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STRUCTURAL CONVERSION OF CYTOSOLIC STEROID RECEPTORS BY AN AGE-DEPENDENT EPIDIDYMAL PROTEASE

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Summary—Epididymes from sexually mature rabbits contain a factor that induces a discrete reduction in the sedimentation coefficient of cytosolic estrogen receptors from various tissues (rabbit epididymis and accessory sex organs; rabbit, rat and mouse uterus) and of cytosolic progesterone receptors from the rabbit uterus. The factor is not species-specific since a similar activity was detected in extracts of mature rat epididymes. Although present in cytosol, the factor is obtained in much higher yield in hypertonic extracts of the nucleomyofibrillar fraction of mature rabbit epididymal tissue. Using rabbit uterine estrogen receptor as substrate, we have determined the following details about the rabbit epididymal factor: (1) it is tissue-specific (undetectable in extracts from rabbit accessory sex organs, testis, uterus, liver, lung, kidney and intestine); (2) it is age-dependent (undetectable in extracts from sexually immature rabbit epididymes); (3) its maintenance is testis-independent following its post-pubertal induction or activation; (4) it is primarily localized in the caput region of the epididymis; (5) it is inactivated by elevated temperature; (6) it is macromolecular in nature; (7) it is DNase- and RNase-resistant; (8) it is irreversibly inactivated by leupeptin, indicating that it is a protease; and (9) it is effective on unoccupied and occupied receptors.

INTRODUCTION

Studies from this laboratory on the hormonal regulation of the male reproductive tract have demonstrated that exogenous estrogen administration has marked effects on the weights and histology of the epididymis and accessory sex organs (vesicular gland, prostatic complex and bulbourethral gland) of sexually immature rabbits [1]. That such responses represent a direct action of estrogen is supported by the presence of estrogen receptors in the cytosolic fraction of these organs [2], and by the ability of estrogen to cause depletion of cytosolic receptors with subsequent accumulation in the nuclear fraction under *in vitro* [3] and *in vivo* conditions [4].

Although initial studies suggested that estrogen receptors were present only in cytosol from epididymes of sexually immature rabbits [2], subsequent studies performed under different experimental conditions demonstrated detectable but considerably lower levels of this receptor in epididymal cytosol from sexually mature animals [5]. Of further interest was the observation of an age-dependent change in the sedimentation behavior of the cytosolic estrogen receptor from the rabbit epididymis [5]. That is, under hypotonic conditions, the receptor sedimented primarily as a >8 S species when obtained from sexually immature animals, but almost exclusively as an ≃4 S species when obtained from sexually mature animals. The age-dependent change in the sedimentation behavior of the epididymal cytosolic estrogen receptor was not accompanied by a measurable alteration in its ligand affinity or specificity [6]. In the accessory sex organs an age-dependent decrease in estrogen-specific binding sites was observed, but no change occurred in the sedimentation coefficient of the estrogen receptor [6].

We developed two alternative hypotheses to account for the age-dependent alteration in the sedimentation coefficient of the estrogen receptor in the epididymis, namely: (1) that, as the animals aged, changes in the receptor occurred that did not allow it to assume the oligomeric state; or (2) that a factor present in the epididymis of adult rabbits modified the "native" oligomeric receptor. In this communication, we demonstrate that a salt-extractable macromolecular protease, apparently unique to the epididymis of sexually mature animals, is responsible for the >8 S to <4 S shift in the sedimentation coefficient of the estrogen receptor. We shall refer to this alteration in the sedimentation properties of the receptor as receptor conversion. Our hypothesis is that the appearance of the protease and its subsequent digestion of the estrogen receptor is responsible for down-regulation of estrogen action in the epididymis of adult rabbits.

EXPERIMENTAL

Chemicals

New England Nuclear Corp. (Boston, Mass., U.S.A.) was the source of [2,4,6,7-^{3}H]estradiol (90–115 Ci/mmol), [17α-methyl-^{3}H]R-5020 (70–

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87 Ci/mmol) and [^{14}C]formaldehyde (10 mCi/mmol). Unlabeled estradiol and cortisol were purchased from Steraloids (Wilton, N.H., U.S.A.). Neutralized charcoal (Norit-I), dextran-60C, $CaCl_2$ (Grade I), Tris (base), NaSCN, sucrose (Grade I), ribonuclease-A (RNase, protease and salt-free, type II-A from bovine pancreas), deoxyribonuclease-I (DNase, non-crystalline from bovine pancreas), bovine serum albumin, bovine γ-globulins, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, synthetic leupeptin and monothioglycerol were from Sigma (St. Louis, MO, U.S.A.). Glycerol, KCl, Triton X-100, dimethylsulfoxide (DMSO), isopropanol and dialysis tubing were from Fisher (Fair Lawn, N.J., U.S.A.). Spectrafluor was from Amersham (Arlington Heights, Ill., U.S.A.), EDTA was from Matheson, Coleman & Bell (Norwood, Ohio, U.S.A.) and hydroxylapatite (HAP, DNA grade) was from Bio-Rad Labs (Richmond, Calif., U.S.A.). Pepstatin and diisopropyl fluorophosphate (sealed in glass capillary tubes) were gifts from Drs T. Inagami and A. M. Burt, respectively, of Vanderbilt University School of Medicine.

Biological materials

Sexually mature (at least 6-months old) and sexually immature (approx. 4- to 6-weeks old) male New Zealand White rabbits, mature male (retired breeders) and immature female (21–23 days) Sprague–Dawley rats and mature female mice (C57BL/6N) were used in this study. Animals were killed by decapitation or with a lethal dose of Nembutal and the tissues of interest were promptly removed. Frozen tissues (uteri from young rabbits and epididymes from mature rabbits) were purchased from Pel-Freez Biologicals (Rogers, Ark., U.S.A.), stored at −20°C, and thawed in ice-cold TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 7.4, at 23°C) when needed. Castration and testicular efferent duct ligation were performed *via* the scrotal route on mature rabbits under Nembutal anesthesia.

Tissue fractionation

Unless otherwise stated, all procedures were performed at 0–4°C. Tissues were dissected free of fat and connective tissue (uterus and intestine were also slit longitudinally), rinsed in TE, blotted dry, weighed, minced with scissors and homogenized in 4 vol of buffer (TE with or without additional agents) usually with three 5 s bursts of a polytron PT 10 ST (Brinkman Instruments, Westbury, N.Y., U.S.A.). Homogenates were centrifuged at 249,000 **g** for 30 min in a Beckman L2-65B centrifuge and resultant supernatant fluids (cytosol) were collected. Cytosol containing estrogen and progesterone receptors were utilized immediately. The various extracts from other tissues (see below) were often stored at −20°C until assayed for receptor-converting activity.

Mature rabbit epididymal tissue, homogenized as described above, was also subjected to fractionation by differential centrifugation. The homogenate was first centrifuged at 800 ***g*** for 10 min to yield a nucleomyofibrillar pellet. An aliquot of the post-nucleomyfibrillar supernatant was removed and the remainder separated (10,000 ***g*** for 1 h) into a mitochondria- and lysosome-enriched pellet and a post-mitochondrial supernatant. An aliquot of this supernatant was removed and centrifuged (249,000 ***g*** for 30 min) to yield a microsome-enriched pellet and a particle-free supernatant (cytosol). Both of the post-nucleomyofibrillar pellets were washed twice and resuspended to the original homogenate volume with TE and all fractions were subsequently assayed for receptor-converting activity, i.e. the ability to cause a decrease in the sedimentation coefficient of receptors on hypotonic sucrose gradients.

Alternative homogenization media

In one experiment, cytosol was prepared from mature rabbit epididymal tissue which had been homogenized in the standard hypotonic buffer (TE) or an isotonic buffer (10 mM Tris, 0.5 mM $CaCl_2$, 0.25 M sucrose); both, with and without 0.1% Triton X-100. In other experiments, various tissues from immature and mature rabbits were homogenized in hypotonic TE buffer or in TE buffer made hypertonic in 0.4–1.5 M KCl or NaSCN. The resultant cytosols were desalted by dialysis for 16–24 h against 2 × 4 l. of TE buffer using a membrane with a cutoff of 12,000–14,000 daltons.

Salt extraction of converting factor from particulate fraction of epididymal tissue

Mature rabbit epididymal tissue was homogenized in isotonic buffer (TE + 0.25 M sucrose) with five strokes of a glass/glass homogenizer and separated (800 ***g*** for 10 min) into a nucleomyofibrillar pellet and post-nucleomyofibrillar supernatant. The pellet was resuspended and washed four times with half the homogenate volume of isotonic TE. Portions of the particulate material were resuspended to the original homogenate volume with isotonic TE or hypertonic TE (final concentration of 0.4 M KCl or NaSCN) and incubated at 0–4°C for 1 h with periodic agitation. The initial supernatant and particulate extracts were centrifuged at 249,000 ***g*** for 30 min and the resultant supernatants were dialyzed and stored as described above.

Protease inhibitor screening study

Samples of TE buffer or mature epididymal cytosol were left untreated or treated at 25°C for 1 h with the appropriate volume of the following stock solutions of protease inhibitors: PMSF, 10 mM in isopropanol; DFP, 10 mM in TE buffer (made up immediately prior to use); 1,10-phenanthroline, 10 mM in TE buffer; pepstatin, 1 mM in DMSO; leupeptin, 10 mM in TE buffer. All samples were then analyzed, as described below, for their effects on rabbit uterine cytosol estrogen receptors.

Analytical techniques

Before or after combination, cytosol and tissue extract samples were incubated at 0–4°C for at least 2 h with 5 nM [^{3}H]estradiol or 20 nM [^{3}H]R-5020 plus 4 μM cortisol (to saturate CBG-like binders) alone, or together with a 100-fold excess of unlabeled estradiol or R-5020, respectively. Free and loosely-bound steroid was removed from all samples by treatment ([^{3}H]estradiol, 10 min; [^{3}H]R-5020, 30 s) with a pellet from an equal volume of a 0.5% charcoal–0.05% dextran suspension. The charcoal was sedimented by centrifugation for 5 min at 1500 ***g***, and aliquots (200 μl) of the supernatants were layered onto sucrose gradients. Linear 5–20% sucrose gradients containing 10% glycerol, 12 mM monothioglycerol and 0.01 m KCl were prepared, run and analyzed as previously described [7]. The data were plotted as radioactivity (cpm)/fraction. To estimate the amount of steroid-binding activity present in sucrose gradients, areas (mm^2) of the plotted binding peaks were calculated using a Bioquant II (E. Leitz Inc, Rockleigh, N.J., U.S.A.).

The hydroxylapatite technique [8] was used to measure estrogen receptors in individual gradient fractions. Fractions (approx. 250 μl) were collected at 0–4°C, brought up to 500 μl with TE, and either immediately (fractions without additional steroid) or 2 h later (fractions exposed to 5 nM [^{3}H]estradiol) received 200 μl of HAP suspension (10 g/100 ml in TE). All samples were incubated at 0–4°C for 45 min with frequent agitation. HAP was pelleted, washed twice with 1.0 ml TE, extracted twice with 500 μl ethanol and the combined ethanol extracts were analyzed for radioactivity.

Sedimentation standards, bovine serum albumin (4.4 S) and bovine γ-globulin (7.1 S), were labeled with ^{14}C according to the method of Rice and Means[9]. Further experimental details are given in the Results section and figure legends.

Miscellaneous techniques

Radioactivity in the samples was determined after the addition of a scintillation fluid containing toluene, Triton X-100 and Spectrafluor (2365:1230:100). The counting efficiency for ^{3}H was approx. 32% and that for ^{14}C was approx. 48%.

RESULTS

Identification of receptor-converting factor in epididymal cytosol from sexually mature rabbits

When cytosol was prepared identically from epididymes of sexually immature and mature rabbits and analyzed on hypotonic sucrose gradients, the estrogen-binding profiles shown in Figs 1A and 1B were obtained. Consistent with previous observations [5, 6], estrogen receptor from the immature epididymis sedimented primarily at >8 S (Fig. 1A), while the receptor from the mature epididymis sedimented at <4 S (Fig. 1B). To determine whether these differences represented an inherent age-dependent alteration in the structure of the epididymal estrogen receptor, or were due to modification of the receptor by factors present in mature epididymal tissue, we performed a mixing experiment. Following combination of equal volumes of immature and mature epididymal cytosol, recovery of binding activity was quantitative (100% according to charcoal assay just prior to sedimentation analysis, and >94% according to calculation of binding peak areas), however much of the activity was shifted from the >8 S to <4 S form (Fig. 1C). These data demonstrate the presence of a factor in mature rabbit epididymal tissue that is capable of modifying the structure of endogenous estrogen receptors.

No evidence of receptor-converting activity was observed in cytosol prepared from pooled accessory sex organs (seminal vesicle, prostate and bulbourethral gland) of the same animals (Figs 1D–1F). Despite the age-dependent decrease in cytosol estrogen receptor concentration in these organs (Figs 1D vs 1E), it remained in the >8 S state. Furthermore, when cytosols from the accessory sex organs of both age groups were combined (Fig. 1F), recovery of estrogen-binding activity was again quantitative (94% by charcoal assay, and 100% by peak area), and remained in the >8 S state. Sensitivity to conversion is not unique to the epididymal receptor since the >8 S receptor present in accessory sex organ cytosol from both age groups shifted to the <4 S form after exposure to mature, but not immature, epididymal cytosol (data not shown). All observations were confirmed in at least three separate experiments.

Comparative effects of mature rabbit epididymal cytosol on estrogen and progestin receptors

To determine if the factor would act on estrogen receptors in other tissues, cytosol from frozen rabbit uteri (Fig. 2A), was used. Similarly prepared cytosol from mature rabbit epididymes contained a negligible amount of estrogen-specific binding sites (Fig. 2B), but it effectively converted the uterine receptor from the >8 S to <4 S state (Fig. 2C). Sensitivity to conversion is not unique to the estrogen receptor from rabbit tissues since estrogen receptors obtained from rat and mouse uteri were also converted (data not shown).

Figure 3 demonstrates that cytosol from frozen rabbit uteri also contains a high concentration of progestin-specific binding sites (Fig. 3C) which sediment slightly slower (7–8 S) than the estrogen receptor (Fig. 3A) under hypotonic conditions. The majority of these two receptor species shift to a <4 S state after exposure to mature rabbit epididymal cytosol (Figs 3B and 3D). Thus the progesterone receptor is also sensitive to the converting factor. All observations were confirmed in at least three separate experiments.

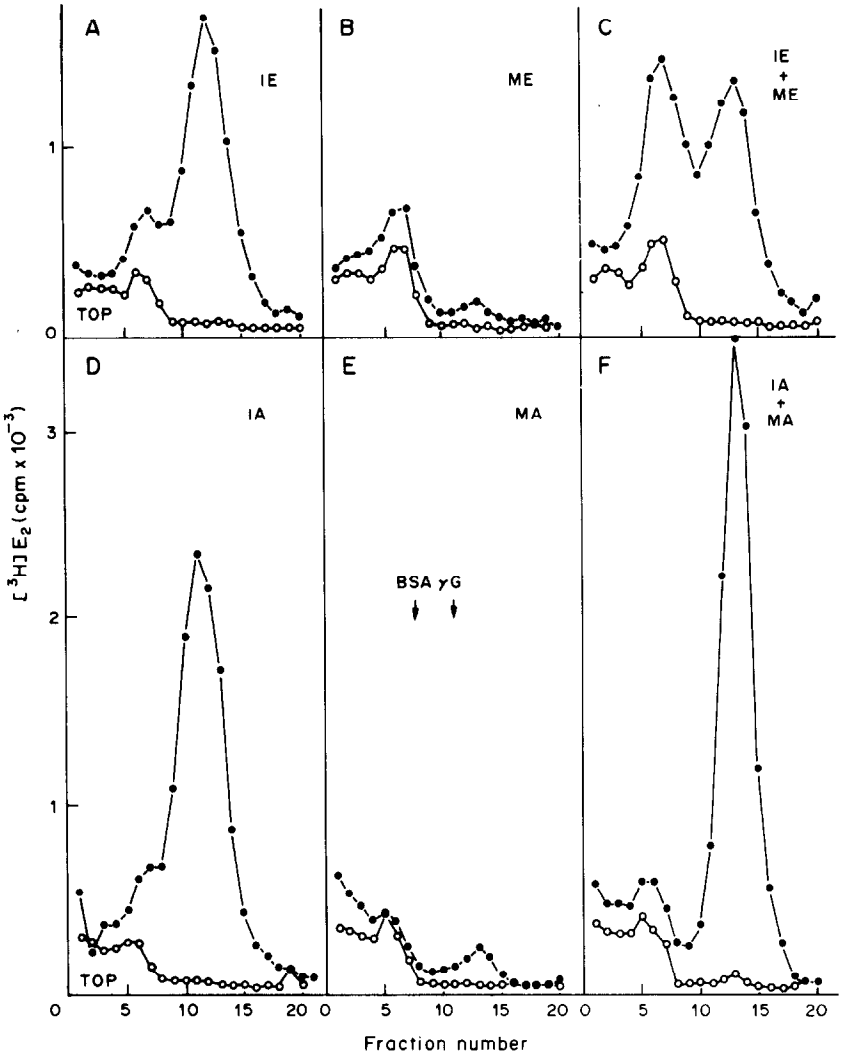

Fig. 1. Sedimentation analysis of estrogen-binding activity in cytosol samples prepared from entire epididymes and pooled accessory sex organs of sexually immature and sexually mature rabbits. Cytosol aliquots (at 0–4°C) were combined either with equal volumes of buffer or with each other, and exposed to [^{3}H]estradiol without (●) or with (○) unlabeled estradiol. Two hours later, all samples were charcoal-stripped and then analyzed on hypotonic (0.01 M) sucrose gradients: individual immature epididymal (A) and immature accessory sex organ (D) cytosol, individual mature epididymal (B) and mature accessory sex organ (E) cytosol, combined immature plus mature epididymal (C) and combined immature plus mature accessory sex organ (F) cytosol. The arrows indicate the sedimentation position of ^{14}C-labeled bovine serum albumin (BSA) and bovine γ-globulin (γG). IE, immature epididymis; ME, mature epididymis; IA, immature accessory sex organs; MA, mature accessory sex organs.

General characteristics of receptor-converting factor in epididymal cytosol

Summarized below are the results of studies utilizing the converting activity assay system described above (i.e. the >8 S to <4 S conversion as monitored on low-salt sucrose gradients) with rabbit uterine cytosol, a rich source of estrogen receptor, as substrate. All observations listed below were confirmed in at least three separate experiments.

(1) *Tissue-specific.* Receptor-converting activity is undetectable in rabbit accessory sex organ or testicular cytosol, or blood plasma from either immature or mature rabbits.

(2) *Age-dependent.* Receptor-converting activity was never detected in epididymal cytosol prepared from sexually immature rabbits (>10 observations), but was always present in that prepared from mature animals (>100 observations).

(3) *Not species-specific.* Cytosol prepared from the adult rat epididymis also contained receptor-converting activity, but on a tissue weight basis, it was less effective than that obtained from the rabbit.

(4) *Regionally distributed in rabbit epididymis.* Since the rabbit epididymis is regionally differentiated, both anatomically and with respect to estrogen receptor levels [5, 10], we assayed cytosol

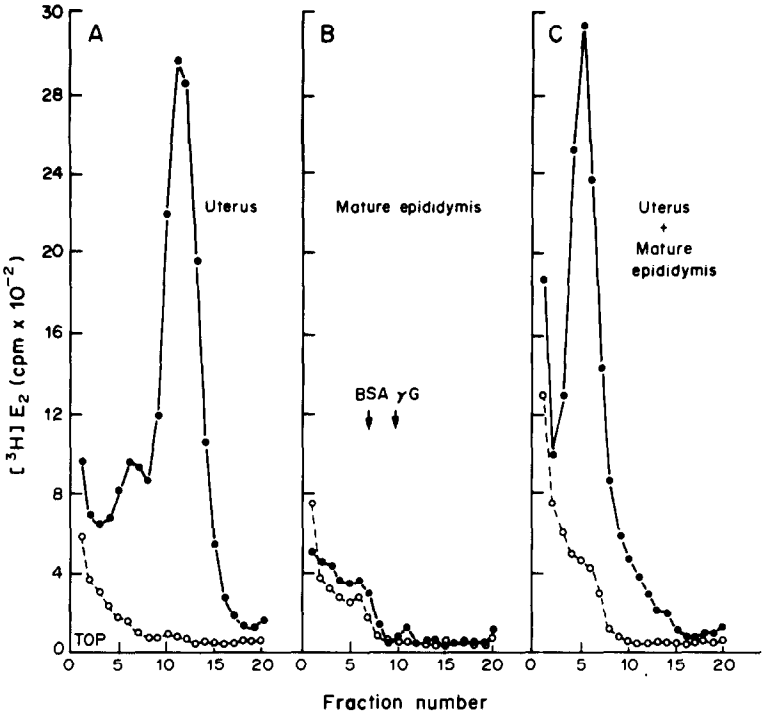

Fig. 2. Sedimentation analysis of estrogen-binding activity in cytosol samples prepared from rabbit uterus and mature rabbit epididymis: individual uterine (A) and mature epididymal (B) cytosol, combined uterine plus epididymal (C) cytosol; [^{3}H]estradiol alone (●), [^{3}H]estradiol plus unlabeled stradiol (○). See legend to Fig. 1 for experimental details.

prepared from the major anatomical segments (caput, corpus, cauda) of the mature rabbit epididymis for converting activity. In three separate experiments, activity was present in caput cytosol but was virtually undetectable in cauda cytosol.

(5) *Post-pubertal maintenance is not testis-dependent.* To test whether the factor might be regulated by endocrine or exocrine products of the testis, groups of 3 adult rabbits either had their testes removed bilaterally (to remove testicular endocrine and exocrine products) or had their ductuli efferentes ligated unilaterally (to prevent testicular exocrine products from entering the epididymis). Cytosol prepared from one epididymis removed at the time of surgery served as the control for castrated animals. Two weeks after surgery, all animals were killed and the cytosol prepared individually from the remaining testes and epididymides was assayed for receptor-converting activity. According to same animal comparisons, neither castration nor duct ligation had any apparent positive or negative effect on the level of converting activity recovered in epididymal cytosol 2 weeks after surgery, nor was activity detectable in cytosols prepared from testes with ligated efferent ducts. These results confirm that the converting factor is of epididymal origin and indicate that, once induced, its maintenance is not dependent on endocrine or exocrine secretions of the testis.

(6) *Temperature effects.* To examine the temperature sensitivity of the converting factor in adult rabbit epididymes, aliquots of epididymal cytosol were incubated at various temperatures, chilled and then assayed for converting activity. Although completely inactivated by a 10 min treatment at 100°C, the factor was only partially inhibited by a 1 h treatment at 60°C and unaffected by a 1 h treatment at 37°C. Furthermore, the factor was stable for at least 3 months at −20°C, and remained active after repeated freezing and thawing.

(7) *Macromolecular in nature.* Mature rabbit epididymal cytosol was subjected to ultrafiltration using a membrane with a molecular weight limit of 10,000 daltons (PM-10, Amicon Corp.). That converting activity was present in the high molecular weight cytosol retentate, but absent in the low molecular weight filtrate provides evidence that the factor is either itself macromolecular or is tightly associated with a macromolecule. This conclusion is further supported by the maintenance of converting activity in epididymal extracts following extensive dialysis (see below).

(8) *DNase- and RNase-resistant.* Since treatment of the factor with RNase or DNase at levels up to 500 μg/ml for 1 h at 37°C failed to affect its converting activity, we conclude that it is a protein.

Influence of protease inhibitors

To test the hypothesis that the epididymal factor might be proteolytic in nature, we analyzed the ability of several protease inhibitors, with specifications covering the four protease classes [11], to block receptor-converting activity. In the absence of any treatment

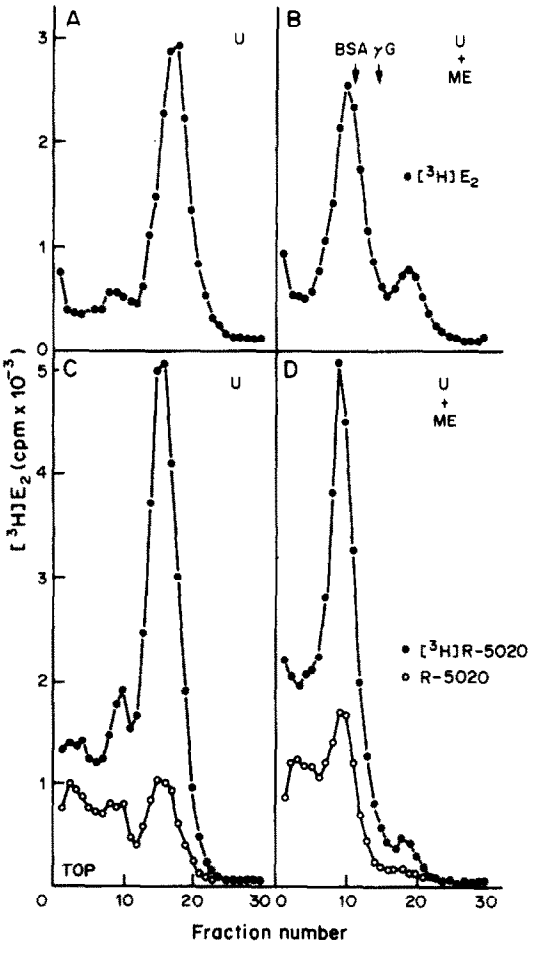

Fig. 3. Sedimentation analysis of estrogen and progestin-binding activity in rabbit uterine cytosol before and after exposure to mature rabbit epididiymal cytosol. Aliquots of uterine cytosol labeled with [^{3}H]estradiol alone (A, B) or [^{3}H]R-5020 and unlabeled cortisol without (●) and with (○) unlabeled R-5020 (C, D) were charcoal-stripped, incubated at 0–4°C for 2 h with equal volumes of buffer (A, C) or epididymal cytosol (B, D) and analyzed on hypotonic (0.01 M KCl) sucrose gradients. U, uterus.

(Fig. 4A), a sample of epididymal cytosol converted most of the >8 S estrogen receptor in rabbit uterine cytosol to the <4 S form. Using samples of buffer or epididymal cytosol which had been pretreated either with two potent inhibitors of serine proteases (1.0 mM PMSF or DFP), a metal chelator known to inactivate metalloproteases (1.0 mM 1,10-phenanthroline), or the highly specific inhibitor of aspartic proteases (0.1 mM pepstatin), no inhibition of conversion was obtained. Thus the factor appears not to be a serine, metallo or aspartic protease. The ability of the epididymal factor to convert receptor was inhibited by 1.0 mM leupeptin (Fig. 4B), a bacterial tripeptide known to have an inhibitory influence on thiol proteases [12]. Leupeptin itself had no effect on the sedimentation behavior of the receptor. Recovery of binding activity, according to calculation of binding peak areas in leupeptin treated combined samples, matched that (>80%) in the untreated combined samples (Fig. 4A).

In other experiments (results not shown), highly active preparations of the epididymal factor were treated or not with 1.0 mM leupeptin. A portion of the leupeptin-treated extract was dialyzed against TE before being combined with samples of cytosol estrogen receptor. Activity in the untreated epididymal extract was sufficient to convert all the >8 S receptor in the cytosol sample to the <4 S form, but no converting activity was detected in either the non-dialyzed or dialyzed leupeptin-treated extracts. These data indicate that leupeptin interacts directly with the epididymal factor and cannot be dissociated from it by dialysis.

Optimization of protease recovery

Since we observed considerable variability in the amount of converting activity obtained from various preparations of epididymal cytosol, we attempted to optimize and standardize recovery of the protease. In initial experiments (data not shown), we detected no apparent enhancement of converting activity in: (1) any of the post-nucleomyofibrillar fractions of epididymal tissue; (2) in cytosol prepared from epididymal tissue homogenized either in buffered isotonic sucrose with $CaCl_2$ to favor integrity of plasma membranes and particulate cell structures [13], or in the standard hypotonic TE buffer which leads to lysis, fragmentation and stripping of cell structures [13]; or (3) in samples which contained 0.1% Triton X-100 in the homogenization buffer to solubilize membrane-bound components [13]. These

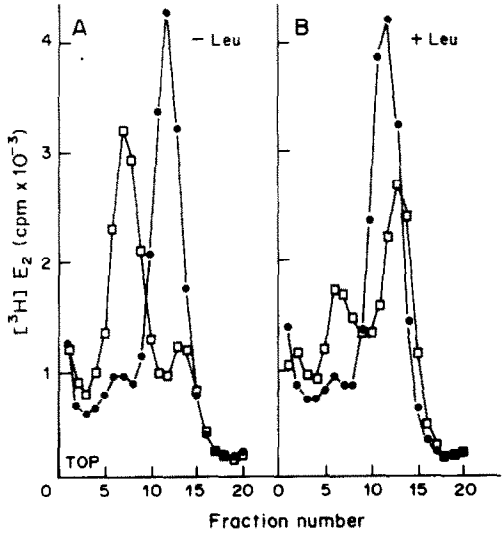

Fig. 4. Leupeptin inhibition of the receptor-active epididymal factor. Aliquots of [^{3}H]estradiol-labeled, then charcoal-stripped, rabbit uterine cytosol were incubated at 0–4°C for 2 h with equal volumes of buffer (●) or mature rabbit epididymal cytosol (□) which had been either left untreated (A), or treated with 1.0 mM leupeptin at 25°C for 1 h. All samples were analyzed on hypotonic (0.01 M KCl) sucrose gradients. Leu, leupeptin.

results indicate that the protease is not derived from non-nucleomyofibrillar particulate cellular components nor from the bulk of plasma membranes which would cosediment with the nucleomyofibrillar fraction in the absence of detergent [14, 15].

Notides *et al.*[16] have identified a human uterine protease capable of converting the rat uterine estrogen receptor from an 8 S to a 4.5 S sedimenting form. This protease was only partially recovered in cytosol prepared from tissue homogenized under hypotonic (50 mM KCl) conditions, but was fully recovered in cytosol prepared using hypertonic (1.5 M KCl) homogenization conditions. Preparation of epididymal cytosol using similar hypertonic homogenization conditions (followed by dialysis to remove salt) yielded enhanced recovery of receptor-converting activity (Fig. 5C) compared to cytosol prepared under standard hypotonic conditions (Fig. 5B). Hypertonic homogenization conditions failed to extract measurable receptor-converting activity from: mature rabbit uterus; mature male rabbit liver, intestine, kidney or lung; or immature rabbit epididymis (data not shown). These results confirm the age-dependent and tissue-specific nature of the epididymal protease, and are in sharp contrast to the nearly ubiquitous distribution of steroid receptor-active proteases in tissues of the calf [17] and chick [18].

Since the epididymal protease did not appear to be associated with cellular organelles or plasma membranes, but was solubilized by salt treatment of whole tissue, we reasoned it must be a component of the nucleomyofibrillar fraction. Considering also that Murayama and Fukai[19] have reported that gilt uterine nuclei contain an estrogen receptor-active protease that is extracted with 0.4 M NaSCN but not 0.4 M KCl, we compared the levels of receptor-converting activity in samples of epididymal cytosol and in various extracts of the washed nucleomyofibrillar fraction of epididymal tissue. As shown in Fig. 6, an isotonic extract of the nucleomyfibrillar fraction was devoid of converting activity (Fig. 6C), whereas dialyzed, hypertonic extracts of the same material (Figs 6D and 6E) were more active than the cytosol fraction (Fig. 6B) and still demonstrated sensitivity to elevated temperature (Fig. 6F). We conclude that the variable levels of converting activity normally recovered in epididymal cytosol represent contamination of cytosol with a protease originally associated with the nucleomyofibrillar fraction of mature rabbit epididymal tissue. In contrast to the KCl resistance of the receptor-active protease in gilt uterine nuclei [19], the epididymal protease was solubilized equally well by KCl (Fig. 6D) and NaSCN (Fig. 6E).

Effects of the epididymal protease on occupied and unoccupied estrogen receptors

The receptor-active protease extracted from human uterine tissue preferentially converts estrogen receptor which is occupied by [^{3}H]estradiol [16]. This is not the case with the epididymal protease. As shown in Fig. 7, >8 S to <4 S conversion of the rabbit uterine estrogen receptor occurred to the same

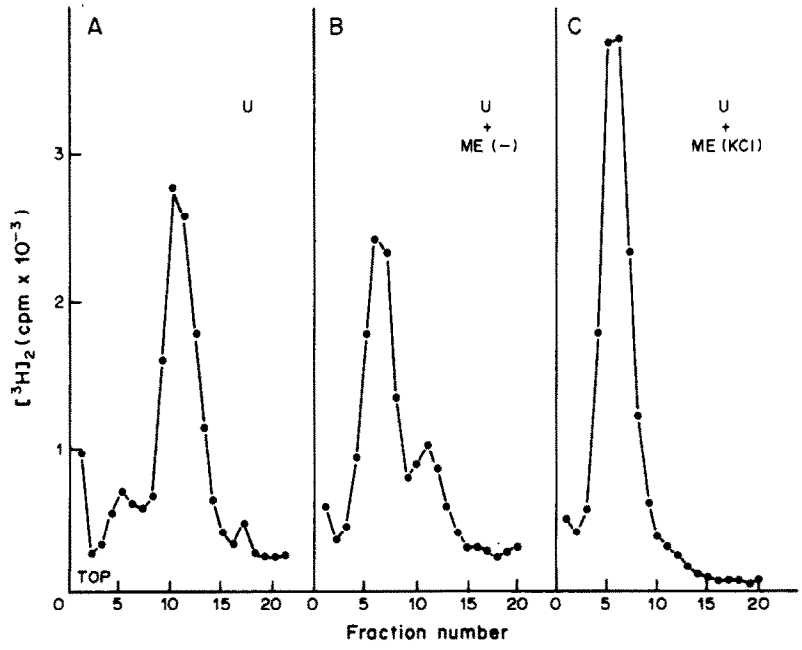

Fig. 5. Sedimentation analysis of estrogen-binding activity in rabbit uterine cytosol before and after exposure to hypotonic and hypertonic extracts of mature rabbit epididymes. Aliquots of [^{3}H]estradiol-labeled then charcoal-stripped uterine cytosol were incubated at 0–4°C for 2 h with equal volumes of buffer (A) or the dialyzed soluble fraction of epididymal tissue homogenized in the absence (B) or presence (C) of 1.5 M KCl and analyzed on hypotonic (0.01 M KCl) sucrose gradients.

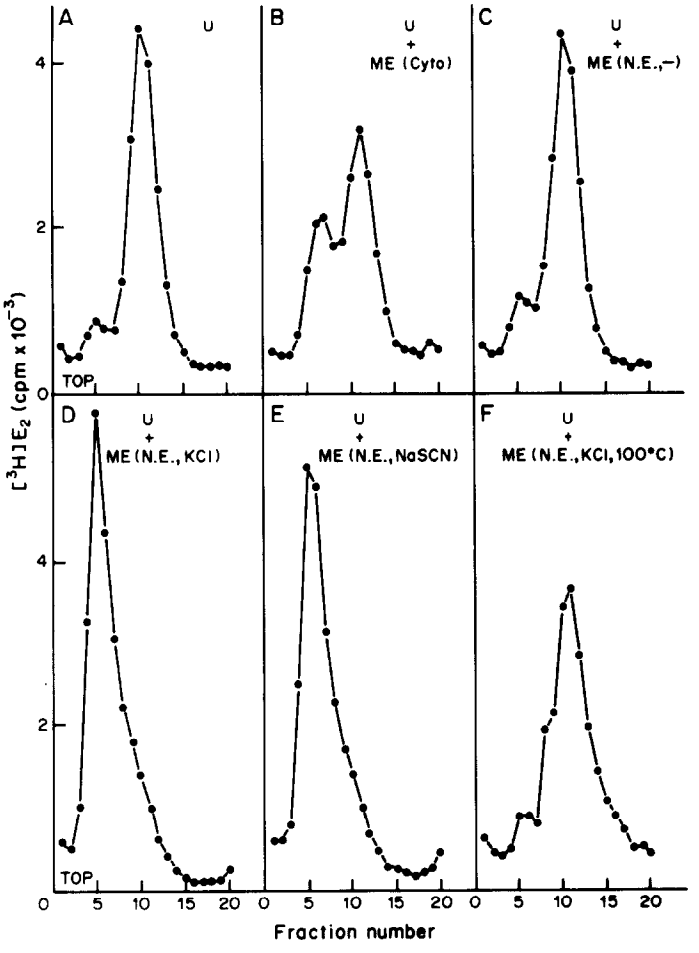

Fig. 6. Sedimentation analysis of estrogen-binding activity in rabbit uterine cytosol before (A) and after (B–F) exposure to the cytosol fraction (cyto) or various extracts of the nucleomyofibrillar fraction (NE) of mature rabbit epididymal tissue (ME). Experimental procedure was the same as described for Fig. 5, except that epididymal tissue was processed as follows: tissue was homogenized under isotonic conditions and separated into cytosol (B) and a washed nucleomyofibrillar fraction; portions of the nucleomyofibrillar material were extracted (1 h at 0–4°C) with isotonic buffer (C) or buffer containing 0.4 M KCl (D) or 0.4 M NaSCN (E) and an aliquot of the KCl extract was also heated to 100°C for 10 min (F); all epididymal preparations were dialyzed against TE buffer before being combined with uterine cytosol.

extent regardless of whether the receptor was exposed to ^{3}H-labeled ligand before (Fig. 7A), before and after (Fig. 7B) or only after (Fig. 7C) being exposed to the epididymal protease. The difference in binding levels observed between prelabeled and post-labeled receptor populations is a consequence of the dissimilar ligand:receptor ratios associated with each labeling technique.

DISCUSSION

Our data indicate that the previously observed [5, 6] age-dependent difference in the sedimentation behavior of the rabbit epididymal estrogen receptor represents modification of the receptor by a protease present in epididymal tissue from sexually mature animals and indicate that the protease is different from several previously identified steroid receptor-active proteases [16–19]. Two lines of evidence mitigate against the possibility that the epididymal protease is a non-specific enzyme. First, the estrogen and progesterone receptor conversion event is always discrete in that we never observe intermediate sedimenting forms. Furthermore, the epididymal protease cleaves the steroid-binding subunit of the glucocorticoid receptor to a single detectable fragment that retains the ligand-binding domain [20]. Interestingly, no glucocorticoid receptor-active proteolytic activity similar to that present in rat liver and kidney [21] is detectable in the liver or kidney of the rabbit (W. J. Hendry, B. J. Danzo and R. W. Harrison, unpublished data). Secondly, although the protease acts on intracellular steroid receptors, it does not act on androgen-binding protein, since this protein sediments the same (approx. 4 S) whether it is isolated from Sertoli cells [22] or from the caput epididymidis of sexually immature or mature rabbits [7, 22]. In addition, the epididymal protease does not affect the Stokes radius of thyroglobulin, ferritin, chymotrypsinogen, aldolase or ribonuclease,

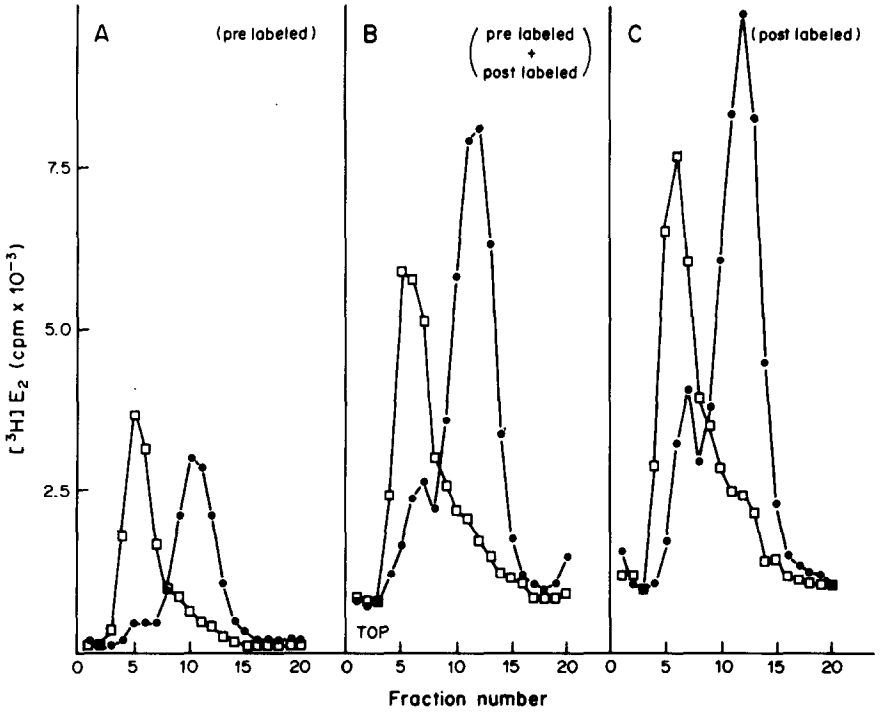

Fig. 7. Sedimentation analysis of occupied and unoccupied rabbit uterine cytosol estrogen receptors before and after exposure to partially purified protease from mature rabbit epididymal tissue. Aliquots of uterine cytosol already labeled (A, B) or not (C) with [^{3}H]estradiol were combined with equal volumes of buffer (●) or the dialyzed material extracted from the nucleomyofibrillar fraction of epididymal tissue with 0.4 M KCl (□) and sedimented on hypotonic (0.01 M KCl) sucrose gradients. Gradient fractions were collected at 0–4°C into buffer without (A) or with [^{3}H]estradiol (B, C). Receptor-bound radioactivity in each fraction was measured either immediately (A) or 2 h later (B, C) by the hydroxylapatite method.

as assessed by chromatography of the proteins on Sephacryl S-300 alone or after combining them with the epididymal protease under the same incubation conditions that caused steroid receptor conversion (data not shown).

Structural modification of steroid receptors often has profound effects on their functional activities. For instance, reduction of the sedimentation coefficient and molecular weight of receptors by the action of proteases can result in either a negative or positive influence on subsequent DNA- or nuclear-binding activity [23, 24]. Furthermore, Murayama *et al.*[25–27] have presented evidence that the "native" cow uterine estrogen receptor, when separated from various "binding factors", sediments at 4 S under both hypotonic and hypertonic conditions [25], and is inhibited from undergoing translocation into nuclei when complexed back with the "binding factors" [26, 31]. Thus it appears that steroid holoreceptors are composed of distinct regulatory domains or subunits that serve to permit or hinder the participation of the receptor in the various events involved in the mechanism of steroid hormone action. For these reasons, it will be important to determine the receptor subunit(s) or domain(s) affected by the epididymal protease, and assess the functional consequences of such action. The results of such studies may indicate ways in which the epididymal protease may be utilized as a reagent to probe structure/function relationships in steroid receptor systems.

The post-pubertal induction or activation of the protease suggests that it is involved in, or is a consequence of, developmental events in the rabbit epididymis. To ascertain whether the factor is an *in vivo* modulator of estrogen receptor function in the epididymis, it will be important to determine whether the protease resides in estrogen target cells, and if so, whether the subcellular localization of the protease is such that it would be capable of interacting with receptor *in vivo*. To answer these questions it will be necessary to develop techniques for separating and characterizing tissue-specific cell types, and/or reliable methods for the histological localization of receptor and protease. Our localization of the protease to the nucleomyofibrillar fraction of epididymal tissue suggests that it may reside in the nuclei of cells comprising that fraction. Although classic dogma asserts that steroid receptors are present in the cytoplasm of target cells and undergo translocation to the nucleus only after complexing with ligand [28], new evidence suggests that, *in vivo*, steroid receptors always reside in the nuclear compartment [29, 30] and that unoccupied receptors undergo redistribution when cells are disrupted under hypotonic conditions [31]. Thus the possibility exists that both

occupied and unoccupied epididymal estrogen receptors would have access to the converting factor. As we have shown, both forms of the receptor are sensitive to the protease *in vitro*. Current results do not exclude the possibility that the protease is a component of the connective tissue extracellular matrix and thus unavailable to act on the receptor *in vivo*.

The final question is whether the epididymis of immature and mature animals responds differently to hormonal stimuli in terms of estrogen receptor dynamics and organ morphology. We have demonstrated in the immature rabbit [1] that the epididymis and accessory sex organs respond to exogenous estrogen treatment with a pattern of growth that is organ-specific and with histological changes that are tissue-specific. Estrogen receptor dynamics (nuclear accumulation and processing) following hormonal treatment also varied among these organs [4]. We have also noted that estrogen-stimulated weight gain by the epididymis of castrated adult rabbits is much less than that which occurs in castrated immature rabbits. Estrogen treatment results in a 4.5-fold increase in the weight of the epididymis of immature rabbits over castrated control values [1], but in only a 1.3-fold increase over castrated controls in adult rabbits (unpublished observations). This decrease in estrogen sensitivity is accompanied by the appearance or activation of the epididymal protease that is able to convert the "native" oligomeric >8 S receptor to a fragment (<4 S) which has reduced affinity for nuclei (B. J. Danzo, unpublished data). Our working hypothesis is that the effects of the protease are responsible for the decreased estrogen sensitivity of the adult epididymis. Studies are underway to test this hypothesis.

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