Localization of cellular retinol-binding protein in several rat tissues
(vitamin A/imunolocalization/radioimmunoassay/liver/epididymis)

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ABSTRACT  The distribution of cellular retinol-binding protein (CRBP) in rat liver, ileum, and epididymis was examined by the peroxidase-antiperoxidase immunolocalization technique. Positive cytoplasmic staining was seen in the liver when antiserum prepared against purified CRBP was used but not when antiserum absorbed with purified CRBP was used. Ileal mucosa, a tissue that contains no detectable CRBP, showed no positive staining. The epididymis showed strong positive staining in the caput but not in the cauda. Staining was present in principal and basal cells but not in peritubular or interstitial cells. Radioimmunoassay revealed that the CRBP within the caput epididymidis was localized in the initial segment and proximal area, regions known to be involved in the synthesis and secretion of factors necessary for sperm maturation. The results demonstrate that the expression of CRBP may vary within the same tissue, as well as between different cell types within the same tissue.

Vitamin A is known to be essential for the support and differentiation of most epithelial tissues (1). Although the mechanism of retinol (vitamin A alcohol) action is unknown, an increasing body of evidence suggests that its effects are mediated in part by cellular retinol-binding protein (CRBP) (2, 3). CRBP is present in all retinol-sensitive tissues of the body but at widely varying levels (4). In addition, the levels of CRBP within a tissue can change during perinatal development (5) and also with the induction of neoplasia (6–8). This variation might be due to differing levels of CRBP, differing populations of cells that contain CRBP, or both.

Previous attempts to examine this question have been by fractionation of the various cell types, followed by measurement of CRBP content through the ability to bind retinol (9). This cannot be done for all tissues and is particularly ineffective for cell types of limited abundance. To overcome this, we have identified CRBP in intact tissue sections, using an immunohistochemical technique. We report here the immunolocalization of CRBP in several rat tissues known to contain high levels of CRBP. In addition, immunolocalization in one organ showed an unexpected cellular distribution of CRBP, which was verified both by retinol binding and by radioimmunoassay.

MATERIALS AND METHODS

Preparation of CRBP. CRBP was purified from rat liver as described (10).

Production and Detection of Antibodies. Specific antibodies to CRBP were raised in male New Zealand rabbits by methods described earlier (4).

Radioimmunoassay of CRBP. Tissues were prepared and CRBP levels measured as described (4). Liver, ileum, and epididymis were obtained from normal, chow-fed rats (Harlan Industries, Indianapolis, IN). The epididymis was dissected into its various regions, each of which was then assayed separately. Protein content was determined by the method of Lowry et al. (11).

Immunolocalization of CRBP. The peroxidase-antiperoxidase (PAP) technique of Sternberger was used (12). Rats were killed by decapitation, and tissues were removed and quick-frozen in OCT compound (Ames, Elkhart, IN). Frozen sections (6 μm) were cut and fixed overnight in 95% ethanol. Sections were incubated for 30 min in 0.3% H2O2 in 100% methanol, hydrated, and incubated with 10% normal goat serum for 10 min. Sections were next incubated with anti-CRBP (appropriately diluted in 1% normal goat serum) for 24–48 hr at 4°C, then with goat anti-rabbit IgG (diluted 1:20 in 10% normal goat serum) for 30 min at room temperature, and finally with rabbit PAP complex (diluted 1:50 in 1% normal goat serum) for 30 min at room temperature. Goat sera and rabbit PAP were obtained from Cappel Laboratories (Cochraville, PA). All incubations were done in a humid chamber, and sections were washed three times with phosphate-buffered saline between steps. The immunoreactive substances were stained by using diaminobenzidin and H2O2 as substrates for the peroxidase. In some cases, the sections were counterstained for 1 min with hematoxylin. The slides were then dehydrated and covered with coverslips.

In control slides, anti-CRBP was replaced with either normal rabbit serum or antiserum treated with CRBP, added in 10-fold excess over the binding capacity of the antiserum as determined by its ability to bind [3H]CRBP (4). The antiserum was incubated with the CRBP for 48–72 hr at 4°C before use.

RESULTS

Liver was chosen as an initial tissue in which to attempt to immunolocalize CRBP by the PAP method, as it was the source for the purification of CRBP (10). Liver has the highest reported level of CRBP as measured both by retinol binding and by radioimmunoassay (4). Optimum staining was obtained by using frozen sections fixed in 95% ethanol. Fig. 1 shows the localization of CRBP in frozen sections of rat liver. Positive staining was granular and cytoplasmic; no nuclear staining was observed. Moreover, staining was homogeneous throughout the cytoplasm and not associated with any one organelle. No positive staining was detected when antiserum treated with CRBP was substituted for the primary antiserum (Fig. 1b). Likewise, substitution of anti-CRBP with normal rabbit serum gave no immunostaining (results not shown).

To establish the specificity of the staining, a tissue presumed to contain no CRBP was submitted to the same procedure. The mucosa of the ileum contains no detectable CRBP as determined by either a retinol-binding assay or radioimmunoassay (4). As shown in Fig. 2, the ileal mucosa showed no staining.

Abbreviations: CRBP, cellular retinol-binding protein; PAP, peroxidase-antiperoxidase.
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FIG. 1. Immunolocalization of CRBP in rat liver. Serial 6-μm frozen sections of normal rat liver were stained by the PAP method with anti-CRBP antiserum (a) or anti-CRBP antiserum pretreated with purified rat CRBP (b). (×2,900.)

when treated with anti-CRBP. Serial sections stained with normal rabbit serum or anti-CRBP pretreated with CRBP gave identical results (results not shown). These observations confirmed that staining was specific for CRBP.

Next to liver, the epididymis has the highest levels of CRBP as measured by radioimmunoassay (4). The epididymis consists

FIG. 2. CRBP is not detected by immunolocalization in the ileum. Serial 6-μm frozen sections of normal rat ileum were stained with hematoxylin and eosin (a) or by the PAP method with anti-CRBP antiserum (b). (×1,160.)
of a single convoluted tubule divided into three histologically, biochemically, and functionally distinct regions: the caput, corpus, and cauda epididymidis (13). Unpublished observations in our laboratory suggested that CRBP, as measured by retinol-binding capacity, resided primarily in the caput and not the corpus or cauda epididymidis. To examine this, we attempted immunolocalization on sections from the caput and cauda epididymidis. The results are shown in Fig. 3. The epithelium in the...

**FIG. 3.** Immunolocalization of CRBP in rat epididymis. Serial 6-μm frozen sections of normal rat caput (a and b) and cauda (c and d) epididymidis were stained by the PAP method with anti-CRBP antiserum (a and c) and anti-CRBP antiserum pretreated with purified rat CRBP (b and d). (Inset) Higher magnification (×2,900) of the same section in a; basal cells are visible (arrowhead).
caput epididymidis showed strongly positive staining with anti-
serum directed against CRBP. Staining was exclusively cyto-
plasmic (Fig. 3Inset) and appeared present in basal cells (ar-
row) as well as principal cells, but not in peritubular cells or the
interstitium (Fig. 3a). No staining was observed when anti-
serum pretreated with CRBP was used (Fig. 3b). In contrast,
sections from the cauda epididymidis showed little positive
staining (Fig. 3c and d), suggesting that it contains much lower
levels of CRBP.

Closer examination of the sections from the caput epididy-
midis revealed an uneven regional distribution of positive cells.
A histologically distinct region consistently showed a higher level
of staining than did the remainder of the sections. This prompted
us to dissect the epididymis into various segments and deter-
mine the CRBP content of each by radioimmunoassay. As can
be seen in Fig. 4, the initial segment b and proximal region c
of the caput epididymidis contained high levels of CRBP, whereas
the other parts of the epididymis were low in CRBP content.
The levels in these segments were 10- to 200-fold higher than
in any other region of the epididymis. Calculation of the CRBP
levels based on protein content rather than tissue weight did
not change the results. Most interesting is the >20-fold dif-
ference seen between the proximal and distal caput, regions
difficult to distinguish morphologically (13). This considerable
difference within the caput epididymidis is reflected by the re-

ditional distribution observed by immunolocalization.

DISCUSSION

Previous work has demonstrated that CRBP is intimately in-
volved in retinol action (for review see refs. 2 and 14). Con-
The epididymis also showed cytoplasmic staining identical to that seen in the liver, but the pattern of staining was not homogeneous throughout the organ. Immunolocalization demonstrated that only the epithelial cells and not the peritubular or interstitial cells contained detectable CRBP. Thus, CRBP clearly showed a cell-specific distribution within the tissue. In addition, immunolocalization suggested that the level of CRBP was higher in the epithelial cells of the caput than in the cauda epididymidis and even differed regionally within the caput itself. This was confirmed by radioimmunoassay of cytosols prepared from dissected segments. A large difference in CRBP levels was observed between the proximal and distal caput epididymidis. The principal cells from these regions are similar morphologically (13). The results suggest that the expression of CRBP can vary dramatically within the same cell type as well as between different cell types within the same tissue. Therefore, both of these possible causes for differing CRBP levels must be considered in comparing different tissues. In addition, both represent possible mechanisms for the alterations of CRBP content seen in perinatal development and neoplasia.

The epididymis plays an essential role in the process of sperm maturation, as acquisition of the ability to fertilize an ovum occurs during transit of spermatozoa through the organ (for review see refs. 18 and 19). In the rat, maturation is complete by the time spermatozoa have reached the cauda epididymidis, where they are stored (18). Thus, the major morphological and physiological changes that spermatozoa undergo take place in the caput and corpus epididymidis. Considerable glycoprotein synthesis and secretion, essential for this process, occurs in the caput epididymidis (20–24). The correlation between such activity and the highly specific localization of CRBP reported here raises the possibility that retinol plays some role in this process.

It has been suggested that one function of retinol is to act as a cofactor in the glycosylation of glycoproteins (25). In the mouse epididymis, the incorporation of labeled mannose, galactose, and fucose has been shown to be highest in the regions distal to the proximal caput (26, 27), areas where the CRBP content is quite low. In contrast, incorporation of labeled sugars is relatively low in the proximal caput, where CRBP content is highest. Consequently, the distribution of CRBP in the epididymis does not appear to correlate with the actual process of glycosylation.

Whereas a role for retinol in directing differentiation of epithelial cells has been recognized for many years, its involvement in supporting the function of terminally differentiated cells rests on more recent discoveries. Histological studies in retinol-deficient rat testes clearly demonstrate that already differentiated ciliated and goblet cells undergo changes unrelated to the development of the metaplastic state, which results from the improper differentiation of basal cells (28). The molecular mechanisms for these two apparently separate roles of retinol are not known, but a review of previous studies in combination with the results presented here raises an interesting possibility. First, retinol has been shown to influence RNA metabolism in a number of tissues (29–31). Alterations in genomic expression of vitamin A-deficient rat testis occur 1 hr after refeeding retinol (32). This rapid response suggests that the effects of retinol are independent of cell replication and, therefore, probably would occur at the level of transcription or processing of specific mRNAs. These effects may be mediated by CRBP, which is capable of delivering retinol to specific binding sites within the nucleus (33). Second, it has been shown that in the mouse epididymis, incorporation of [3H]retinol is highest in the initial segment of the caput epididymidis and decreases rapidly the more distal the segment examined (34). This activity coincides with the localization of CRBP reported here. Finally, the liver, a transcriptionally active tissue, has one of the highest levels of CRBP of any organ (4). The evidence developed here invites the suggestion that one of the major functions of CRBP, and thus retinol, is maintaining the high rates of genomic expression that occur in some fully differentiated cells. The mechanism of retinol action in differentiated cells and its exact role in the process of cellular differentiation remain to be established.

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