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CHARACTERIZATION OF ESTROGEN RECEPTORS AND ASSOCIATED PROTEIN KINASE ACTIVITY BY HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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SUMMARY

We have determined that high-performance hydrophobic-interaction chromatography (HPHIC) with weakly hydrophobic columns permit the rapid separation of the labile isoforms of estrogen receptor proteins. Previously we reported the use of the SynChrom propyl 500 column for HPHIC of steroid receptors. However, due to the strongly hydrophobic characteristics of the ligand, [^{125}I]iodoestradiol-17β, and the receptor protein, organic solvent was required in the mobile phase for greater recovery of receptor proteins. Here, we report separation of steroid receptors from human breast tumors and rat uteri, using the Beckman CAA-HIC, a non-ionic polyether-bonded column, without the need for organic solvents and with virtually 100% recoveries. Receptors were extracted in 10 m*M* phosphate buffer (pH 7.4). Maximum resolution and separation were achieved when a descending salt gradient of ammonium sulfate in phosphate buffer (pH 7.4) was used (2–0 *M* in 30 min). Estrogen receptor (ER) was resolved into two isoforms with t_R = 22 ± 1 min (n = 16, designated as peak I) and 27.5 ± 0.5 min (n = 14, designated as peak II) and a purification of five- to twenty-fold in a single pass. Free steroid was eluted at t_R = 35 ± 1 min (n = 4). Separation was dependent on adjusting the ionic strength of cytosol to 1.5 *M* ammonium sulfate. ER, purified by HPHIC, retained ligand binding capacity and exhibited protein kinase activity, which was dominant in the less hydrophobic peak I (t_R = 22 min) when immunoprecipitated with the monoclonal antibody D547. This method of rapidly purifying ER with high retention of biological activity may now be applied to the study of the molecular interrelationships of steroid receptor isoforms.

INTRODUCTION

High-performance hydrophobic-interaction chromatography (HPHIC) of proteins is widely gaining recognition in the rapidly growing field of high-performance liquid chromatography (HPLC) since the introduction of microparticular rigid packing materials[1,2]. Among the various modes of HPLC such as reversed-phase (RPLC), ion-exchange (HPIEC), size-exclusion (HPSEC) and chromatofocusing (HPCF),

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HPHIC represents the mildest means of separating protein molecules with complete retention of their biological activity[3]. This fact, coupled with recoveries of these biopolymers of almost 100%, indicates that HPHIC is a favorable choice of methods for separating such complex molecules[4–6]. An additional feature which merits mention is that HPHIC, unlike most RPLC procedures may be performed at physiological pH.

HPHIC relies on the interaction of hydrophobic patches, present in the protein, with the stationary phase. Hydrophobic patches are present on the surface and in the interior of the molecule and therefore must be exposed. This is achieved by promoting such interaction in the presence of high-ionic-strength buffers used as the initial mobile phase. The latter exposes the hydrophobic patches buried within the protein molecule. Subsequent elution of the protein is obtained by lowering the salt concentration in a gradient mode and thus selectively dissociating the proteins from the stationary phase. The least hydrophobic protein will be eluted earlier in the separation gradient.

The recent advances in molecular biology requiring protein purification suggest that the mild conditions of HPHIC in combination with the short analysis time will make it a preferred protein separation technique. Currently, we are involved in optimizing various HPLC separation modes for characterization of proteins[7,8]. We have previously reported that HPHIC rapidly separates steroid receptors with retention of their biological activity[9,10].

Here, we report our results on optimizing conditions for HPHIC of steroid receptors, performed on a recently developed, polyether-bonded stationary phase, which is non-ionic in nature[11]. This appears to be a suitable column material for HPHIC of receptors, since these proteins are highly charged molecules which show retention on both ion-exchange and size-exclusion columns[12]. We report further that, although ER separates into two isoforms following HPHIC, only the receptor dissociated by high-salt (less hydrophobic) was eluted together with a Mg^{2+}-dependent protein kinase activity. Protein kinase activity was immunoprecipitated with monoclonal antibodies raised against the estrogen receptor. To our knowledge, this is the first report where HPLC in the hydrophobic-interaction mode is coupled with immunoprecipitation to demonstrate the presence of protein kinase activity in only one of the two isoforms of ER.

EXPERIMENTAL

Materials

HPLC-grade ammonium sulfate and all the material used for gel electrophoresis were obtained from Bio-Rad laboratories (Richmond, CA, U.S.A.). The ligand, [16-α^{125}I]iodoestradiol-17β (1500–2000 Ci/mmol) and γ-labeled [^{32}P]ATP ($\approx$ 2000 Ci/mmol) were obtained from New England Nuclear/DuPont (Boston, MA, U.S.A.). Disodium ethylenediaminetetraacetic acid (EDTA) and glycerol were purchased from Fisher Scientific (Louisville, KY, U.S.A.). Unlabeled diethylstilbestrol (DES), which was used as an estrogen inhibitor, Norit A, Dextran T-70, and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.).

Female Sprague Dawley rats (weighing *ca.* 250 g), were obtained from Laboratory Supplies, Indianapolis, IN, U.S.A. Animals were sacrificed by cervical dislo-

cation, and their uteri were removed. All experiments reported here were performed with fresh rat uteri. Human breast tumor tissues from patients were provided by the various surgeons and pathologists at the local hospitals, cooperating with the Hormone Receptor Laboratory. The tissues were brought to the laboratory on dry ice and were kept frozen at −86°C until analyzed. Only residual from clinical receptor analyses was used in this study.

Preparation and labeling of soluble estrogen receptor

All procedures were performed at 4°C. Rat uterine tissue (1 ml per uterus) or human breast tumors (*ca.* 200–400 mg/ml) were homogenized in P_{10}EDG [10 m*M* phosphate–1.5 m*M* EDTA–1 m*M* DTT–10% (v/v) glycerol, pH 7.4]. Homogenization was performed in two 10-s bursts in a Brinkman Polytron homogenizer (Westbury, NY, U.S.A.).

Soluble fractions were prepared by centrifugation of the homogenate for 30 min at 40000 rpm in a Beckman (San Ramon, CA, U.S.A.) Ti 70.1 rotor. The supernatant was removed carefully, avoiding the layer of fat at the top. The soluble fractions were labeled with 2–3 n*M* [16α-^{125}I]iodoestradiol-17β in the presence and absence of a 200-fold excess of diethylstilbestrol for 2–4 h at 4°C, unless otherwise stated. The reaction was terminated by removing unbound steroid with a pellet, derived from the dextran-coated charcoal suspension (1% charcoal, 0.05% dextran). The labeled cytosol was applied to the charcoal pellet, mixed, and allowed to stand for 5 min at 4°C. Dextran-coated charcoal was then removed by centrifuging the sample for 5 min at 1000 *g*. Cytosol protein concentrations were determined by the method of Bradford[13], using bovine serum albumin as the standard. The protein concentrations generally ranged from 4 to 8 mg/ml.

HPHIC

Chromatography was performed in a Puffer-Hubbard cold box (Ashville, NC, U.S.A.) at 4°C. All buffers were filtered under vacuum through Millipore 0.45-μm HAWP filters (Bedford, MA, U.S.A.) before use. Free steroid or estrogen receptor complexes were applied to the polyether-bonded, non-ionic silica-based Spherogel CAA-HIC column (300 A), obtained from Beckman/Altex using an Altex Model 210 sample injection valve. Elution was carried out with a Beckman Model 114 solvent delivery module, including a Model 421 system controller.

Unless otherwise stated, the gradient program for the elution consisted of an initial elution with eluent A (P_{10}EDG, containing 2 *M* ammonium sulfate, pH 7.4) at a flow-rate of 1 ml/min. Following sample injection, a descending salt gradient was developed to reach P_{10}EDG (eluent B) in the next 30 min. Eluent B was then continued at a flow-rate of 1 ml/min for the next 30 min before stopping and re-equilibrating to eluent A. There was a gradient delay period of *ca.* 5 min. This time period was not subtracted from the t_R values shown. The above described gradient elution program was used in most cases, but the nature of some experiments dictated use of other gradient elution conditions. These are described in the various figure legends.

Following chromatography, the eluted steroid (free and protein-bound) was collected as 1 ml fractions and detected radiometrically in a Micromedics 4/600 gamma radioisotope detector (Rohm & Haas, Cleveland, OH, U.S.A.). The counting

efficiency was 65%. Since the nonspecific binding (radioactivity eluted from cytosols labeled in the presence of DES) showed mainly base-line levels and represented between 5 and 10% of the total binding, these are usually not shown in the figures. Recovery of total radioactivity and injected protein was almost always 75–100%.

Immunoprecipitation and detection of protein kinase activity

Following HPHIC, fractions which showed eluted protein-bound radioactivity and fractions from other elution positions within the gradient were incubated with D547-immobilized monoclonal antibody (MAb), obtained from Abbott Labs. (Chicago, IL, U.S.A.) in the form of an ER-EIA kit[14]. Two MAb-coated beads were added directly to each of the test tubes, containing labeled receptor proteins and other fractions (controls), and incubated for 18 h. The beads were removed and one of these was developed for quantitation (mass) of ER, associated with MAb, as recommended by the manufacturer. The second bead was used for protein kinase activity measurements and was processed exactly as described in previous publications[15,16].

Briefly, the beads were washed first with distilled water, then with P_{10}EDG, containing 0.05% NP-40 followed by P_{10}EDG and incubated at 30°C for 30 min with 5–10 μCi γ-labeled ATP in the presence of $\approx$ 10 μg phosvitin (Sigma), which served as exogenous substrate for transfer of ^{32}P from ATP to a polypeptide. The phosphorylated polypeptides were eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions in 7.5% slab gels as described by Laemmli[17]. Molecular-weight markers for SDS-PAGE were obtained from Sigma.

RESULTS AND DISCUSSION

With the recent developments in molecular biology, there has been a general trend toward increased production of biochemically engineered proteins. In this respect, the use of HPLC as a method of separation, purification, and analysis of such proteins is expected to gain even further popularity. Mainly, this is because HPLC rapidly separates many structural proteins with high recoveries. However, problems are experienced in its application to more sensitive regulatory proteins under retention of their biological characteristics[12,18]. In contrast, conventional column chromatography may take from hours to days for similar results.

Both structure and regulation of steroid hormone receptors have been extensively explored. Recently the gene for ER has been cloned[19]. The complexity of steroid receptors, especially with respect to the various domains (such as the DNA, ligand binding and the recently described protein-kinase domain[15]), make this protein accessible to biochemists for study by application of the powerful technique of site-directed mutagenesis. Therefore, purification methodology of these recombinant proteins forms a central part of detailed molecular studies.

Our laboratory has developed HPLC separation in single and multistep purification procedures for steroid receptors and other proteins with high retention of their biological activity[7,8,12,18]. Here, we have characterized the chromatograms of estrogen receptors, eluted from recently introduced Beckman/Altex Spherogel CAA-HIC, a non-ionic polyether coated, silica-based column. This stationary phase

provided a very mild environment for selective elution of estrogen receptor isoforms, which retained their biological activity with respect to ligand binding. In addition, when immunoprecipitated with monoclonal antibody D547, raised against estrogen receptors, only one isoform (a less hydrophobic protein) demonstrated an associated protein kinase activity.

Effect of initial ionic strength on separation of ER by HPHIC (gradient 1.0–0 M ammonium sulfate)

When ER was analyzed on the CAA-HIC column with a linear gradient of 1.0–0 *M* ammonium sulfate, developed in 30 min, the majority of the receptor was eluted in the void volume (t_R = 3–4 min), a minor portion being eluted as two peaks with t_R = 16 min and t_R = 25 min (Fig. 1A). However, when the sample was first adjusted to the ionic strength of the initial mobile phase (in this case, 1 *M* ammonium sulfate, see Fig. 1B), a relatively lower proportion of receptor was found in the void volume, while the remainder was found at t_R = 16 min and 25 min. All the receptor isoforms were specific, as judged by DES inhibition of [^{125}I]iodoestradiol radioactivity, associated with the ER. This provided the first clue that either unfolding of receptor structure was important for promoting its hydrophobic bonding with the stationary phase or that the hydrophilic groups on the surface of the protein are neutralized and only the hydrophobic groups are left to interact with the column matrix.

Under the conditions used for the chromatography of ER, it is imperative to establish the elution profile of the ligand itself, in our case [^{125}I]iodoestradiol-17β

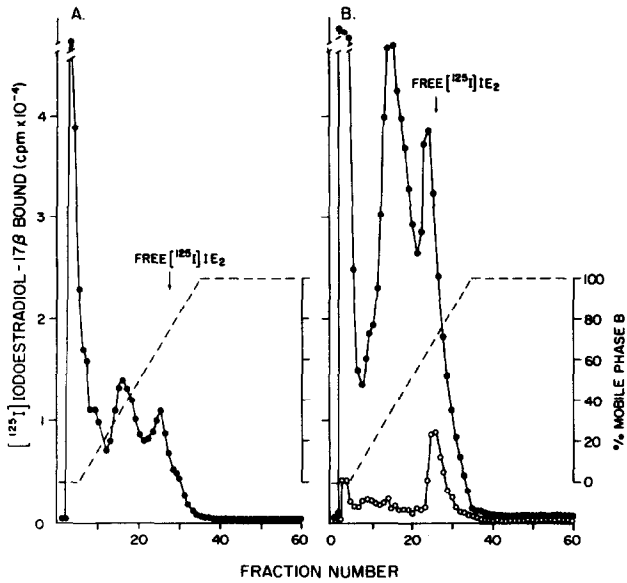

Fig. 1. Influence of the initial ionic strength on the separation of ER isoforms in rat uterine cytosol with a 1–0 *M* gradient of ammonium sulfate. Rat uterine cytosol was injected into the CAA-HIC column (A, control) without or (B) with adjustment of the sample to 1.0 *M* with respect to ammonium sulfate. (●) Total cpm/fraction, (○) cpm/fraction in the presence of DES. Recovery of radioactive iodine was 87% in A and 100% in B.

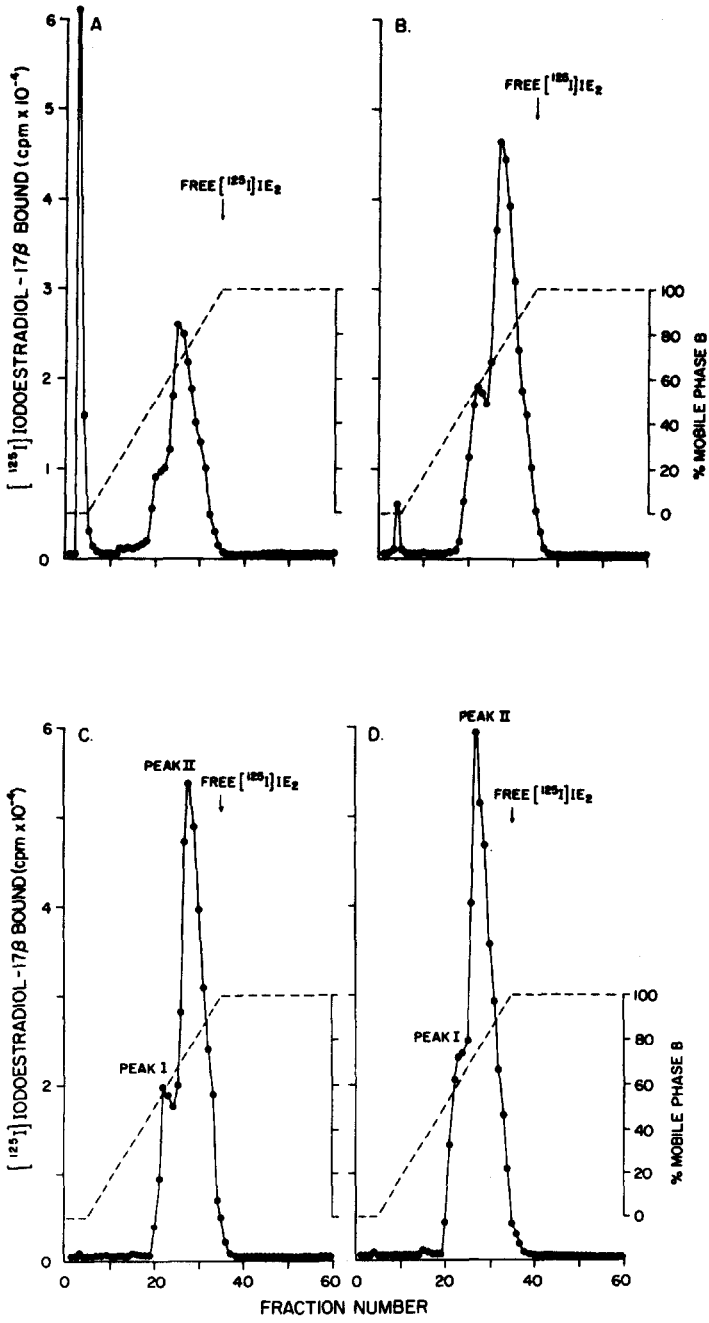

Fig. 2. Influence of initial ionic strength on the separation of ER isoforms from rat uterine cytosol with 2–0 *M* ammonium sulfate. Rat uterine cytosol was injected onto the CAA-HIC column (A, control) without or with adjustment of the sample to either (B) 1.0 *M*, (C) 1.5 *M* or (D) 2.0 *M* with respect to ammonium sulfate. (●) Total cpm/fraction. For clarity, chromatograms of non-specific binding profiles are not shown.

(ref. 9). We used this ligand because of its high specific radioactivity and also because we usually monitor all assays in-line with a Beckman Model 170 flow-through radioactivity detector as an additional control to confirm the presence of poorly resolved components which would not be identified by fraction collection[12]. Experience has shown that such ligands themselves show a certain affinity for the hydrophobic matrices and are retained by the column[9]. Therefore, their release during chromatography may be incorrectly attributed to receptor elution. Under the chromatographic conditions described, free ligand was eluted at t_R = 27 min, *i.e.* at some distance from any of the specific receptor peaks observed.

Effect of initial ionic strength on separation of ER (gradient 2–0 M ammonium sulfate)

To confirm an observation which is described in the earlier experiment (Fig. 1A and B), regarding the inability of receptor to interact with the stationary phase due to the lack of exposure of hydrophobic patches on the protein molecule, receptor was separated by using an elevated salt concentration (2 *M*) in the initial mobile phase with increasing ionic concentration in the sample injected (Fig. 2A–D). When cytosol was injected without altering its ionic strength, some specific receptor was eluted in the void volume as a result of the lack of interaction with the stationary phase (Fig. 2A). One other distinct component was present at t_R = 25 min together with a trailing edge. However, increasing the ionic strength of the sample to 1.0–1.5 *M* ammonium sulfate prior to injection (Fig. 2B and C) completely eliminated the specific bound radioactivity in the void volume and resolved the receptor into two peaks of t_R = 22 min (peak I, t_R = 22 ± 1 min, n = 16) and t_R = 28 min (peak II, t_R = 27.5 ± 0.5 min, n = 14). Further increase of ionic strength in the sample to 2

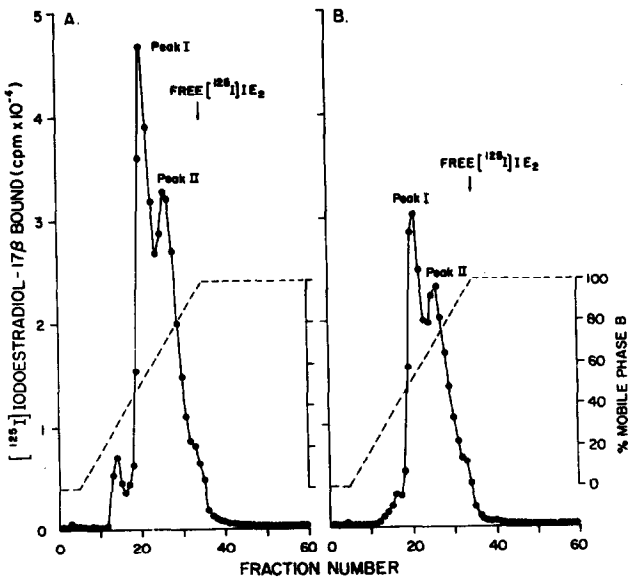

Fig. 3. Influence of prior exposure to ammonium sulfate on separation of ER isoforms. Following adjustment of rat uterine cytosol to 1.5 *M* with respect to ammonium sulfate, the sample (200 μl) was either (A) injected immediately or (B) was injected (150 μl) after 1 h. For clarity, only total cpm/fraction (●) are shown.

M ammonium sulfate prior to injection led to a loss in resolution (Fig. 2D). Our data suggest that this loss of resolution is better described as a slow interconversion of peak I to peak II (see results on time-dependent changes in receptor profiles Figs. 4–7). We chose to adjust all of our cytosol preparation to 1.5 *M* ammonium sulfate prior to injection.

It must be stressed that the concentrations of peak I and peak II were variable from one rat uterine sample to the next. We cannot be certain at present whether this was due to the fact that we used mature rats, irrespective of their stage of oestrous cycle, or whether some other factors, such as proteolysis or different degree of association with other molecules, may account for this variability. In two experiments, we detected peak I exclusively (*cf.*, *e.g.*, Fig. 7).

Effect of incubation time of cytosol with ammonium sulfate prior to HPHIC

Following addition of ammonium sulfate (1.5 *M* final concentration) to cytosol, there was no significant difference in the elution pattern of receptor, whether analyzed immediately or after a 1-h incubation with ammonium sulfate prior to injection (Fig. 3). This suggested that the differences observed in the relative proportions of receptor in peak I and peak II are not due to time variations following ammonium sulfate addition, which in every case was kept to a minimum. We can also rule out that the stationary phase altered receptor separation, since both injections were made from the same cytosol preparation. The relative differences, therefore, appear real in terms of their presence before chromatography and may represent

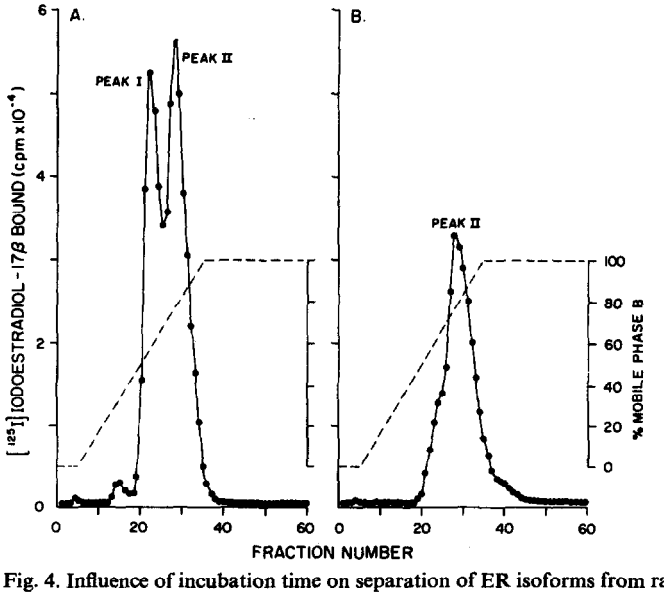

Fig. 4. Influence of incubation time on separation of ER isoforms from rat uterus by HPHIC. Rat uterine cytosol was prepared as described in Experimental and incubated with 3 n*M* [16-α^{125}I]iodoestradiol in the absence or presence of a 200-fold excess of DES. Following a 3-h incubation, one sample was injected into a CAA-HIC column for analysis (A, control). A second sample was injected after 24 h incubation with steroid (B). For clarity, only total cpm/fraction (●) is shown since non-specific cpm/fraction was virtually undetectable.

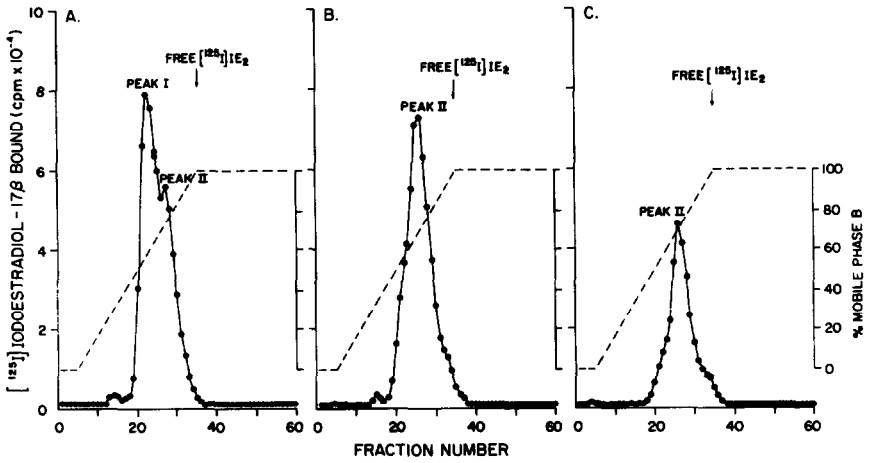

Fig. 5. Influence of incubation time on separation of ER isoforms from rat uterus by HPHIC. Cytosol was prepared as described in Experimental and incubated with 3 n*M* [16-α^{125}I]iodoestradiol in the absence or presence of 200-fold excess DES. Following a 3-h incubation, one sample was injected into a CAA-HIC column for analysis (A, control). A second sample was injected after 24 h incubation with steroid (B). A third sample was injected after 96 h of incubation with steroid (C). For clarity, only total cpm/fraction (●) is shown since non-specific binding was virtually undetectable.

certain aspects of distribution of receptor isoforms as a result of tissue differentiation[20]. However, other explanations are possible.

Effect of time of incubation of cytosol with radioactive steroid prior to HPLC analysis

Steroid receptors are known to undergo structural changes which are ligand- and time-dependent[21]. It has been known, for example, that liganded receptors are much more stable than unliganded receptors. In addition, we have found that the unliganded estrogen receptor is more prone to structural alterations following HPLC in the ion-exchange mode, leading to reduced association of receptors with specific monoclonal antibodies[22]. Some of the time-dependent alterations are related to receptor activation, *i.e.* the receptor affinity for binding to DNA increases[23]. We used HPHIC to study the time-dependent alteration of receptor structure to ascertain isoform conversion.

Because of our observation that cytosols from different uteri exhibit different profiles following HPHIC, especially in terms of their relative proportions of peak I and peak II, we analyzed four different types of cytosols, exhibiting either a dominant or exclusive peak I, dominant peak II, and cytosols exhibiting both peak I and peak II in approximately similar proportions. Fig. 4 represents a profile where two isoforms of ER were present in approximately similar concentrations (Fig. 4A). However, after an overnight incubation, only peak II was observed, and there was a decrease in the total amount of receptor present in the cytosol, probably due to degradation overnight. Fig. 5 represents a chromatogram in which peak I was abundant after 3 h of incubation with [^{125}I]iodoestradiol and subsequent analysis (Fig. 5A). However, following overnight incubation with the ligand, cytosol predominantly exhibited peak II with little or no free ligand (Fig. 5B). However, there was some

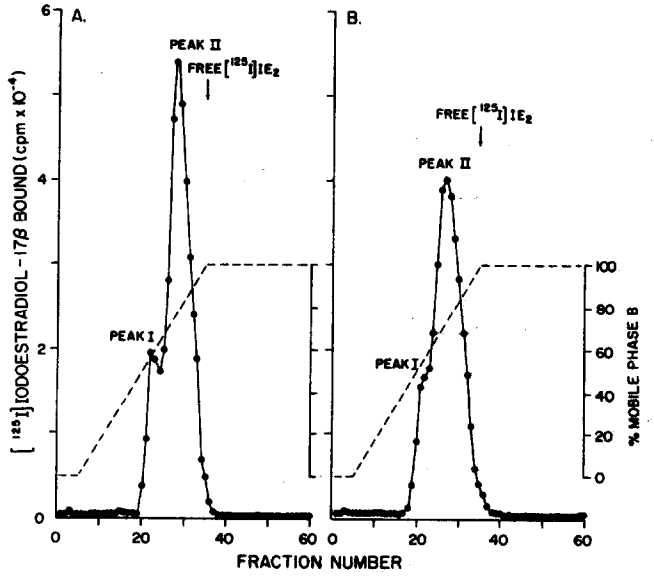

Fig. 6. Influence of incubation time on separation of ER isoforms from rat uterus by HPHIC. Cytosol was prepared as described in Experimental and incubated with 3 n*M* [16-α^{125}I]iodoestradiol in the absence or presence of 200-fold excess DES. Following a 3-h incubation, one sample was injected into a CAA-HIC column for analysis (A, control). A second sample was injected after 24 h incubation with steroid (B). For clarity, only total cpm/fraction (●) is shown since non-specific binding was virtually undetectable.

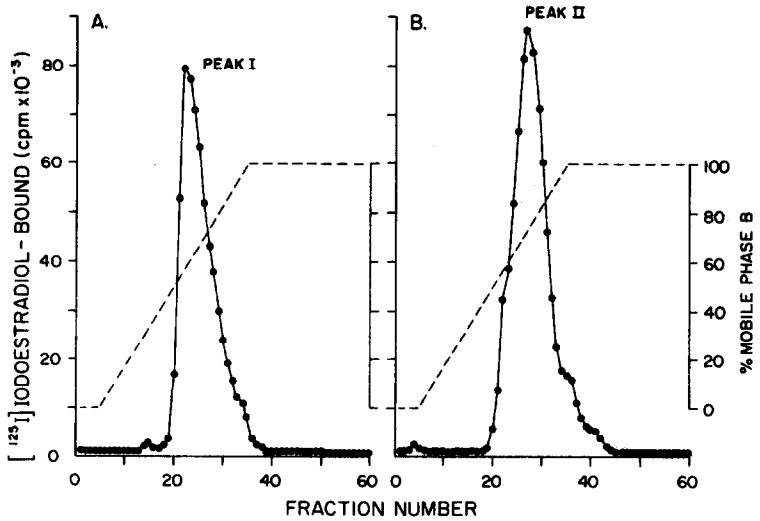

Fig. 7. Influence of incubation time on separation of ER isoforms from rat uterus by HPHIC. Cytosol was prepared as described in Experimental and incubated with 3 n*M* [16-α^{125}I]iodoestradiol in the absence or presence of 200-fold excess DES. Following a 3-h incubation, one sample was injected into a CAA-HIC column for analysis, (A, control). A second sample was injected after 24 h of incubation with steroid (B). For clarity, only total cpm/fraction (●) is shown since non-specific binding was virtually undetectable.

loss of total receptor. The profile was consistent, even after 96 h of incubation, although there was a greater loss of specific binding (Fig. 5C). If peak II represented activated receptor (ability to bind DNA avidly), as has been suggested to be the case after long-term incubations, then the results favor the suggestion of Aranyi[24] that, once activated (peak II), the receptor declines in terms of its ligand binding activity. Our previous results with human endometrial estrogen receptor, analyzed on ion exchange columns do not show such time-dependent changes[7].

Fig. 6 shows a representative chromatogram from a tissue dominant in peak II receptor form after 3 h of incubation with [^{125}I]iodoestradiol-17β and subsequent analysis by HPHIC (Fig. 6A). Similar conditions as described for experiments illustrated in Figs. 4 and 5 led to a conversion of peak I isoform to the peak II component. Finally, Fig. 7 shows an exclusive peak I-type profile after 3 h of incubation with steroid and HPHIC analysis (Fig. 7A). This figure shows the most remarkably complete conversion of the isoform in peak I to that in peak II when analyzed by HPHIC after an overnight incubation. Thus, we believe that peak II represents the active isoform of the estrogen receptor.

Effect of warming cytosol on the HPHIC profile of ER

It is known that activation of receptor may be carried out following a brief exposure of cytosol to elevated temperatures, such as 25–30°C, for 20–30 min[25]. We found that this temperature effect renders the receptor more hydrophobic (Fig. 8),

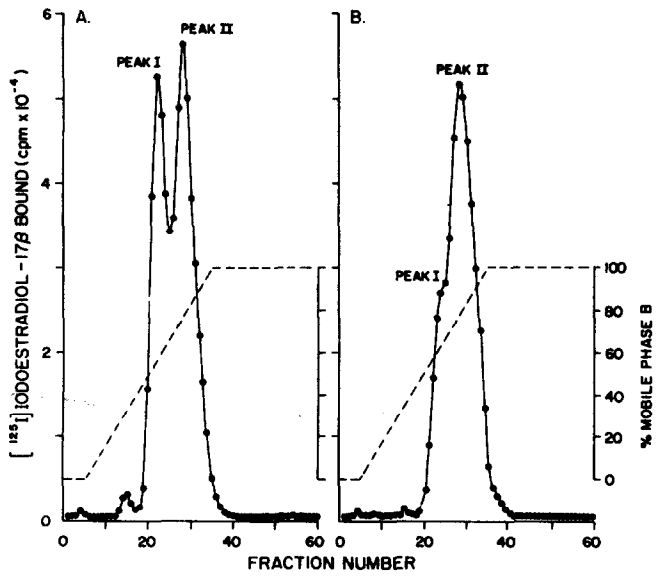

Fig. 8. Influence of warming of cytosol on the HPHIC profile of ER isoforms from rat uterus. Cytosol was prepared and incubated with steroid as described in Experimental. Following a 3-h incubation, one set was treated with DCC, adjusted with ammonium sulfate, and injected into a CAA-HIC column (A, control). Another aliquot was warmed to 25°C for 30 min to activate the receptor, cooled to 4°C, treated with DCC, and, following adjustment to 1.5 *M* ammonium sulfate, injected into the CAA-HIC column (B). For clarity, only total cpm/fraction (●) is shown.

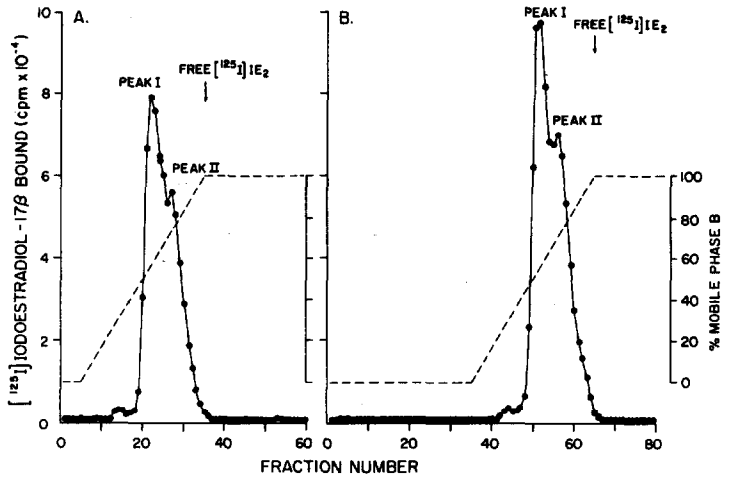

Fig. 9. Influence of stationary phase contact time with ER on HPHIC separation profile of ER isoforms from rat uterus. Labeled cytosol was cleared of excess steroid with DCC, as described in Experimental, and chromatographed on a CAA-HIC column with a 30 min linear gradient of ammonium sulfate from 2–0 *M* (A, control). A second sample of the same cytosol was injected and 2 *M* ammonium sulfate was first delivered isocratically for 30 min, followed by a 30 min gradient to 0 *M* ammonium sulfate (B). For clarity, only total cpm/fraction (●) is shown.

leading to a delayed elution of receptor peaks (t_R = 27 min) from the hydrophobic column. This result agrees with time-associated changes, shown in Figs. 4–7, and once again indicates that the activated isoform of the receptor is more hydrophobic. Such an increase in hydrophobicity may also result from aggregation of proteins during incubation, providing greater surface area for protein stationary phase interaction.

Influence of time on the interaction of receptor with the stationary phase in HPHIC

Steroid receptors are labile proteins which are prone to aggregation and/or thermal degradation when incubated with or without steroid or chromatographed on strong hydrophobic matrices[26]. We were not sure whether the molecular heterogeneity observed following elution from the CAA-HIC column was due to time-dependent conformational changes taking place while the receptor was in contact with the stationary phase. However, we found that if the salt gradient was started immediately after sample injection (Fig. 9A) or if mobile phase was first released isocratically for 30 min after sample injection (Fig. 9B) before gradient elution was initiated, the separation profiles obtained were the same. Therefore, we may conclude that the stationary phase itself does not contribute to receptor heterogeneity. Importantly, the elution of receptor isoforms was dependent upon ionic strength and not time. It should be noted that the CAA-HIC column also may be utilized in an isocratic mode as a size-exclusion column with a different mobile phase[27]. We have not evaluated receptor stability after longer time periods because these do not appear necessary in purification procedures. Because of the absence of stationary phase-induced conformational changes, one may inject multiple volumes of protein to increase sample loads.

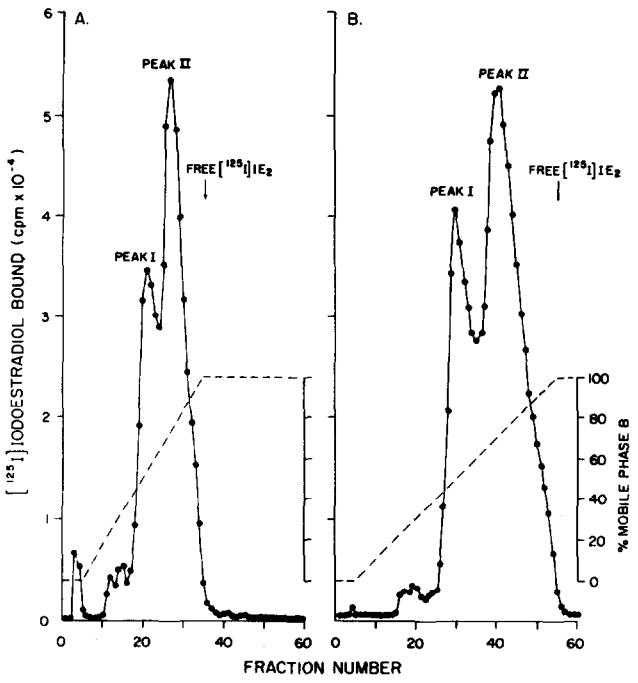

Fig. 10. Influence of gradient development time on HPHIC separation of ER isoforms from rat uterus. DCC-treated rat uterine cytosol was injected into a CAA-HIC column and chromatographed with either (A, control) a 2–0 *M* gradient of ammonium sulfate in 30 min or (B) 2–0 *M* ammonium sulfate gradient in 50 min. For clarity, only total cpm/fraction (●) is shown.

Influence of length of gradient on separation of ER isoforms

Fig. 10 shows the effect of gradient length on the separation of estrogen receptors by HPHIC. When the gradient developing time was increased from 30 to 50 min, it led to improved resolution of the two peaks and band broadening, as expected. No further distinct receptor species were seen. However, in other experiments, where the two receptor isoforms were less well resolved, increasing the gradient time did not improve separation. Routinely, we use a gradient elution time of 30 min to speed up the assay.

Separation of human breast cancer ER isoforms by HPHIC

The methodology developed for the separation and characterization of ER from rat uteri was also applied to ER from human breast cancer. An example is presented in Fig. 11. The separation characteristics shown are almost the same as those described for rat uteri (*e.g.* Fig. 4A). Again, two receptor isoforms were eluted with the same retention times as those of ER from rat uteri indicating the commonality between the polypeptides from these different tissues from two widely different species. Characterization of ER from human breast cancer by HPHIC is a subject of another study[28] and will not be discussed further. Suffice it to say that results were quite similar to those seen for rat uterine ER.

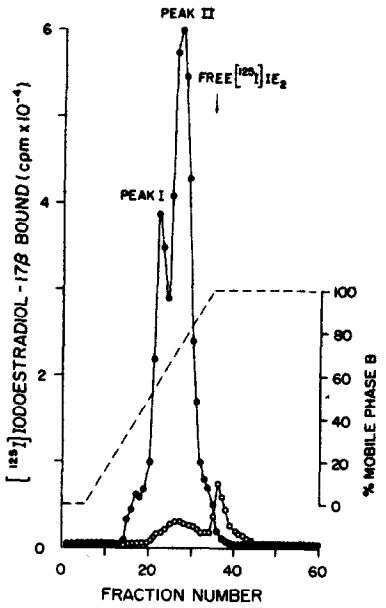

Fig. 11. Separation of ER isoforms from human breast cancer cytosol by HPHIC. Cytosol was prepared, labeled and cleared with DCC, as described in Experimental. Cytosol, adjusted to 1.5 *M* ammonium sulfate, was injected into a CAA-HIC column and chromatographed with a linear gradient of ammonium sulfate from 2–0 *M* in 30 min. (●) Total cpm/fraction, (○) nonspecific cpm/fraction.

Protein kinase activity associated with ER isoforms separated by HPHIC

Current reports from our laboratory have demonstrated a Mg^{2+}-dependent protein kinase activity, associated with immunopurified ER from human breast cancer cells (MCF-7)[15,16]. It was shown that receptors eluted by HPIEC retained this kinase activity[29]. We investigated whether retention of kinase activity was also possible following HPHIC. Fig. 12A illustrates a typical isoform chromatogram of ER, separated from rat uteri and used for analysis of protein kinase activity associated with ER. These separations resulted in a five- to twenty-fold purification for each isoform, depending upon the relative proportion present. In this experiment, both components were purified *ca.* fifteen-fold following a single pass. Karger's group[3] has had similar success in resolving two labile enzymes on the CAA-HIC column under retention of their activities, confirming its mild nature.

To demonstrate protein kinase activity, fractions from the receptor peaks (fractions 22 and 28) and two control points at fractions 12 and 50 were incubated directly with polystyrene beads, linked to D547 monoclonal antibodies against ER (Abbott ER-EIA kit). A separate bead was incubated with non-fractionated receptor in P_{10}EDG buffer for each experiment. After an overnight incubation, these antibodies were washed (see Experimental) and then one bead was analyzed for ER content by the EIA procedure and the other was used for protein kinase assay with phosvitin as the exogenous substrate. Histones were also used successfully as substrates. Fig. 12B is an autoradiogram demonstrating that only the ER eluted in fraction 22 immunoprecipitated with monoclonal antibody D547, exhibited protein kinase activity.

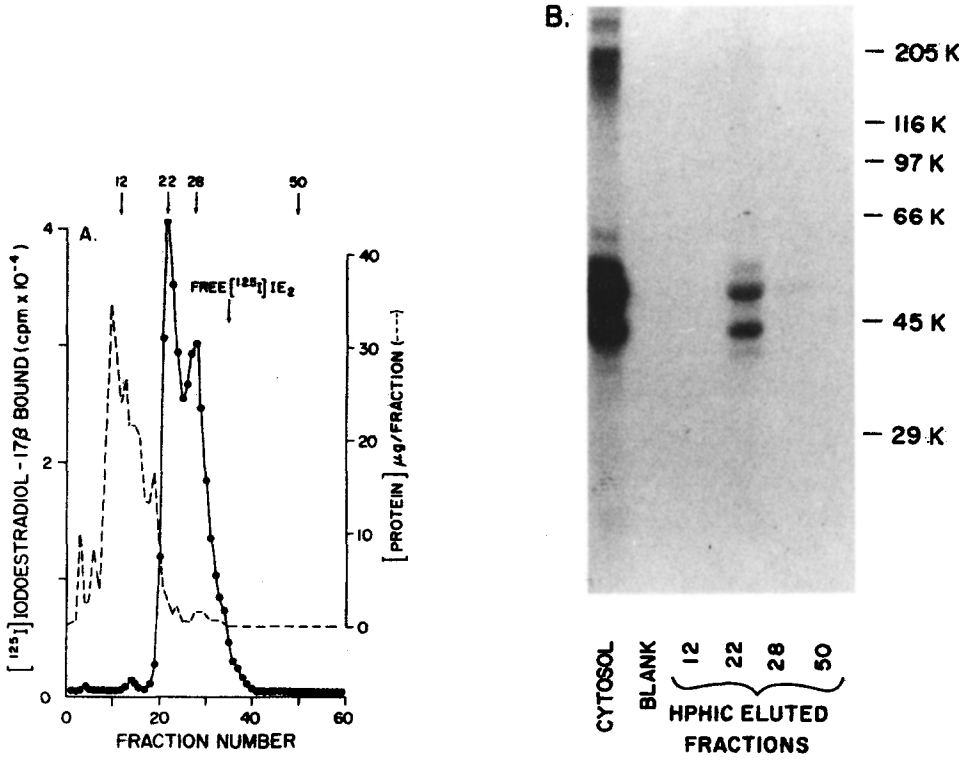

Fig. 12. Protein kinase activity, associated with ER isoforms, separated by HPHIC. (A) Rat uterine cytosol was chromatographed on the CAA-HIC column as described in Experimental. (●) Total cpm/fraction. (——) protein profile, as determined by the Bradford procedure[13]. For clarity, the non-specific binding profile which was virtually undetectable is omitted. (B) Fractions 12, 22, 28, and 50 from the HPHIC-separated sample (shown in A) were directly incubated with monoclonal antibody (D547), which was coated on polystyrene beads. A non-fractionated control sample was also incubated with the monoclonal antibody complex bead. Following an 18-h incubation and subsequent washing, one bead was analyzed for ER content (mass) by an EIA procedure and the second bead was tested for protein kinase activity, as described in Experimental. The receptor content associated with the monoclonal antibody in fmol/bead was 0 in fraction 12, 1.4 in fraction 22, 2.1 in fraction 28 and 0 in fraction 50. The control bead contained 7 fmol of receptor from the unfractionated cytosol in this representative experiment.

Importantly, no reaction was observed when the monoclonal antibody was allowed to interact with fraction 12, where most of the proteins were eluted.

Unlike previous studies with human breast cancer cells[15], we have not been able to demonstrate autophosphorylating activity of ER from rat uterus. The latter result agrees with that obtained by Ahrens *et al.*[30]. In the present experiments (Fig. 12) both isoforms were purified to the same extent (*ca.* fifteen- to sixteen-fold) and yet only isoform I (peak I) exhibited protein kinase activity. This exciting finding suggests that putative regulatory components are associated with these ER isoforms to a different extent, which may be due to varied affinities. The protein kinase activity associated with purified isoform I may even be an intrinsic property of the receptor molecule. Extensive investigation is required to resolve this question.

Several investigations of other steroid hormone receptors suggest that phosphorylation/dephosphorylation reactions play an important role in the activity of these regulatory proteins. For example, Weigel *et al.*[31] demonstrated that the purified subunits of the chicken oviduct progesterone receptor was phosphorylated by a cAMP-dependent protein kinase. Earlier, Toft's group reported this receptor was phosphorylated *in vivo* on serine residues[32]. These data clearly support the view that the progestin receptor in chick oviduct is a phosphoprotein. The glucocorticoid receptor also has been reported to be phosphorylated[33,34] and, more germaine to our study, to contain an associated protein kinase activity when purified from rat liver[35]. In contrast, Sanchez and Pratt[36] published evidence that the glucocorticoid receptor in L-cells was not a protein kinase itself. Finally, Auricchio's group[37] has reported that protein kinases added *in vitro* to the estrogen receptor bring about phosphorylation on tyrosine residues. This result is in contrast to our recent results showing that the immunopurified estrogen receptor from MCF-7 breast cancer cells is phosphorylated on serine residues[29].

CONCLUSION

Our results demonstrate a methodology that can be used for the separation and characterization of steroid-hormone receptors, which are labile regulatory proteins, without loss in their biological activity. Resolution may be achieved in the absence of organic solvents with virtually 100% recoveries of both the protein and the radioactivity associated with the receptors. This methodology appears promising for discerning subtle conformational changes associated with estrogen receptor isoforms such as reported for other protein molecules[27]. It is intriguing that only one isoform of these receptors demonstrated the protein kinase activity. This may be an integral part of the receptor molecule itself or of another very tightly associated protein with the less hydrophobic receptor species. HPHIC thus represents a mild chromatographic separation procedure for the rapid isolation of steroid receptor in a partially purified form for further analysis. These data, the retention of steroid-binding activity and the identification of additional functions (protein kinase) suggest that HPHIC is useful in resolving the composition of the estrogen receptor molecule and may have application to other types of steroid hormone receptor.

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