Purification and Partial Characterization of Rat Epididymal Retinoic Acid-binding Protein, and Its Immunohistochemical Localization

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Summary Two types of retinoic acid-binding proteins, designated as epididymal retinoic acid-binding protein (ERABP) types A and B, have been purified to homogeneity from rat genital organs. ERABP types A and B had molecular weights of 19,000 and 18,500 and isoelectric points of 5.72 and 5.90, respectively. The absorption spectrum of ERABP complex with retinoic acid had two peaks at 277 and 354 nm, and showed similar uncorrected fluorescence spectra to that of cellular retinoic acid-binding protein (CRABP). These ERABPs were identified as major androgen-dependent epididymal proteins by amino acid sequences analyses. Furthermore, immunohistochemical examinations revealed that the ERABPs exist in the epithelium of the proximal portion of the epididymis and in the lumen of epididymal canal and seminal tract leading from the epididymis. The existence of ERABP strongly suggests that retinoic acid might be involved in the maturation of spermatozoa.

Key Words rat, epididymis, epididymal retinoic acid-binding protein, retinoic acid-binding protein, androgen-dependent secretory protein, retinoic acid

It is known that vitamin A (retinol) is essential for the maintenance of reproductive functions in higher animals (1). Animals which are fed retinol-deficient diets develop various disorders in the testes. These include atrophy of the...

Abbreviations: ERABP, epididymal retinoic acid-binding protein; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
testes, reduction of the deferent duct, deterioration of germinal epithelial cells, disappearance of Sertoli cells and cessation of spermatogenesis, and are recovered after supplementation with retinol (2). However, supplementation with retinoic acid does not reverse the disorders in testes, except for the decreased secretion of testosterone (3).

Both retinol and retinoic acid have their specific binding proteins in the cytoplasm, i.e. cellular retinol-binding protein (CRBP) (4) and cellular retinoic acid-binding protein (CRABP) (5). Both proteins are present in many organs including genital organs (6, 7). Besides CRBP and CRABP, some kinds of organ-specific retinoid-binding proteins have been identified, such as cellular retinal-binding protein, interstitial retinol-binding protein in the retina (8, 9), and CRBP (type II) in the small intestine (10). These proteins are supposed to have their specific functions in the cells.

In this report, we found and purified novel retinoic acid-binding proteins which exist only in the epididymis and were copurified with CRBP and CRABP. The purified proteins are designated epididymal retinoic acid-binding protein (ERABP) types A and B and have similar physiochemical properties to CRABP. The amino acid sequence of ERABP type B was identical to a major androgen-dependent secretory protein in the epididymis (11). These results are in good agreement with the recent report by Newcomer and Ong (12). Since the androgen-dependent epididymal proteins are supposed to be important for maturation of spermatozoa and fertilization, retinoic acid may have some effects on these activities. The new biological function of retinoic acid is discussed in this paper.

EXPERIMENTAL PROCEDURES

Materials. All-trans-[15-3H]retinol (20.4 Ci/mmol) and all-trans-[11,13-3H] retinoic acid (46.0 Ci/mmol) were purchased from New England Nuclear, Boston, Mass., U.S.A. All-trans-[15-14C]retinoic acid (46.0 Ci/mmol) was obtained from Amersham Co., Buckinghamshire, U.K. Unlabeled all-trans-retinol was a product of Nakarai Chemical Co., Kyoto, Japan and unlabeled all-trans-retinoic acid, all-trans-retinal and all-trans-retinyl palmitate were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Peroxidase-antiperoxidase complex was a product of DAKO Corp., Carpinteria, Ca., U.S.A.

Purification of epididymal retinoic acid-binding proteins. Testes, epididymides and seminal vesicles from 350 rats were lyophilized and broken into pieces in 5 liters of ice-cold acetone in a Waring Blender®. The homogenate was spread on a paper filter and dried to a powder. The powder was homogenized in a Polytron® with 4 liters of 50 mM Tris-HCl, pH 8.4, containing 4 mM EDTA, 0.04% NaN₃, 150 mM NaCl, 12 mM monothioglycerol and 0.05% gabexate mesilate and centrifuged at 20,000 × g for 60 min. The supernatant was titrated to pH 5.2 with glacial acetic acid and recentrifuged at 20,000 × g for 60 min. The supernatant fluid was immediately titrated to pH 8.4 with 5 N NaOH, and incubated at 4°C overnight with
[\textsuperscript{14}C] retinoic acid to give a final concentration of 20 \textmu M.

The retinoic acid-binding proteins were purified by monitoring the radioactivity of [\textsuperscript{14}C] retinoic acid from this solution through a column chromatography series: DEAE-Sepharose, pH 8.4; Sephacryl S-300; DEAE-cellulose, pH 8.4; SP-Sephadex, pH 4.9; Sephadex G-50 and SP-Sephadex, pH 4.9. The proteins were dialyzed against distilled water, lyophilized and kept at −70°C until later analyses. CRBP and CRABP were also copurified during these purification procedures.

Characterization of epididymal retinoic acid-binding proteins. The purified ERABPs were analyzed by electrophoresis in three systems. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli (13), using a 14% polyacrylamide slab gel. Nondenaturing gel electrophoresis was performed using a 10% polyacrylamide slab gel. Isoelectric focusing electrophoresis was conducted according to the method of Righetti and Drysdale (14) using a 6% polyacrylamide disc gel containing 2% Ampholine®.

The amino acid sequence of ERABP type B was analyzed according to the method of Takashima et al. (15). The peptides were sequenced on an Applied Biosystems gas-phase sequencer 470 A. Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC on an SSC-SEQ-4 column (4.6 × 300 mm) with gradients of acetonitrile in sodium acetate buffer (16).

The ligand specificity of the ERABPs was determined by competitive binding assay as described previously (17). Briefly, 1 \mu g of ERABP in 100 \mu l of 200 mM phosphate buffer (pH 7.2) was incubated with 700 nM of [\textsuperscript{3}H] retinoic acid in the presence or absence of 200-fold molar excess of unlabeled retinal, retinoic acid, retinal or retinyl palmitate for 10 h at 4°C in the dark. After binding equilibrium had reached, the samples were subjected to a TSK G3000SW column (Toyo Soda Kogyo Co., Tokyo, Japan) on a high-performance liquid chromatography, and 0.5 ml fractions were collected at a flow rate of 1 ml/min. The radioactivity of each fraction was counted with a Beckman LS-7500 liquid scintillation counter.

Immunohistochemical staining. Antisera against ERABP type B were obtained by the method of Kato et al. (18). Purified ERABP type B (3 mg) was conjugated with 0.3 mg of bovine serum albumin in 5% glutaraldehyde solution and injected intra- or subcutaneously in multiple locations on the back of male rabbits with complete or incomplete Freund's adjuvant. Rabbit anti-ERABP sera were obtained 7 weeks after the first injection.

The tissues were prepared from 10-week-old male Fisher rats as described by Kato et al. (18). Immunohistochemical staining was carried out by the unlabeled peroxidase-antiperoxidase method of Sternberger et al. (19).

Other procedures. Fluorescence spectra were analyzed on a Hitachi 204 fluorescence spectrophotometer and absorbance spectra on a Hitachi 220A spectrophotometer. Protein concentrations were measured by the method of Lowry et al. (20). The concentration of ERABP was determined by single radial immunodiffusion. The contents of carbohydrates of purified ERABPs were measured by
the phenol-sulfuric acid method (21).

RESULTS

Purification of epididymal retinoic acid-binding proteins

From 145 g of soluble proteins, three kinds of retinoic acid-binding proteins were purified to homogeneity, namely, CRABP and ERABP types A and B. The three proteins were purified together until the third step of column chromatography on DEAE-cellulose. The ERABPs were clearly separated from CRABP by the next column chromatography on SP-Sephadex with a linear gradient of 10 to 200 mM sodium acetate, pH 4.9. In the final step of chromatography on SP-Sephadex, 22 mg of ERABP type A, which were eluted with a lower concentration of sodium acetate, and 109 mg of ERABP type B, with a higher concentration, were separated with a linear gradient of 50 to 250 mM sodium acetate, pH 4.9 (Fig. 1). The overall purification was 962-fold and the recovery was 25.9%, being estimated by a single radical immunodiffusion assay.

Physiochemical properties of ERABPs

Both purified ERABPs gave single bands in three different gel electrophoresis experiments. The migration rates of ERABP types A and B on an SDS-PAGE were consistent with molecular weights of 19,000 and 18,500, respectively (Fig. 2A). Both proteins showed prealbumin mobility on a nondenaturing PAGE, while type

Fig. 1. Final step in purification of ERABP on SP-Sephadex column chromatography. ERABP was separated into two peaks with a linear gradient of 50 to 250 mM sodium acetate buffer, pH 4.9. Peak A represents ERABP type A and peak B represents ERABP type B.

Fig. 2. Polyacrylamide gel electrophoresis of ERABPs in three different systems. 
(A) SDS-PAGE: lane 1, ERABP type A; lane 2, ERABP type B; lane 3, molecular weight standards. 
(B) Nondenaturing PAGE: lane 1, ERABP type A; lane 2, ERABP type B; lane 3, rat serum. 
(C) Isoelectric focusing gel electrophoresis: tube 1, ERABP type A; tube 2, ERABP type B.

Fig. 3. Absorption spectrum of ERABP type B complex with retinoic acid. The concentration of ERABP type B was 0.48 mg/ml. The absorption spectrum of type A was the same as that of type B.

A had a slightly higher mobility than type B (Fig. 2B). The isoelectric points of types A and B were 5.72 and 5.90, respectively, being estimated by the migration rates of the standard proteins by isoelectric focusing gel electrophoresis (Fig. 2C).

The absorption spectrum of ERABP type B had two major peaks of absorbance at 277 nm and 354 nm (Fig. 3). The ratio of $A_{354}/A_{277}$ of type B ERABP was
Fig. 4. Uncorrected fluorescence spectra of ERABP type B complex with retinoic acid. The emission spectrum was determined by monitoring with excitation at 370 nm and the excitation spectrum was determined by monitoring emission at 470 nm. The fluorescence spectra of type A was the same as type B.

1.37 when saturated with retinoic acid, and was found to be slightly lower value than 1.7 for CRABP. The uncorrected fluorescence spectra for ERABP type B complex with retinoic acid are shown in Fig. 4. When ERABP was excited at 370 nm, a peak of emission was observed at 470 nm with a shoulder at 437 nm. The excitation spectrum of ERABP, which was monitored at 470 nm for emission, had a peak at 370 nm with minor shoulders at 300 and 405 nm. The absorption and fluorescence spectra of type A were identical to those of type B.

The 30 residues of the amino-terminal of ERABP type B are shown in Table 1. The sequence of ERABP type B was identical to the deduced amino acid sequence of androgen-dependent secretory B protein of rat epididymis, which was reported by Brooks et al. (11).

Figure 5 shows the ligand specificity of ERABP with retinoic acid. The

Table 1.

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<th>(A) AVVKDFDISKFLGF<em>Y</em>IAFASKMGTPGLA</th>
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<td>(B) AVVKDFDISKFLGFWEIAFASKMGTPGLA</td>
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Amino acid sequence of ERABP type B (A) and deduced amino acid sequence of rat epididymal androgen-dependent B protein (B) reported by Brooks et al. (11). * represents an amino acid which could not be determined.

Fig. 5. Specific binding of ERABP with retinoic acid as determined by gel filtration on TSK G3000SW by high-performance liquid chromatography. The peak of radioactivity of [\textsuperscript{3}H]retinoic acid associated with ERABP type B (●) was competitively replaced by 200 molar excess of unlabeled retinoic acid (○), but not by unlabeled retinol (○), retinal (○) or retinyl palmitate (●).

Radioactivity of [\textsuperscript{3}H]retinoic acid was found to be associated with ERABP at an elution volume of 23.5 ml in the absence of unlabeled compounds. The radioactivity of [\textsuperscript{3}H]retinoic acid with ERABP was competitively replaced by unlabeled retinoic acid, but not by retinol, retinal or retinyl palmitate.

ERABPs are assumed to be glycoproteins, because ERABPs reacted with periodic acid-Schiff reagent (data not shown). The carbohydrate contents were 1.8% (w/w) for type A and 0.9% for type B.

**Immunohistochemical staining**

The antisera against ERABP type B reacted with type A and B ERABPs with the same potency, and not with rat serum, serum retinol-binding protein, CRBP, or CRABP by Ouchterlony’s diffusion method (data not shown). Using this anti-ERABP sera, immunohistochemical localization was studied in various rat organs. Specific immunostaining for ERABP was localized in the epithelium and lumen of the epididymal canal. However, no staining was observed in other tissues: testis, brain, lung, liver, spleen, kidney or gastrointestinal tract. In the proximal portion of the epididymis, namely the caput epididymis, some epithelial cells, which might be principal cells, showed strong positive staining for ERABP (Fig. 6A), and the number of positive staining cells decreased in the distal portion of the epididymal canal.
Fig. 6. Immunohistochemical localization of ERABP in rat epididymis was studied by the unlabeled peroxidase-antiperoxidase method. A, caput epididymis (×87); B, cauda epididymis (×87); C, higher magnification of caput epididymis (×350).

dymis, that is the cauda epididymis (Fig. 6B). On the other hand, specific immune staining in the lumen was observed throughout the epididymal canal and seminal tract leading from the epididymis.

Figure 6C shows a higher magnification of the epididymal canal of caput epididymis. In the epithelial cells, intensive staining was observed on the apical side rather than the basal, and also in the stereocilia. Striated staining was found in the lumen of the epididymal duct, which might be associated with spermatozoa. These results indicate that ERABP are secreted from the principal cells of the caput epididymis and attached to the surface of spermatozoa.

DISCUSSION

In order to purify CRBP and CRABP, we started to purify both proteins from testes, epididymides and seminal vesicles which contain relatively higher amounts of both proteins. During this purification, we found and purified proteins distinct from CRBP and CRABP, which associated with \[^{14}\text{C}]\text{retinoic acid. The novel proteins have different molecular weights and isoelectric points from the other kinds of retinoid-binding proteins which have been reported so far. However, the absorption and fluorescence spectra of the novel proteins were similar to CRBP and CRABP. Furthermore, the fact that by immunohistochemical study, the proteins exist only in the epididymis, we designated them epididymal retinoic acid-binding proteins.}
proteins (ERABPs). Recently, Newcomer and Ong also purified novel retinoic acid-binding proteins from the epididymis, which were identical to the major androgen-dependent epididymal proteins. Amino acid sequence analyses revealed that our purified ERABPs were also identical to androgen-dependent proteins.

The family of androgen-dependent proteins in the epididymis has been reported under various designations, such as, androgen-controlled specific proteins (22), acidic epididymal glycoproteins (23), specific epididymal proteins (24), forward motility proteins (25) or surface glycoproteins of spermatozoa (26). These proteins are acidic glycoprotein, and secreted mainly from the principal cells in the epithelium of the caput epididymis and attach to the surface of spermatozoa for their maturation. Although the expression of androgen-dependent proteins in rats is usually regulated by the serum androgen level, it was reported that a marked increase of the gene expression is not associated with androgen or androgen receptor mRNA at an early age in rats (27). Therefore, it is worthwhile to study the influence of retinoic acid on the expression of androgen-dependent proteins, namely ERABPs.

The present study has revealed that ERABPs are exclusively localized in the epididymis. This would indicate that ERABPs belong to tissue-specific retinoid-binding proteins and are different from CRBP and CRABP. Other tissue-specific retinoid-binding proteins include rhodopsin, cellular retinal-binding protein and interstitial retinol-binding protein, which exist only in the retina and have important functions in vision, and CRBP (II), found in only the small intestine, which is supposed to be involved in the absorption of retinol (10).

On the other hand, CRBP and CRABP are widely distributed in various organs. The distribution of CRBP and CRABP has been intensively studied in the genital organs of rats (6, 7). CRBP exists in the proximal portion of the epididymis, in the seminiferous tubules and Sertoli cells in testes in larger amounts. CRABP is present mainly in the seminal vesicle, vas deferens and testes. In the epididymis, CRABP is detected mainly in the distal portion rather than the proximal portion, which differs from the distribution of ERABPs. Therefore, it is difficult to assume that CRABP provides retinoic acid to ERABPs.

Like CRABP, ERABPs have a strict ligand specificity to retinoic acid. Although we have not yet studied how many molecules of retinoic acid bind endogenously to ERABP, the fact that androgen-dependent proteins bind to retinoic acid indicates that retinoic acid might play an important role in maturation of spermatozoa or in fertilization. It will be of interest to study if retinoic acid-deficient rats or anti-ERABP-treated rats really have decreased fertilization rates.

Recently, it has been reported that retinoic acid has a primary and potent morphogenic activity in the developing chick limb bud (28, 29). It is possible that some kinds of novel retinoic acid-binding proteins exist in embryonic animals and are involved in a series of regeneration, maturation of spermatozoa, fertilization and morphogenesis processes of individuals. Further study on the biological role of
retinoic acid in genital organs would provide a new understanding of retinoic acid in regeneration.

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