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HIGH-RESOLUTION SEPARATION OF MOLYBDATE-STABILIZED PROGESTIN RECEPTORS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Molecular heterogeneity of the human uterine progestin receptor was investigated employing sucrose density gradient centrifugation and high-performance liquid chromatography (HPLC) in size-exclusion (HPSEC), ion-exchange (HPIEC) and chromatofocusing (HPCF) modes. Synthetic progestomimetic ligands, [^{3}H]R5020 and [^{3}H]ORG-2058, were used to identify these receptors. Rapid centrifugation with a vertical tube rotor showed both 8–9 S and 4–5 S receptor species in the presence of 10 m*M* sodium molybdate with a 90–96% recovery. [^{3}H]R5020 displayed greater non-specific binding than [^{3}H]ORG-2058. When separated receptor preparations were labeled, each with a different ligand, mixed and separated on optimized gradients, at least two receptor isoforms were identified in the components sedimenting at 8–9 S. HPSEC confirmed the presence of receptor isoforms displaying different molecular size and shape dependent upon the progestin ligand used. When the surface charge properties were examined by HPIEC using AX-1000, two distinct species were observed irrespective of the radioactive ligand. The first peak appeared in the void volume similar to the position of free steroid, indicating the possibility of ligand stripping by the column. The second peak bound steroid specifically and eluted with 100 m*M* phosphate. If either 8-S or 4-S progestin receptors were first separated by gradient centrifugation then by HPIEC, both receptor isoforms eluted with 60 m*M* phosphate. Re-chromatography of these on HPIEC also gave the isoform eluting at 60 m*M* phosphate. HPCF of ligand-bound receptors on AX-500 columns also identified one isoform eluting at pH 5.6–6.1. Using a combination of HPLC techniques and sucrose gradient centrifugation, heterogeneity of the progestin receptor has been demonstrated.

INTRODUCTION

The determination of steroid hormone receptors as predictive indicators of an endocrine-responsive breast tumor, and as prognostic markers of the clinical course of the patient are widely utilized (e.g. refs. 1–3). Although our knowledge of estrogen receptor behaviour and clinical utility is more extensive than that of

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the progestin receptor, it appears that the latter is becoming increasingly important in therapeutic rationale [2, 4–6]. Progestin receptors may be of particular significance, not only in the identification of responsive patients [4–6], but in the primary selection of premenopausal patients for subsequent hormonal therapy [3]. The importance of this measurement has been confirmed in a recent trial where tamoxifen was incorporated in a cytotoxic chemotherapy regime [5]. Other studies strongly support this recommendation [6].

Our knowledge of the structural properties of the progestin receptor is imperative to an understanding of the mechanism of its biological action. Many investigators have studied the role of the subunit structure of progestin receptors in a variety of mammalian and avian systems [7–13]. Buller et al. [14] were among the first to suggest the subunit nature of the progestin receptor from chick oviduct and its role in nuclear binding. Horwitz and co-workers [11, 12] have identified two hormone-binding subunits of progestin receptors in both cytosol and nucleus using [^{3}H]R5020 affinity labeling of breast cancer cell lines. The behaviour of rabbit uterine progestin receptors has been characterized on conventional ion-exchange chromatography and DNA-cellulose columns in the molybdate-stabilized, non-transformed state and following receptor activation [15]. Evidence for heterogeneity of the receptor was demonstrated employing chromatography on DE-52 cellulose and QAE-Sephadex.

The classicial, overnight sucrose density gradient technique for separation and characterization of receptors is being replaced by vertical tube rotor methods [16–18]. This method is reliable in the separation of the labile 8–9 S and 4–5 S receptors in a rapid yet reproducible manner.

Further evidence concerning the heterogeneity of steroid hormone receptors has been derived from high-performance liquid chromatographic (HPLC) techniques. Among these applications has been size-exclusion (HPSEC) [19–23], ion-exchange (HPIEC) [24, 25] and chromatofocusing (HPCF) [26]. An application report on the use of the alternative ligand, ORG-2058, as opposed to the classical promegestone (R5020) and high-performance techniques has appeared [27].

In this study, we investigated the heterogeneity of the progestin receptor in human uterus employing HPLC and sucrose gradient centrifugation using a vertical tube rotor. Sodium molybdate was used to provide minimal disturbance of the untransformed receptor which was labeled with two different progestin ligands. The results provide new information on the size, shape and surface charge properties of progestin receptor isoforms.

EXPERIMENTAL

Reagents and chemicals

The tritium-labeled ligands utilized in this investigation were 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (R5020, susequently referred to as compound I) obtained from New England Nuclear/DuPont (Boston, MA, U.S.A.) and 16-α-ethyl-21-hydroxy-19-norpregn-4-en-3,20-dione (ORG-2058, subsequently referred to as compound II) procured from Amersham (Arlington Heights, IL,

U.S.A.). Unlabeled ligands for competitive inhibition were obtained from the same firms supplying the labeled ligands. Other chemicals required for the buffers were obtained from Sigma. Polybuffers 96 and 74 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Purified proteins used as markers were purchased as ^{14}C-labeled compounds from NEN/DuPont. Marker proteins chosen for the study were γ-globulin (7.1 S) and bovine serum albumin (4.3 S). These markers reference the molecular weight range of 69 000 through 150 000. Other markers such as ferritin, cytochrome c and thyroglobulin were employed in certain experiments.

Tissues

All human tissues were obtained through the aegis of pathologists at local hospitals and represent excess material from specimens sent to the Hormone Receptor Laboratory for routine steroid receptor analysis. Tissue reference powders from the University of Louisville were evaluated as a quality assurance measure of the standard procedures.

Preparation of cytosol and ligand-binding reactions

Fresh tissue was minced at 0–4°C whereas frozen tissue was sliced into thin sections with a scalpel blade before mincing. Pulverized tissue was mixed directly with buffer prior to homogenization. Homogenization was carried out at 0–4°C utilizing a Brinkman Polytron. A homogenization buffer termed PEGM was used [10 m*M* Na_2HPO_4–NaH_2PO_4, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10 m*M* monothioglycerol, 10% (v/v) glycerol and 10m*M* sodium molybdate]. Homogenates were centrifuged at 105 000 *g* for 30 min to sediment nuclei, organelles and cellular debris. The infranates were separated from the lipid-rich layer and used immediately in binding studies.

The cytosolic protein concentration was determined by the method of Waddell [28] using bovine serum albumin as a standard. With cytosols prepared in the presence of molybdate, the Waddell method was modified since molybdate interferes with this procedure. In this case, a dual-beam spectrophotometer was utilized and initially balanced using molybdate-containing buffers diluted to the same extent as the unknowns (1:100). Protein standards were prepared in molybdate-containing buffer, read at the appropriate wavelength and the standard curve constructed.

All ligand-binding reactions were carried out at 0–4°C. Cytosols were incubated with a 5 n*M* concentration of one of the labeled ligands either in the presence or absence of a 200-fold excess of unlabeled competitor to estimate nonspecific (low affinity, high capacity) binding. The reactions were terminated after 1 h by the addition of the reaction mixture to pellets of dextran-coated charcoal which removed unbound ligand from solution. In order to prevent stripping of bound ligand, the incubate was exposed to dextran-coated charcoal for only 30 s prior to a 4-min, high-speed centrifugation using a Beckman Microfuge for precipitation of the charcoal.

Sucrose density gradient centrifugation

Linear gradients of 18–20% (v/v) sucrose were formed by pre-layering equal volumes of 18, 19 and 20% sucrose, prepared in their respective homogenization buffers, into 13×57 mm Beckman Quick-seal cellulose nitrate centrifuge tubes. Each layer was snap-frozen at −86°C prior to addition of the subsequent concentration. Tubes were stored at −20°C until use whereupon they were allowed to equilibrate overnight at 4°C. Centrifugation was accomplished in a Beckman VTi-65 rotor at 369 000 *g* for 180 min in an L8-70M ultracentrifuge, essentially as previously described [29]. Gradients were fractionated in either 80- or 100-μl volumes by piercing the bottom of the gradient tube using peristaltic pump feed.

Calculation of specific binding capacity

Gradient or chromatography fractions containing tritium-labeled steroid were counted in Beckman LS-9000 and 3801 liquid scintillation counters giving an average counting efficiency of 35–39%. Specific binding capacity was expressed as fmol steroid bound per mg cytosol protein using the difference between the accumulated total binding and the binding in the presence of excess unlabeled ligand.

High-performance size-exclusion chromatography

All chromatography was performed in a Puffer Hubbard cold box (0–5°C) utilizing the Spherogel TSK-3000SW (Toyo Soda, Tokyo, Japan) size-exclusion column (700 mm×7.5 mm) with a Beckman Model 322 HPLC system equipped with an in-line Hitachi Model 100-40 spectrophotometer [21, 23]. The chromatographic column was comprised of two units, a short (100 mm×7.5 mm) TSK 3000SW guard column and, immediately downstream, the longer (600 mm×7.5 mm) TSK 3000SW size-exclusion column [21]. Reactions were applied in 100–250 μl volumes using a Hamilton syringe and the Model 210 sample injection valve. Essentially no difference in resolution has been noted when injections of 2–12 mg protein per ml were used.

The elution buffers were either $TEGK_{100}$ [10 m*M* Tris–HCl, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 100 m*M* potassium chloride], $TEGK_{400}$ [10 m*M* Tris–HCl, pH 7.4 at 4°C, containing 1.5 m*M* EDTA, 10% (v/v) glycerol, 400 m*M* potassium chloride] or $PEGK_{100}$ [25 m*M* NaH_2PO_4–Na_2HPO_4 buffer, pH 7.4 at 4°C, containing1.5 m*M* EDTA and 10% (v/v) glycerol, 100 m*M* potassium chloride]. The $PEGK_{100}$ buffers appear to give similar results on the HPSEC system when used for incubation and elution. All buffers were pre-filtered with a 0.45-μm HAWP filter (Millipore). Elution was carried out at a flow-rate of 0.7 ml/min. The column effluent was collected as 1-min fractions. Following a day of chromatography, the column was washed overnight with filtered, distilled and deionized water. The entire column was washed weekly with a filtered solution of 15% dimethyl sulphoxide in methanol whereas the TSK 3000SW guard column was flushed periodically with a solution of 6 *M* urea. The chromatographic system was stored under filtered, distilled, deionized water.

High-performance chromatofocusing

All chromatography was performed in the coldbox at 0–5°C and buffers were filtered as described for HPSEC. Free steroid or the labeled cytosols were applied

to a SynChropak AX-500 (250 mm×4.1 mm I.D.) anion-exchange column (SynChrom, Linden, IN, U.S.A.) utilizing the Altex Model 210 sample injection valve [26]. Elution was carried out using Altex Model 112 pumps.

Two different column equilibration and elution regimes were used depending upon the initial buffer conditions of the receptor preparations [26]. The columns were initially equilibrated to the starting pH (slightly above the desired upper limit) using a common cationic buffer. In the case of chromatofocusing on AX-500 columns, we used 25 m*M* Tris–HCl containing 1 m*M* dithiothreitol and 20% (v/v) glycerol adjusted to pH 8.1–83 at 4°C [26]. For HPCF of the molybdate-stabilized receptor components, 10 m*M* sodium molybdate was included in the column equilibration buffer.

Cytosols prepared in PEGM homogenization buffer were eluted with a 30:70 mixture of polybuffers 96 and 74. This polyampholyte solution was diluted ten- to twenty-fold with 20% glycerol, filtered with a 0.45-μm filter (Millipore) and adjusted to between pH 4.0 and 5.0 at 4°C. Various dilutions of polybuffer 96–74 were utilized for each of the columns.

High-performance ion-exchange chromatography

A sample of incubate cleared of unbound ligand by charcoal precipitation was applied to an Altex 322 (Beckman) chromatograph equipped with an AX-1000 (Synchrom) anion-exchange column. All chromatography was performed in a Puffer Hubbard cold box at 4±1°C as described earlier [25]. Each column was equilibrated previously with PEGM buffer. A 30-min wash of the column with buffer was followed by elution with a linear gradient of potassium phosphate, pH 7.6, which approached 500 m*M* at 90 min after gradient initiation. Subsequently the column was returned to starting conditions. All buffers were filtered prior to use employing a 0.45-μm Millipore filter. Approximately 1-ml fractions of the eluate were collected and counted for the presence of tritium. The phosphate concentration was determined by measuring individual fraction conductivity on a pre-calibrated standard curve of phosphate solutions in glycerol.

RESULTS AND DISCUSSION

Progestin receptors are known to exist in untransformed (8–9 S) as well as transformed states in which subunit dissociation to 4–5 S forms is thought to be relevant (e.g. refs. 7–15). The importance of rapid manipulation of this labile receptor under strictly controlled conditions to study the untransformed state cannot be over-emphasized. For this reason, cytosol was exposed to ligand for 1 h only, as had been previously suggested [30]. Walters [30] utilized other forms of stabilization prior to analysis and indicated that molybdate failed to stabilize the rat uterine progestin receptor. We evaluated the 30-s exposure to charcoal in the separation of bound and free steroid as well as the incubation time and high protein concentrations as suggested. In contrast to receptor from rat uterus, the progestin receptors from human uterus appeared stabilized and exhibited grossly different results as reported here.

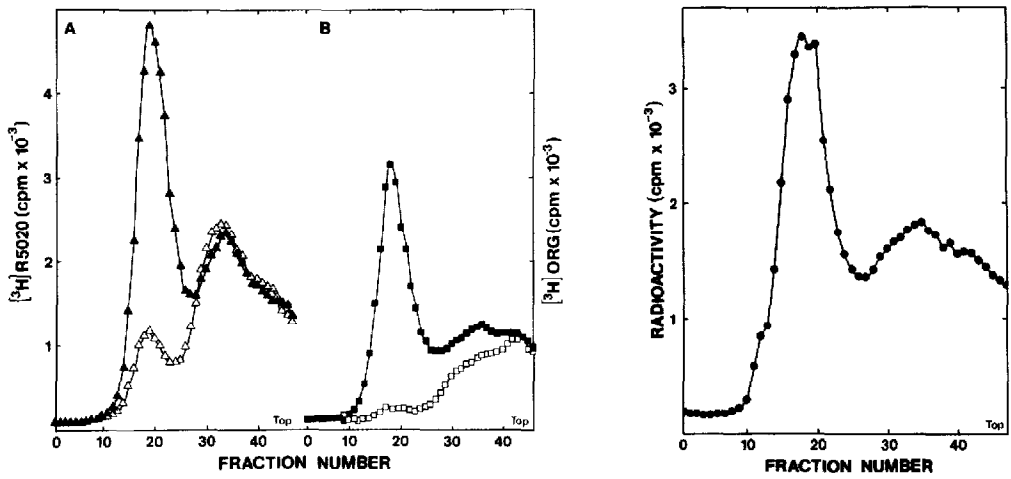

Fig. 1. Separation of progestin receptor species from human uterus by sucrose density gradient centrifugation using a vertical tube rotor. Cytosol was incubated with 5 n*M* [^{3}H]I in the presence (△) or absence (▲) of excess unlabeled compound I (A). A 100-μl aliquot of incubate was treated with dextran-coated charcoal and applied to an 18–20% sucrose gradient. Similarly, [^{3}H]II was utilized as ligand under identical conditions (B). The total bound (■) and non-specifically bound steroid (□) are indicated. The peak at fraction 20 represents the heavier 8–9 S binding components of the progestin receptor.

Fig. 2. Separation of progestin receptor species in a mixture of ligand-bound receptors which were combined prior to sucrose density gradient centrifugation. Cytosol from human uterus was incubated separately with 5 n*M* of either labeled compound I and II and cleared of free steroid with dextran-coated charcoal. A 50-μl aliquot from each incubate was layered on 18–20% linear gradients prior to centrifugation at 369 000 *g* for 3 h. Only specific bound steroid is shown.

Sucrose density gradient ultracentrifugation

The integrity of stabilized receptor was retained so that sedimentation values of the different molecular-weight species could be determined under the conditions of receptor preparation as outlined in the Experimental section. In fresh uterus, [^{3}H]I and [^{3}H]II showed binding to two distinct 8–9 S and 4–5 S receptor species. Compound I displayed a significantly higher non-specific binding in the 4–5 S region than exhibited by compound II (Fig. 1). Compound II showed virtually only specific binding in the 8–9 S region when compared to compound I and one could discern a small amount of specific binding in the 4–5 S region as well (Fig. 1B). These data suggest the manner of association of the two ligands with non-receptor proteins (non-specific binding) may be dissimilar. Titration data from our laboratory showed similar affinity constants (K_d values) for association of compounds I and II with progestin receptors from human uterus [31].

Since both ligands under invstigation were tritium-labeled, it was difficult to identify these from each other when gradients were run with mixtures of cleared cytosol. An example of a profile is shown in Fig. 2. Cytosols were incubated separately with each ligand, then treated with dextran-coated charcoal prior to com-

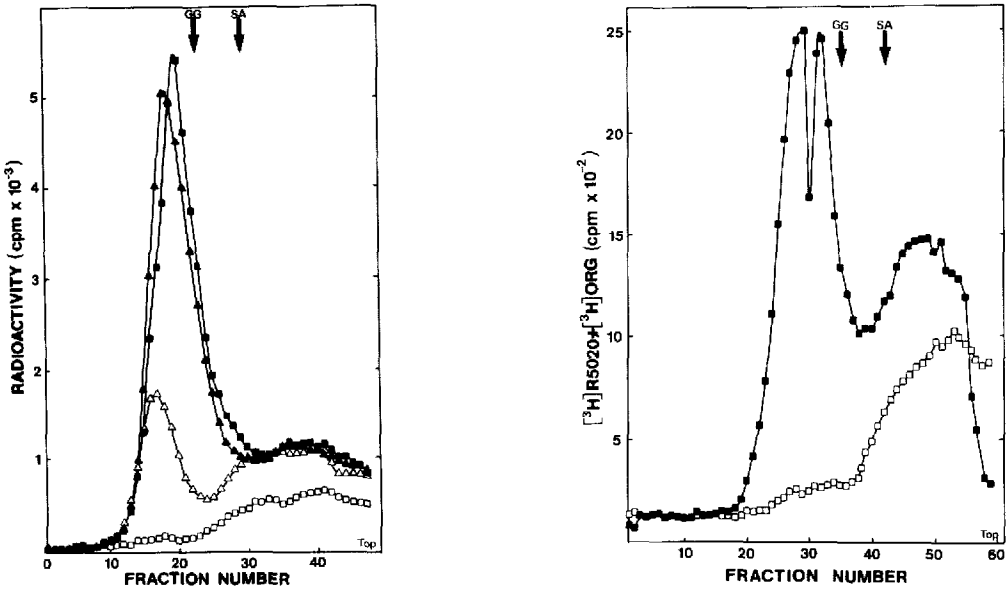

Fig. 3. Sucrose gradient separation of progestin receptors using two different ligands. Human uterine cytosol was incubated with 5 n*M* [^{3}H]I in the presence (△) and absence (▲) of excess unlabeled compound I. Separately cytosol was also incubated with [^{3}H]II in the presence (□) and absence (■) of excess unlabeled steroid. A 100-μl aliquot of each incubate was cleared with dextran-coated charcoal prior to centrifugation in separate gradients. ^{14}C-Labeled markers are γ-globulin (GG) and bovine serum albumin (SA).

Fig. 4. Separation of a mixture of progestin receptor species bound to compounds I and II using optimized gradient conditions. Cytosol extracted from human uterus was incubated separately with 5 n*M* of either of the labeled compounds. Following clearance of the free ligand with charcoal-coated dextran, 50 μl of each incubate were mixed prior to layering on an 18–20% gradient. Both total bound (■) and non-specific bound (□) steroid are shown. ^{14}C-Labeled markers were fractionated as indicated: γ-globulin (GG) and bovine serum albumin (SA).

bining equal volumes of 50 μl each, and separating on gradients as described under Experimental. Curiously these mixed samples exhibited heterogeneity in the 8–9 S region (Fig. 2).

To clarify the possibility of ligand interaction, each steroid was reacted with cytosol separately and then sedimented under identical conditions in separate gradients using markers (Fig. 3). The data in Fig. 3 are representative of our experience with nine separate experiments. They indicate that although a certain amount of overlap existed between the proteins sedimenting in the same region (8–9 S), the sedimentation velocities of the peaks appear different. Compound I appeared to bind to a heavier component while compound II was associated with a lighter receptor species. Recent studies in our laboratory with estrogen receptors immunopurified by monoclonal antibodies suggest the presence of receptor-associated proteins such as protein kinase which may contribute to the molecular heterogeneity [32, 33]. This is currently being investigated with progestin receptors.

In order to achieve greater resolution, the gradient profiles were expanded by collecting 60 fractions (Fig. 4) again using the marker proteins. When these gradients were analysed, multiple-binding components were discernible in the 8–9 S region (Fig. 4). This indicated that [^{3}H]I and [^{3}H]II either interacted with different receptor isoforms or altered the conformation of a single isoform in a different fashion.

Since the density gradient centrifugation results supported the possibility of multiple forms of receptors under molybdate-stabilized conditions we employed the more sophisticated HPLC techniques [21, 23, 25, 26] for analysis of progestin-receptor binding.

High-performance size-exclusion chromatography

Initially each radioactive-free steroid was subjected to HPSEC to determine the position at which free ligand appears. This control was included to eliminate the possibility of (1) incomplete separation of bound from free ligand by dextran-coated charcoal, (2) ligand dissociating from receptor during the progression of the column separation and (3) cleavage or steroid binding to the column matrix. Both free ligands eluted toward the end of the separation as predicted by the molecular sieving effect (data not shown).

HPSEC was performed utilizing 30-cm TSK-3000SW columns as well as longer 60-cm columns. On the shorter column, receptor-bound ligand appeared as two peaks, using either compound I or II. The primary peak associating specifically with compound I appeared consistently between fractions 30 and 35 in a number of uterine cytosols (Fig. 5B). This receptor isoform represented the majority ($>70\%$) of specific binding applied to the column. Recoveries on the 30-cm TSK-3000SW columns were consistently 87–93%. Column calibration with a wide range of marker proteins suggested this primary peak to be a very large species of >80 Å eluting near the position of thyroglobulin.

A small but distinct and reproducible secondary peak (Fig. 5B) was demonstrable between fractions 50 and 60 with compound I (ca. 35 Å), but only represented 10–15% of the specifically bound radioactivity. No free steroid radioactivity was demonstrated in these runs, indicating that dextran-coated charcoal clearance of the free ligand was complete and that no discernible column-induced dissociation of steroid occurred. Compound II also appeared bound specifically by two isoforms with virtually identical ratios as observed for compound I. However, with compound II as ligand, the primary receptor isoform was observed between fractions 40 and 47 (Fig. 5A) representative of a protein of ca 55 Å eluting between the positions observed for bovine serum albumin (40 Å) and ferritin (60 Å). The smaller, secondary receptor peak appeared between fractions 73 and 83 with compound II. The non-specific binding was minimal with compound II when compared with that of compound I, similar to the density gradient sedimentation data shown.

These experiments not only demonstrated the integrity of the receptor based on size and shape but indicated that at least two different isoforms exist. The most exciting observation, however, lay in the fact that the distribution of these isoforms appeared to be a function of the ligand used in the study.

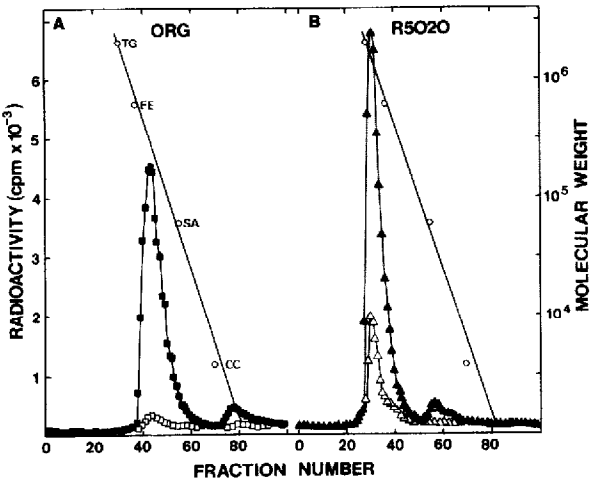

Fig. 5. Separation of progestin receptor isoforms from human uterus using HPSEC. Labeled cytosolic proteins were applied to and eluted from a 30-cm TSK-3000SW column as described in the text. (A) Progestin receptors labeled with [^{3}H]II and incubated in the presence (□) and absence (■) of excess compound II. (B) The receptor isoform pattern eluting under identical conditions using [^{3}H]I as ligand in the presence (△) and absence (▲) of excess unlabeled steroid. The HPSEC system was pre-calibrated with a series of pure proteins, thyroglobulin (TG), ferritin (FE), bovine serum albumin (SA) and cytochrome c (CC).

Use of the 60-cm TSK-3000SW column showed that its dimensions permitted separation of receptor species not discernible by the use of the shorter column. To investigate the difference between receptors binding to the different progestin ligands, both [^{3}H]steroids were tested in a mixture, then separately with cytosol.

Fig. 6 represents cytosol incubated for 1 h in the presence of 10 m*M* molybdate with each ligand separately, then cleared of free steroid and combined. The mixture of progestin-labeled isoforms as applied to the column were resolved into four distinct radioactive peaks (Fig. 6). The components separating with the major peak, fractions 40–60, clearly indicated additional heterogeneity. When the steroid–receptor complexes were formed separately and then applied to the same column individually (Fig. 7), [^{3}H]I bound to isoforms which eluted as five different peaks that were readily distinguishable from each other. Using [^{3}H]II as ligand on the identical cytosol preparation, six different peaks of specifically bound steroid were observed (Fig. 7).

Upon superimposition of the patterns it appeared that at least four of the radioactive peaks, represented as B, C, D and E in Fig. 7, were similar regardless of the ligand employed. These results clearly indicated that multiple receptor isoforms of variable size and shape were present in human uterine cytosol and that these were easily separated on HPSEC columns. At this stage it was unclear if these binding components represented true isoforms of the progestin receptor or whether some of them arose from modification of the receptors during the homogenization and binding steps or in the column run as we discussed in detail earlier [23].

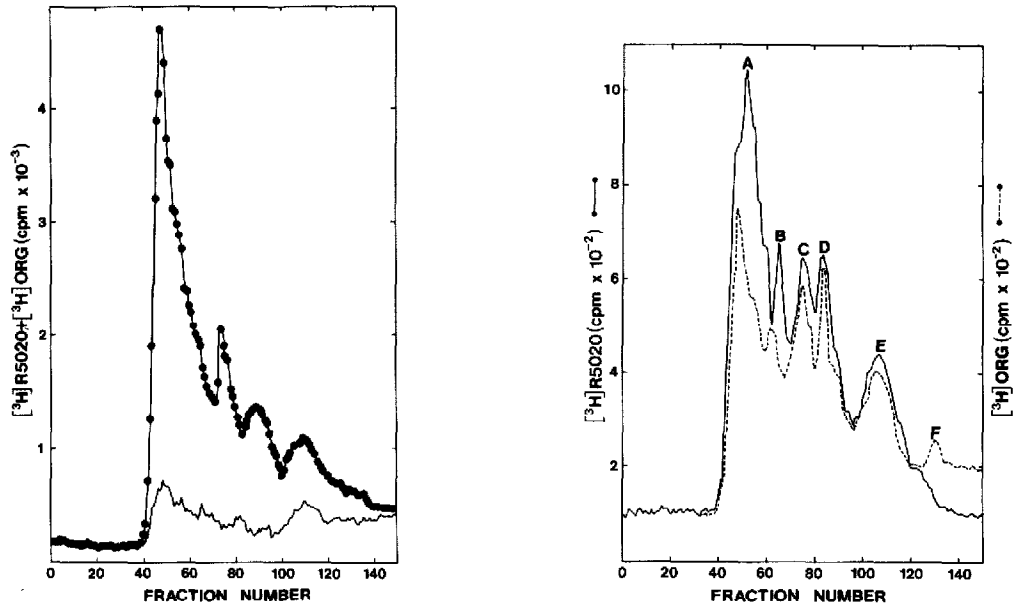

Fig. 6. Progestin receptor isoforms detected in a mixture of cytosol labeled with either tritiated compound I or II and separated by HPSEC. Cytosols were labeled separately and cleared of unbound steroid prior to mixing and chromatography on a 60-cm TSK-3000SW column. Total binding (●) and non-specific binding (—) indicated at least four binding components.

Fig. 7. HPSEC of progestin receptor isoforms identified by their binding to different radioactive ligands. Cytosols from human uterus were incubated separately with 5 n*M* concentration of either [^{3}H]II or [^{3}H]I for 1 h at 4°C prior to clearance of free ligand with dextran-coated charcoal. The incubates were injected separately and eluted from 60-cm TSK-3000SW columns. Only specifically bound tritiated compound I (solid line) and II (dashed line) are depicted.

High-performance ion-exchange chromatography

HPIEC of progestin receptors was accomplished employing the AX-1000 column [25]. Peaks of bound radioactivity were separated on HPIEC. The primary peak, representing approximately 70% of the total radioactivity, eluted from the column near the void volume at fractions 5–10 (Fig. 8). The same component was observed when either labeled compound was used as ligand. Consideration of the profiles generated in the presence of excess unlabeled steroid indicated this peak was primarily composed of non-specifically bound steroid and free ligand stripped from the receptor by interaction with the column matrix. When either unbound labeled compound I or II was analyzed by HPIEC with the AX-1000 column, each progestin eluted at this position (data not shown). This supports the idea that changes occurred in the progestin-bound receptor with subsequent dissociation of the steroid from the binding site. Further support is provided by the observation that the primary peak appeared prior to the generation of the gradient and is, therefore not charge-dependent.

The secondary peak appeared to contain a specific progestin receptor isoform at 95–100 m*M* phosphate (Fig. 8). This isoform was observed in all of the uterine tissues examined by HPIEC analysis. Furthermore this isoform bound either

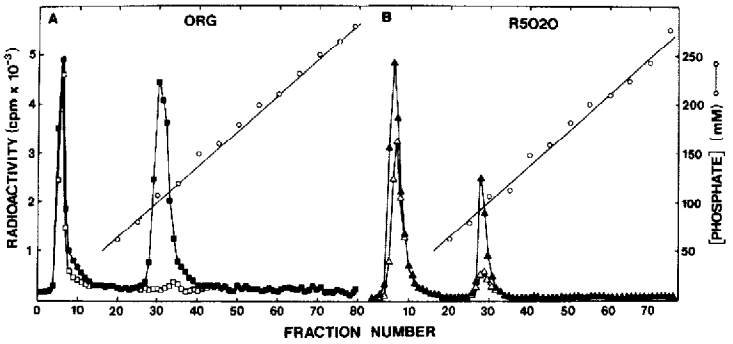

Fig. 8. Separation of progestin receptors by HPIEC. Cytosol from human uterus was incubated with 5 n*M* [^{3}H]II in the presence (□) and absence (■) of excess unlabeled ligand for 1 h at 4°C (A). Similarly [^{3}H]I was incubated either in the presence (△) or absence (▲) of unlabeled compound I (B). Following clearance of free ligand, the incubates were separately injected and eluted from an AX-1000 ion-exchange column.

[^{3}H]II (Fig. 8A) or [^{3}H]I (Fig. 8B) specifically and eluted at an identical ionic strength. Recoveries of radioactivity with HPIEC have consistently been of the order of 97%. When HPIEC of the labeled cytosol was followed immediately by collection of the progestin receptor isoform eluting at 100 m*M* phosphate (Fig. 8) and rechromatographed in an identical system, some free steroid appeared in the void volume with reappearance of the isoform at 100 m*M* phosphate (data not shown). These data are in contrast to our findings employing sucrose density gradient centrifugation as the first purification procedure.

Sucrose density gradient centrifugation was conducted with the vertical tube rotor as a primary receptor purification procedure. As shown in Fig. 9A, specific progestin receptors sedimented at 8–9 S and 4–5 S in the preparation used. Portions of the 4–5 S isoforms were subsequently separated by HPIEC (Fig. 9B). Although free steroid was again observed at fractions 5–10, indicating dissociation of progestin receptor complexes, a receptor isoform was eluted at 60 m*M* phosphate (Fig. 9B).

When the 8-9 S progestin receptors were collected from sucrose gradients (Fig. 9A) and subsequently separated based upon surface charge properties by HPIEC, the receptor isoform was detected again eluting at 60 m*M* phosphate (Fig. 9C). Some labeled compound II appeared to be dissociated from the receptor during separation as shown by the radioactivity in fractions 5–10. The isoform at 60 m*M* phosphate was collected and reapplied to an HPIEC column (Fig. 9D). The receptor isoform retained its charge characteristics again eluting at 60 m*M* phosphate. Apparently steroid was stripped from the receptor under these conditions in spite of the presence of 10 m*M* molybdate. When these receptor isoforms are separated based upon surface charge properties, they appear to be relatively consistent in nature. When the initial purification step employed sucrose density gradient centrifugation of receptor species in cytosol, the surface charge properties of the isoform appeared altered compared to that purified first by HPIEC. The latter isoform eluted at 100 m*M* phosphate. The reasons for this shift in elution properties are unclear but suggest that one of these techniques removes a

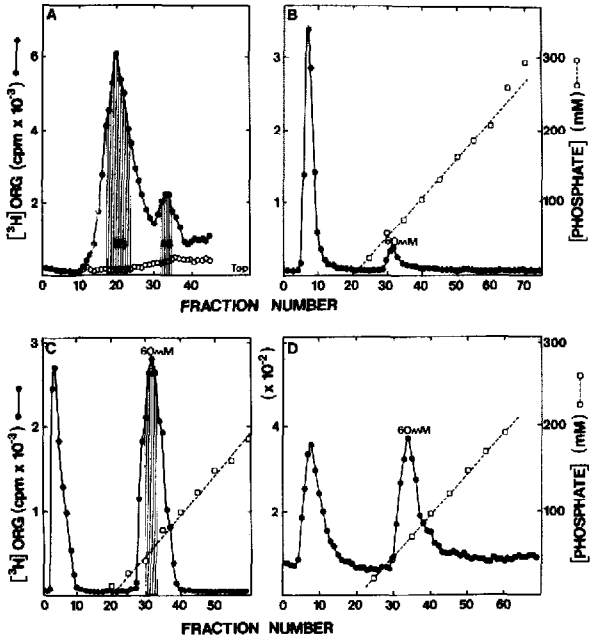

Fig. 9. Characterization of progestin receptor isoforms from human uterus employing a combination of sucrose density gradient centrifugation and HPIEC. Cytosol prepared from human uterus was incubated with [^{3}H]II in the presence (○) and absence (●) of excess unlabeled compound II. Aliquots (100 μl) of incubate were cleared with dextran-coated charcoal and applied to 18–20% sucrose density gradients (A). Subsequently either the 8–9 S (fractions 17–24) or 4–5 S (fractions 32–35) receptor isoforms from gradients were pooled, injected and eluted on an AX-1000 HPIEC column as described under Experimental. (C) The 8–9 S isoform profile; (B) the 4–5 S isoform. Collection of the specific bound fraction from the first HPIEC run, i.e. fractions 30–33 (C) was rechromatographed on HPIEC and fractionated (D).

receptor-associated species conferring ionic charges to these isoforms. This may involve the 90 000 heat shock proteins [13] or protein kinase activities [32, 33] which appear to be associated with receptor.

High-performance chromatofocusing

HPCF was accomplished primarily on the AX-500 column as described previously [26]. The AX-500 column in the HPCF mode displayed the identical phenomenon found with the HPIEC system, namely that a radioactivity peak appeared just after the void volume and prior to application of the pH gradient (Fig. 10). Again, this peak was in the exact location in which free steroid eluted (Fig. 10).

Even in the presence of 10 m*M* molybdate, the column appeared to strip labeled steroid from the receptor. However, a secondary peak appeared at a pH of 5.6–6.1 which contained specifically bound steroid (Fig. 10). The results presented with [^{3}H]I in Fig. 10 were virtually identical to those observed when [^{3}H]II was used as ligand. Thus, based on pH, the progestin receptor isoform focused at a p*I* value that did not distinguish one ligand binding species from the other.

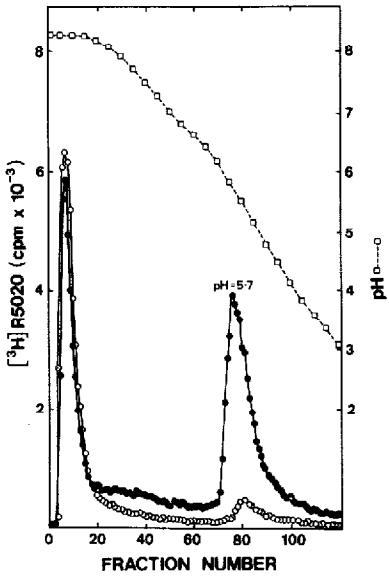

Fig. 10. HPCF of progestin receptor isoforms in human uterus. Cytosol obtained from human uterus was incubated with tritiated compound I in the presence (○) and absence (●) of excess unlabeled compound I. Following clearance of the free steroid, the incubate was injected and eluted on an AX-500 column in a chromatofocusing mode as described under Experimental. Note the presence of a specific binding species eluting at pH 5.7.

The origin and significance of progestin receptor polymorphism remains obscure. Although certain components may represent distinct physiological species, proteolytic cleavage may occur with a labile receptor [23, 34]. Dougherty et al. [13] reported the presence of two 8-S forms of the progesterone receptor from chick oviduct. Interestingly each form contained a 90 kDa component now shown to be a heat shock protein and either a 75 kDa or 110 kDa steroid binding species. Various combinations of these components could give rise to considerable size heterogeneity as we observed with receptors from human uterus.

The possibility of receptor–column association or of enhanced dissociation of the receptor–ligand complex from mammals has previously been reported with HPSEC [21]. A progesterone receptor component of the chick oviduct could not be recovered following HPSEC analysis [35] further suggesting their lability. Both of these studies and our data imply that caution be exercised in analyzing steroid receptors by HPLC.

The results of the present study indicate polymorphism in the human uterine progestin receptor as analysed by sucrose density gradient centrifugation and HPSEC. Interestingly the size and shape properties of these receptors appear to be highly dependent upon the progestin (either compound I or II) used as labeled ligand. The isoform associating with compound I had a molecular mass of 190 000 while that binding to compound II had a molecular mass of 173 000 by sucrose density gradient centrifugation. By HPSEC analysis, an even greater difference was observed.

These data suggest for the first time that these ligands may transform (activate) the progestin receptor in a different fashion even in the presence of sodium molybdate. Additional experiments to discern the characteristics of nuclear binding and DNA-dependent RNA-polymerase activation are needed to ascertain the native and activated isoforms. Regardless of the ligand, the surface charge properties appear similar by HPIEC and HPCF. A provocative finding was made that prepurification on sucrose density gradients gave isoforms on HPIEC that exhibited different surface charge properties from those initially separated by HPIEC. These data suggest a non-ligand-binding component was removed by one of these procedures; this entity apparently conferred surface charge characteristics on the progestin receptor. It is not clear if this is the 90 000 heat shock protein which does not bind steroid reported by Dougherty et al. [13].

In summary, a combination of HPLC methods in size-exclusion, ion-exchange and chromatofocusing modes have demonstrated progestin receptor heterogeneity. Our experience described here and with estrogen receptors [36] suggests a multidimensional approach combining these methodologies would be fruitful especially utilizing sucrose gradient centrifugation or HPSEC with HPIEC in the early stages and HPCF in later steps. Thus it should be possible to purify and rapidly characterize these isoforms in order to relate their molecular properties to their biological function in target organs such as the uterus.

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REFERENCES

1 J.C. Allegra, M.E. Lippman, E.B. Thompson, R. Simon, A. Barlock, L. Green, K.K. Huff, H. My T. Do and S.C. Aitken, Cancer Res., 39 (1979) 1447.
2 K.B. Horwitz and W.L. McGuire, Steroids, 25 (1975) 497.
3 J.L. Wittliff, Cancer, 53 (1984) 630.
4 K.B. Horwitz and W.L. McGuire, in W.L. McGuire, J.P. Raynaud and E.E. Baulieu (Editors), Progesterone Receptors in Normal and Neoplastic Tissues, Raven Press, New York, 1977, p. 103.
5 B. Fisher, C. Redmond, A. Brown, D.L. Wickerham, N. Wolmark, J.C. Allegra, G. Escher, M. Lippman, E. Savlov, J. Wittliff and E.R.Fisher, J. Clin. Oncol., 1 (1983) 227.
6 G.M. Clark, W.L. McGuire, C.A. Hubay, O.H. Pearson and J.S. Marshall, N. Engl. J. Med., 309 (1983) 1343.
7 D. Philibot, T. Ojasoo and J.P. Raynaud, Endocrinology, 101 (1977) 1850.
8 W.A. Coty, W.T. Schrader and B.W. O'Malley, J. Steroid Biochem., 10 (1979) 1.

9 P.A. Boyd and T.C. Spelsberg, Biochemistry, 18 (1979) 3679.
10 R.G. Smith, M. d'Istria and N.T. Van, Biochemisry, 20 (1981) 5557.
11 B.A. Lessey, P.S. Alexander and K.B. Horwitz, Endocrinology, 112 (1983) 1267.
12 K.B. Horwitz and P.S. Alexander, Endocrinology, 113 (1983) 2195.
13 J.J. Dougherty, R.K. Puri and D.O. Toft, J. Biol. Chem., 259 (1984) 8004.
14 D.O. Buller, D.O. Toft, W.T. Schrader and B.W. O'Malley, J. Biol. Chem., 250 (1975) 801.
15 D.J. Lamb and D.W. Bullock, Endocrinology, 114 (1984) 1833.
16 B. Powell, R.E. Garola, G.C. Chamness and W.L. McGuire, Cancer Res., 39 (1979) 1678.
17 V.C.Jordan and G. Prestwich, Mol. Cell Endocrinol., 8 (1977) 179.
18 B. Freedman and R.A. Hawkins, J. Endocrinol., 86 (1980) 431.
19 E.J. Pavlik, J.R. van Nagell, M. Muncey and C.S. Donaldson, Biochemistry, 21 (1982) 139.
20 E.J. Pavlik, J.R. van Nagell, E. Donaldson and C.S.Donaldson, J.Steroid Biochem., 17 (1982) 553.
21 R.D. Wiehle, G.E. Hofmann, A. Fuchs and J.L. Wittliff, J. Chromatogr., 307 (1984) 39.
22 N.A. Shahabi, T.W. Hutchens, J.L. Wittliff, S.D. Halmo, M.E. Kirk and J.A. Nisker, Prog. Cancer Res. Ther., 31 (1984) 63.
23 J.L. Wittliff and R.D. Wiehle, in V.P. Hollander (Editor), Hormonally Sensitive Tumors, Academic Press, New York, 1985, pp. 383–428.
24 B.S. Leung, O.Deleon and T. Madhock, Endocr. Soc. Meet. Abstr., 212A (1983) 715.
25 R.D. Wiehle and J.L. Wittliff, J. Chromatogr., 297 (1984) 313.
26 T.W. Hutchens, R.D. Wiehle, N.A. Shahabi and J.L. Wittliff, J. Chromatogr., 266 (1983) 115.
27 A. Heubner, B. Manz, H. Grill and K. Pollow, J. Chromatogr., 297 (1984) 301.
28 W.J. Waddell, J. Lab. Clin. Med., 48 (1956) 311.
29 D.J. Ledden, R.C. Feldhoff, P.W. Feldhoff, P.H. Heidemann and J.L. Wittliff, Clin. Chem., 27 (1981) 1357.
30 M. Walters, Arch. Biochem. Biophys., 225 (1983) 656.
31 W. Mujaji, L.A. van der Walt, N.A. Shahabi, S.M. Hyder and J.L. Wittliff, J. Clin. Lab. Anal., submitted for publication.
32 A. Baldi, D.M. Boyle and J.L. Wittliff, Biochem. Biophys. Res. Commun., 135 (1986) 597.
33 S.M. Hyder, N. Sato and J.L. Wittliff, J. Chromatogr., 397 (1987) 251.
34 M.R. Sherman, L.A. Pickering, F.M. Rollwagen and L.K. Miller, Fed. Proc., Fed. Am. Soc. Exp. Biol., 37 (1978) 167.
35 N.L. Weigel, C.J. March, W.T. Schrader and B.W. O'Malley, Endocr. Soc. Meet. Abstr., (1983) 224.
36 N.A. Shahabi, S.M. Hyder, R.D. Wiehle and J.L. Wittliff, J. Steroid Biochem., 24 (1986) 1151.