Journal of Chromatography, 444 (1988) 225–237
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1328

HIGH-PERFORMANCE HYDROPHOBIC INTERACTION CHROMATOGRAPHY AS A MEANS OF IDENTIFYING ESTROGEN RECEPTORS EXPRESSING DIFFERENT BINDING DOMAINS

SALMAN M. HYDER and JAMES L. WITTLIFF*
Hormone Receptor Laboratory, Department of Biochemistry, and James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40292 (U.S.A.)

SUMMARY

Methodology for high-performance hydrophobic interaction chromatography (HPHIC) of estrogen receptors (ER) was developed, utilizing a polyether-bonded stationary phase, which was non-ionic in nature. Using a descending salt gradient (2 *M* to 0 *M* ammonium sulphate in 40 min), ERs from human breast cancer separated into two isoforms, which retained ligand-binding domains. The same isoforms were observed with ER preparations from rat uterus. When sodium molybdate, a stabilizer of receptor structure, was incorporated into the mobile phase, it altered the ER characteristics, producing an earlier elution of one component, while the other one remained unchanged. Treatment of breast cancer cytosol with RNase A did not alter ER elution from either the hydrophobic or size-exclusion (TSK 3000 SW) columns. Modification of cysteine residues with N-ethylmaleimide led to a broad elution pattern of receptor from the hydrophobic column, implying the existence of multiple conformations of ER. Limited trypsin treatment of ER, which removes the DNA binding domain, led to the elution of only one receptor peak from the hydrophobic column. The receptor eluted at 24 min both in the presence and in the absence of sodium molybdate. Thus, at least one mechanism of the sodium molybdate effect must involve its direct interaction with ER to influence the sequence between the DNA-binding domain and the N-terminus. This also indicates that the most hydrophobic species of ER (sodium molybdate sensitive) may arise due to the interaction of the DNA-binding site with the stationary phase. Other possibilities, such as differential post-translational modifications of the receptor protein could also account for the two isoforms of ER, observed in HPHIC analysis.

INTRODUCTION

Analysis of estrogen receptors (ER) by high-performance hydrophobic-interaction chromatography (HPHIC) has led to the development of a rapid separation technique for steroid receptors[1–3]. Separation based on the hydrophobicity of proteins has revealed two isoforms, and this substantiates our hypothesis of receptor polymorphism, based on receptor size, shape, and surface charge properties[4–6]. Cloning

0021-9673/88/$03.50

of the ER gene[7] has led to the assignment of certain functional domains on the protein molecule. These mainly fall into three broad areas: (1) the N-terminus region with suggested function related to protein–protein interaction; (2) the DNA-binding domain, which is rich in cysteine residues; (3) and the domain near the C-terminus of the protein, which encodes the ligand-binding of receptor. The latter region also contains the highest number of hydrophobic residues.

Analysis of ER in the presence of sodium molybdate[8], a receptor-stabilizing reagent, indicates selective interaction of this oxyanion with one of the two isoforms detected. When the receptor was prepared without sodium molybdate and chromatographed with a mobile phase containing sodium molybdate, a selective decrease of the more hydrophobic isoforms was observed. There was a concomitant increase in a previously undetected component, which was the least hydrophobic in nature. Since sodium molybdate is believed to interact with the DNA binding domain of the receptor[9], it appears that the most hydrophobic component in the absence of sodium molybdate interacts with the stationary phase through the DNA-binding domain of receptor. Therefore, the isoform uninfluenced by sodium molybdate appears to interact with the stationary phase via the C-terminus region of the receptor or via the association with another protein. Although the C-terminus region is the most hydrophobic, based on cDNA deduced protein sequence[7], the tertiary structure may not be fully exposed to interact with the stationary phase. Thus, application of HPHIC with reagents such as sodium molybdate and trypsin permits separation and probing of receptor components expressing various binding domains.

EXPERIMENTAL

Materials and methods

HPLC-grade ammonium sulphate was obtained from Bio-Rad (Richmond, CA, U.S.A.). The ligand [16α-^{125}I]iodoestradiol-17β (*ca.* 2200 Ci/mmol) (IE) was obtained from New England Nuclear/DuPont (Boston, MA, U.S.A.). Sodium molybdate, disodium ethylenediaminetetraacetic acid (EDTA), and glycerol were purchased from Fisher Scientific (Louisville, KY, U.S.A.). Unlabeled diethylstilbestrol (DES), Norite A, Dextran T-70, dithiothreitol (DTT), trypsin and trypsin inhibitors were obtained from Sigma (St. Louis, MO, U.S.A.). RNase was obtained from two different sources: Worthington (Freehold, NJ, U.S.A.) and Boehringer Mannheim (Indianapolis, IN, U.S.A.). N-Ethylmaleimide (NEM) was purchased from United States Biochemical Co. (Cleveland, OH, U.S.A.).

Human breast tumor tissues were provided by the various surgeons and pathologists at local hospitals, cooperating with the Hormone Receptor Laboratory. The tissues were brought to the laboratory on dry ice and kept frozen at −86°C until analyzed. Only residual tissue from clinical receptor analyses was used in this study. Female Sprague-Dawley rats (*ca.* 250 g) were obtained from Laboratory Supplies (Indianapolis, IN, U.S.A.). Animals were sacrificed by cervical dislocation, and their uteri were removed. All uteri used were fresh.

Preparation and labeling of soluble estrogen receptors

All procedures were performed at 4°C. 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 containing 10% (v/v) glycerol (pH 7.4)]. Homogenization was performed with two 10-s bursts in a Brinkman (Westbury, NY, U.S.A.) Polytron homogenizer. Rat uteri were homogenized in 1 ml per uterus.

Soluble fractions were prepared by centrifugation of the homogenate for 30 min at 40 000 rpm (147 000 g at r_{max}) in a Beckman Ti 70.1 rotor (San Ramon, CA, U.S.A.). The supernatant was carefully removed, avoiding the layer of fat at the top. The soluble fractions were labeled with 2–3 n*M* IE in the presence and absence of a 200-fold excess of DES for 2–4 h at 4°C. Free steroid was removed with 1% (w/v) dextran-coated charcoal (DCC). DCC was then removed by centrifuging the sample for 5 min at 1000 *g*. Cytosol protein concentrations were determined by the method of Bradford[10], 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 ER complexes were applied to the polyether-bonded, non-ionic silica-based Spherogel CAA-HIC column (pore size 300 Å), obtained from Beckman/Altex (San Ramon, CA) using an Altex Model 210 sample injection valve. All samples were adjusted to 1.5 *M* ammonium sulphate prior to injection. Elution was carried out with a Beckman Model 114 solvent delivery module including a Model 421 system controller.

Unless otherwise stated, the gradient program consisted of preliminary wash with eluent A (P_{10}EDG), containing 2 *M* ammonium sulphate (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 40 min. Eluent B was then maintained at a flow-rate of 1 ml/min for the next 20 min before re-equilibration with eluent A. In experiments which required sodium molybdate in the mobile phase, both eluents A and B contained 10 m*M* sodium molybdate.

Fractions (1 ml) were collected, and the free and protein-bound steroid were detected radiometrically in a Micromedics 4/600 gamma radioisotope detector (Rohm & Haas, Cleveland, OH, U.S.A.), having a counting efficiency of 65%. Since the non-specific binding (radioactivity eluted from cytosols labeled in the presence of DES) showed mainly base levels and represented no more than 5–10% of the total binding, these are not shown in the figures. Recovery of total radioactivity and injected protein was almost always 75–100%, with a receptor purification factor of *ca.* 5–20.

High-performance size exclusion chromatography (HPSEC)

Analytical size-exclusion columns (Spherogel TSK-3000 SW), particle size 10 μm (600 × 7.5 mm) from Beckman Altex Instruments, were used for steroid receptor separation, as described previously[11]. HPLC was performed at 4°C with a Beckman 114 solvent delivery module, including a Model 421 system controller and injector block. Cytosols were applied in 100–250 μl volumes with a Hamilton syringe. The elution buffer (pH 7.4) at 4°C, was $PEDGK_{100}$ (10 m*M* phosphate buffer–1.5 m*M* EDTA–1 m*M* DTT–100 m*M* potassium chloride, containing 10% (v/v) glycerol). All buffers were filtered through a 0.45-μm filter (Millipore). Elution was carried out at

a flow-rate of 0.7 ml/min. Fractions were collected at 0.5-min intervals in 12 × 75 mm tubes. Recoveries were in the range 75–100%.

Limited tryptic digestion of ER from human breast cancer

Our procedure used was as described earlier[12]. The cytosol from cancer tissues was labeled with 3 n*M* IE, both in the absence and in the presence of a 200-fold excess of DES at 4°C. After 2–4 h of incubation, one reaction mixture of labeled cytosol was adjusted to 40 μg trypsin/mg cytosol protein, while the other one was kept as a control. Mixtures were incubated further for 1 h at 4°C, and then the tryptic digestion was stopped by adding soybean trypsin inhibitor (2.5 × trypsin concentration). Reaction mixtures were kept at 4°C for an additional 30 min, then treated with a DCC pellet to remove the free steroid from the protein-bound steroid. The bound fraction (*i.e.* IE–receptor) was used for HPHIC analyses.

Influence of RNase A on ER from human breast cancer

Following incubation with IE for 2–4 h at 4°C one set of aliquots was adjusted to various concentrations of RNase A, as indicated in the figure legends. Mixtures were incubated further for 40 min at 4°C then DCC treated to remove free steroid from protein-bound steroid. The bound fraction was used for HPHIC analyses.

NEM treatment of ER from human breast cancer

After incubation of cytosol with IE for 2–4 h at 4°C, a set of aliquots was adjusted to different concentrations of NEM (indicated in the figure legends) for 1 h. Following incubation, the cytosols were treated with DCC, and the bound fraction was used for HPHIC analyses.

RESULTS AND DISCUSSION

Steroid hormone receptors are regulatory proteins[13], which are present in sex steroid target organs in femtomolar concentrations. Thus, sensitive methods are required for their purification in studies of their structure–function relationships. Our laboratory has developed several HPLC procedures for this, both in single and multidimensional modes[1–6,14]. The protein structure of ER predicted from its gene sequence indicates potential hydrophobic patches on the receptor molecule. These could be utilized for purification, particularly since HPHIC is gentle enough for labile proteins. We have already demonstrated the utility of this approach for receptor purification[1–3,8,13]. In this report, we present data to show the influence of certain protein modifying reagents on receptor hydrophobic domains, which allow resolution of isoforms retaining binding activities.

Effect of NEM on ER

Protection of sulfhydryl groups on the estrogen receptor are known to preserve certain ligand-binding properties. The DNA binding domain of ER is rich in cysteine residues[7]. In the absence of sulfhydryl-modifying reagents, the receptor was eluted at t_R = 26 min (peak I) and t_R = 34 min (peak II), as shown in Fig. 1A. Some free ligand was present (t_R = 46 min). The presence of free ligand suggests that the stationary phase may promote dissociation of steroid and receptor; this phenomenon

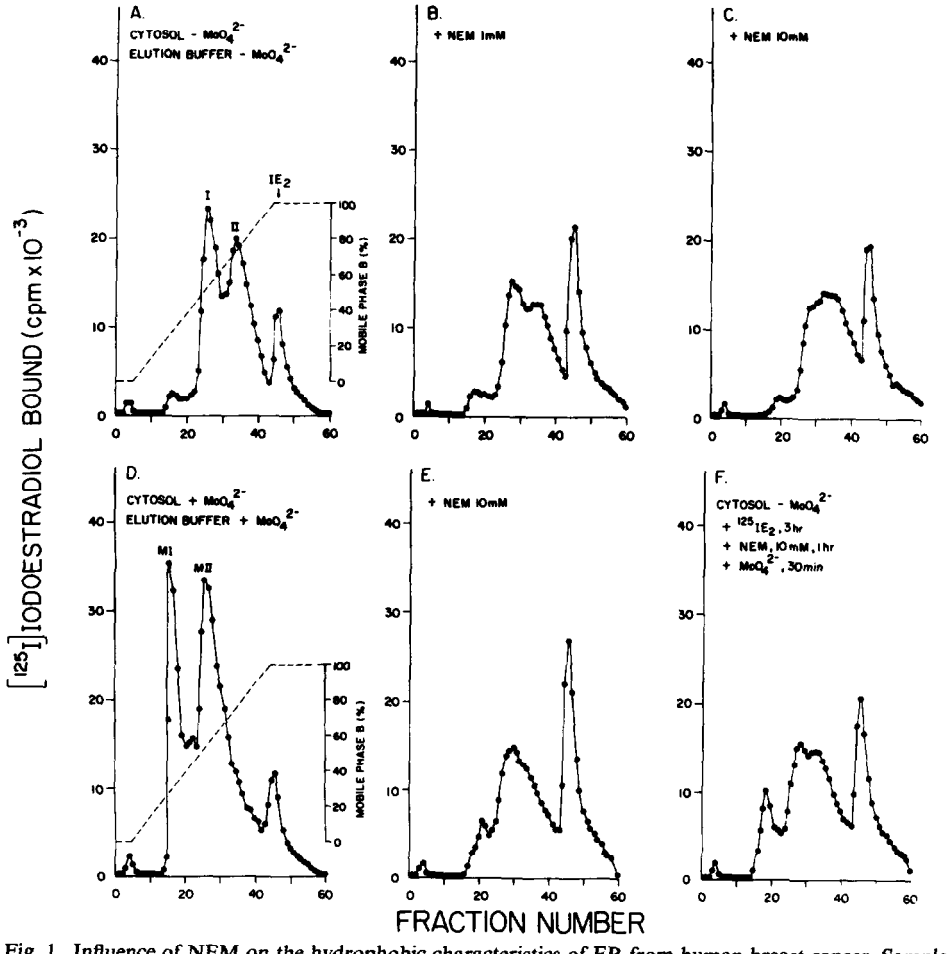

Fig. 1. Influence of NEM on the hydrophobic characteristics of ER from human breast cancer. Samples were prepared in buffers, either with (D–F) or without 10 m*M* sodium molybdate (A-C). These were labeled with [^{125}I]iodoestradiol-17β as indicated in *Materials and methods*. One set of aliquots was then treated with the concentration of NEM indicated for 1 h at 4°C. Following removal of free steroid with charcoal, samples were injected into the column. The elution conditions are indicated on each graph. The elution position of [^{125}I]iodoestradiol is represented by IE (A).

varied from column to column. The Spherogel CAA-HIC column used in our earlier study[2] did not show this effect. In the presence of the sulfhydryl-modifying reagent, NEM, there was a dose dependent loss of peak resolution (Fig. 1B and C). There appeared to be a larger loss of peak I than of peak II with 10 m*M* NEM. This produced a broad peak possibly owing to multiple conformations of receptor. Peak II appears to be due to the interaction of the DNA-binding domain of the receptor with the stationary phase[8]. These results suggests that receptors in peak II are less susceptible to the NEM effect than to those in peak I. This is despite the fact that peak II may contain more sulfhydryl groups, which may be modified. Receptor activation, which exposes the DNA-binding domain of the receptor, is known to be a

temperature-, time- and ionic-strength-dependent process. In our experiments, the samples were adjusted to a high ionic strength just prior to injection, and this is likely to expose the DNA-binding domain. Prior to this treatment, sulfhydryl residues are unable to react with NEM, since they appear to be buried within the protein molecule. The period between increasing the ionic strength of the sample and injection (<2 min) probably was insufficient for NEM to modify the sulfhydryl groups of the DNA-binding domain. In addition to loss of peak resolution in the presence of NEM, there was a reduction in the quantity of receptors, based on steroid-binding domains. Surprisingly, this loss was greater when sodium molybdate was present in the cytosol (20–30%) than when sodium molybdate was absent (5–10%). In addition, the presence of NEM in cytosols also led to increased stripping of ligand during chromatography. This appears to reflect the importance of sulfhydryl groups in maintaining the high affinity of the receptor for the ligand.

The presence of sodium molybdate in buffers did not prevent the NEM effect (Fig. 1D and E). Once again, multiple conformations of receptors were observed. Since sodium molybdate promotes interaction of the mol.wt. 90 000 heat-shock protein (HSP) with receptors[15] and the NEM effect may involve this receptor-associated protein, we used the following procedure (Fig. 1F). The receptor was extracted from tissues in the absence of molybdate and labeled with IE. Following labeling, NEM was added to modify the receptor, and just prior to chromatography, sodium molybdate was added to the cytosol. HPHIC once again revealed no effect of this treatment. Our results indicate that sulfhydryl groups present on the receptor protein, detected in both peaks I and II are readily modified by NEM, but sodium molybdate addition does not alter this influence.

Effect of sodium molybdate on ER

In our earlier work[8] we observed that sodium molybdate reduced the hydrophobicity of ER. In contrast to the chromatogram shown in Fig. 1A (peaks I and II), we observed components labeled MI and MII in the presence of sodium molybdate (Fig. 1D); MI was the least hydrophobic protein(s). Based on binding-domain specificity and similarity in chromatographic behavior of peaks I and MII, we suggested that they represented the same isoform of ER. In over 50% of the human breast tumor cytosols examined, there was a decrease of peak II with a concomitant increase in isoforms MI when chromatographed in the presence of sodium molybdate. However, in other specimens there was also an increase in isoforms MII(I), suggesting that the transformation of peak II to MI may involve MII(I) as an intermediate. This may indicate that receptor molecules in peak II, which are modified by sodium molybdate, may be associated with sodium molybdate-independent species in peak I. Fortunately, a system exists where this can be demonstrated directly. In 15% of rat uterine cytosols, only isoform I is present[2]. No similar chromatograms have been obtained with more than 25 human breast tumor cytosols. The occurrence of only one isoform after HPHIC may be a result of the stage of differentiation of the rat uterus[16].

Fig. 2A provides an example of the presence of only one isoform in rat uterine cytosol. When chromatographed with buffers containing sodium molybdate, isoform MI also appeared with an unchanged total quantity of binding domains in the two species (Fig. 2B). Extensive washing of immobilized receptors with sodium molyb-

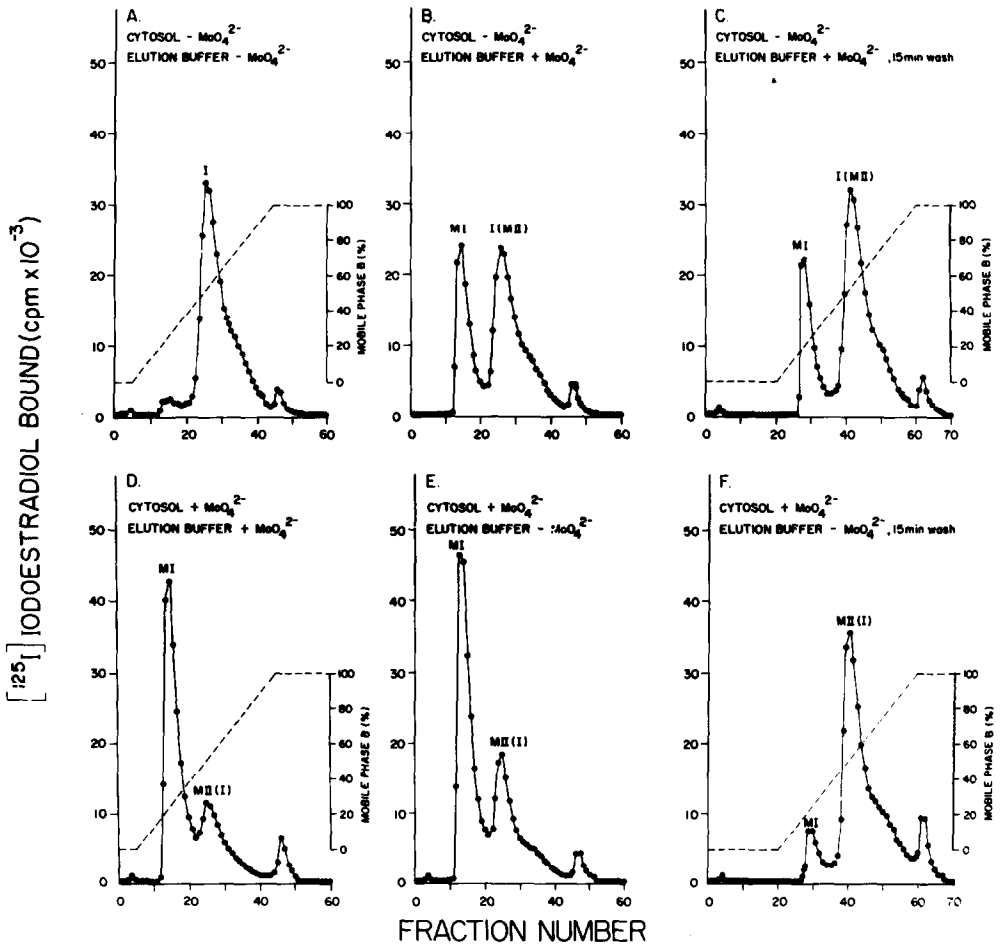

Fig. 2. Influence of sodium molybdate on the hydrophobic characteristics of ER from the rat uterus. Rat uteri were homogenized, and the cytosol was prepared in P_{10}EDG, as described in *Materials and methods*. One set of aliquots was then made 10 m*M* with respect to sodium molybdate and labeled with [^{125}I]iodoestradiol. The elution buffers in each case are indicated in the figure. In C and F, the column was washed with eluent A for an extended period prior to initiation of the gradient, which reached 100% eluent B in 40 min.

date-containing buffers did not significantly alter peak I (Fig. 2C). However, when the cytosol was adjusted to 10 m*M* sodium molybdate and chromatographed with sodium molybdate-containing buffers, there was a sharp increase in MI (Fig. 2D). This indicates that, in the absence of sodium molybdate, of all of the receptors with a steroid-binding domain attached to the stationary phase only certain receptor molecules (presumably those with intact DNA-binding domains) were influenced by sodium molybdate (Fig. 2A–C). When sodium molybdate-containing cytosol was chromatographed with buffers lacking sodium molybdate, using an extended column wash, MI was lost, while isoform I(MII) was increased (Fig. 2F). Without additional washing in the absence of sodium molybdate, there was little conversion (Fig. 2E).

These results imply that isoform I(MII) may also contain sites that are sodium molybdate sensitive. It must be stressed that results shown in Fig. 2 are from rat uterine tissue. Although human and rat uterine receptors behave similarly on the HPHIC column, subtle differences do exist[2]. This may be due to sequence differences in the ER molecules from the two species. Only the human ER gene has been cloned[7]; obviously, other explanations are possible.

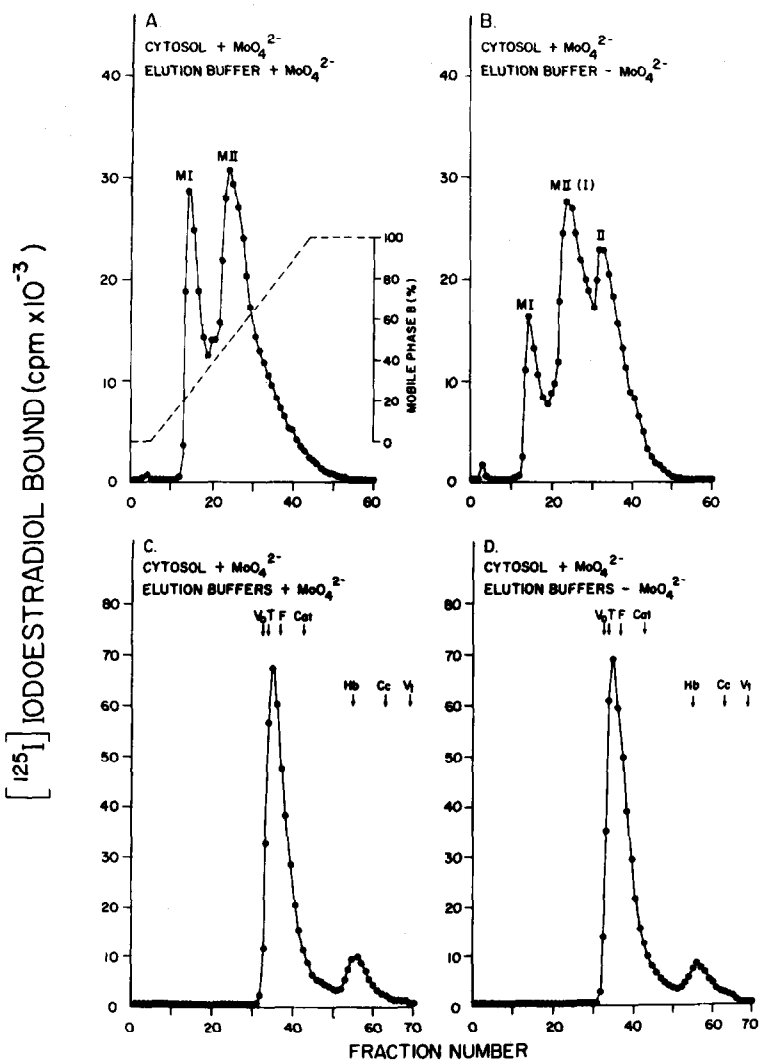

Fig. 3. Influence of sodium molybdate on the hydrophobic and size properties of ER from human breast cancer. Cytosol was prepared in P_{10}EDG and then immediately made 10 m*M* with respect to sodium molybdate. After labeling with [^{125}I]iodoestradiol and removal of free steroid, samples were injected into either the hydrophobic column (A,B) or the size-exclusion column (C,D). The receptor was then eluted with buffer either containing (A,C) or lacking sodium molybdate (B,D).

Resolving power of the hydrophobic column

The reproducibility of the resolution of isoforms MI and MII(I), prepared and chromatographed in the presence of sodium molybdate was consistently excellent (Fig. 3A). The detection of three hydrophobic forms of receptors in molybdate-containing cytosol, which was chromatographed in the absence of molybdate (Fig. 3B) raises intriguing possibilities for ER composition. To ascertain whether these changes in receptor hydrophobicity were the result of changing the mobile phase, which may alter receptor size, HPSEC was employed simultaneously (Fig. 3C and D). Under both conditions of separation, a predominant isoform at *ca.* 80Å was observed in HPSEC, a minor component being found at *ca.* 30Å. When the same cytosol without sodium molybdate was analyzed by HPSEC in the absence of sodium molybdate, a similar chromatogram was obtained (not shown). On the HIC column, various conformers with associated non-receptor proteins (*e.g.* HSP) may be formed as a consequence of the change in mobile phase with time. This is not the case with HPSEC. We now have evidence that peak MI and MII originate as a result of association of the receptor with other macromolecules, *e.g.* HSP-90, while peak II appears to be a monomer of estrogen receptor. In other words, sodium molybdate selectively promotes the interaction of HSP with receptor[17]. Collectively, our results suggest that HPHIC has greater isoform-resolving power than HPSEC, and that estrogen receptors separated either in the absence or presence of sodium molybdate are eluted as high-molecular-weight complex in HPSEC, suggesting that they represent the native form. The latter conclusion is based on the observation that HPSEC is the most rapid method for analyzing proteins with the least formation of homogenization artifacts. Our study shows the utility of HPLC in rapidly separating ER (< 1 h) in its native state, whether or not sodium molybdate is employed. However, the protective effect of sodium molybdate is only observed when manipulations are prolonged[18].

Effects of trypsin on hydrophobic properties of receptor

Limited trypsin treatment of ER reduces the receptor size to *ca.* mol.wt. 35 000 with a loss in its DNA-binding properties[12,19]. The hydrophobic characteristics of ER in the intact (Fig. 4A) and trypsin-treated (or mero-receptor) states (Fig. 4B) were compared. Trypsinized receptor (I′) was consistently eluted earlier (t_R = 23–24 min) but similarly to isoform I (t_R = 26–28 min). This suggests that the steroid-binding domain near the C-terminus region of the receptor must contribute significantly to the interaction with the stationary phase. Further evidence for this suggestion is provided by the trypsin study conducted in the presence of sodium molybdate (Fig. 4C and D). Although receptor was resolved into two isoforms in the presence of sodium molybdate (Fig. 4C), after trypsin treatment, a single isoform (MII′, t_R = 24 min) was observed (Fig. 4D), similar to isoform I′, detected in the absence of sodium molybdate (Fig. 4B). These results indicate that the chromatographic behavior of trypsin-treated receptor was independent of the sodium molybdate effect and reaffirm that isoform II involves interaction of the DNA-binding domain with the stationary phase. Furthermore, sodium molybdate does not prevent proteolysis of ER under the conditions used and should not be considered an inhibitor of receptor-modifying proteases.

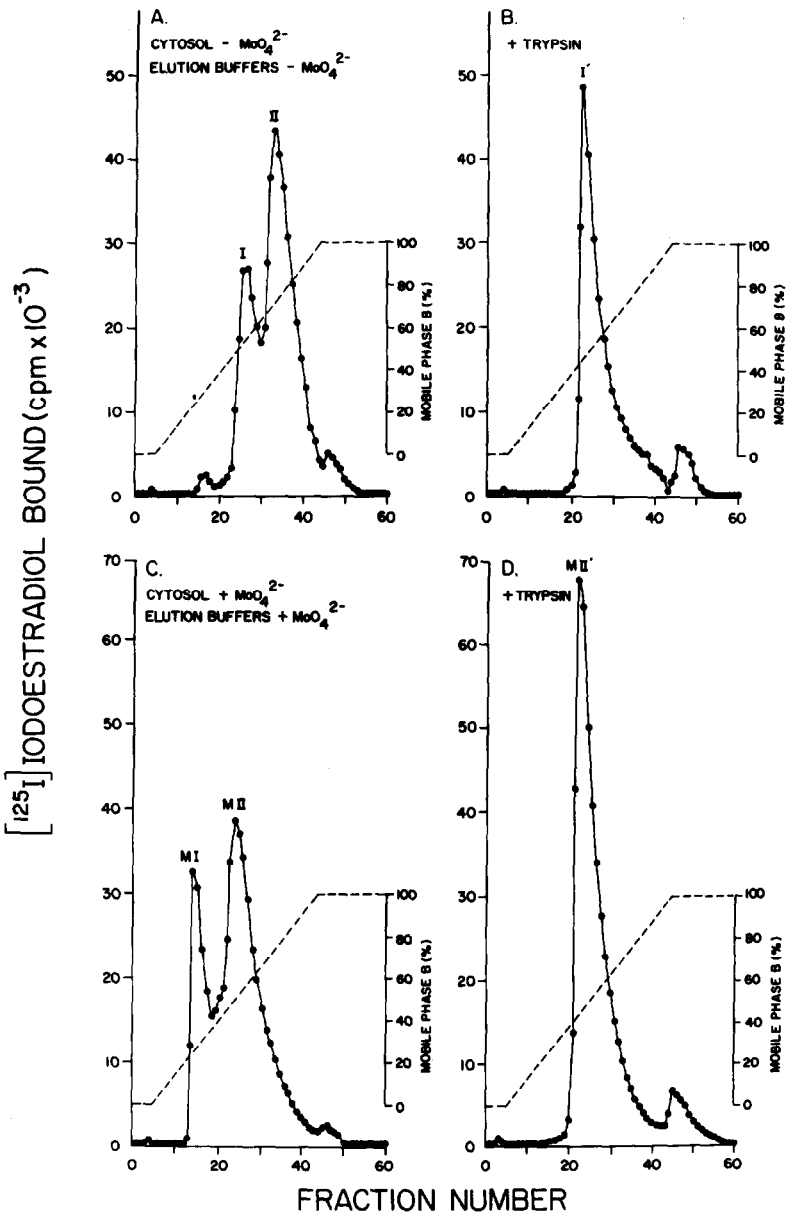

Fig. 4. Influence of trypsin on the hydrophobic properties of ER from human breast cancer. Cytosol was prepared in P_{10}EDG, and an aliquot was made 10 m*M* with respect to sodium molybdate. Following incubation with [^{125}I]iodoestradiol, one set of aliquots (B,D) was subjected to limited trypsin digestion (*Materials and methods*). Free steroid was then removed with charcoal, and samples were injected into the hydrophobic column. The eluents are indicated in the figure panel.

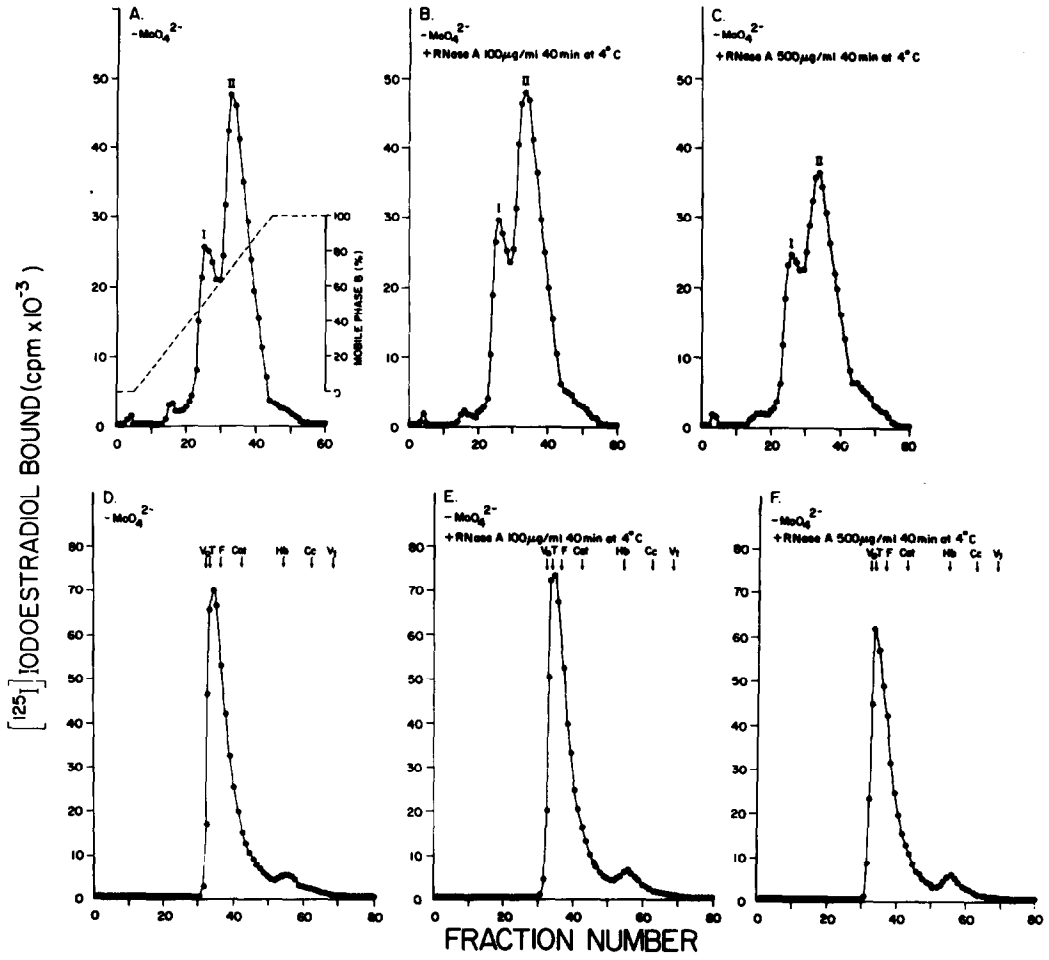

Fig. 5. Influence of RNase A on the size and hydrophobic properties of ER from human breast cancer. Cytosol was prepared in P_{10}EDG and labeled with [^{125}I]iodoestradiol (*Materials and methods*). Aliquots were then treated with different concentrations of RNase, A as indicated in the figure. Following removal of free steroid from the cytosol, samples were injected into either the hydrophobic (A–C) or the size-exclusion columns (D–F). Receptor was then eluted with the buffers indicated in the figure.

Effect of RNase A on receptor hydrophobicity

It has been suggested that steroid receptors are associated with RNA[20,21]. However, it is now clear that RNA is not associated with receptor, as it is normally extracted from the cell. Rather, the receptor protein complex first undergoes dissociation, followed by reassociation with RNA molecule(s)[22]. This may be observed when receptors are fractionated according to their size. Removal of RNA with RNase led to a form of glucocorticoid receptor migrating more slowly in sucrose density gradients[20]. We demonstrated that the high-molecular-weight forms of ER do not contain RNA molecule(s) (Fig. 5D–F) and that RNase treatment does not alter the hydrophobic properties of the receptor (Fig. 5A–C). When the effect of RNase A of

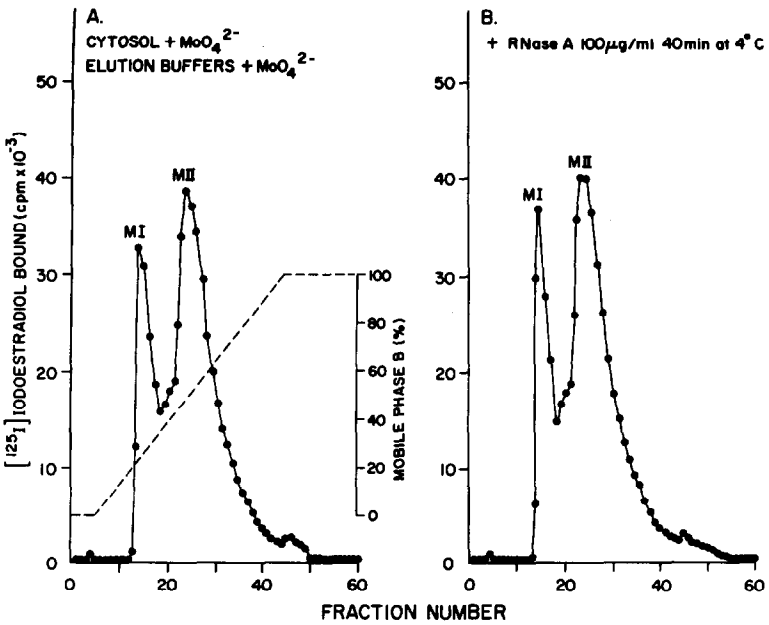

Fig. 6. Influence of RNase A on the hydrophobic properties of ER from human breast cancer. Cytosol was prepared in P_{10}EDG and immediately made 10 m*M* with respect to sodium molybdate. Following incubation with [^{125}I]iodoestradiol, one set of aliquots was treated with RNase A (B). Free steroid was then removed with charcoal, and samples were injected into the hydrophobic column, which was eluted with sodium molybdate containing buffers.

the highest purity from two different sources was studied in concentrations up to 10 mg/ml, no effect was observed on receptor hydrophobic or size characteristics compared to a control. In addition, the presence of sodium molybdate in the reaction mixture also did not influence the hydrophobic properties of ER either (Fig. 6A and B). These data rule out the possibility that the appearance of isoform MI is due to interaction of receptor RNA complexes with the stationary phase via the nucleic acid. However, it cannot be ruled out that selective complexes, *e.g.* receptor–RNA–receptor (or receptor–RNA–another protein), interact with the stationary phase. Collectively, our results show that high-molecular-weight forms of ER exist independent of exposed RNA. It is unknown whether the high-molecular-weight ER complex contains RNA buried within the protein structure. Results from other studies with glucocorticoid receptors have indicated that this is not the case[22,23].

Conclusions

Our results demonstrate the effectiveness of HPHIC[1,2] not only in the purification of ER and other labile proteins but also in the elucidation of their structure–function relationships. The present study suggests that two isoforms are separated, based on hydrophobic properties resulting from exposure of different binding domains: the steroid-binding domain near the C-terminus (isoform I) and the DNA-binding domain (isoform II) of receptor. Exposure of these domains is dictated not only by the conditions of the stationary phases of the HPHIC system,

but also by various properties of the receptor molecule. These may include different phosphorylation state(s) and the association of receptor with either lipids or proteins or a combination of these. Detailed analysis of the receptor isoforms in purified states should explain the origin of this polymorphism and assist in our understanding of their biological significance.

ACKNOWLEDGEMENTS

This research was supported in part by USPHS grants CA-32102, CA-37429 and CA-42154 from the National Cancer Institute and by a grant from the Phi Beta Psi Sorority. Salman M. Hyder was a research fellow of the Graduate School.

REFERENCES

1 S. M. Hyder, R. D. Wiehle, D. W. Brandt and J. L. Wittliff, *J. Chromatogr.*, 327 (1985) 237.
2 S. M. Hyder, N. Sato and J. L. Wittliff, *J. Chromatogr.*, 397 (1987) 251.
3 S. M. Hyder and J. L. Wittliff, in F. Ausubel, R. Brant, R. Kingston, D. Moore, J. A. Smith, J. Seidman and K. Strubl (Editors), *Current Protocols in Molecular Biology*, Greene Publishing Associate, NY, 1987, pp. 10.15.1.
4 J. L. Wittliff, *LC-GC Mag. Liq. Gas Chromatogr.*, 4(11) (1986) 1092.
5 J. L. Wittliff, N. A. Shahabi, S. M. Hyder, L. A. van der Walt, L. Myatt and J. L. Wittliff, in James L'Italien (Editor), *Modern Methods in Protein Chemistry*, Plenum Press, NY, in press.
6 J. L. Wittliff, R. Wiehle, N. A. Shahabi, L. A. van der Walt and S. M. Hyder, in A. R. Kerlavage (Editor), *Receptor Methodology*, in press.
7 S. Green, P. Walter, V. Kumar, A. Krust, J. M. Burnest, P. Argos and P. Chambon, *Nature (London)*, 320 (1986) 134.
8 S. M. Hyder, N. Sato, W. H. Hogancamp and J. L. Wittliff, *J. Steroid Biochem.*, 29 (1988) 197.
9 W. de Boer, J. Bolt, A. O. Brinkmann and E. Mulder, *Biochem. Biophys. Acta*, 889 (1986) 240.
10 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
11 R. D. Wiehle, G. E. Hofmann, A. Fuchs and J. L. Wittliff, *J. Chromatogr.*, 307 (1984) 39.
12 N. Sato, S. M. Hyder, L. Chang, A. Thais andd J. L. Wittliff, *J. Chromatogr.*, 359 (1986) 475.
13 S. M. Hyder and J. L. Wittliff, *BioChromatography*, 2 (1987) 121.
14 N. A. Shahabi, S. M. Hyder, R. D. Wiehle and J. L. Wittliff, *J. Steroid Biochem.*, 24 (1986) 1151.
15 A. Ziemiecki, M. G. Catelli, I. Joab and B. Moncharmont, *Biochem. Biophys. Res. Commun.*, 138 (1986) 1298.
16 S. M. Hyder, R. D. Wiehle and J. L. Wittliff, *Fed. Proc.*, (1985) 1474.
17 S. M. Hyder and J. L. Wittliff, in preparation.
18 N. A. Shahabi, S. M. Hyder and J. L. Wittliff, *Endocr. Soc. Abs.*, (1988) No. 1311.
19 G. Greene, N. Sobel, W. King and E. V. Jensen, *J. Steroid Biochem.*, 20 (1984) 51.
20 J. Tymoczko and M. M. Phillips, *Endocrinology*, 112 (1983) 142.
21 M. Feldman, J. Kallos and V. P. Hollander, *J. Biol. Chem.*, 256 (1981) 1145.
22 W. V. Vedeckis, *BioChromatography*, 2 (1987) 121.
23 S. Kasayama, K. Noma, B. Sato, M. Nakao, Y. Nishizawa, K. Matsumoto and S. Kishimoto, *J. Steroid Biochem.*, 28 (1987) 1.