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Plasma and cellular retinoid-binding proteins and transthyretin (prealbumin) are all localized in the islets of Langerhans in the rat

(retinoids/vitamin A/immunohistochemistry/radioimmunoassay)

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ABSTRACT **The immunohistochemical localization of plasma retinol-binding protein (RBP), cellular retinol-binding protein (CRBP), and transthyretin (TTR) was studied in rat pancreas. The studies employed antibodies purified by immunosorbent affinity chromatography, permitting the specific staining and localization of each antigen by the unlabeled peroxidase-antiperoxidase method. Specific immunostaining for each of these three proteins was found localized to the islets of Langerhans. Both RBP and CRBP were localized in cells that were peripherally distributed within the islets, with an anatomic distribution that resembled that of the glucagon-containing A cells. Immunoreactive TTR was localized in cells that were more centrally distributed in the islets, with an anatomic distribution that resembled that of the insulin-containing B cells. These findings were confirmed by radioimmunoassay of a homogenate of isolated rat islets. By using sensitive and specific radioimmunoassays for each antigen, unusually high levels of CRBP, RBP, TTR, and cellular retinoic acid-binding protein (CRABP) were found in rat islets. The physiological significance of the localization of RBP, CRBP, CRABP, and TTR in the islets is not known. The findings suggest that retinoids and their binding proteins may play important metabolic roles within islet cells, and hence that they may be involved in some way in the biological, endocrine, function of the islets.**

Retinoids with vitamin A biological activity influence a variety of biological processes. Retinoids are necessary for the support of growth, health, and life of higher animals. Retinoids are essential for vision, for reproduction, and for the maintenance of differentiated epithelia and of mucus secretion. The molecular mechanisms whereby retinoids achieve these biological effects are not known, except for the well-documented role in vision (1). It is likely that retinoids affect gene expression in target cells (2).

Specific binding proteins for retinoids exist in plasma and in the intracellular compartment in tissues. Plasma retinol-binding protein (RBP) is the specific transport protein that serves to mobilize retinol from the liver and transport it to extrahepatic target tissues (see ref. 3 for a recent in-depth review). RBP in plasma interacts with another protein, transthyretin (TTR, also more commonly known as prealbumin) and normally circulates as a 1:1 molar RBP·TTR complex. Retinol delivery may involve cell surface receptors that recognize RBP (4, 5). Within many tissues, retinol is present bound to a soluble intracellular protein, cellular retinol-binding protein (CRBP) (6). A related intracellular protein, cellular retinoic acid-binding protein (CRABP) is also widely distributed in tissues. Although the biological roles that CRBP and CRABP play within cells have not been established, it has been suggested that these binding proteins may be involved in the biological expression of retinoid activity within cells (see ref. 6 for a recent review).

We have recently reported studies on the immunohistochemical localization of RBP, TTR, and CRBP in rat liver and kidney (7). Each of these proteins was found to be localized in specific cells within each organ. Highly specific localization of CRBP has also been observed in the testis and epididymis (8, 9). These studies provide information concerning the specific cells and anatomic loci, within different organs, where retinol and/or its binding proteins may be playing important biological roles.

We now report the immunohistochemical localization of RBP, of TTR, and of CRBP in the islets of Langerhans in the rat. The immunohistochemical findings have been confirmed by radioimmunoassay studies, which showed a marked enrichment in islets of CRBP, RBP, TTR, and CRABP.

MATERIALS AND METHODS

Antigens and Antibodies. RBP and TTR were isolated from rat serum and CRBP and CRABP were isolated from rat testis homogenates, by procedures described recently in detail (7).

The immunohistochemical study employed rabbit antisera against purified rat RBP, TTR, and CRBP, prepared as described elsewhere (7). Purified monospecific antibodies against each antigen (RBP, TTR, or CRBP) were obtained (7) by immunosorbent affinity chromatography of the IgG fractions prepared from the specific antisera against each of these proteins. In each case, the affinity chromatography procedure used the respective pure antigen coupled to Sepharose 4B as the immunosorbent. Similarly, specific goat antibodies against rabbit IgG were obtained by immunosorbent affinity chromatography on rabbit IgG linked to Sepharose; crossreactive antibodies against rat IgG were removed from the purified goat anti-rabbit IgG by a further immunosorbent affinity chromatography step on rat IgG coupled to Sepharose (7).

The radioimmunoassays of CRBP and CRABP employed turkey antibody preparations against each of these proteins, prepared as described elsewhere (10, 31). Purified monospecific turkey IgG against CRBP and against CRABP were obtained by immunosorbent affinity chromatography of the whole turkey IgG fraction with the respective antigen (CRBP or CRABP) linked to Sepharose 4B.

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Abbreviations: RBP, retinol-binding protein; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; TTR, transthyretin; PAP, peroxidase-antiperoxidase.

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The specificities of the purified antibodies against RBP, TTR, CRBP, and CRABP were examined extensively by double immunodiffusion and by enzyme-linked immunosorbent assays (ELISA) (7). Each antibody preparation reacted only with the antigen against which it had been prepared, and not at all with the other three proteins (antigens) under study. There was no crossreactivity among RBP, TTR, CRBP, and CRABP in any of the four specific radioimmunoassays used to measure the levels of these four proteins. CRBP, CRABP, and liver Z-protein have been reported to show homology with each other (11, 12). However, no crossreactivity was observed among these three proteins (Z-protein kindly provided by I. Arias, Tufts University) when they were examined by either ELISA or radioimmunoassay procedures.

Rabbit anti-human glucagon, guinea pig anti-human insulin, rabbit anti-guinea pig immunoglobulins, and peroxidase-antiperoxidase complex (PAP) were purchased from Accurate Chemicals (Westbury, NY). The anti-glucagon serum (1 ml) was absorbed with an immunosorbent consisting of bovine insulin (4 mg) bound to Sepharose 4B (2 ml). The anti-guinea pig immunoglobulin serum (1 ml) was absorbed with two immunosorbents, consisting of the proteins of normal rat serum and of normal goat serum bound to Sepharose 4B (proteins of 100 μl of serum bound to 1 ml of Sepharose).

Preparation of Rat Pancreas for Immunohistochemistry. Three male weanling rats of the Holtzman strain were fed a purified diet (13) containing 2.4 μg of retinyl ester per g of diet (and hence a normal vitamin A content) for 6 months. The rats were anesthetized with diethyl ether; blood was removed, and the organs were fixed by perfusion of the whole animal through the left ventricle (with exit through the right atrium) with an ice-cold solution of Hepes-buffered saline (10 mM Hepes buffer, pH 7.4/122 mM NaCl/6.6 mM KCl/1.2 mM $CaCl_2$) containing heparin (10 units/ml) and aprotinin (500 kallikrein inhibitor units/ml) for 5 min, followed by ice-cold Perfix (Fisher) for 15 min. Perfusion was performed at a constant rate of 20 ml/min. The pancreas was then removed from each animal and fixation was continued for 2 hr at 4°C. The fixed tissues were washed with 95% (vol/vol) ethanol three times (8 hr each time at 4°C) and embedded in paraffin. Serial sections of 4- to 5-μm thickness were mounted on glass slides.

Islet Isolation. Islets were isolated from normal Sprague–Dawley male white rats strain from (Hilltop Laboratories, Scottsdale, PA) weighing 350–400 g and fed a chow diet ad lib, as described elsewhere (14, 15). In brief, pancreata were excised from anesthetized rats, immediately minced, and digested with 0.7% collagenase, and the islets were isolated in Krebs/Ringer bicarbonate medium, pH 7.4. A sample of 1000 islets from six rats was washed four times in phosphate-buffered saline, pH 7.4, and then rapidly frozen and stored at −80°C for 18 hr and then at −40°C until used for radioimmunoassay.

Immunohistochemical Staining for RBP, TTR, CRBP, Glucagon, and Insulin. The unlabeled PAP method of Sternberger *et al.* (16) was used as described (7). The following incubations were performed with sections of tissue from which the paraffin had been removed: (*i*) 0.3% H_2O_2 in absolute methanol for 20 min; (*ii*) phosphate-buffered saline containing 10% normal goat serum for 30 min; (*iii*) primary antibody (purified anti-RBP, anti-TTR, or anti-CRBP, each at 25 μg/ml in phosphate-buffered saline; or anti-glucagon, diluted 1:400, or anti-insulin, diluted 1:4000) for 120 min; (*iv*) purified goat anti-rabbit IgG antibody (referred to as the "bridge antibody"), 200 μg/ml in phosphate-buffered saline, for 60 min; and (*v*) PAP, diluted 1:40 in phosphate-buffered saline, for 60 min. In the staining procedure for insulin, a sixth incubation, with rabbit anti-guinea pig immunoglobulins (diluted 1:2000 with phosphate-buffered saline) for 60 min, was performed between incubations *iii* and *iv*. The sections were washed with phosphate-buffered saline three times after each incubation. They were then rinsed in 50 mM Tris·HCl buffer, pH 7.6, and allowed to react with a solution of 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 50 mM Tris·HCl buffer, pH 7.6, containing 0.003% H_2O_2 for 5 min. The tissues were counterstained with diluted (1:4) hematoxylin (Gill formulation no. 1, Fisher) in 25% (vol/vol) ethylene glycol. Under these conditions, background peroxidase staining or endogenous peroxidase activity was not observed.

Various control experiments, described elsewhere (7), have established the specificity of the same immunohistochemical procedures used for these same antibody preparations against RBP, TTR, and CRBP. No specific immunostaining was observed after omission of the primary antibody from the staining procedure or when one or another of the following components of the staining procedure was omitted: the bridge antibody, PAP, or the final substrate for the peroxidase reaction. When tested with sections of tissue, the specific staining associated with the antibodies against RBP, TTR, and CRBP disappeared after absorption with the particular antigen against which the antiserum had been raised.

A preliminary study was conducted to evaluate and to determine effective procedures for specific immunostaining of rat pancreatic islets for glucagon and insulin. Rabbit anti-human glucagon, used directly as purchased, showed moderate staining of both A and non-A cells of the islets. Absorption of this antiserum with insulin linked to Sepharose (see above) eliminated the immunostaining of the non-A cells. Similarly, the preliminary study with anti-human insulin showed both specific staining of the B cells and nonspecific staining of other islet cells. The nonspecific staining was found to be due to crossreactive antibodies in the rabbit anti-guinea pig immunoglobulin serum, and it was eliminated by absorption of this latter antiserum with the proteins of normal rat serum and of normal goat serum (see above).

Radioimmunoassays for RBP, TTR, CRBP, and CRABP. The islets (4.7 mg estimated wet weight) were thawed and homogenized with a Polytron homogenizer (Brinkmann) in 1.0 ml of a solution (hereafter referred to as "RIA buffer") of 50 mM imidazole hydrochloride buffer, pH 7.4, containing 0.79% NaCl, 0.03% bovine serum albumin, 0.1% thimerosal, 0.01% leupeptin (Peninsula Laboratories, San Carlos, CA), and 1% Triton X-100. The islet protein concentration of the homogenate, determined by measuring the protein concentration of the homogenate [by the method of Bradford (17)] and subtracting that of the RIA buffer, was found to be 548 μg of islet protein per ml of homogenate. RBP and TTR levels were determined by radioimmunoassay as reported previously (18, 19). CRBP and CRABP levels were determined by recently developed sensitive and specific radioimmunoassays for each protein (31). The CRBP radioimmunoassay represents a modification (9) of the method previously reported (10). The radioimmunoassays for CRBP and CRABP employed identical protocols; both assays can accurately detect 1–10 ng of protein (CRBP or CRABP) per assay tube.

RESULTS

Specific immunohistochemical staining for RBP, for TTR, and for CRBP was observed in cells of the pancreatic islets (Figs. 1 and 2). None of these three proteins was found to be localized in other cells of the pancreas (Fig. 1). Both RBP (Figs. 1*D* and 2*D*) and CRBP (Figs. 1*C* and 2*C*) were found to be localized in cells that were peripherally distributed within the islets. The anatomic distribution of these RBP and CRBP containing cells resembled the distribution of the

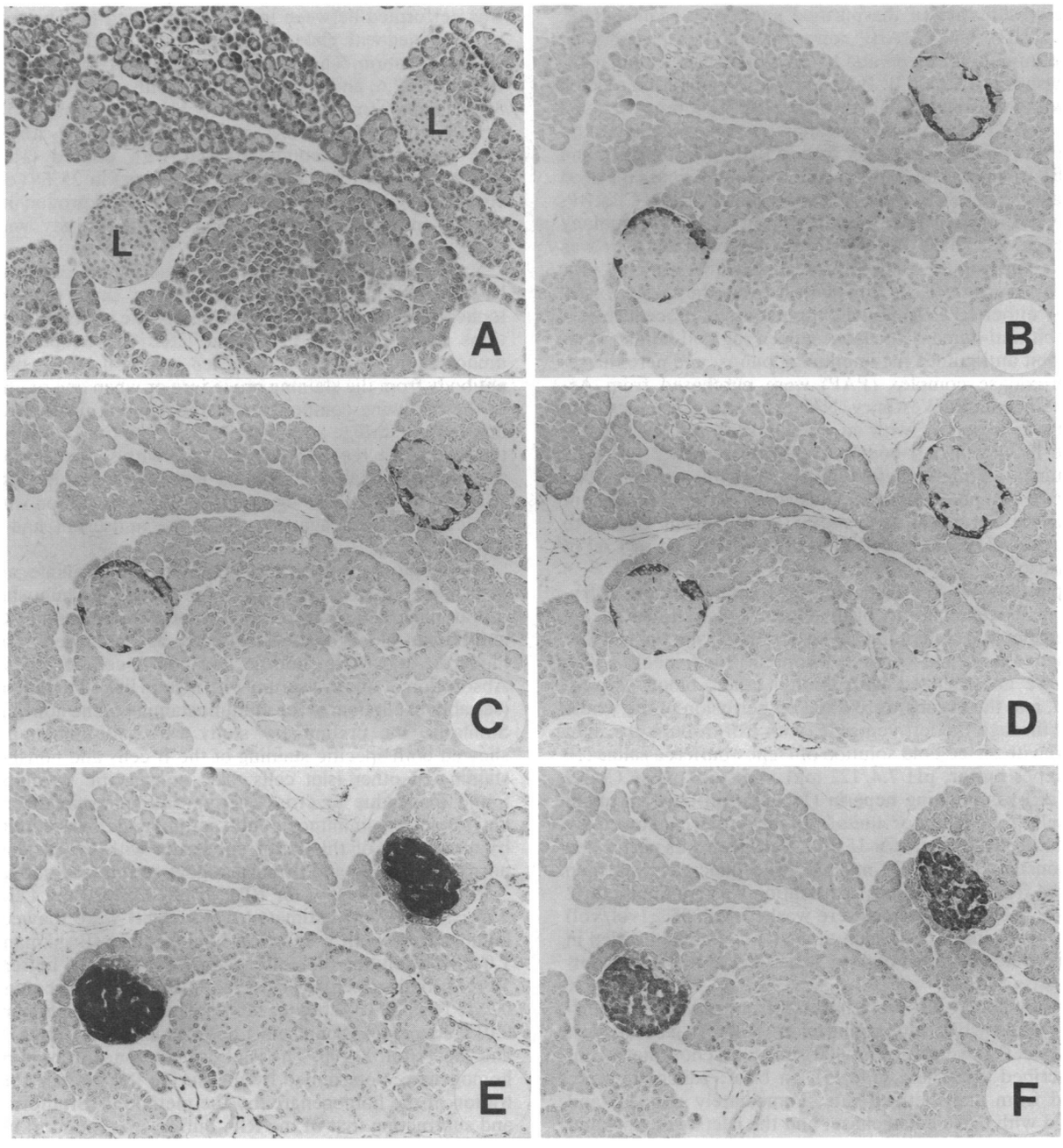

FIG. 1. Localization of immunoreactive RBP, TTR, and CRBP in rat pancreas. Adjacent sequential sections of pancreas were stained with hematoxylin/eosin (*A*) or specifically immunostained with antibodies against glucagon (*B*), CRBP (*C*), RBP (*D*), TTR (*E*), or insulin (*F*). All of these antigens were localized to the islets of Langerhans (labeled L in *A*). (×90.)

glucagon-containing A cells (Figs. 1*B* and 2*B*). In contrast, immunoreactive TTR was localized in cells that were distributed throughout the main central portion of the islets, and the islets showed very strong specific immunostaining for TTR (Figs. 1*E* and 2*E*). The anatomic distribution of the TTR-positive cells in the islets resembled the distribution of the insulin-containing B cells (Figs. 1*F* and 2*F*). The immunostaining for TTR (Fig. 2*E*) appeared, however, rather more diffuse in the islets and within islet cells than did the immunostaining for insulin (Fig. 2*F*).

The levels of immunoreactive RBP, TTR, CRBP, and CRABP found in the islet homogenate, as determined by specific radioimmunoassays for each of these four proteins, are shown in Table 1. Very high levels of each of the four proteins (compared to the levels found in homogenates of whole pancreas, Table 1) were found in the homogenate of isolated islets. The level of CRBP was particularly high, much higher than the levels of CRBP that we or others have observed in other studies (10, 20, 31), in any other rat tissue. The radioimmunoassay data thus confirm the immunohistochemical results, by showing a marked localization of immunoreactive RBP, CRBP, and TTR in the islets.

DISCUSSION

The immunohistochemical studies reported here demonstrated that RBP, CRBP, and TTR were all strikingly localized in the islets of Langerhans in the pancreas of the rat. These findings were confirmed by radioimmunoassay of a homogenate of isolated rat islets, which demonstrated unusually high levels of RBP, of CRBP, and of CRABP, and a high level of TTR in rat islets.

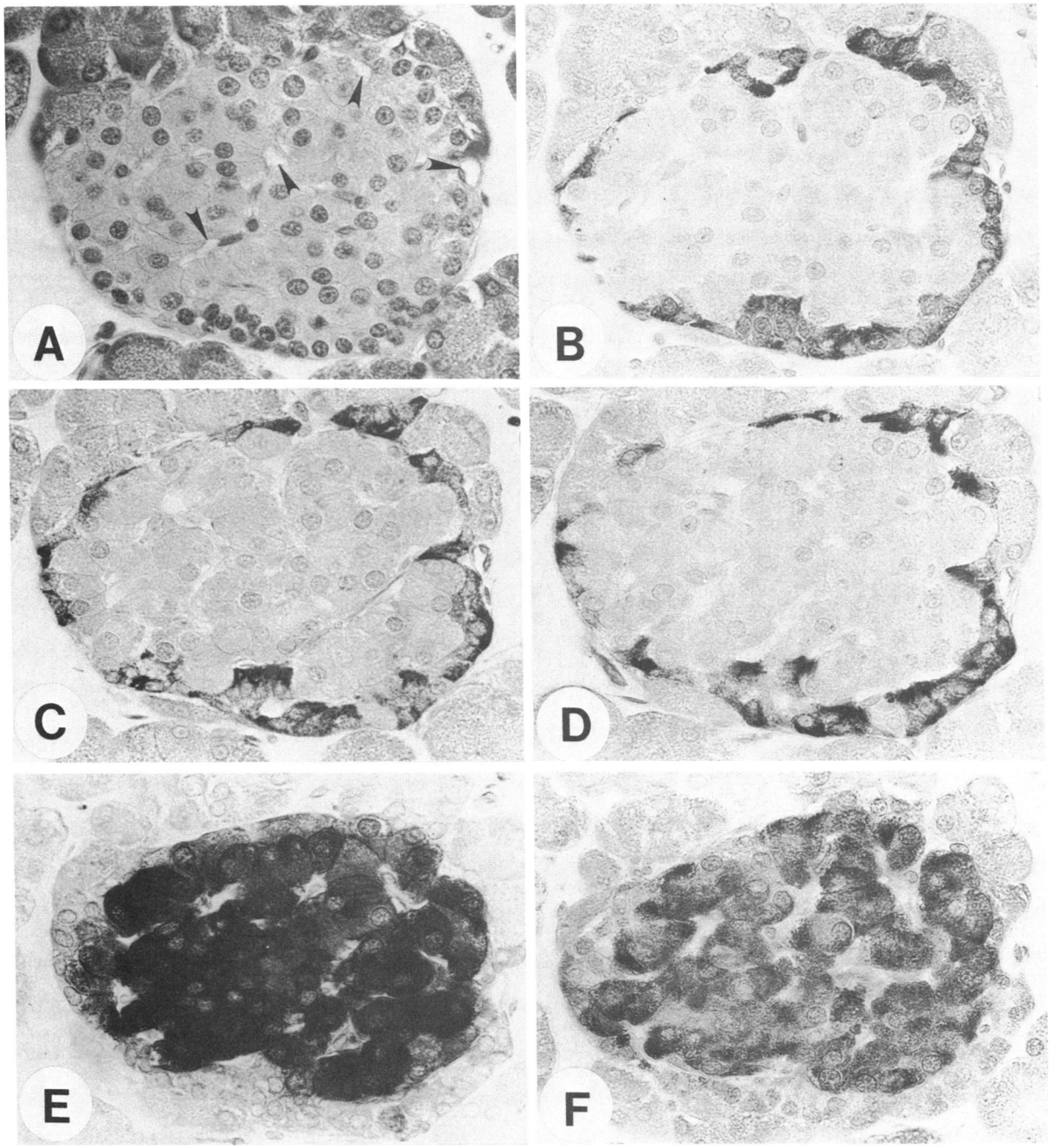

FIG. 2. Localization of immunoreactive RBP, TTR, and CRBP in rat islets of Langerhans. (*A–F*) Higher-magnification micrographs of the islet shown in the upper right portion of each of Fig. 1 *A–F*. See Fig. 1 legend for the sequence of specific immunostaining (*B–F*). *A* (hematoxylin/eosin staining) shows evidence of successful perfusion, which has completely washed out blood from the capillaries (arrowheads). (×430.)

Several investigators have reported recently the immunohistochemical localization of TTR (prealbumin) in human islets and in pancreatic tumors composed of hormone-producing (islet-derived) cells (21–23). The cellular distribution of TTR immunoreactivity (immunostaining) was similar to that of the glucagon-producing A cells of the islets (21–23); the immunostaining was, however, not blocked by glucagon (21, 22). It has been reported that the amino acid sequence of TTR shows significant homology to the sequences of glucagon and of related gastrointestinal hormones (including secretin and vasoactive intestinal peptide) (24). These observations elicited the suggestion (23, 24) that the immunostaining of islet cells for TTR might represent the binding of antibodies against TTR to antigenic determinants present in homologous hormones or prohormones. In the present work, the distribution of TTR immunoreactivity in islets resembled that of insulin rather than that of glucagon. The reason for this difference is not apparent, but it may be due to differences between species (rat vs. human islets) and/or methodological differences between the present and previous studies (e.g., the use of purified monospecific antibodies in the present studies vs. the use of commercially obtained antisera in the previous studies).

The specific immunostaining of rat islet A (alpha) and B (beta) cells with antisera against human glucagon and human insulin, respectively, was achieved only after appropriate absorption of the commercial antisera to eliminate nonspecific staining. Previous studies by other investigators have clearly demonstrated that both insulin and glucagon can be immunohistochemically demonstrated in islets of various

Table 1. Concentrations of retinoid-binding proteins and TTR in rat islets, as determined by radioimmunoassay

Protein	Concentration	
	μg/mg of protein	μg/g wet wt*
RBP	1.3 (0.096)	155 (4.70)
TTR	0.80 (0.300)	93 (14.60)
CRBP	4.7 (0.052)	549 (2.50)
CRABP	0.15 (0.007)	17 (0.32)

Values for whole pancreas homogenates of normal male rats, as determined in other studies (31), are given in parentheses.
*The islet values given in this column are considered less accurate than the values given in units of μg/mg of protein, because of the lower accuracy inherent in the estimate of the wet weight of the islets studied. These values are, however, useful for comparison with values available for other tissues in these units (9, 10, 20, 31).

vertebrate species by using heterologous antibodies that were prepared against the hormones from a single (different) species (25, 26).

We were surprised to find a dramatic localization of both RBP and CRBP in islets, with a cellular distribution of immunoreactivity for both binding proteins that very closely resembled that of glucagon. Somatostatin-containing D cells are also usually peripherally distributed in islets. No amino acid sequence homologies have been found between either RBP or CRBP and any known gastrointestinal hormone or other hormone (3, 27, 28). CRBP and CRABP have been reported to show homology with both liver Z-protein and nerve myelin P2 protein (12). In the present work, the antisera used against both CRBP and CRABP did not show immunological crossreactivity with purified liver Z-protein or with each other.

The radioimmunoassay study confirmed the conclusion from the immunohistochemical studies, namely, that RBP, TTR, and CRBP were all localized and concentrated in the islets. The concentration of each protein was measured by using sensitive radioimmunoassays specific for each protein. Of the proteins assayed, CRBP was found in highest concentration; in fact, the CRBP level in islets was much higher than the levels we have observed in other rat tissues (31), where the highest mean CRBP levels were found in the head of the epididymis (67 μg/g wet wt) and the liver (40 μg/g wet wt). In another study (9) a CRBP level of 117 μg/g wet wt was found in the proximal portion of the head of the epididymis. The level of RBP in the islets was as high as or higher than levels found in liver in retinol-deficient rats (29) and much higher than the levels of RBP found in any tissue of normal rats (29). Further studies are needed to determine whether RBP is actively synthesized in islet cells or whether islet RBP originates from circulating RBP produced in the liver. The level of CRABP was an order of magnitude less than the levels of CRBP or RBP, but it was still higher than the levels of CRABP that we have observed recently (31) in any of a large number of rat tissues, except for the seminal vesicles (mean value, 30.4 μg/g wet wt) and the vas deferens (17.4 μg/g wet wt). Thus, all three of these retinoid-binding proteins are highly enriched and concentrated in the islets.

The physiological significance of the localization of plasma and cellular retinoid-binding proteins in the islets is not known. Retinoids have been observed to influence several aspects of islet ultrastructure and function, including insulin release, in previous *in vitro* studies (14, 15, 30). The present findings suggest that retinoids and their binding proteins may play important metabolic roles within islet cells, and hence that these moieties may be involved in some way in the biological, endocrine, functions of the islets. Further studies are needed to explore possible relationships among retinoids and their binding proteins and the structure and function of the islets.

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