leu)RBP; RBP_2, truncated RBP (residues 1-181), des(^{182}Leu-^{183}Leu)RBP; CRF, chronic renal failure; Ig, immunoglobulin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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internalized RBP might be secreted for possible recycling (16, 17). Such a process might alter RBP which, in turn, accumulates in the plasma of CRF patients. The discrepancy observed between RBP-gene and protein with regard to the C-terminal amino acid, and the alteration of RBP levels in patients with CRF prompted us to use CRF sera as a model for studying possible new RBP forms. Two post-translational serum RBP species modified at the C-terminus were characterized and their putative physiological roles in retinol metabolism are discussed.

EXPERIMENTAL PROCEDURES

Materials

The sera obtained from ten normal subjects and from ten patients with chronic renal failure (under hemodialysis) were analyzed immediately or stored at −20°C. Activated CH-Sepharose 4B, Sephadex G-75 were from Pharmacia (Uppsala, Sweden). The rabbit anti-goat IgG Fab' fragments were purchased from Cappel (Cochranville, PA). Nitrocellulose membranes were obtained from Schleicher & Schuell (Zürich, Switzerland). Healthy kidney and liver biopsies were obtained from donors for organ transplantation. Subcutaneous adipose tissue and skin samples were obtained during abdominal plastic surgery.

Antiserum was raised in a goat with RBP purified from normal serum, as described earlier (18), and the IgG fraction was purified on a DEAE-cellulose column after sodium sulfate precipitation (19). RBP amounts were estimated by radial immunodiffusion technique (LC-partigen, Behring, Hoechst-Pharma, Switzerland). All other chemicals were of the highest grade available.

SDS-PAGE-immunoblotting analysis

RBP species were analyzed by SDS-PAGE-immunoblotting with an acrylamide concentration of 15% and 0.2% of N,N'-diallyltartardiamide as crosslinker (20). After electrophoresis, proteins were transferred to nitrocellulose membranes in Tris-glycine-methanol buffer (25 mM:192 mM:20%), pH 8.3, and immunoreactive bands were visualized after treatment with goat anti-human RBP serum, horseradish peroxidase-labeled rabbit anti-goat IgG Fab' fragments and diaminobenzidine as substrate (21, 22).

Purification of serum RBPs from patients with chronic renal failure

RBP was isolated from normal human serum as previously described (23). RBP from serum of patients with chronic renal failure was purified by affinity chromatography using anti-RBP IgG bound to Sepharose 4B. IgG (48 mg) from the goat antiserum was coupled to 15 ml of swollen activated CH-Sepharose 4B at room temperature according to the manufacturer's instructions. Serum of patients, 20 ml, representing 4.3 mg of RBP, was applied to an IgG-Sepharose column (10 × 1 cm) equilibrated in phosphate-buffered saline containing 0.5 M NaCl at pH 7.2. The flow-through serum fraction was reapplied three times to the column. Unbound proteins were removed with 30 ml of equilibration buffer until no more protein was eluted (followed by absorbance at 280 nm). Bound proteins were then desorbed with 3 M potassium thiocyanate in phosphate-buffered saline (without 0.5 M NaCl) at pH 7.2 in a single fraction and dialyzed against 1 mM Tris-HCl, 0.02% NaN_3, pH 8.4. The affinity gel was used several times without significant loss of RBP-binding capacity and was stored in phosphate-buffered saline containing 0.5 M NaCl, 0.02% NaN_3, pH 7.2, at 4°C.

RBP fractions (3.6 mg of total protein/ml) were further purified on a Sephadex G-75 column (1 × 85 cm) equilibrated with the dialysis buffer at a flow rate of 0.1 ml/min. The column was calibrated with proteins of known molecular weight (aprotinin 6.5 kDa, carbonic anhydrase 29 kDa, transthyretin 55 kDa, and purified RBP 21 kDa). Fractions of 2 ml were collected and those containing free RBP were pooled and concentrated using Centricon-10 centrifugal microconcentrators (Amicon, Wallisellen, Switzerland) with a 10 kDa cut-off. Because the RBP from CRF serum obtained in this way showed a doublet at 21 kDa when analyzed by SDS-PAGE, these two RBP species were purified by semi-preparative SDS-PAGE under conditions similar to those of analytical SDS-PAGE except that the gel was 3 mm thick instead of 1.5 mm. Protein bands in the gel were negatively stained with 0.3 M $ZnCl_2$ in water according to Dzandu, Johnson, and Wise (24). The RBP bands were excised, cutting for purity, and the gel pieces were washed with 250 mM EDTA, 250 mM Tris, pH 9.0, to remove excess Zn^{2+}. The proteins were electro-eluted with a Bio-Rad electroeluter according to the manufacturer's instructions.

Edman degradation

Purified RBP from normal serum and from CRF patients (and tryptic peptides isolated from digests of these samples) were analyzed on an Applied Biosystems Model 477A or model 473A sequencer.

Mass spectrometry

Mass spectra were obtained using a Trio 2000 instrument equipped with a 3000 amu rf generator (VG BioTech, Altrincham, UK) and operated under computer control using Lab-Base software. Samples were diluted with methanol and acetic acid in order to obtain the following composition: water–methanol–acetic acid 49.5:49.5:1 (by vol.). They were infused into the electrospray source at 2 μl/min. The range m/z 500–1600 or 900–1800 was

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scanned every 10 sec and multiple spectra were averaged until a satisfactory signal to noise ratio was obtained, usually within about 4 min. Calibration was achieved by infusing a solution of horse heart myoglobin. Masses, in the context of mass spectrometry, refer to the average mass of uncharged species. Electrospray ionization gives rise to several multiply charged forms of proteins. The signal due to each form permits the mass of the protein to be determined, so experimentally determined masses are given ± the standard deviation based on these multiple determinations.

Analysis of normal RBP treated with carboxypeptidase A

The change in electrophoretic mobility of normal purified RBP treated with carboxypeptidase A (Sigma) was analyzed by SDS-PAGE-immunoblotting. Five units of the commercially available enzyme suspension was washed twice with 1 ml water and centrifuged. The pellet was dissolved in a small volume of 2 M NH_4HCO_3 to obtain an enzyme activity of 5 U/ml and 0.1 unit of the same suspension was added to a solution of purified RBP dissolved in 0.2 M N-methylmorpholine acetate buffer containing 1% SDS at pH 8.5 to give a molar ratio of enzyme to substrate of about 1:100. Digestion was performed at room temperature during 5 min. Proteolysis was stopped by adding a solution of EDTA to obtain a final concentration of 5 mM.

Tissues and cells supernatants

Normal human skin was keratomized (180 μm) and treated as described (21). Fresh or frozen normal human biopsies of liver, kidney, adipose tissue, and fresh cultured HepG2 cells (16) were homogenized in ice-cold 100 mM Tris/HCl buffer containing 50 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 10 μM aprotinin, pH 7.4 using a Polytron-PT7 (Kinematica, Luzern, Switzerland) tissue homogenizer. Supernatants were obtained by ultracentrifugation at 100,000 *g* at 4°C for 60 min.

RESULTS

RBP from CRF serum shows a doublet on SDS-PAGE-immunoblotting

Analysis of normal serum shows only one RBP band (25) at 21 kDa (band A), (**Fig. 1, lane 1**), with mobility identical to that of *E. coli*-expressed human RBP (lane 5). CRF serum shows a doublet of RBP bands near 21 kDa (Fig. 1, lane 2, representative of the results with samples from ten different patients). Band A is the member of this doublet with mobility identical to that of RBP from normal serum, and band B corresponds to the faster migrating band. Band A was present in all sera analyzed, whereas band B was particularly strong in CRF sera. The relative amounts of the band B varied a little from one CRF patient to another, but its level was always higher than the very low amounts found in normal serum. The use of N,N'-diallyltartardiamide instead of N,N'-methylenebisacrylamide as cross-linker in the running gel improves the separation of proteins of very similar molecular weight (20).

Purification of RBP from bands A and B

RBPs from CRF serum were purified by affinity chromatography using IgG raised against human normal RBP. About 27% of total RBP in CRF serum was recovered by this procedure. Affinity chromatography isolates both the free RBP and RBP-transthyretin complex, thus a second purification step was needed to dissociate the complex and to separate RBP from transthyretin, which was achieved by gel filtration. From a total of 100 ml of CRF serum (21.5 mg of RBP), 1.1 mg of purified RBP was isolated, representing a yield of 5.2%. SDS-PAGE stained with Coomassie blue showed that purified RBP contained the doublet at 21 kDa (Fig. 1, lane 6) in similar proportions to the unpurified material (Fig. 1, lane 2).

The low recovery of RBP during purification may be due to partial loss of immunoreactivity during immobili-

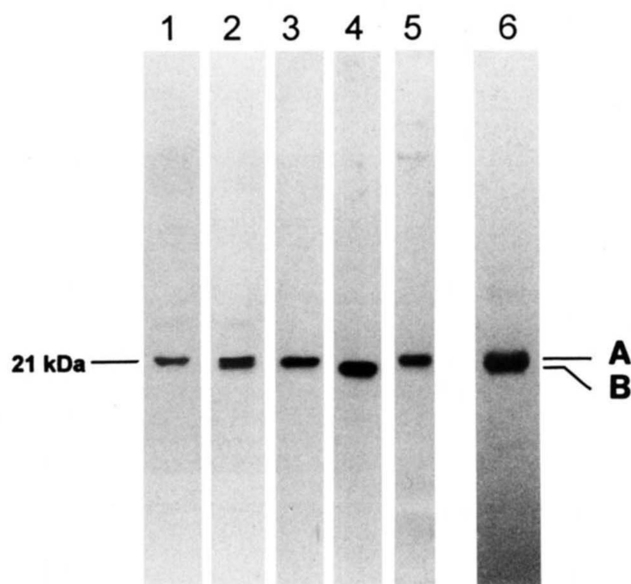

Fig. 1. SDS-PAGE-immunoblotting of RBPs. Aliquots (1 μl) of normal and CRF sera were analyzed by SDS-PAGE-immunoblotting with a goat antiserum to human RBP (to improve protein separation, gel matrix was constituted with 15% acrylamide and 0.2% N,N'-diallytartardiamide as crosslinker, see the Experimental section). Lane 1, normal serum; lane 2, CRF serum showing a doublet of RBP bands, bands A and B; lanes 3 and 4 show the purified band A and band B, respectively; lane 5, *E. coli*-expressed human RBP as standard. Lane 6, RBP obtained after affinity chromatography and Sephadex G-75, proteins are stained with Coomassie blue.

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zation of the anti-RBP antibody on the affinity gel matrix. Indeed, the purification procedure used might introduce bias. However, analysis of RBPs after affinity purification of normal serum closely reflects the ratio of RBP forms found in whole serum, and the affinity purification procedure yielded the major band A with a similar yield (5.4%) to that obtained for the doublet isolated from CRF serum (5.2%).

Semi-preparative SDS-PAGE was used to separate RBP species. By cutting carefully for purity, a complete separation of band A from band B was obtained, as shown by analytical SDS-PAGE-immunoblotting in Fig. 1 (lanes 3 and 4).

Carboxypeptidase A treatment of RBP from normal serum

Purified RBP from normal serum, corresponding mainly to band A, was digested with carboxypeptidase A and degradation products were analyzed by SDS-PAGE-immunoblotting. As shown in **Fig. 2,** the proteolysis almost totally converts band A into band B. Addition of 5 mM EDTA as inhibitor of carboxypeptidase A significantly inhibits the proteolytic activity. This result suggests that band B results from the loss of amino acid residues from the C-terminus of the polypeptide chain of RBP. Carboxypeptidase A rapidly releases nonpolar residues from the C-terminus of polypeptides while asparagine is released more slowly (26). As the last four amino acid residues of RBP are -Arg-Asn-Leu-Leu, one may expect that digestion will be slowed after removing the two Leu residues. Therefore, RBP species from band B should correspond to band A species that have lost the two C-terminal Leu residues. It is interesting to note that SDS-PAGE analysis can separate species that differ by only one amino acid residue. One possible explanation of this surprising result, further confirmed by mass spectrometry, could be that the penultimate lipophilic Leu residue leads to interactions with the gel matrix and its deletion significantly increases the mobility of the protein.

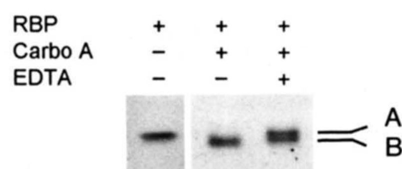

Fig. 2. Digestion of purified RBP from normal serum by carboxypeptidase A. Aliquots of purified RBP (1.9 μg) from normal serum were treated with or without carboxypeptidase A (0.1 units) for 5 min at room temperature. In control experiments, carboxypeptidase A activity was inhibited with 5 mM EDTA (final). Protease activity was followed by measuring the conversion of band A into band B by SDS-PAGE-immunoblotting analysis.

Edman degradation

Bands A and B of RBP from CRF serum were isolated by semi-preparative SDS-PAGE (see above and Fig. 1, lanes 3 and 4). Edman degradation of both bands gave the same clear N-terminal sequence Glu-Arg-Asp-(blank)-Arg-Val-Ser-Ser-Phe-Arg-Val-Lys-Glu-, in complete agreement with the sequence deduced from the cDNA (6); Cys, predicted for cycle four, gave no phenylthiohydantoin as reduction and alkylation of the protein was not performed prior to degradation of the intact protein. Tryptic peptide mapping of both bands (data not shown) produced peptides that could be aligned with most of the sequence deduced from the cDNA sequence, and no peptides were found that could not be aligned with the expected sequence. However, we were unable to isolate the C-terminal predicted peptide -Asn-Leu-Leu, probably because of its very low ultraviolet absorbance; its yield would have been low because of the truncated forms present, and under the conditions used it would have eluted in or close to the front. The tryptic peptide found that was closest to the C-terminus had the sequence -Leu-Ileu-Val-His-Asn-Gly-Tyr-Cys-Asp-Gly-Arg, corresponding to residues 167–177; in this case Cys was positively identified since reduction and alkylation preceded digestion with trypsin.

Mass spectrometry

RBP isolated from normal human serum was subjected to analysis by mass spectrometry (**Fig. 3A**). Groups of signals due to multiply charged forms of two major components and of one minor component are visible with approximate relative intensities of 3:3:1. The determined masses of these components are 21063.46 ± 1.88, 20952.20 ± 1.62 and 20838.06 ± 2.85, which correspond very well to the masses calculated for species containing residues 1–183 (21065.6), i.e., full length RBP, and for the C-terminally truncated forms RBP(1-182), i.e., des(^{183}Leu)RBP (20952.4) and RBP(1-181), i.e., des(^{182}Leu-^{183}Leu)RBP (20839.3), named RBP, RBP_1, and RBP_2, respectively. RBP isolated from CRF serum was also examined by mass spectrometry, (Fig. 3B). Two major components are evident, with masses 20951.98 ± 1.02 and 20839.88 ± 1.46. These values correspond very closely to those calculated for polypeptides containing residues 1–182 (20952.4) and 1–181 (20839.3), RBP_1 and RBP_2, respectively. The full-length chain RBP, the major species identified in normal human serum, is present only in very small amounts in this spectrum.

Peptide mapping and Edman degradation of normal and CRF RBPs and their fragments were performed (discussed above). Results confirmed that both normal and CRF RBPs were very similar (no sequence differences were found) and that both start with N-terminal Glu. The polypeptide chain determination extends at least up to residue 177, so the very close agreement between observed masses and those calculated for the unmodified polypeptide chain shows that the protein as isolated carries no

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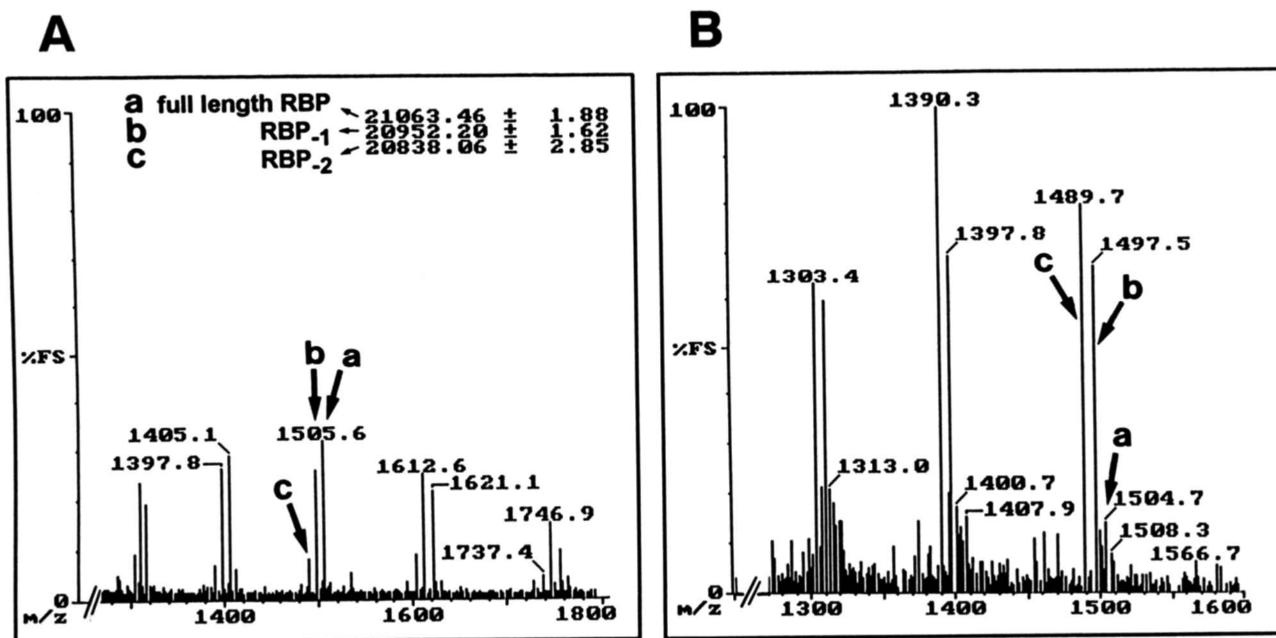

Fig. 3. Mass spectra of purified RBPs. (A) RBP purified from normal serum; (B) RBP purified from CRF serum. Sample preparation and analytical parameters are described in the Experimental Procedures section. Arrows indicate representative triplets of signals (a, b, c), several of which are shown. Each triplet corresponds to a particular charge state of the three RBP species present. Signals a, b, c are due to full-length RBP (residues 1–183), to truncated RBP_1 (residues 1–182), i.e., des(^{183}Leu)RBP, and to truncated RBP_2 (residues 1–181), i.e., des(^{182}Leu-^{183}Leu)RBP, respectively.

post-translation modification such as phosphorylation, sulfation, or glycosylation.

Given the great similarity between full-length RBP and the two truncated species found (the differences concern one and two uncharged Leu residues attributed to RBP_1 and RBP_2, respectively), the ionization efficiencies of the three proteins should be very similar and so the mass spectra should fairly accurately represent the relative amounts of the species present.

As normal serum RBP has very little band B material present (Fig. 1, lane 1), both major components (i.e., full-length RBP and truncated RBP_1) visible in Fig. 3 A must be associated with band A, and the very minor component visible in Fig. 3A, i.e., RBP_2, is the candidate form responsible for band B.

CRF serum possesses bands A and B in similar amounts (Fig. 1, lane 2), and mass spectrometry shows (Fig. 3 B) two major components: truncated forms RBP_1 and RBP_2. For consistency with the interpretation of the results obtained with normal serum, we attribute full-length RBP and RBP_1 to band A and RBP_2 to band B. This is also consistent with the results of carboxypeptidase digestion, which transforms band A into band B (Fig. 2).

Tissue and cell expression of RBP species

RBP species were analyzed by comparing the intensity of bands A and B after SDS-PAGE-immunoblotting of protein extracts of normal tissues and cultured hepatoma carcinoma cells (HepG2) (**Fig. 4**). Liver extract (lane 1), adipose tissue (lane 3), and normal skin (lane 4) contained higher amounts of band B, whereas kidney extract (lane 2) expressed higher levels of band A in addition to an unknown fast migrating band. This band, also present in normal urine (unpublished results), represents partially degraded RBP. Two to three biopsies per tissue were analyzed for RBP species and band B was significantly present in all samples. To assess the extent of proteolysis during handling of biopsies, the cytosolic fraction from fresh HepG2 cells was analyzed for comparison. As seen in lane 5, band B was detected in higher amounts than band A. All samples analyzed contained a cocktail of antiproteases to inhibit nonspecific RBP degradation.

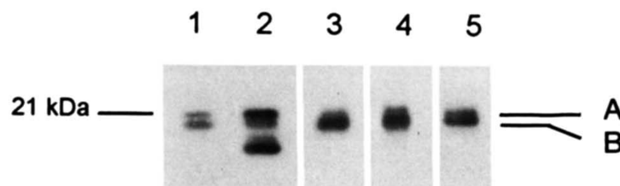

Fig. 4. SDS-PAGE-immunoblotting analysis of RBP species in various normal human tissues and cells. Tissues and cell supernatants obtained as described in Experimental Procedures were subjected to SDS-PAGE-immunoblotting to analyze expression of full-length RBP and RBP_1 (band A) and expression of RBP_2 (band B). Lane 1, liver (21 μg of protein extract); lane 2, kidney (35 μg); lane 3, adipose tissue (66 μg); lane 4, normal skin (146 μg); lane 5, HepG2 cells (44 μg). Representative experiment showing the results obtained with two to three different biopsies per tissue. No significant differences in band intensities were observed.

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DISCUSSION

We postulated that the serum of CRF patients contains large amounts of RBP species involved in retinol recycling that are usually recycled via the kidneys in normal subjects. We have demonstrated by SDS-PAGE-immunoblotting technique, using N,N'-diallyltartardiamide as cross-linker, that serum RBP is present as a doublet (band A and band B) with a molecular weight near 21 kDa. Band A was present in all sera analyzed, whereas band B was particularly elevated in CRF sera. The relative amounts of band B varied slightly from one CRF patient to another, but its expression was always significantly higher than the low amounts found in normal serum. In addition, band B was detected in significant amounts only in sera of patients with kidney diseases (CRF) and not liver diseases (cirrhosis) (unpublished data).

In addition to the native full-length RBP (C-terminus -Asn-Leu-Leu), mass spectrometric analyses of normal and CRF serum showed the presence of two other RBP species differing by the loss of one or two C-terminal amino acid residues, (RBP_1, C-terminus -Asn-Leu) and (RBP_2, C-terminus -Asn), respectively. This analysis also demonstrated that RBP is not phosphorylated, sulfated, or glycosylated.

From the results obtained by Edman degradation, peptide mapping, mass spectrometry, digestion with carboxypeptidase A, and SDS-PAGE-immunoblotting analysis, it is clear that full-length RBP and truncated RBP_1 species migrate together on SDS-PAGE as band A, whereas the truncated RBP_2 species migrates as band B. Our results demonstrate that the major forms of RBP in normal serum are the full-length polypeptide of RBP and the RBP_1 form, whereas CRF serum shows additional high levels of RBP_2 species. The failure to detect Leu 183 in amino acid sequence analysis reported in previous studies (3, 7) may now be explained; indeed, the RBP used for sequencing was from the urine of patients with tubular proteinuria (27) which contains high levels of the 1-182 species (unpublished observation).

Interestingly, truncated RBP_2 was also present at different levels in healthy vitamin A target tissues and in HepG2 cells, suggesting a specific cell processing of RBP by a carboxypeptidase. It seems that this proteolytic activity must be specific to the removal of the two Leu, as no RBP species of lower molecular masses was detected except in the kidney, a tissue involved in protein catabolism. Whether the RBP_2 generated in the cell is derived from serum RBP or from RBP synthesized by the cell remains to be determined.

As the C-terminal segment of RBP is flexible and located at the surface of the molecule (5), it could be the signal domain involved in uptake/release mechanisms of RBP which is further processed by a specific carboxypeptidase A. A similar mechanism was reported for a membrane carboxypeptidase A which inactivates neuropeptides by lowering their interaction with receptors (28). Moreover, transmembrane carboxypeptidases were involved in precursor processing within the secretory pathway (29, 30). Further studies are necessary to determine whether or not the presence of two post-translational RBP forms in CRF serum is due to the complex health status of these patients. Interestingly, fresh or CRF serum left at room temperature for 2 days did not show any additional RBP bands of lower molecular masses on SDS-PAGE, and the intensity of band B did not increase significantly with length of serum storage. These observations strongly suggest that RBP_2 is not formed by unspecific proteolysis in CRF serum. Although the role of RBP_2 in the transport of retinol remains unclear, it seems likely that RBP_2 is formed in tissues (and in HepG2 cells in vitro), and released into the blood circulation where, in healthy individuals, it is rapidly cleared by the kidney, explaining its low levels in normal serum. In patients with CRF, RBP_2 accumulates in their serum due to the alteration of kidney function such that RBP_2 is no longer catabolized by this organ. This hypothesis is strengthened by preliminary observations showing that RBP_2 levels decrease rapidly to normal levels within 2–3 days after kidney transplantation (manuscript in preparation). We thus suggest that RBP_2 may play an important physiological role in retinol transport and/or recycling. ■

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REFERENCES

1. Lohnes, D., A. Dierich, N. Ghyselinck, P. Kastner, C. Lampron, M. LeMeur, T. Lufkin, C. Mendelsohn, H. Nakshatri, and P. Chambon. 1992. Retinoid receptors and binding proteins. *J. Cell Sci.* **103 Suppl. 16:** 69–76.
2. Goodman, D. S. 1984. Plasma retinol-binding protein. *In* The Retinoids. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, Inc., New York. 41–88.
3. Blaner, W. S. 1989. Retinol-binding protein: the serum transport protein for vitamin A. *Endocr. Rev.* **10:** 308–316.
4. Soprano, D. R., K. J. Soprano, and D. S. Goodman. 1986. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J. Lipid Res.* **27:** 166–171.

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5. Newcomer, M. E., T. A. Jones, J. Åqvist, J. Sundelin, U. Eriksson, L. Rask, and P. A. Peterson. 1984. The three-dimensional structure of retinol-binding protein. *EMBO J.* **3:** 1451–1454.
6. Colantuoni, V., V. Romano, G. Bensi, C. Santoro, F. Costanzo, G. Raugei, and R. Cortese. 1983. Cloning and sequencing of a full length cDNA coding for human retinol-binding protein. *Nucleic Acids Res.* **11:** 7769–7776.
7. Rask, L., H. Anundi, J. Fohlman, and P. A. Peterson. 1987. The complete amino acid sequence of human serum retinol-binding protein. *Ups. J. Med. Sci.* **92:** 115–146.
8. Lewis, K. C., M. H. Green, J. B. Green, and L. A. Zech. 1990. Retinol metabolism in rats with low vitamin A status: a compartmental model. *J. Lipid Res.* **31:** 1535–1548.
9. Blomhoff, R., M. H. Green, J. B. Green, T. Berg, and K. R. Norum. 1991. Vitamin A metabolism: new perspectives on absorption, transport, and storage. *Physiol. Rev.* **71:** 951–990.
10. Tsutsumi, C., M. Okuno, L. Tannous, R. Piantedosi, M. Allan, D. S. Goodman, and W. S. Blaner. 1992. Retinoids and retinoid-binding protein expression in rat adipocytes. *J. Biol. Chem.* **267:** 1805–1810.
11. Mogielnicki, R. P., T. A. Waldmann, and W. Strober. 1971. Renal handling of low molecular weight proteins. I. L-Chain metabolism in experimental renal disease. *J. Clin. Invest.* **50:** 901–909.
12. Berggård, I. 1970. Plasma proteins in normal human urine. *In* Proteins in Normal and Pathological Urine. Y. Manuel, H. Revillard, and H. Beutel, editors. Karger, Basel. 7–19.
13. Smith, F. R., and D. S. Goodman. 1971. The effects of diseases of the liver, thyroid, and kidneys on the transport of vitamin A in human plasma. *J. Clin. Invest.* **50:** 2426–2436.
14. Båvik, C-O., U. Eriksson, R. A. Allen, and P. A. Peterson. 1991. Identification and partial characterization of a retinal pigment epithelial membrane receptor for plasma retinol-binding protein. *J. Biol. Chem.* **266:** 14978–14985.
15. Båvik, C-O., C. Busch, and U. Eriksson. 1992. Characterization of a plasma retinol-binding protein membrane receptor expressed in the retinal pigment epithelium. *J. Biol. Chem.* **267:** 23035–23042.
16. Malaba, L., G. M. Kindberg, K. R. Norum, T. Berg, and R. Blomhoff. 1993. Receptor-mediated endocytosis of retinol-binding protein by liver parenchymal cells: interference by radioactive iodination. *Biochem. J.* **291:** 187–191.
17. Senoo, H., S. Smeland, L. Malaba, T. Bjerknes, E. Stang, N. Roos, T. Berg, K. R. Norum, and R. Blomhoff. 1993. Transfer of retinol-binding protein from HepG2 human hepatoma cells to cocultured rat stellate cells. *Proc. Natl. Acad. Sci. USA.* **90:** 3616–3620.
18. Siegenthaler, G., and J-H. Saurat. 1987. Loss of retinol-binding properties for plasma retinol-binding protein in normal human epidermis. *J. Invest. Dermatol.* **88:** 403–408.
19. Johnstone, A., and R. Thorpe. 1982. Purification of immunoglobulins, constituent chain and fragments. *In* Immunochemistry in Practice. Blackwell Scientific Publications, Oxford, England. 41–76.
20. Hahn, E. C., and P. S. Hahn. 1987. Properties of acrylamide gels cross-linked with low concentrations of N,N'-diallyltartardiamide. *J. Virol. Methods.* **15:** 41–52.
21. Siegenthaler, G., I. Tomatis, D. Chatellard-Gruaz, S. Jaconi, U. Eriksson, and J-H. Saurat. 1992. Expression of CRABP-I and -II in human epidermal cells. Alteration of relative protein amounts is linked to the state of differentiation. *Biochem. J.* **287:** 383–389.
22. Siegenthaler, G. 1990. Gel electrophoresis of cellular retinoic acid-binding protein, cellular retinol-binding protein, and serum retinol-binding protein. *Methods Enzymol.* **184:** 299–307.
23. Kanai, M., A. Raz, and D. S. Goodman. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47:** 2025–2044.
24. Dzandu, J. K., J. F. Johnson, and G. E. Wise. 1988. Sodium dodecyl sulfate-gel electrophoresis: staining of polypeptides using heavy metal salts. *Anal. Biochem.* **174:** 157–167.
25. Siegenthaler, G., and J-H. Saurat. 1987. Retinol-binding protein in human serum: conformational changes induced by retinoic acid binding. *Biochem. Biophys. Res. Commun.* **143:** 418–423.
26. Allen, G. 1981. Determination of the carboxy-terminal residue. *In* Laboratory Techniques in Biochemistry and Molecular Biology. Sequencing of Proteins and Peptides. T. S. Work and R. H. Burdon, editors. Elsevier, Holland. vol. 9: 38–42.
27. Peterson, P. A., and I. Berggård. 1971. Isolation and properties of a human retinol-transporting protein. *J. Biol. Chem.* **246:** 25–33.
28. Bunnett, N. W., S. M. Goldstein, and P. Nakazato. 1992. Isolation of a neuropeptide-degrading carboxypeptidase from the human stomach. *Gastroenterology.* **102:** 76–87.
29. Fuller, R. S., A. J. Brake, and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science.* **246:** 482–486.
30. Cooper, A., and H. Bussey. 1992. Yeast Kex1p is a Golgi-associated membrane protein: deletions in a cytoplasmic targeting domain result in mislocalization to the vacuolar membrane. *J. Cell Biol.* **119:** 1459–1468.

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