Cellular retinol-binding protein (type two) is abundant in human small intestine

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Abstract Human small intestine was found to contain a retinol-binding protein similar to the gut-specific cellular retinol-binding protein, type two [CRBP (II)], described in the rat. This newly detected human protein was immunochemically distinct from human cellular retinol binding protein previously described but immunochemically similar to rat CRBP (II). The partially purified protein bound retinol and exhibited fluorescence excitation and emission spectra distinct from those spectra for retinol bound to pure human CRBP but similar to the spectra for retinol bound to rat CRBP (II). Human CRBP (II) could be localized to the villus-associated enterocytes by immunohistochemistry, using antiserum against rat CRBP (II). The protein was abundant representing 0.4% of the total soluble protein in a jejunum mucosal extract. This protein may play an important role in the absorption and necessary intestinal metabolism of vitamin A.—**Ong, D. E., and D. L. Page.** Cellular retinol-binding protein (type two) is abundant in human small intestine. *J. Lipid Res.* 1987. **28:** 739—745.

Supplementary key words enterocytes • rat cellular retinol-binding protein • immunohistochemistry • vitamin A

Vitamin A is required not just for vision but for the maintenance of proper differentiation of most, if not all, of the epithelial tissues of animals (1). Consequently, upon ingestion, vitamin A must be absorbed and distributed, not just to the eye and the liver, the central storage organ, but to virtually all tissues of the body. The physiological forms of vitamin A implicated in function, transport or storage include retinol, retinoic acid, and fatty acyl esters of retinol. All of these forms are quite hydrophobic. This hydrophobicity is circumvented by the existence of several carrier proteins that specifically bind and solubilize these different compounds (except for retinyl esters).

Six such proteins have been discovered and well characterized (reviewed in ref. 2). These six are: retinol-binding protein, RBP, found in serum (3); cellular retinol-binding protein, CRBP, and cellular retinoic acid-binding protein, CRABP, found within cells that utilize vitamin A (4); cellular retinal-binding protein, CRALBP, and interphotoreceptor retinol-binding protein, IRBP, both unique to visual tissue (5, 6); and cellular retinol-binding protein, type two, CRBP (II), present in the small intestine (7, 8). These proteins appear to transport their noncovalently bound ligands, either extracellularly or intracellularly, to sites of action or metabolism.

All of the above proteins except CRBP (II) have been shown to be present in the appropriate human tissues. CRBP (II) has been described only for rat (7). It is a small protein of molecular weight 16,000 that has been demonstrated to have retinol as an endogenous ligand. In the adult rat, CRBP (II) was found almost solely in the small intestine, restricted to the villus-associated enterocytes, strongly suggesting a role in vitamin A absorption (7, 8). Interestingly, it has considerable sequence homology with the more widely distributed CRBP, with 55% identity (9). Here we present evidence that human small intestine also contains a cellular retinol-binding protein, type two, that is distinct from human CRBP previously described (10). This new protein was localized in the mature absorptive cells in the jejunum.

METHODS

Human tissue

Human tissue used for demonstration of the presence of human CRBP (II) was obtained at autopsy from subjects who died of trauma. Jejunal samples, either scraped mucosa or sections including muscle, were taken. Tissue samples were removed at time of autopsy and frozen in air-tight containers at −70°C until preparation for study. Postmortem interval to collection varied from 5 to 32 hr.

Specimen slides examined by immunohistochemical techniques were prepared from pre-existing blocks of formalin-fixed paraffin-embedded tissue obtained by endoscopic biopsy as part of the clinical evaluation of abdominal pain and diarrhea. The histologic appearance was normal.

Abbreviations: CRBP, cellular retinol-binding protein; RBP, retinol-binding protein; PBS, 0.14 M NaCl in 0.01 M sodium phosphate, pH 7.5.

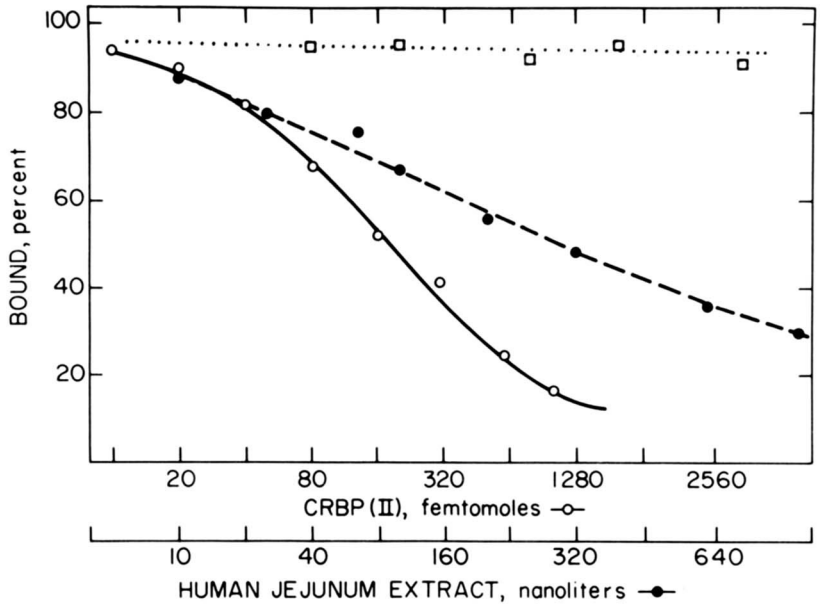


Fig. 1. Radioimmunoassay for rat CRBP (II) indicating presence of cross-reacting material in extracts of human jejunal mucosa. The steeper displacement curve was produced when increasing amounts of pure rat CRBP (II) were added to a standard incubation mixture containing ^{125}I-labeled CRBP (II) and antiserum against rat CRBP (II). Radioactivity precipitated by addition of immobilized protein A was determined and expressed as percent of radioactivity precipitated when no rat CRBP (II) was added (○). The shallower curve was produced when increasing amounts (expressed in nanoliters) of an extract of soluble protein of human jejunal mucosa were added to the same incubation mixture (●). The placement of the scale of the abcissa for this addition in relation to the scale for pure rat CRBP (II) was made arbitrarily. No displacement was observed for pure human CRBP (□). The scale on the abscissa for CRBP (II) should be multiplied by 10 to produce the correct scale for human CRBP.

Collection of samples was approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Tissue extracts

Samples of jejunal mucosa were homogenized in 4–10 volumes (wt/vol) of 0.14 M NaCl in 0.01 M sodium phosphate, pH 7.5 (PBS), using a Polytron homogenizer (Brinkman Instruments, Inc., Westbury, NY). The homogenate was centrifuged at 105,000 *g* for 60 min and the supernatant liquid was collected. This extract was used after appropriate dilution for investigation of immunoreactive material.

For examination of retinol binding, 93 g of jejunum (including muscle) from a 38-year-old male was minced then homogenized in 10 volumes (v/w) of acetone at −20°C in a Waring blender. The acetone powder was recovered by filtration and rehomogenized in the same volume (above) of acetone. The powder was recovered by filtration and dried under vacuum. The powder was stored at −20°C until use.

Soluble proteins were extracted from 6.75 g of acetone powder (equivalent to 41.5 g of jejunum) by suspending the powder in 100 ml of PBS, homogenizing in a Waring blender, then stirring for 30 min at 4°C. The mixture was

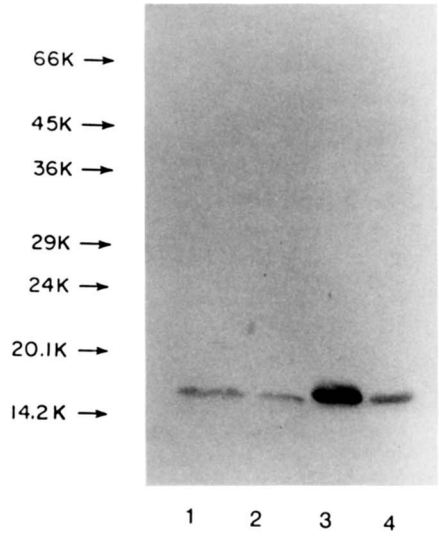


Fig. 2. Detection of immunoreactive protein in an extract of human jejunal mucosa. Aliquots of mucosal extract and of pure rat CRBP (II) were subjected to electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel system and then transferred to nitrocellulose. Immunoreactive proteins were detected as described in Methods. Migration position of proteins of defined molecular weight are shown at the arrows. Lanes 1 and 2, 10 and 20 μliters extract; 3 and 4, 5 and 2.5 μg pure rat CRBP (II).

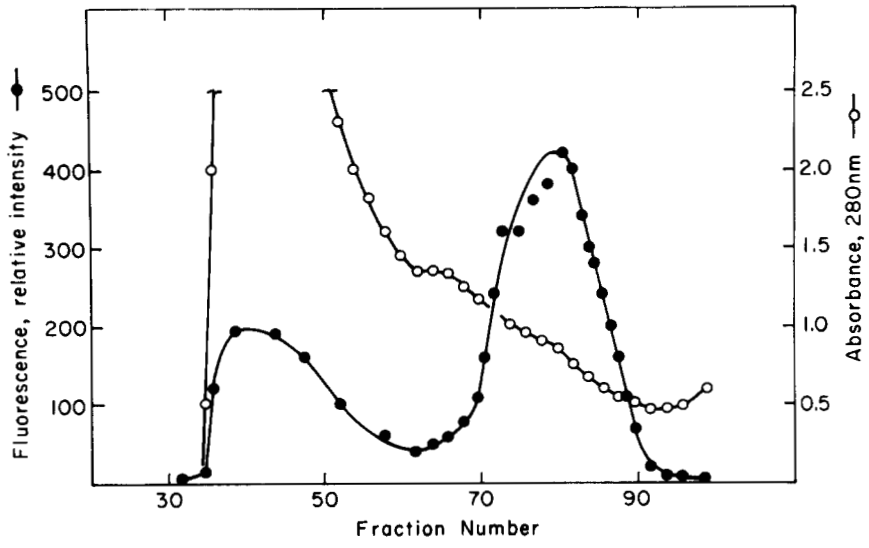


Fig. 3. Detection of the retinol-binding protein after gel filtration. An aliquot of soluble protein from human jejunum was submitted to gel filtration on Sephadex G-75. Protein was monitored at 280 nm. Retinol-like fluorescence (excitation 348, emission 490 nm) was determined after addition of 100 nmol retinol to each fraction of 15 ml.

centrifuged at 24,000 rpm for 25 min and the supernatant liquid was collected. The well-packed pellet was rehomogenized in 100 ml of PBS and stirred for 30 min. The supernatant liquid was collected after centrifugation as above and combined extracts were concentrated by ultrafiltration before gel filtration.

Detection of immunoreactive material

Diluted aliquots of extracts of jejunal mucosa were examined by the radioimmunoassay for rat CRBP (II) as previously described for extracts of rat tissue (7).

Immunoreactive proteins were also demonstrated after

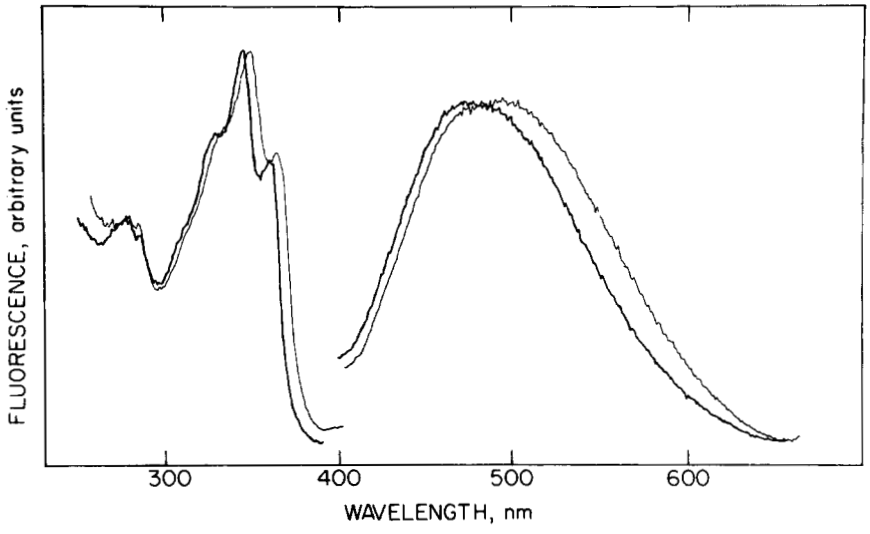


Fig. 4. Fluorescence excitation and emission spectra of retinol bound to the putative human CRBP (II) compared to retinol bound to pure human CRBP. The bold line shows the corrected fluorescence excitation and emission spectra obtained from fractions 72–90 (Fig. 3). For the excitation spectrum, emission was monitored at 490 nm. For the emission spectrum, excitation was at 348 nm. The thinner line shows the corrected fluorescence spectra for retinol bound to pure human CRBP with emission monitored at 510 nm and excitation at 350 nm.

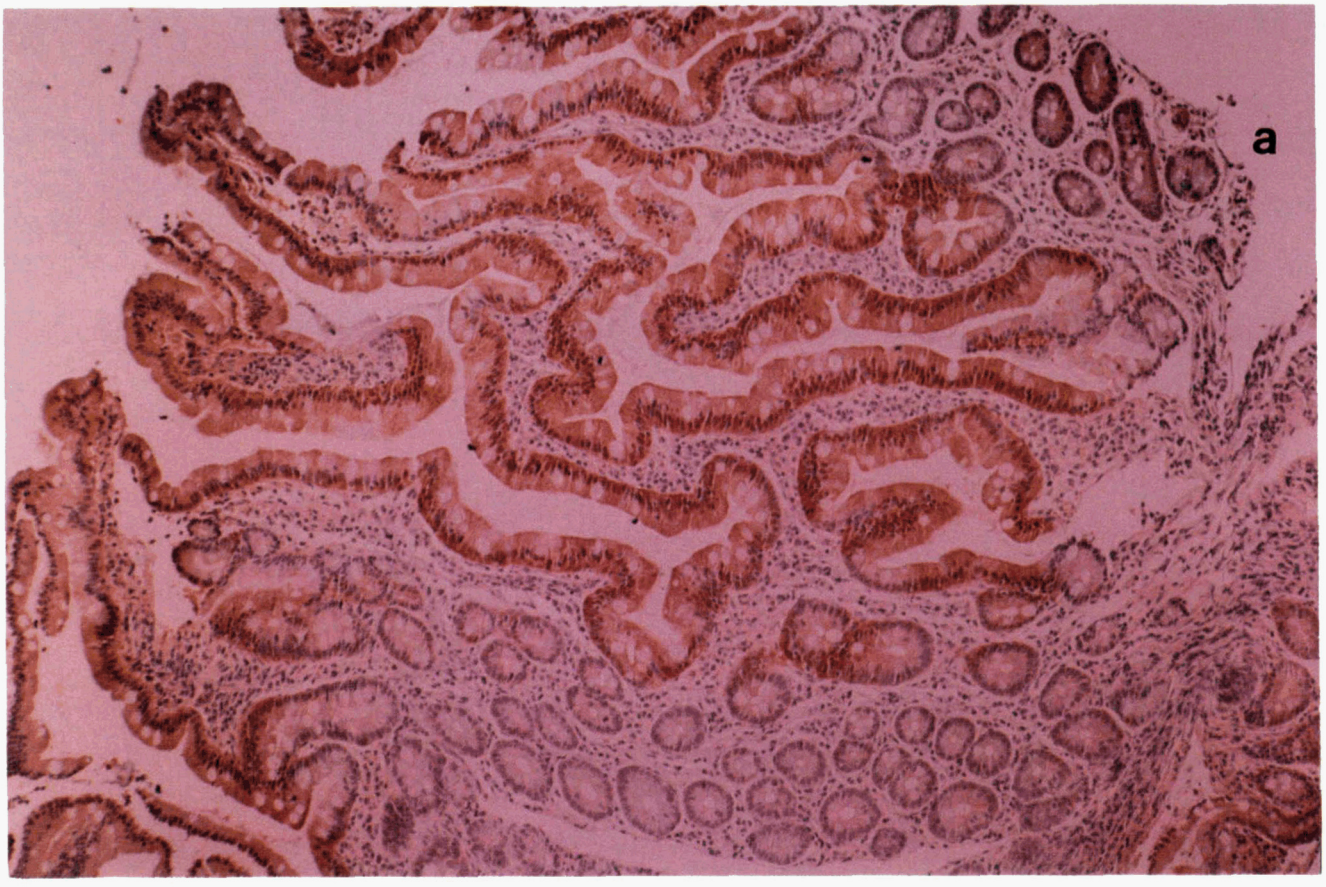


Fig. 5. a: low power view of normal proximal jejunal biopsy marked immunochemically for CRBP (II), represented by brown deposits. Villi extend to left and are covered with cells with positively staining cytoplasm. Note that crypts are negative (120 ×). b and c: Higher power views of 5a. Dense marking of jejunal villus epithelial cells, particularly in basal epithelium, is evident. Note that staining of goblet cells is not present, including the basal (between nucleus and basement membrane) portion of cytoplasm as seen in center of picture (5b, 490 ×; 5c, 1200 ×).

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% gel) by a modification of the method of Towbin, Stachelin, and Gordon (11) as previously described (8). Immunohistochemical localization was by the ABC method described by Hsu, Raine, and Fanger (12), modified as described for rat CRBP (II) with the exception that the antiserum was diluted 1/250 rather than 1/1000 (8).

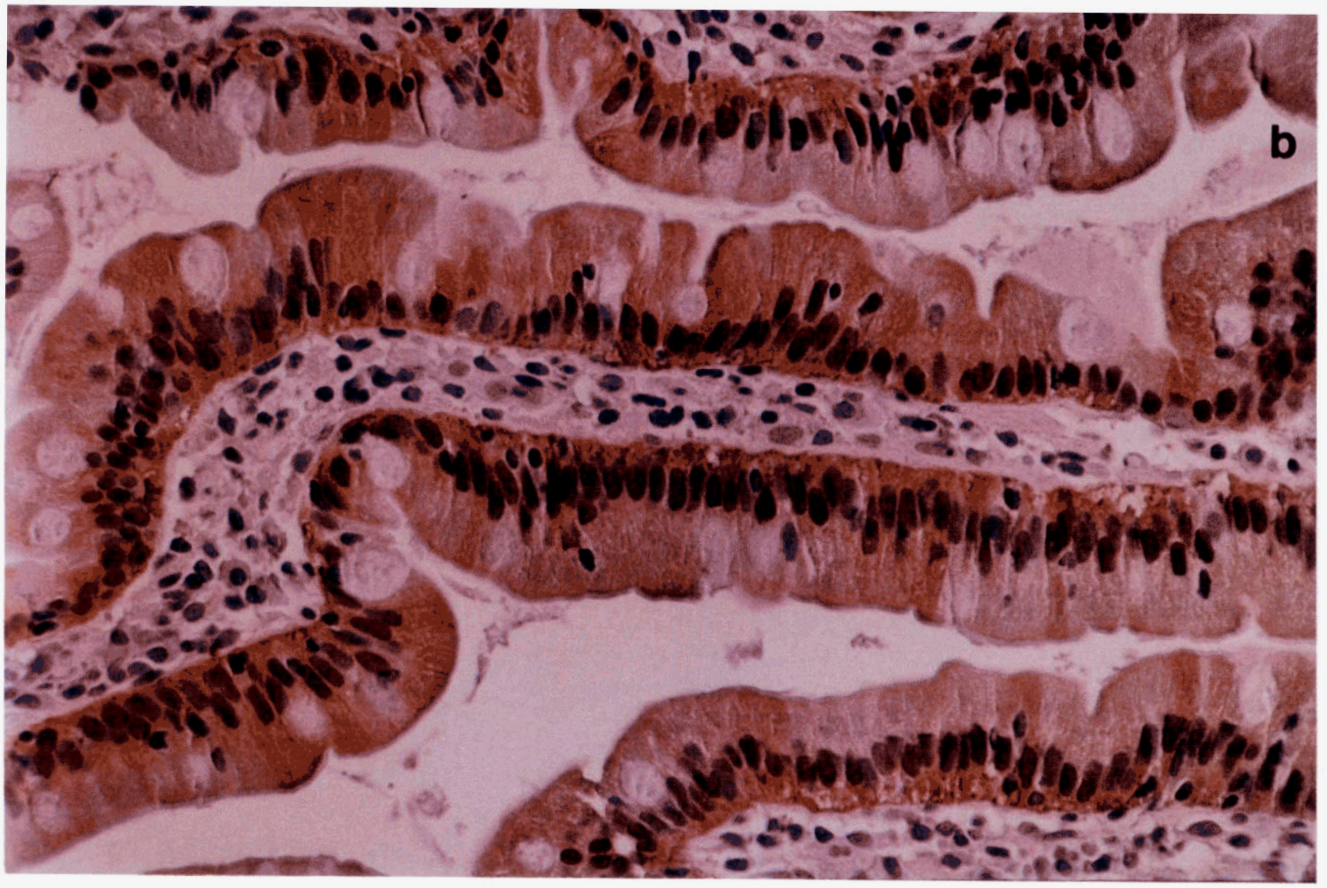
Gel filtration

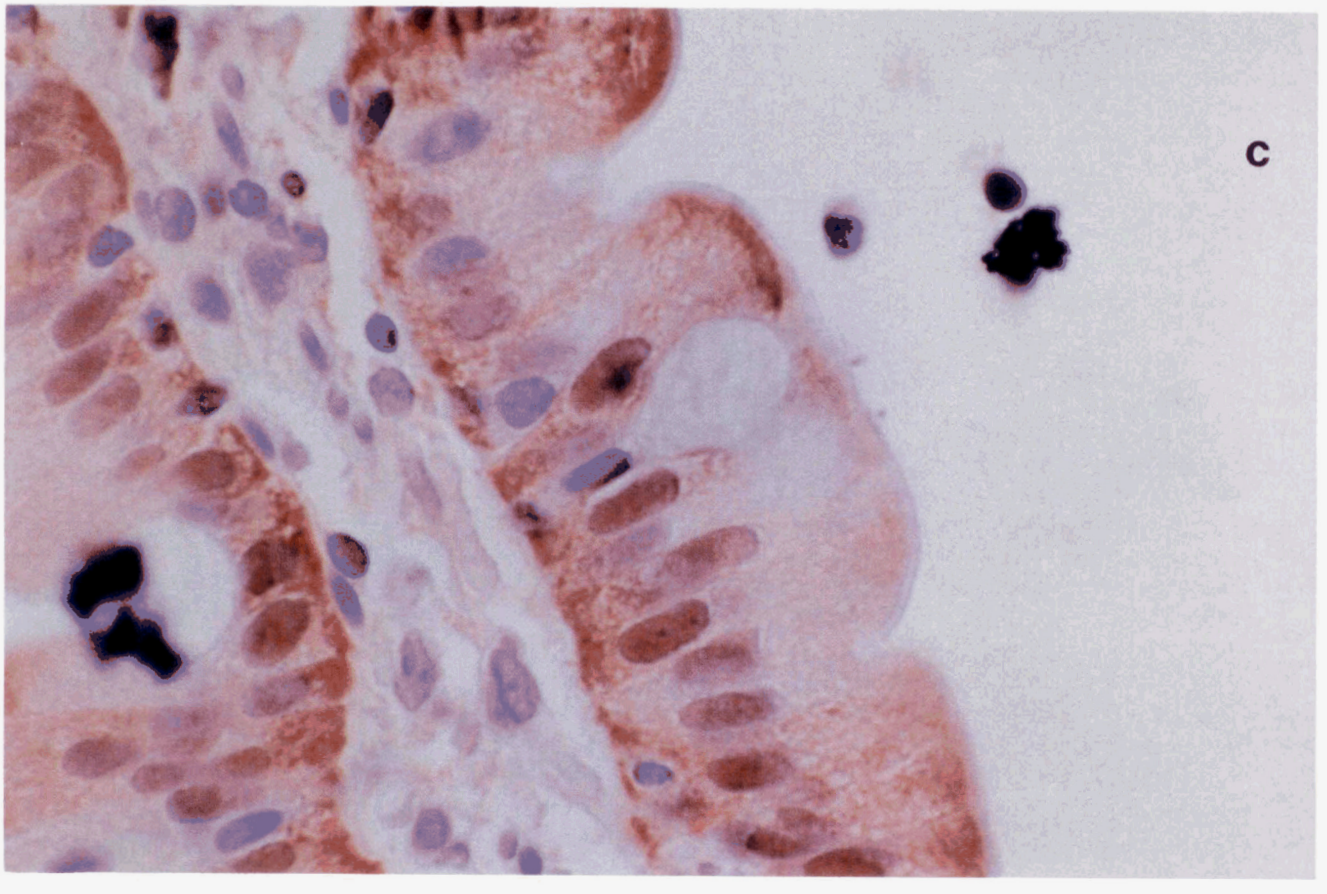
Gel filtration of 100 ml of jejunum extract, equivalent to 41.5 g of tissue including muscle, was performed on a 5 × 70 cm column of Sephadex G-75, medium (Pharmacia) equilibrated and run in PBS. The column was calibrated with a molecular weight calibration kit of aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A (Pharmacia). Fractions of 15 ml were collected and absorbance was monitored at 280 nm. Retinol in dimethyl sulfoxide (100 nmol in 10 μl) was added to each fraction, mixed, and the fluorescence was determined (excitation at 348 nm, emission at 490 nm) in an SLM-500C spectrofluorometer. The fluorescence spectra were established with the same instrument. Human CRBP for comparison spectra was purified as previously described (10).

RESULTS AND DISCUSSION

Presence in gut of a protein that cross-reacts with anti-rat CRBP (II) serum

When aliquots of an extract of soluble protein from human jejunal mucosa were examined in the radioimmunoassay system specific for rat CRBP (II) (7), the displacement curve shown in **Fig. 1** was observed. The curve, shallower than obtained with rat CRBP (II), was indicative of the presence of a species capable of considerable cross-reaction with the antibodies present, but with a lower affinity than rat CRBP (II). Over 90% displacement could be achieved with concentrated, partially purified material described later (data not shown). When





pure human CRBP was examined in the same way, amounts up to and including 100 pmol showed no significant displacement. Consequently, in order for the displacement observed for the extract to be due to the presence of human CRBP, the concentration would need to be in excess of 100 pmol per 10–20 nl or greater than 100 mg CRBP per ml. This point is stressed as CRBP and CRBP (II) of rat have very significant sequence homology (9).

The nature of the immunoreactive material was examined by submitting aliquots of the extract to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to resolve the proteins on the basis of molecular weight. The resolved proteins were then transferred to nitrocellulose paper and probed for immunoreactivity with the anti-rat CRBP (II) serum. As shown in **Fig. 2,** only one band was detectable in the extract and its position corresponded to a molecular weight of 16,000. Also shown is the detection of pure rat CRBP (II) run on the same gel, demonstrating a mobility identical to the human protein. In this system human CRBP is found at a lower position, slightly above the arrow at 14.2 K.

Presence in gut of retinol-binding activity distinct from CRBP

If the immunoreactive material detected above were indeed a human CRBP (II), it should be possible to demonstrate the protein by its retinol-binding ability. This was examined by submitting an extract of the soluble proteins of human jejunum to gel filtration on a column of Sephadex G-75. Retinol binding characteristic of CRBP (II) was detected after addition of an aliquot of free retinol to each fraction. A considerable increase in retinol fluorescence occurs when retinol is bound by CRBP (II) (7). When this was done, as shown in **Fig. 3,** a prominent peak of binding activity was seen centered at fraction 80. The potential contribution of free retinol to the fluorescence was less than 2% of the fluorescence observed. This was the elution position expected for a protein of molecular weight 16,000 and was identical to the elution position previously observed for rat CRBP (II).

Fractions 72–90 shown in Fig. 3 were collected and concentrated 10-fold by ultrafiltration which removed much of the free retinol. The fluorescence excitation and emission spectra of the concentrated fractions are shown in **Fig. 4** by the bold line. For comparison the spectra for retinol bound to pure human CRBP are shown by the fine line. The excitation spectrum observed is quite similar to that previously observed for retinol bound to rat CRBP (II). The spectrum is considerably altered from the relatively smooth peak with λ max at 325–330 nm observed for retinol dissolved in organic solvents. The bound retinol has a red-shifted spectrum with fine structure, the major peak being at 348 nm with two distinct lesser peaks at about 332 nm and 365 nm. This spectrum is quite similar to, but reproducibly distinct from, the excitation spectrum of retinol bound to pure human CRBP. That spectrum is displaced about 2 nm further to the red as shown in Fig. 4. A similar 2-nm difference has been observed between the spectra of retinol bound to rat CRBP and rat CRBP (II), illustrated previously by absorption spectra rather than fluorescence excitation spectra (7). The 2-nm difference is insensitive to pH and has always been observed when the two retinol-protein complexes have been compared. A difference in λ max in the emission spectra can also be seen in Fig. 4.

The partially purified preparation was reexamined in the radioimmunoassay as previously done for the whole extract. A displacement curve was generated which was indistinguishable from that observed for the whole extract and from other extracts of jejunal mucosa only (data not shown). The recovery of displacement ability was about 80% of that displayed by the whole extract. This was equivalent to the expected recovery of material on such columns and suggested that all of the immunoreactive material was found in this peak, consistent with the single band observed in Fig. 2. The retinol-binding capacity of the partially purified material was quantitated using the assumption that the fluorescence observed was equivalent in intensity to that obtained when retinol binds to pure rat CRBP (II). This allowed assignment of pmoles bound per microliter. By the further assumption that all immunoreactive material could bind retinol, pmoles of retinol bound was equated with pmoles of binding protein present. This allowed a new displacement curve to be generated using the partially purified material with defined binding protein content. An extract of jejunal mucosa from a 46-year-old male was then examined. The displacement observed was equivalent to 250 pmol of CRBP (II) per mg of soluble protein. Using the indicated molecular weight of 16,000, this means that CRBP (II) represented 0.4% of the total protein. In rat jejunal mucosa extracts from 250-g rats maintained on standard chow, CRBP (II) is about 1% of the total protein (7).

Demonstration of the immunoreactive protein in the absorptive cell

The specificity and reactivity of the anti-rat CRBP (II) serum suggested it would be usable for immunohistochemical localization studies in human tissue. The results of such studies are shown in **Fig. 5.** Brown staining indicative of the presence of immunoreactive material can be seen in the epithelial cell layer of the villi shown in Fig. 5a. Little or no staining is apparent in the epithelial cells of crypts, shown in cross-section in the lower part of the micrograph. The higher magnification shown in Fig. 5b shows no staining of cells within the lamina propria, but distinct staining of the enterocytes. The field shown in Fig. 5c contains a goblet cell which does not appear to have staining in its cytoplasm found compressed below the mucigen globule. No staining was observed for serial

sections when nonimmune serum or antiserum passed over a column of immobilized rat CRBP (II) was substituted for the immune serum.

These results duplicate what we have previously observed for rat: the presence of cellular retinol-binding protein, type two, in the mature, villus-associated enterocytes, a low or nonexistent amount in the maturing enterocytes still in the crypts, and no apparent CRBP (II) in the goblet cells.

This cellular location suggests quite strongly that this protein may play an important role in the necessary movements and metabolism of retinol that must occur in the absorptive process. A possible role would be to receive newly absorbed retinol and present it to an enzyme for the obligatory esterification that takes place prior to incorporation into chylomicrons. Such a reaction has been demonstrated for retinol bound to rat CRBP (II) presented to microsomes prepared from rat small intestine (13).

This work was supported by grant AM-32642 from the United States Public Health Service.

Manuscript received 10 November 1986 and in revised form 22 December 1986.

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