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ASSESSMENT OF ESTROGEN RECEPTOR–MONOCLONAL ANTIBODY INTERACTION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High performance liquid chromatography (HPLC) was employed as a means of analyzing estrogen receptor (ER)–antibody recognition. This technique takes advantage of the fact that the majority of γ-globulin–antigen complexes do not interact with the anion-exchange resins selected. A variety of monoclonal (MAb) and polyclonal antibodies (PAb) raised against ER and ER-associated proteins were assessed for their chromatographic behaviour after association with charged ER, based upon properties of size, shape, and surface charge. ER exhibits polymorphism, several isoforms being present in target cells. The monoclonal antibody H222Spγ demonstrated discrete specificity for the 150 m*M* ER isoform (normally eluting at 150 m*M* phosphate) from the high-performance ion-exchange chromatography column which was eluted unretained when complexed with antibody. However, the monoclonal reagent D547Spγ interacted directly with anion-exchange columns (SynChropak AX-1000 and Altex DEAE-5PW), complicating a clear evaluation of ER–MAb association. Only 50–60% of the 150 m*M* ER isoform was eluted at a lower salt concentration. Few conclusions could be drawn with respect to MAb interaction with the 50–60 m*M* ER isoform (normally eluting at 50–60 m*M* phosphate) since the antibody–receptor complex was also eluted at the same phosphate concentration. In addition, polyclonal and monoclonal antibodies to the ER and ER-associated proteins were assessed by HPLC. At present, heat shock proteins and protein kinase activity have been shown by other techniques to be associated with the ER. Size-exclusion resins, such as TSK 3000 SW, were employed in a fast method of determining ER isoform–antibody recognition. Thus, HPLC may be used to analyze soluble antibody–antigen interactions rapidly, with high recovery of biological activity.

INTRODUCTION

Although the estrogen receptor (ER) has been studied extensively, a clear understanding of its macromolecular composition and architecture has not been achieved. However, we have established that the ER exhibits polymorphism, with several protein components (isoforms) retaining steroid-binding domains[1–3] as well as an associated protein kinase activity[4]. If ER is prepared in low-ionic-strength

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buffers, then the cytosol usually exhibits two principal ionic isoforms[2], although greater molecular heterogeneity has been detected[5]. These ER isoforms are eluted with 50–60 m*M* and 150 m*M* phosphate buffer in high-performance ion-exchange chromatography[6] (HPIEC). Their chemistry and biological role in the intracellular mechanism of estrogen action is the subject of our research.

When the separation is performed in the presence of sodium molybdate, the ER is eluted as a single component with 100 m*M* phosphate buffer[2,3]. This allows one to differentiate the molybdate-stabilized ER from the ER isoforms. Recent studies with the molybdate stabilized isoform have demonstrated the association of a 90-kilodalton heat shock protein (HSP) with all types of molybdate-stabilized steroid receptors[7–9] and with tyrosine kinase oncogene products[10]. It has been hypothesized that dissociation of the 90-kilodalton HSP results in "activation" or "transformation" of the receptor into a DNA-binding protein[11,12].

Previous studies, employing monoclonal antibodies (MAb) raised against ER from the MCF-7 human breast cancer cell line, examined their interaction with only the high-ionic-strength, 4–5 S forms of the ER, separated by sucrose density gradient centrifugation[2,13–17]. In some of these experiments, two immunodistinct forms of ER[15–17] were observed, with functions related to the state of activation[16] or a minor modified form of the ER[17]. In the following study, MAb H222Spγ and D547Spγ, raised against the ER, and MAb AC88 and 4F3, raised against the 90-kilodalton HSP were used to probe the immunochemical relationships of ER-isoforms prepared in the presence and absence of molybdate, by means of high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and chemicals

The ligands [16α-^{125}I]iodoestradiol-17β and [^{3}H]estradiol-17β were obtained from DuPont/New England Nuclear Products (Boston, MA, U.S.A.). The purity of labeled steroids was checked by thin-layer chromatography in two solvent systems; only those with purities of >95% were used. Other unlabeled ligands such as diethylstilbesterol (DES) were purchased from Sigma (St. Louis, MO, U.S.A.).

The purified marker proteins, namely thyroglobulin, ferritin, catalase, hemoglobin, ovalbumin, myoglobin, and cytochrome *c*, were obtained from Sigma. All other chemicals were of reagent grade.

Sample preparation

All procedures were carried out at 0–4°C. Residual specimens of human breast cancer biopsies from clinical studies in our laboratory[18] were homogenized, using a Brinkman Polytron® (New York, NY, U.S.A.) (three 5-s bursts) in four to five volumes of either phosphate buffer (10 m*M* potassium hydrogenphosphate–10 m*M* EDTA–1.5 m*M* dithiothreitol–10% (v/v) glycerol pH 7.4) or phosphate buffer containing 0.6 *M* potassium chloride, depending upon the chromatographic conditions. Cytosols were prepared by centrifugation of the homogenates for 15 min at 75000 rpm in a Beckman (Palo Alto, CA, U.S.A.) TLA 100.2 rotor using a Beckman TL-100 tabletop ultracentrifuge. The supernatant lipid was separated from the cytosol layer. Samples were incubated at 4°C for 4 h with [16α-^{125}I]iodoestradiol (final con-

centration, 6 n*M*) in the presence and absence of a 200-fold molar excess of DES. After a reaction with ligand, samples were incubated with a two- to four-fold excess of monoclonal antibodies or with control rat IgG for 12–16 h. Under high-salt conditions, all samples were maintained at 0.4 *M* potassium chloride by addition of a 2.0 *M* potassium chloride solution. The incubations were terminated by removing unbound steroid with a pellet derived from an equal volume of a 1% dextran-coated charcoal suspension (1% charcoal, 0.5% dextran). Samples were mixed with the charcoal pellet and allowed to stand for 5 min under high-salt conditions or for 10 min under low-salt conditions. Cytosol protein concentrations were determined by the method of Bradford[19], using a kit from Bio-Rad Labs. (Richmond, CA, U.S.A.).

High-performance liquid chromatography

Chromatography was performed at 4°C in a cold room or cold-box. Beckman (Altex, San Ramon, CA, U.S.A.) Model 114M two-pump solvent delivery systems were used[3].

High-performance size-exclusion chromatography (HPSEC) was performed with a Spherogel TSK-3000SW column (600 × 7.5 mm), coupled to a guard column (Upchurch, Oak Harbor, WA, U.S.A.)[20]. Samples were applied in 100-μl to 250-μl volumes, using a Hamilton syringe and a Model 210 sample injection valve. The elution buffer was phosphate containing 0.4 *M* potassium chloride. All buffers were filtered through a 0.22-μm Millipore (Bedford, MA, U.S.A.) filter. Elution was performed at a flow-rate of 0.4 ml/min, and 1.0 min fractions were collected. The void volume and total volume were determined by Blue Dextran and [^{3}H]water (DuPont/New England Nuclear) respectively. The column was calibrated with purified proteins, purchased from Sigma.

High-performance ion-exchange chromatography (HPIEC)[6] was performed with a Synchropak AX-1000 column (250 × 4.6 mm) (SynChrom, Lafayette, IN, U.S.A.) or an ALTEX Spherogel TSK DEAE 5 PW column (750 × 7.5 mm) coupled to a guard column (Upchurch). Samples were applied in 100-μl to 250-μl volumes of phosphate buffer, and proteins were eluted with a programmed linear gradient from 10 to 500 m*M* phosphate buffer. Fractions of approximately 1.0 ml were collected.

Sucrose density centrifugation

Appropriate samples of 200 μl were applied to a 60 × 11 mm linear 10–40% sucrose gradient, prepared in phosphate buffer with either 0.4 *M* potassium chloride or 10 m*M* sodium molybdate. Tubes were centrifuged at 60000 rpm for 16 h in a Beckman SW60Ti swinging bucket rotor in a Beckman L8-70 ultracentrifuge. Gradients were fractionated with a Beckman fraction recovery system, and five-drop fractions were taken.

Radioactivity

The [^{125}I]iodoestradiol-17β-labeled receptor complexes, nonspecific binding components, and free steroids in each fraction were detected radiometrically in a Micromedics 4/600 γ-counter (Horsham, PA, U.S.A.). The counting efficiency was approximately 65%.

RESULTS

Monoclonal antibody titers were determined by various means. For the MAb D547Spγ, sucrose density gradient centrifugation (SDG) was employed. ER demonstrated a dose-dependent shift from a 4 S species to a larger, *ca.* 8 S species when associated with the MAb (Fig. 1). However, typically, 10–15% of the specific ligand-binding activity did not shift to the larger-molecular-weight (MW) species even upon higher doses of antibody (Fig. 1C). The titer used in further studies was 0.1 μg D547Spγ per fmol ER. The titer of the MAb H222Spγ was based upon information from the ER-EIA assay kit (Abbott Labs., Chicago, IL, U.S.A.). To ascertain if the MAb AC88 and 4F3, raised against the chick oviduct 90-kilodalton HSP, would interact with the human breast cancer ER, MAb concentrations were set such that high-affinity ($K_d \geqslant 10^{-8}$ *M*) antibodies would be saturated (100 μg/ml).

The HPSEC separation of proteins is based upon size and shape with a linear

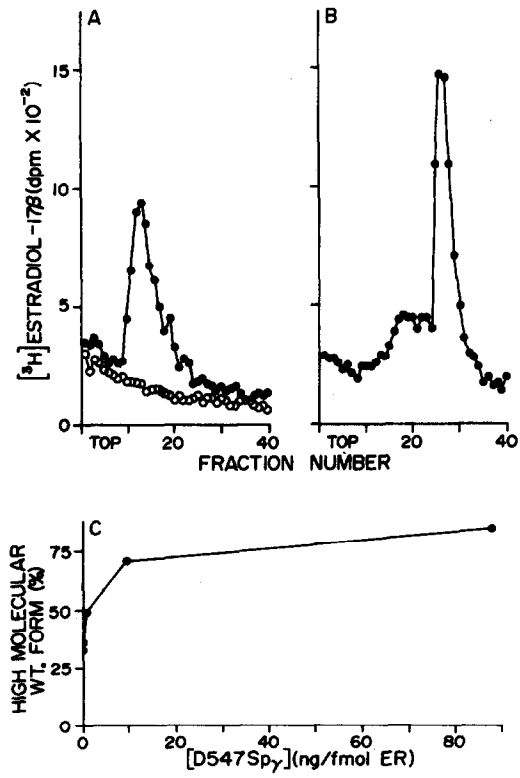

Fig. 1. Titration of estrogen receptors from human breast cancer with D547Spγ monoclonal antibody. In Panel A, [^{3}H]estradiol-17β receptor complexes were incubated overnight with non-specific rat IgG, followed by centrifugation for 16 h at 105000 *g* in 10–40% sucrose gradients, containing 0.4 *M* potassium chloride; (●) total ligand binding, (○) non-specific binding. In Panel B, receptor complexes were incubated with 85 ng D547Spγ per fmol receptor and centrifuged as described in A. In Panel C, receptor complexes were incubated with varying amounts of D547Spγ and the percent shift to the higher-molecular-weight species was determined and plotted. The titer was taken to be 10 ng per fmol receptor.

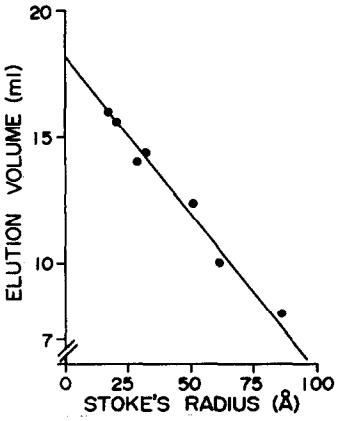

Fig. 2. The calibration curve for TSK-3000SW column was obtained as described in the Experimental section, using purified proteins: thyroglobulin, ferritin, catalase, hemoglobin, bovine serum albumin, ovalbumin, myoglobin and cytochrome *c*.

relationship between Stokes radius (Å) and elution volume (Fig. 2). When ER was chromatographed in 0.4 *M* potassium chloride, it was eluted mainly as a 25 to 35 Å species, as previously described[2,20] (Fig. 3A and 4A). This allowed a clear separation of H222Spγ and D547Sp-ER complexes from unreacted ER (Fig. 3B and 4B), as shown by the shift in Stokes radius of the eluted species. The MAb H222Spγ produced a broad peak when complexed with ER, but appeared to interact with virtually all ER. More clearly defined MAb–ER complexes were seen in the presence of D547Spγ eluted at 60–70 Å. However, a shoulder was observed in the 25- to 35-Å

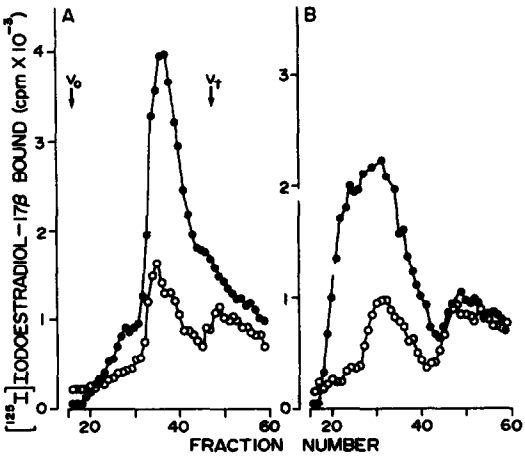

Fig. 3. HPSEC evaluation of estrogen receptor interaction with H222Spγ monoclonal antibody conjugate. In Panel A, [^{125}I]iodoestradiol-17β receptor complexes (50 fmol) were incubated with non-specific rat IgG (0.2 μg/ml) overnight and then separated on a TSK 3000SW column, as described in Experimental; (●) total ligand binding, (○) non-specific binding. Panel B was a parallel run of an incubate of ER with H222Spγ conjugate as described in Experimental.

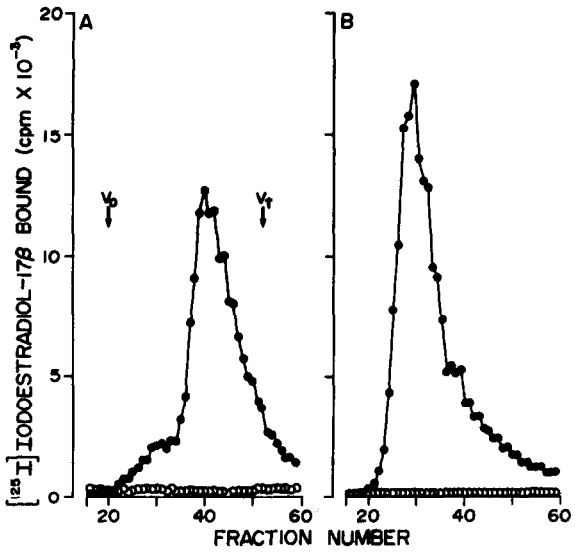

Fig. 4. HPSEC evaluation of estrogen receptor interaction with D547Spγ monoclonal antibody. [^{125}I]Iodoestradiol-17β receptor complexes were incubated with either rat IgG (Panel A) or with D547Spγ (Panel B) at a titer of 0.1 μg per fmol receptor in phosphate buffer, containing 0.4 *M* potassium chloride, and separated on a TSK 3000SW column. (●) Total ligand binding, (○) non-specific binding.

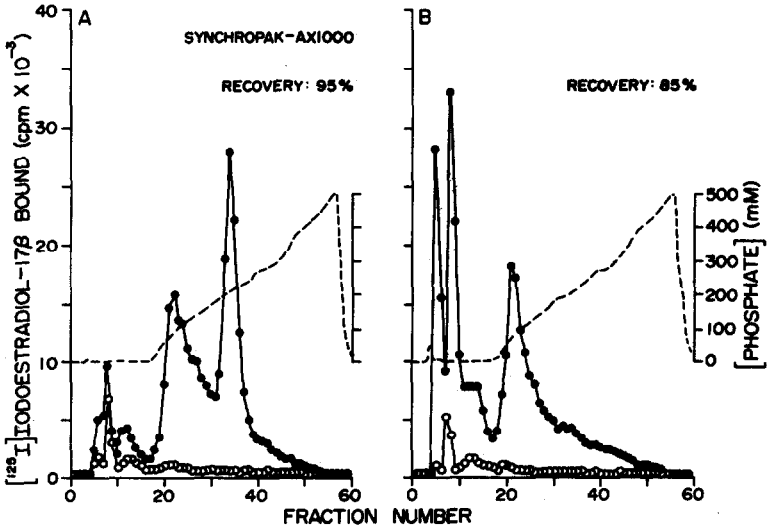

Fig. 5. HPIEC evaluation of estrogen receptor interaction with H222Spγ monoclonal antibody conjugate. In Panel A, [^{125}I]iodoestradiol-17β receptor complexes were incubated with non-specific rat IgG overnight in phosphate buffer, then separated on a SynChropak AX-1000 column, as described in Experimental. Panel B was a parallel incubation with H222Spγ conjugate and separation by HPIEC. (●) Total ligand binding, (○) non-specific binding.

region of unreacted ER with a magnitude similar to that seen with SDG (Fig. 4B).

When employing HPIEC, we took advantage of our observation that the majority of γ-globulins did not interact with the AX-1000 anion-exchange resin. Therefore, we predicted that ER–MAb complexes would not interact with the stationary phase and be eluted without retention. ER isoforms not recognized by a MAb would be eluted at characteristic salt concentrations. This appeared to be the case with H222Spγ (Fig. 5). Under control conditions, at least two ER isoforms were observed (Fig. 5A), eluted with appropriate phosphate concentration in the mobile phase. In the presence of MAb, there was a shift of the 150 m*M* ER isoform to be unretained without apparent influence on the elution position of the 50–60 m*M* ER isoform (Fig. 5B). The MAb D547Spγ was unusual, since this γ-globulin appeared to interact with the resin. In the absence of receptor, it was eluted in the same region as the 50–60 m*M* phosphate ER isoform. This would not permit a clear evaluation of this ER isoform. However, with the DEAE-5PW column, a partial shift of the ER isoform eluted with 150 m*M* phosphate into this region was observed (Fig. 6). The magnitude of the shift varied among tumor samples and may be dependent on the MAb concentration. MAb (AC88 and 4F3) did not alter the elution of the ER isoforms under normal conditions (data not shown) which supports the earlier finding from Toft's group[21] that AC88 MAb recognizes HSP from a variety of hormone targets but does not associate with receptors themselves.

When the HPIEC conditions were altered by the addition of 10 m*M* sodium molybdate, the ER was eluted as a single peak with 100 m*M* phosphate concentration (Fig. 7A). If the molybdate-stabilized ER was preincubated with MAb raised against the ER (H222Spγ and D547Spγ) or against receptor-associated 90-kilodalton HSP (AC88 and 4F3), no change was seen in the elution process (Fig. 7B, C). This clearly indicated the influence of molybdate ions on the exposure of epitopes recognized by the MAb to ER.

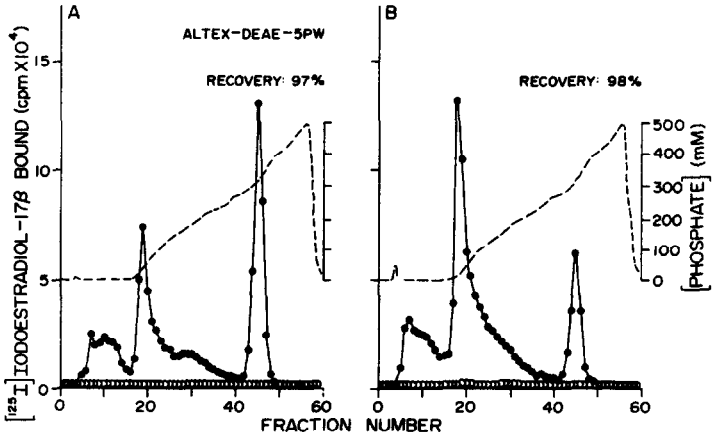

Fig. 6. HPIEC evaluation of estrogen receptor interaction with D547Spγ monoclonal antibody. In Panel A, [^{125}I]iodoestradiol-17β receptor complexes were incubated with non-specific rat IgG overnight in phosphate buffer, then separated on DEAE-5PW column, as described in Experimental. Panel B was a parallel incubation with D547Spγ MAb under the same separation conditions. (●) Total ligand binding, (○) non-specific binding.

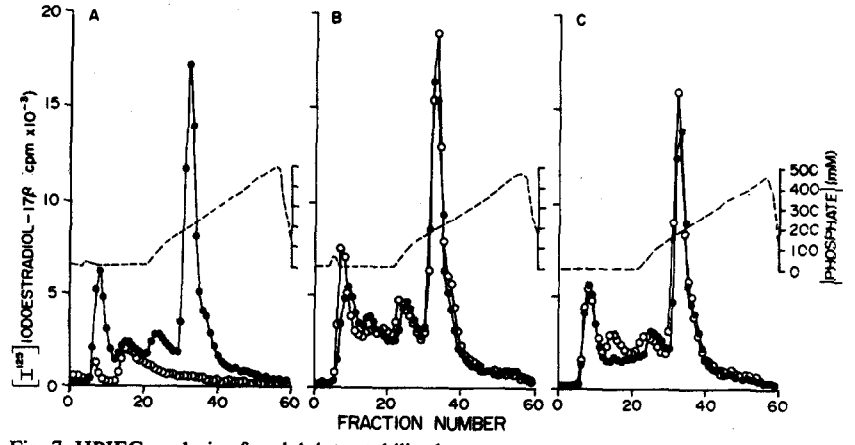

Fig. 7. HPIEC analysis of molybdate-stabilized estrogen receptors interaction with various MAb. In the following panels, non-specific rat IgG (A) MAb raised against the steroid-binding protein (B), and MAb raised to the chick oviduct 90-kilodalton HSP (C) were incubated overnight in parallel with [^{125}I]iodoestradiol-17β receptor complexes, then separated on a SynChropak AX-1000 column, as described in Experimental, in the presence of 10 mM sodium molybdate. (●) Total ligand binding, (○) non-specific binding.

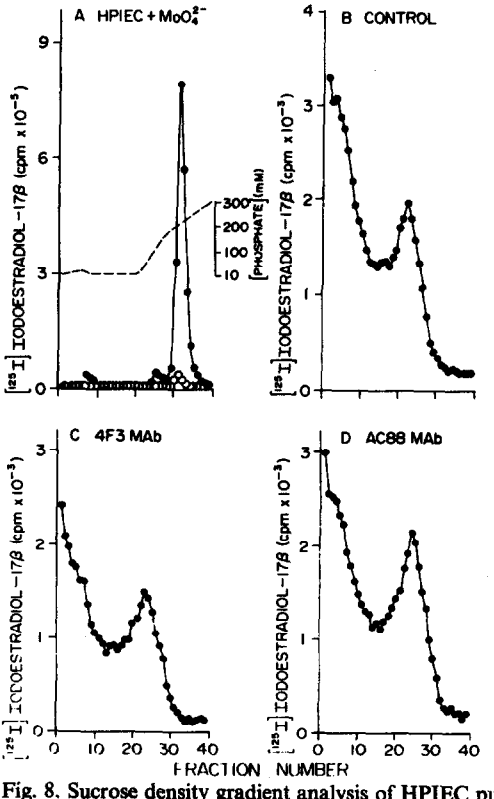

Fig. 8. Sucrose density gradient analysis of HPIEC purified estrogen receptors after interaction with 90-kilodalton heat shock MAb. In Panel A, [^{125}I]iodoestradiol-17β complexes were partially purified on a SynChropak AX-1000 column. Aliquots of the peak fraction were incubated with either the IgG (B) MAb 4F3 (C), or MAb AC88 (D) for 2 h and then subjected to a 10–40% linear sucrose gradient, containing 10 mM sodium molybdate.

In the case of the MAb raised to the 90-kilodalton HSP, excess 90-kilodalton HSP may have interfered with ER interaction, since it is a major cytosol protein[21,22]. Therefore, molybdate-stabilized receptor was separated from free 90-kilodalton HSP by HPIEC (Fig. 8A), incubated with either the AC88 or 4F3 MAb, and analyzed by SDG. No shift to a higher MW species was observed (Fig. 8B–D).

DISCUSSION

In this work we used MAb raised against ER from human breast cancer cells to determine their immunochemical relationships to the ER isoforms, prepared in the presence and absence of a receptor-stabilizing reagent, sodium molybdate. HPSEC and HPIEC were employed to distinguish MAb–ER complexes from unrecognized, immunochemically distinct forms of the ER (Table I).

It was clear from HPSEC data that, under high salt conditions, nearly all ER was recognized by H222Spγ. However, when specificity studies were performed under low salt condition, H222Spγ exhibited selectivity toward the 150 m*M* ER isoform suggesting a difference in the recognition of epitopes on ER Isoform I and II. This was interesting since the 150 m*M* isoform has been shown to be induced by phorbal esters[23] and may contain protein kinase activity[4]. In addition, others have demonstrated that the ER may be hyperphosphorylated in the presence of phorbal esters employing an H222-immobilized immunoabsorbant[24]. These data suggest that the 150 m*M* isoform may exist as a phosphorylated species of the ER.

With the D547Spγ MAb, a small percentage of the ER, separated under high-salt conditions, appeared to be immunochemically distinct. This is similar to that observed in other systems[15–17]. It has been suggested that this subpopulation resulted from some alteration in the ER moiety[17]. Unlike most rat IgG molecules, the MAb D547Spγ interacted with anion-exchange resins used in this study. This did not allow clear conclusions to be drawn with respect to the 50–60 m*M* ER isoform. Sato *et al.*[25] employed the D547Spγ linked to a polystyrene bead, and showed partial interaction with both isoforms. In our experiments, soluble D547Spγ appeared to recognize a portion of the 150 m*M* ER isoform. The extent of recognition varied from tumor to tumor and it is possible that a relationship exists between the immuno-

TABLE I

EVALUATION OF MONOCLONAL ANTIBODY RECEPTOR INTERACTION BY HPLC

Monoclonal antibody	*Elution characteristics of ER isoforms*			
	HPIEC		*HPSEC*	*HPIEC → SDG*
	50–60 mM [P_i]	*150 mM [P_i]*	*(0.4 M [KCl])*	*(+ 10 mM MoO_4^{2-})*
H222γ	±	++++	++++	–
D547γ	?	+++	++++	–
AC88	–	–	N.A.	–
4F3	–	–	N.A.	–

N.A. = not analysed.

distinct subpopulation seen under high-salt conditions on HPSEC and partial recognition of the 150 m*M* ER isoform.

There is mounting evidence that the ER may be a protein complex with nonsteroid binding components[4,7–10]. In particular, the molybdate-stabilized ER may contain a 90-kilodalton HSP. We employed two MAb, raised against the 90-kilodalton HSP from achlya[21] and chick oviduct[8,9] to determine whether these proteins were involved in the chromatographic behavior of the ER isoforms and the molybdate-stabilized ER from human breast tumors. These characteristics may be ascertained based upon properties of size, shape, surface charge and hydrophobicity[2,3]. Surprisingly, neither isoform of ER showed detectable interaction, even when the molybdate-stabilized ER was separated by HPIEC. These data suggest that the molybdate-stabilized ER from human breast cancer cells may assume a conformation with 90-kilodalton HSP which does not permit recognition of HSP epitopes by MAb AC88[21].

In addition, the MAb H222Spγ and D547Spγ did not appear to recognize the molybdate-stabilized ER. Since these MAb recognized two distinct epitopes at some distance in the ER molecule[26] prepared under high-salt conditions, our data suggest that the steroid-binding domain on the ER is inaccessible to the MAb and maybe spacially blocked by nonsteroid binding protein(s) such as the 90-kilodalton HSP. However, since the molybdate-stabilized form and Isoforms I and II are prepared in non-physiological buffers, we cannot determine their relationship to the native ER species *in vivo* at this time. Affinity steroid labels, in combination with MAb, should assist in this regard, when the rapid HPLC procedures we have described are used.

The heterogeneity regarding antibody affinity and specificity poses both advantages and disadvantages in their use as probes for biochemical studies. In this report, we have demonstrated that MAb to ER may be used to study interrelationship of receptor isoforms. Conditions must be carefully considered (in particular, salt concentration) to insure recognition of ER. Furthermore, our data suggest that ER isoforms, resolved by HPIEC, may be the result of protein–protein interaction of nonsteroid-binding components with the component retaining the steroid-binding domain. Thus, HPLC may be used to isolate soluble complexes of MAb estrogen receptors for more detailed characterization of their complicated molecular composition.

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