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CHARACTERIZATION OF AN ANTIESTROGEN-BINDING PROTEIN IN HIGH SALT EXTRACTS OF HUMAN BREAST CANCER TISSUE

OI LIAN KON

Department of Medicine, Memorial University of Newfoundland, Elizabeth Avenue, St John's, Newfoundland A1C 5S7, Canada and [1]Department of Biochemistry, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 0511, Republic of Singapore

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Summary—An antiestrogen binding protein which binds [^{3}H]tamoxifen (1-[4-(2-dimethylaminoethoxy)-phenyl]1,2-diphenylbut-1(Z)-ene) with high affinity ($K_d = 1.1 \times 10^{-9}$ M) is present in high salt (0.6 M KCl) extracts of washed breast cancer tissue pellets. Its concentration in high salt extract is higher than its concentration in cytosol. The characteristics of the antiestrogen binding protein from cytosol and salt extract of breast cancer tissue are indistinguishable. It specifically binds triphenylethylene and other nonsteroidal antiestrogens and displays little or no binding affinity for estrogens, progesterone, dihydrotestosterone and cortisol. The antiestrogen binding protein is of unusually large size as judged by gel filtration on agarose 0.5 m and sedimentation analysis on 5–20% sucrose density gradients. Differential centrifugation studies indicate that it is not principally microsomal in origin. This protein is more thermostable than the estrogen receptor from which it can also be distinguished by ion exchange chromatography. The antiestrogen binding protein was eluted from DEAE-Sephacel by 0.05 M KCl indicating that it is less negatively charged than the estrogen receptor which was eluted by 0.1 M KCl. Lipoprotein fractionation of breast cancer cytosol using potassium bromide density gradients did not reveal specific antiestrogen binding activity associated with any recognized class of lipoprotein. Specific [^{3}H]tamoxifen binding sites were pelleted in potassium bromide gradients consistent with the apparent large size of this protein. The physical characteristics of the antiestrogen binding protein in normal human tissue (myometrium) and neoplastic tissue (breast cancer) are remarkably similar, possibly reflecting a highly conserved structure.

INTRODUCTION

Nonsteroidal antiestrogens are generally believed to act through the estrogen receptor (ER) [1–5]. In addition to the estrogen receptor which binds this class of compounds, several investigators have reported the presence of discrete and specific antiestrogen binding sites in estrogen target as well as nontarget tissues [6–13]. These sites bind nonsteroidal antiestrogens in preference to estrogenic ligands, are protein in nature, and are more thermostable than the estrogen receptor. Unlike the estrogen receptor whose presence is restricted to estrogen target tissues, the antiestrogen binding protein is ubiquitous in those species (rat and human) in which its distribution has been examined [13, 14].

The biological function(s) of this protein is presently unknown. A number of apparently conflicting observations suggest that it may or may not be related to the mechanism of antiestrogen action. For example, the relationship between the structural requirements for ligand binding and for biological activity is a matter of some debate. Using a series of clomiphene (1-[*p*-(β-diethylaminoethoxy)phenyl]-1,2-diphenyl-2-chloroethylene) derivatives, Murphy and Sutherland [15] concluded that the aminoether side chain which is essential for antiestrogenic activity is also essential for binding to the antiestrogen binding protein. Later studies on clomiphene analogues showed some correlation between affinity for the antiestrogen binding site and growth inhibition of MCF 7 cells [16]. Modifications to the structure of tamoxifen (1-[4-(2-dimethylaminoethoxy)phenyl]1,2-diphenylbut-1(Z)-ene) e.g. monohydroxylation, which greatly enhanced the affinity of binding of the estrogen receptor had no effect on affinity for the antiestrogen binding sites [15]. In contrast, Sudo *et al.*[14] found that while tamoxifen analogues lacking the amine side chain did not bind to the antiestrogen specific sites, they were nonetheless still antiuterotrophic in the rat. Furthermore, these workers also noted that the ranking order of a series of antiestrogens in competing with [^{3}H]tamoxifen for the antiestrogen binding site did not parallel their potency as antiestrogens [14].

What role these antiestrogen binding sites may have in mediating antiestrogen action is further clouded by discrepant findings in mammary tumours. A tamoxifen-resistant human breast cancer cell line was found by Faye *et al.* to have virtually undetectable antiestrogen binding sites, in contrast to their presence in the tamoxifen sensitive parent cell line, MCF 7 [17]. Contradictory findings, however, were obtained from similar studies on a tamoxifen responsive and two tamoxifen resistant cell lines, all of which had equivalent levels of antiestrogen binding sites [18].

In addition to the intracellular antiestrogen bind-

[1]Present address.

ing protein, Winneker *et al.* have described a binding site associated with rat serum low density lipoprotein (LDL) which is specific for triphenylethylene antiestrogens [19]. Unlike the intracellular binding protein, the serum binding site had lower affinity for [^{3}H]tamoxifen, was present in similar concentration in sera from male and female rats, and appeared to co-fractionate with rat LDL. What relationship this serum binding site has to the intracellular antiestrogen binding protein is presently unclear.

The subcellular localization of tissue antiestrogen binding sites is unsettled. Sudo *et al.*[14] localized these sites to the microsomal fraction of rat tissues and noted their absence in 100,000 *g* and 180,000 *g* supernatants. Other workers, however, have routinely found these sites to be readily detectable in the high speed supernatant of various tissues [13, 17]. Indeed, the original studies of Sutherland and Foo[6] which led to the identification of these sites were performed on cytosol prepared by high speed (105,000 *g*) centrifugation.

The antiestrogen binding protein has been partly characterized in the cytosol and microsomal fraction of several tissues [13, 14]. Its presence in the nuclear extract of foetal guinea-pig uteri has also been reported [20]. Localization in the nuclear extract is of interest particularly in attempting to elucidate the biological function(s) of this novel protein. The present studies demonstrate that high salt (0.6 M KCl) extracts of human breast cancer tissue are rich in antiestrogen binding sites. These sites have physical characteristics similar to the cytosolic antiestrogen binding protein, and both can be clearly distinguished from the estrogen receptor. Furthermore, these studies on human breast cancer tissue indicate that, unlike rat tissues, the antiestrogen binding sites are not removed by high speed centrifugation and thus may not be microsomal in origin.

Tamoxifen binding activity could not be detected in human serum. Fractionation of breast cancer cytosol into lipoprotein classes failed to demonstrate specific antiestrogen binding sites in any band suggesting that the tissue antiestrogen binding protein is not a lipoprotein.

EXPERIMENTAL

Chemicals

[*N*-Methyl-^{3}H]Tamoxifen (87 Ci/mmol), 17β-[2,4,6,7-^{3}H]estradiol (104 Ci/mmol), [^{14}C]ovalbumin and [^{14}C]-γ globulin were from New England Nuclear Corporation. Purity of tritiated compounds was verified by thin layer chromatography and each was used without further purification. Non-radioactive steroids, diethylstilbestrol, clomiphene (1-[*p*-(β-diethylaminoethoxy)phenyl]-1,2-diphenyl-2-chloroethylene) citrate, α-monothioglycerol, molybdic acid (sodium salt) and all enzymes were from Sigma Chemical Company. Radioinert antiestrogens were generous gifts from ICI Americas Inc. (tamoxifen citrate), the Upjohn Company (nafoxidine hydrochloride, 1-[2-(*p*-[3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl]phenoxy)ethyl]-pyrrolidine hydrochloride), Parke Davis Inc. (CI 628, α-[4-pyrrolidinoethoxy]-phenyl-4-methoxy-α'-nitrostilbene) and Eli Lilly Inc. (trioxifene, [{3,4-dihydro-2-(4-methoxyphenyl)-1-naphthalenyl} [4-2-(1-pyrrolidinyl)-ethoxy}-phenyl], and LY117018, [6-hydroxy-2-(*p*-hydroxyphenyl)-benzo-[*b*]thien-3-yl *p*-{2-(1-pyrrolidinyl)ethoxy}-phenyl ketone.) Bio-Gel A-0.5 m and hydroxylapatite (DNA grade) were from Bio-Rad Laboratories. Blue Dextran 2000, Dextran T70 and DEAE-Sephacel were from Pharmacia Fine Chemicals. All other chemicals and solvents were from Fisher Scientific Company and were of reagent grade or better.

Preparation of cytosol

Histologically confirmed human breast carcinoma tissue was collected in liquid nitrogen and stored at −70°C until analysis. Cytosol was prepared by homogenizing tissue in 6 vol of 10 mM Tris–HCl, 1.5 mM EDTA, 20 mM sodium molybdate, 10% glycerol (v/v), 12 mM monothioglycerol, pH 7.4 at 4°C (TEMG buffer) using a Polytron PT-10 homogenizer. Cytosol was obtained by centrifugation at 100,000 *g* for 1 h.

Preparation of high salt extracts

Crude tissue pellets remaining after preparation of cytosol were washed to remove contaminating cytosol as follows. Pellets were gently but thoroughly redispersed in 10 vol of phosphate buffer (5 mM sodium phosphate, 1 mM monothioglycerol, 10% glycerol (v/v), pH 7.4 at 4°C) using a Dounce homogenizer, mixed well and centrifuged at 10,000 *g* for 15 min. The procedure was repeated for a total of three washes.

The washed pellets were extracted with 0.6 M KCl exactly as described by Garola and McGuire [21], the clear extracts being obtained by centrifugation at 100,000 *g* for 30 min.

Assay for antiestrogen binding sites in high salt extract

The concentration of these sites was determined by multiple point saturation analysis. The antiestrogen binding protein was first preadsorbed on hydroxylapatite as described by Garola and McGuire [21]. Briefly, 250 μl of freshly prepared hydroxylapatite slurry (packed/liquid volume ratio approx 0.7) was added to each tube containing 200 μl high salt extract (protein concentration approx 1 mg/ml) and incubated on ice for 30 min with intermittent vortexing. Tubes were then centrifuged at 800 *g* for 5 min (4°C) and the supernatants discarded. Phosphate buffer containing 0.1–15 nM [^{3}H]tamoxifen plus a 100-fold excess of diethylstilbestrol (to eliminate binding of [^{3}H]tamoxifen to estrogen receptor sites) was added to the hydroxylapatite pellets in a total volume of 500 μl. Nonspecific binding was estimated from parallel incubations containing 100-fold molar excess of

unlabeled tamoxifen. For this purpose, a solution of unlabeled tamoxifen (1 mM) in absolute ethanol was freshly prepared in a light-protected vial and diluted with TEMG buffer to a concentration of 10 μM. Twenty-five μl of this solution (1% ethanol) was added to a final incubation volume of 500 μl. Binding was allowed to proceed over 5 h at 30°C, after which 1 ml phosphate buffer was added and the tubes centrifuged at 800 *g* for 5 min. After washing the pellets twice with 1% Tween 80 (Gibco Laboratories) in phosphate buffer, the pellets were extracted with 2 ml ethanol overnight at room temperature. The ethanol extracts were dried down and counted in 7 ml scintillant (6 g 2,5-diphenyloxazole, 0.07 g *p-bis*-[2-(5-phenyloxazoly)]benzene per litre toluene, Amersham). The counting efficiency for ^{3}H was 50%.

To separately determine if the antiestrogen binding sites in high salt extracts were estrogen competable, the extract was preincubated with 10 nM unlabeled estradiol-17β or vehicle (final ethanol concentration 1%) for 2 h at room temperature, after which it was returned to 4°C and the concentration of binding sites determined as described above.

Assay for cytosol antiestrogen binding sites

The concentration of these sites was also determined by multiple point saturation analysis. Cytosol (4.8 mg protein/ml) was incubated with [^{3}H]tamoxifen (0.1–5 nM) and 250 nM diethylstilbestrol to abolish binding of [^{3}H]tamoxifen to estrogen receptor sites. Nonspecific binding was estimated from parallel incubations containing a 100-fold molar excess of unlabeled tamoxifen. The volume of cytosol was always 150 μl, and the total incubation volume was 270 μl. Binding was allowed to occur over 16 h at 4°C after which 0.5 ml Dextran-coated charcoal slurry (10 mM Tris–HCl, 10% glycerol (v/v), 0.25% charcoal (Norit A) (w/v), 0.025% Dextran T70 (w/v), pH 7.4 at 4°C) was added. Following a 30 min incubation on ice, charcoal was pelleted by centrifugation at 1520 *g* for 15 min at 4°C. Supernatants were decanted into vials containing scintillant as described above.

Binding site concentrations and equilibrium dissociation constants (K_d) were derived by Scatchard analysis [22] using a multiple linear regression program (Hewlett–Packard).

Ligand specificity studies

High salt extract preadsorbed on hydroxylapatite as described above was incubated for 16 h at 4°C with 5 nM [^{3}H]tamoxifen alone and in the presence of a 100-fold molar excess of various competing ligands. Bound [^{3}H]tamoxifen was determined as described above.

Cytosol was incubated with 5 nM [^{3}H]estradiol or 5 nM [^{3}H]tamoxifen for 16 h at 4°C in the absence and presence of a 100-fold molar excess of competing ligands shown in Fig. 3. Bound [^{3}H]-ligand was determined using dextran-coated charcoal as described above.

Sedimentation analysis of high salt extract

High salt extract labeled with 2.5 nM [^{3}H]tamoxifen plus 250 nM diethylstilbestrol for 16 h at 4°C was layered (100 μl) on linear 5–20% sucrose density gradients (4.8 ml) containing 0.5 M KCl prepared as described by Stone[23]. Nonspecific binding was estimated from a parallel gradient in which the sample contained a 100-fold molar excess of radioinert tamoxifen. The gradients were subjected to centrifugation in a vertical rotor (Beckman VTi80) at 243,000 *g* for 1.75 h. Each gradient was fractionated by means of a piercing unit (Buchler Instruments), collecting 28 fractions of 4 drops each. Protein standards were ovalbumin (3.6S) and γ globulin (8S).

Sedimentation analysis of cytosol

Cytosol prelabeled with 2.5 mM [^{3}H]tamoxifen plus 250 nM diethylstilbestrol or with 2.5 nM [^{3}H]estradiol for 16 h at 4°C was analyzed on 5–20% linear sucrose density gradients exactly as previously described [24] except that samples were not preadsorbed with charcoal. Nonspecific binding was estimated from parallel gradients in which samples contained a 100-fold molar excess of unlabeled ligand.

Lipoprotein fractionation

Cytosol prelabeled with 5 nM [^{3}H]tamoxifen plus 500 nM diethylstilbestrol was analyzed for lipoproteins using potassium bromide density gradient centrifugation as described by Terpstra *et al.*[25]. Nonspecific binding was estimated from a parallel gradient containing 250 nM unlabeled tamoxifen in addition. After removing eleven fractions (1 ml each) by tube slicing, the bottom of each tube was also counted.

Gel filtration, ion exchange chromatography and thin layer chromatography

These were performed as previously described [13].

Protein determination

Protein concentration was assayed by the method of Bradford[26] using bovine serum albumin as standard.

RESULTS

Breast cancer cytosol displayed saturable [^{3}H]tamoxifen binding (Fig. 1A) and Scatchard analysis was consistent with a single class of high affinity binding sites ($K_d = 1.1 \times 10^{-9}$ M) [Fig. 1B]. The number of [^{3}H]tamoxifen binding sites in cytosol was not decreased by preincubating cytosol with 5 nM unlabeled estradiol (169 fmol/mg protein in control cytosol vs 213 fmol/mg protein in estradiol pre-

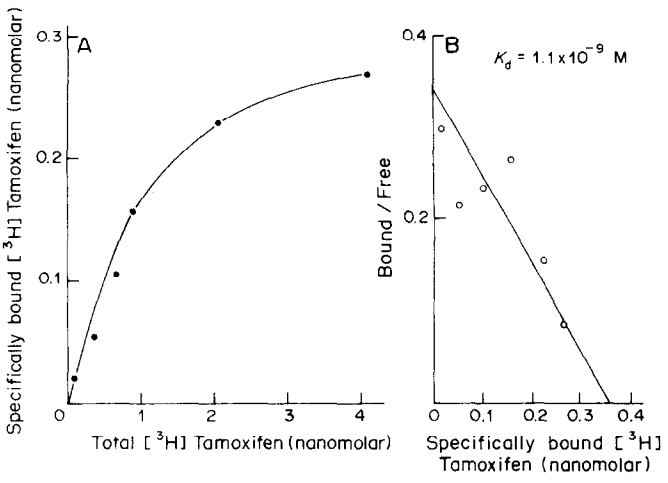

Fig. 1. Panel A shows saturation analysis of [^{3}H]tamoxifen binding to breast cancer cytosol performed as described in Experimental. Panel B is a Scatchard plot of the same data. Specifically bound [^{3}H]tamoxifen is the difference between binding obtained in the absence and presence of a 100-fold molar excess of unlabeled tamoxifen.

saturated cytosol). By contrast, preincubation with 5 nM estradiol had the expected effect of greatly diminishing the number of detectable estrogen receptor sites (98 fmol/mg protein in control cytosol vs 3 fmol/mg protein in estradiol presaturated cytosol).

The high salt (0.6 M KCl) extract of breast cancer tissue pellets also bound [^{3}H]tamoxifen in a saturable manner. Scatchard analysis indicated a single class of high affinity ($K_d = 3.7 \times 10^{-9}$ M) binding sites (Fig. 2). The concentration of [^{3}H]tamoxifen binding sites in the high salt extract was abundant (2042 fmol/mg protein, 4538 fmol/g tissue) in comparison with cytosol (169 fmol/mg protein, 1512 fmol/g tissue). Like the cytosolic sites, [^{3}H]tamoxifen binding sites in high salt extract were not significantly decreased by preincubation with 10 nM unlabeled estradiol (2042 fmol/mg protein in control extract vs 1976 fmol/mg protein in estradiol presaturated extract) [Fig. 2].

The ligand specificity of [^{3}H]tamoxifen binding sites was investigated by comparing the degree of displacement of bound [^{3}H]tamoxifen produced by several unlabeled ligands added in 100-fold molar excess. Bound [^{3}H]tamoxifen in cytosol was weakly displaced (<20%) by all estrogenic ligands tested (estradiol-17β, estradiol-17α, diethylstilbestrol, estriol and estrone). In contrast, nonsteroidal antiestrogens (tamoxifen, nafoxidine, clomiphene and CI628) were considerably more effective and caused 40–50% displacement of bound [^{3}H]tamoxifen. Progesterone, dihydrotestosterone and cortisol did not compete for [^{3}H]tamoxifen binding sites (Fig. 3, upper panel). This pattern was in contrast to that observed when the same panel of unlabeled competing ligands was used to displace [^{3}H]estradiol bound to estrogen receptor sites in cytosol. As expected, estrogenic ligands were, as a group, considerably more effective in competing for bound [^{3}H]estradiol than the non-steroidal antiestrogens while progesterone, dihydrotestosterone and cortisol did not displace bound [^{3}H]estradiol (Fig. 3, lower panel).

[^{3}H]Tamoxifen binding sites in high salt extract displayed essentially the same pattern of ligand

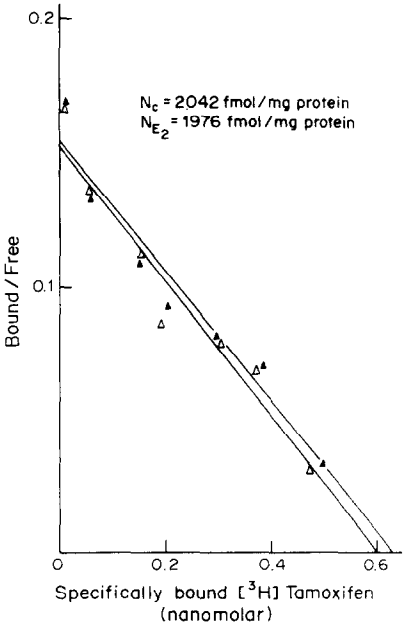

Fig. 2. Scatchard analysis of [^{3}H]tamoxifen binding in 0.6 M KCl extract of washed breast cancer tissue pellet. The extract was preadsorbed on to hydroxylapatite before incubation with concentrations of [^{3}H]tamoxifen ranging between 0.1–15 nM. Specifically bound [^{3}H]tamoxifen was determined as described in Experimental. N_{E2} (△) is the concentration of [^{3}H]tamoxifen binding sites in high salt extract preincubated with 10 nM unlabeled estradiol-17β. N_C (▲) is the concentration of these sites in the same extract preincubated with vehicle (ethanol) alone under the same conditions.

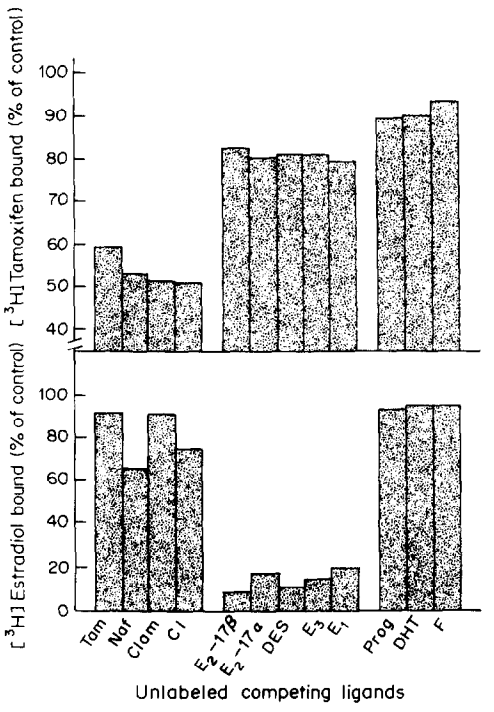

Fig. 3. Ligand specificity of antiestrogen binding sites and estrogen receptor in breast cancer cytosol. Breast cancer cytosol was incubated with 5 nM [^{3}H]tamoxifen (upper panel) or 5 nM [^{3}H]estradiol (lower panel) in the absence and presence of a 100-fold molar excess of the unlabeled competing ligands indicated. Bound ^{3}H-ligand was determined as described in Experimental. Binding obtained in the absence of competitors was set at 100%. (Tam, tamoxifen; Naf, nafoxidine; Clom, clomiphene; CI, CI628; E_2-17β, estradiol-17β, E_2-17α, estradiol-17α; DES, diethylstilbestrol; E_3, estriol; E_1, estrone; Prog, progesterone; DHT, dihydrotestosterone; F, cortisol).

specificity as described above for the cytosolic sites. Nonsteroidal antiestrogens derived from triphenylethylene (tamoxifen, nafoxidine, clomiphene, CI628) as well as those structurally unrelated (LY 117018, trioxifene) all displaced bound [^{3}H]tamoxifen by more than 60% while estrogenic ligands, progesterone, dihydrotestosterone and cortisol exhibited little or no competition for bound [^{3}H]tamoxifen (Fig. 4).

It was observed that "nonspecific binding" of [^{3}H]tamoxifen (i.e. that which remained in the presence of a 100-fold molar excess of unlabeled tamoxifen) was about 40–50% of total bound [^{3}H]-tamoxifen. Increasing the concentration of unlabeled tamoxifen did not lower it. This finding is consistent with the high nonspecific binding of nonsteroidal antiestrogens previously noted by other workers [1].

Characterization of antiestrogen binding sites by gel filtration was performed by fractionating [^{3}H]-tamoxifen prelabeled cytosol on an agarose 0.5 m column. This produced a single peak of radioactivity eluting just after the void volume (Fig. 5, upper panel) which was virtually obliterated by a 100-fold molar excess of unlabeled tamoxifen. It was only slightly diminished by an identical excess of diethylstilbestrol consistent with the findings of ligand specificity studies above indicating that estrogens compete weakly for [^{3}H]tamoxifen binding sites.

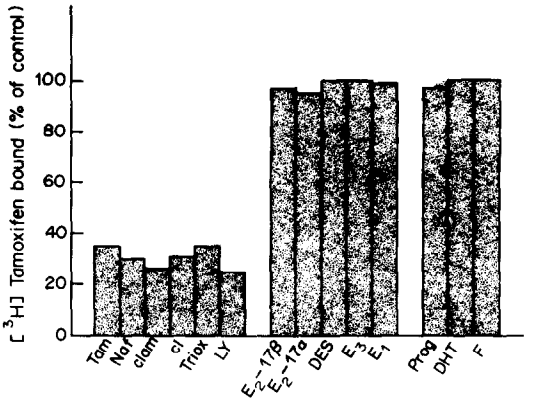

Fig. 4. Ligand specificity of antiestrogen binding sites in 0.6 M KCl extract of washed breast cancer tissue pellets. The extract was preadsorbed on to hydroxylapatite and incubated with 5 nM [^{3}H]tamoxifen alone with the indicated unlabeled ligands present in a 100-fold excess. Bound [^{3}H]tamoxifen was determined as described in Experimental. Binding obtained in the absence of competitors was set at 100%. (Troix, trioxifene; LY, LY117018; other competing ligands as in the legend to Fig. 3.)

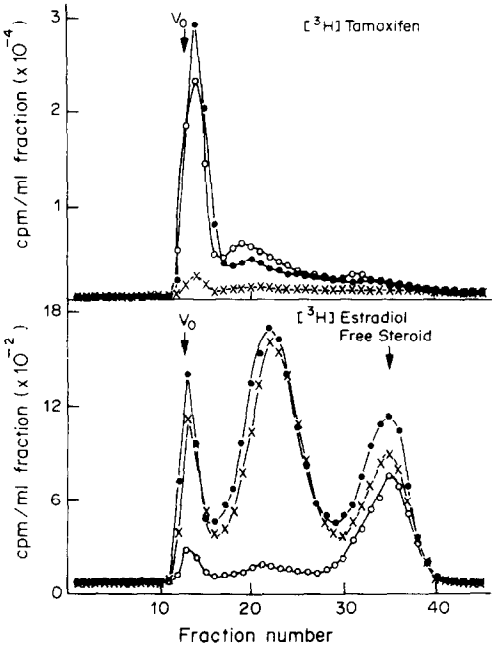

Fig. 5. Gel filtration of antiestrogen binding protein and estrogen receptor from breast cancer cytosol. Six equal aliquots of breast cancer cytosol were labeled with 5 nM [^{3}H]tamoxifen or 5 nM [^{3}H]estradiol, with the following additions: (i) none (●); (ii) 500 nM diethylstilbestrol (○); (iii) 500 nM tamoxifen (×). Each sample (1.5 ml) was charcoal treated [13] just before application to an agarose 0.5 m column (1.6 × 37 cm) equilibrated in 0.5 M KCl TEMG buffer. Flow rate was 1.1 ml/min. Fifty fractions (2.05 ml) were collected and 1 ml of each counted. Void volume was determined by blue dextran 2000. Due to variable recoveries, all data have been normalised to 100% recovery.

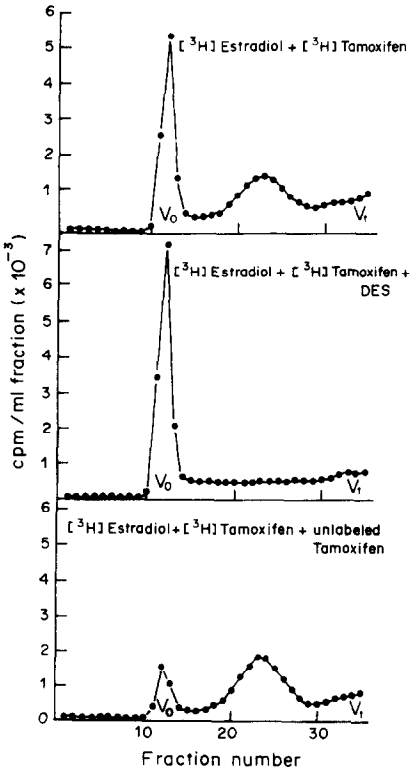

Fig. 6. Gel filtration of antiestrogen binding protein from 0.6 M KCl extract of breast cancer tissue pellets. Three identical aliquots of the extract were prelabeled with 5 nM [^{3}H]tamoxifen and 5 nM [^{3}H]estradiol, with the following additions: (i) none (top panel); (ii) 500 nM diethylstilbestrol (DES) (middle panel); and (iii) 500 nM unlabeled tamoxifen (bottom panel). Each aliquot was charcoal treated [13] just before applying 1.5 ml to an agarose 0.5 m column (1.6 × 37 cm). Flow rate was 1.1 ml/min and 50 fractions (2.05 ml) were collected. Void volume was determined by blue dextran 2000. Data have been normalised to 100% recovery.

A different elution profile was obtained when cytosol estrogen receptor sites were analyzed on the same column. Two peaks of bound [^{3}H]estradiol were eluted (Fig. 5, lower panel). The first coincided with the peak of bound [^{3}H]tamoxifen but was not the antiestrogen binding species since it was effaced by excess diethylstilbestrol and only slightly diminished by excess unlabeled tamoxifen. The latter effect is consistent with the known weaker affinity binding of nonsteroidal antiestrogens to the estrogen receptor [27]. The second peak of bound [^{3}H]estradiol, eluting with a K_{av} of 0.39, resembled the earlier peak in being abolished by an excess of diethylstilbestrol and unaltered by excess unlabeled tamoxifen (Fig. 5, lower panel). Both peaks of bound [^{3}H]estradiol were therefore different molecular weight forms of the estrogen receptor in breast cancer cytosol. Similar heterogeneity of receptor forms in this tissue has previously been reported by other workers [28]. The third peak of [^{3}H]estradiol was free steroid since it was completely adsorbed by dextran-coated charcoal and [^{3}H]estradiol in buffer alone eluted in an identical position.

Gel filtration on the same column of 0.6 M KCl extract prelabeled with 5 nM each of [^{3}H]tamoxifen and [^{3}H]estradiol resulted in two peaks of bound radioactivity (Fig. 6, top panel). The first peak, which eluted in the void volume, was the [^{3}H]tamoxifen binding species since this peak alone was abolished by an excess of unlabeled tamoxifen (Fig. 6, bottom panel). The included peak of bound radioactivity ($K_{av} = 0.47$) represented estrogen receptor sites because it was abolished by an excess of diethylstilbestrol (Fig. 6, middle panel). The apparently large size of the antiestrogen binding component was unlikely to represent protein aggregation since gel filtration was always performed in 0.5 M KCl containing solutions and the elution profile was unchaged in the presence of 3 M urea.

Sedimentation analysis on 5–20% sucrose density gradients containing 0.5 M KCl revealed that the cytosol estrogen receptor migrated in the 3S–4S region and that the antiestrogen binding species was consistently pelleted (Fig. 7). Similarly, the antiestrogen binding activity in the high salt extract was also rapidly sedimenting and was found mainly at the bottom of the gradient (Fig. 8). The above findings from gel filtration and sedimentation analysis indicate an unusually large size, and suggest that the antiestrogen binding sites may reside in a large

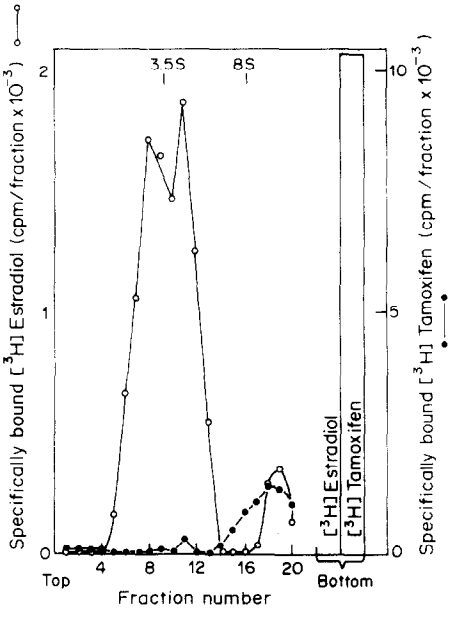

Fig. 7. Sedimentation analysis of antiestrogen binding protein and estrogen receptor from breast cancer cytosol. Breast cancer cytosol (180 μl) labeled with 5 nM [^{3}H]tamoxifen or 5 nM [^{3}H]estradiol was layered on 5–20% sucrose gradients (4.5 ml) [23, 24]. Gradients were centrifuged at 238,000 g for 16 h at 4°C. Twenty fractions (5 drops each) were collected and the bottoms of the polyallomer tubes were cut off and also counted. Internal standards were [^{14}C]ovalbumin (3.5S) and [^{14}C]γ-globulin (8S). Nonspecific binding was determined from parallel analyses of samples containing a 100-fold molar excess of unlabeled ligand. Specific binding of [^{3}H]tamoxifen is denoted by (●) and of [^{3}H]estradiol by (○).

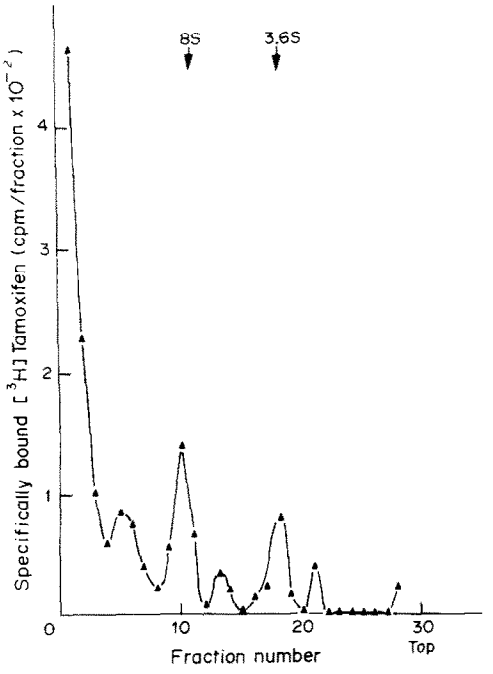

Fig. 8. Sedimentation analysis of antiestrogen binding protein from 0.6 M KCl extract of breast cancer tissue pellet. High salt extract was prelabeled with 2.5 nM [^{3}H]tamoxifen plus 250 nM diethylstilbestrol and layered (100 μl) on 5–20% sucrose gradients followed by centrifugation in a vertical rotor as described in Experimental. Protein standards and specific binding are as described in the legend to Fig. 7.

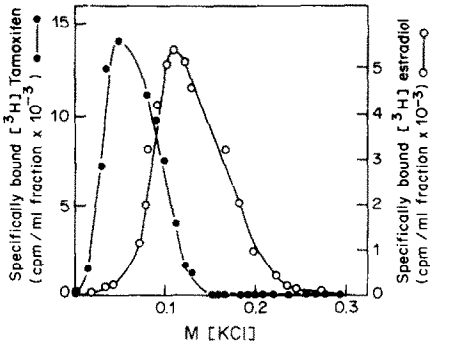

Fig. 9. Ion exchange chromatography of antiestrogen binding protein and estrogen receptor from breast cancer cytosol. Breast cancer cytosol (4 ml) labeled with 5 nM [^{3}H]tamoxifen plus 500 nM diethylstilbestrol or 5 nM [^{3}H]estradiol was applied to a DEAE-Sephacel column (2.6 x 6 cm). After washing with 10 bed vol of TEMG buffer, elution was effected with a KCl gradient (0–0.5 M, 3 bed vol). Flow rate was 1.6 ml/min and 46 fractions (2.05 ml each) were collected. Column fractions were analyzed as previously described [13]. Specifically bound [^{3}H]estradiol is denoted by (○) and [^{3}H]tamoxifen by (●).

complex molecule, or possibly in a subcellular organelle.

The subcellular distribution of the antiestrogen binding sites was examined by quantitating [^{3}H]-tamoxifen binding activity in low- and high-speed cytosol preparations by means of multiple point saturation analyses. Table 1 summarizes the findings which clearly show that these sites are present in equivalent concentrations in both low-speed (800 *g* 10 min, 12,000 *g* 30 min) and high-speed (100,000 *g* 60 min) supernatants. The equilibrium dissociation constants (K_d) were also similar. There was no evidence that the low speed supernatants were enriched in antiestrogen binding sites, or that high speed cytosol was devoid of this activity.

In addition to sedimentation analysis and gel filtration, it was also possible to distinguish antiestrogen binding activity from estrogen receptor sites by ion exchange chromatography. When cytosol prelabeled with 5 nM [^{3}H]tamoxifen plus 500 nM diethylstilbestrol was applied to a column of DEAE-Sephacel, specifically bound [^{3}H]tamoxifen was eluted by 0.05 M KCl (Fig. 9). Identical analysis of [^{3}H]estradiol labeled cytosol resulted in elution of estrogen receptor sites by 0.1 M KCl.

Protease treatment of cytosol destroyed [^{3}H]tamoxifen binding activity while amylase, deoxyribonuclease and ribonuclease had no effect. The antiestrogen binding protein was relatively thermostable compared to the estrogen receptor. Warming cytosol to 37°C completely destroyed [^{3}H]estradiol binding capacity but had relatively slight effect on [^{3}H]tamoxifen binding capacity (95% of control activity remaining).

Thin layer chromatography of [^{3}H]tamoxifen was performed after incubation with cytosol for 16 h at 4°C as previously described [13]. There was a single peak of radioactivity in three solvent systems [benzene–piperidine (9:1), benzene–triethylamine (9:1) and diethylether–triethylamine (98:2)] co-migrating with authentic unlabeled trans-tamoxifen indicating that metabolic conversion of [^{3}H]tamoxifen had not occurred under these conditions. Thus, the ligand bound to the antiestrogen binding protein in the above studies was tamoxifen itself, rather than its metabolites.

In order to ascertain if the specific [^{3}H]tamoxifen binding activity resided in a lipoprotein, breast cancer

Table 1. Subcellular localization of antiestrogen binding protein (AEBP) in breast cancer tissue by differential centrifugation

Speed of centrifugation	Total protein (mg)	Total AEBP (fmol)	Concentration of AEBP		K_d
			fmol/mg protein	fmol/g tissue	$\times 10^{-9}$ M
800 *g*, 10 min	19.6	4922	251	1068	1.8
12,000 *g*, 30 min	9.7	3783	386	810	1.5
100,000 *g*, 60 min	17.1	6969	407	1163	4.4

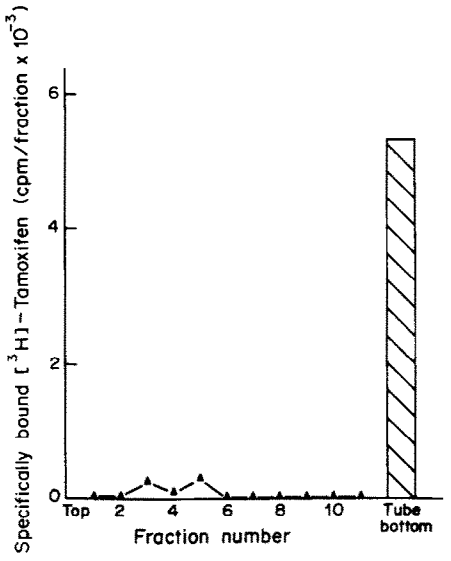

Fig. 10. Lipoprotein fractionation of breast cancer cytosol. Breast cancer cytosol (1.67 ml) labeled with 5 nM [^{3}H]tamoxifen plus 500 nM diethylstilbestrol in the absence and presence of 500 nM radioinert tamoxifen was prestained with Sudan black B and fractionated on a potassium bromide density gradient as described by Terpstra *et al.*[25]. Centrifugation was at 236,000 *g* for 22 h at 20°C. Each gradient was sliced into eleven 1 ml fractions and each fraction was analyzed for bound [^{3}H]tamoxifen using dextran-coated charcoal as described in Experimental. Only specific binding (the difference between binding observed in the absence and presence of excess unlabeled tamoxifen) is shown. The bottoms of the centrifuge tubes were also cut off and counted.

cytosol prelabeled with 5 nM [^{3}H]tamoxifen plus 500 nM diethylstilbestrol was fractionated on a potassium bromide density gradient as described by Terpstra *et al.*[25] in the absence and presence of 500 nM unlabeled tamoxifen. Only one peak of radioactivity displaceable by 100-fold molar excess of unalabeled tamoxifen was observed, and this was found to have pelleted at the bottom of the tube (Fig. 10). It should be noted that bound [^{3}H]tamoxifen was also present in a fraction of density 1.24 g/ml but that this peak was not altered by an excess of unlabeled tamoxifen and was therefore not the specific antiestrogen binding site. Endogenous lipoproteins banded in the expected positions as judged by Sudan Black staining.

Serum from men and premenopausal women was also examined for anti-estrogen binding activity by incubating with 10 nM [^{3}H]tamoxifen plus 1 μM diethylstilbestrol in the absence and presence of 1 μM unlabeled tamoxifen for 16 h at 4°C. Serial dilutions of serum (1:5, 1:10, 1:20 and 1:50) were also made in TEMG buffer and labeled in identical manner. Separation of bound from free [^{3}H]tamoxifen was performed using two different conditions. To one set of tubes, dextran-coated charcoal slurry was added to give a final concentration of 0.83% charcoal (w/v) and 0.083% dextran T70 (w/v). Tubes were then incubated on ice for 30 min prior to centrifugation as described in Experimental. The second set of tubes was exposed for about 1 min to a final concentration of 0.42% charcoal (w/v) and 0.042% dextran T70 (w/v). Displaceable binding of [^{3}H]tamoxifen was not detected using either method.

Fifteen different breast cancer tissue specimens were simultaneously analyzed for estrogen receptor and antiestrogen binding protein levels. Nine tumours were estrogen receptor positive (> 3 fmol/mg protein) and six were estrogen receptor negative ($\leq$ 3 fmol/mg protein). In contrast, all but one of the fifteen tumours had readily measurable specific [^{3}H]tamoxifen binding activity. The single tumour without detectable antiestrogen binding sites was also negative for estrogen receptor. There was a weak correlation between the levels of estrogen receptor and the antiestrogen binding protein (correlation coefficient 0.585, $P < 0.025$ by rank analysis).

DISCUSSION

Although antiestrogen specific binding sites have previously been identified in human breast cancer tissues and in breast cancer derived cell lines, these sites have so far been detected only in cytosol and in microsomes [8, 12, 17, 18]. The studies described above demonstrate the existence of these novel sites in high salt extract of human breast cancer tissue for the first time, and indicate that the physical characteristics of cytosolic and salt extracted sites are highly similar. Furthermore, the methods used to characterize the antiestrogen binding protein provide additional strong support for the separate identity of this protein distinct from the estrogen receptor. The possibility that the antiestrogen binding protein detected in the 0.6 M KCl extract was merely the result of contamination by cytosol appears unlikely for two reasons. Firstly, precautions were taken to remove cytosol as far as possible by redispersing tissue pellets and washing in large volumes of buffer prior to extraction with 0.6 M KCl. Secondly, the concentration of specific [^{3}H]tamoxifen binding sites was much higher in the extract (2042 fmol/mg protein, 4538 fmol/g tissue) than in cytosol (169 fmol/mg protein, 1512 fmol/g tissue).

The physical characteristics of the cytosolic and salt extracted antiestrogen binding proteins are virtually identical. The protein is of unusually large size as judged by its behaviour on gel filtration and sedimentation analysis. The possibility that it may be part of a subcellular organelle cannot be entirely discounted. However, differential centrifugation of breast cancer tissue homogenates in the above studies revealed equivalent concentrations and affinities of these binding sites in low speed (800 *g* 10 min, 12,000 *g* 30 min) and in high speed (100,000 *g* 60 min) supernatants (Table 1). This makes it unlikely that the antiestrogen binding protein is principally a microsomal protein since the operational definition of the microsomal fraction is that which sediments at speeds exceeding

10,000 *g* [29]. These findings in human breast cancer tissue are at variance with those reported for the subcellular localization of antiestrogen binding sites in rat tissues in which these sites were clearly localized to the microsomal fraction [14]. There is no obvious explanation for these discrepant findings which may reflect a true species difference. Nonetheless, the possibility that organelle fragments in cytosol may be the source of antiestrogen binding sites cannot yet be exluded. An alternative explanation for the apparent large size is that the antiestrogen binding site may be part of a large complex molecule such as a proteoglycan or multimeric protein.

The existence of triphenylethylene antiestrogen binding sites in rat serum low density lipoprotein [19] raises the possibility that the intracellular antiestrogen binding protein may also be a lipoprotein. This was not substantiated, however, when lipoprotein fractionation of [^{3}H]tamoxifen labeled breast cancer cytosol was performed using potassium bromide gradient centrifugation. A band of [^{3}H]-tamoxifen within the gradient corresponding to a density of 1.24 g/ml probably represented nonspecific binding since it was not diminished by excess unlabeled tamoxifen. Furthermore, the density of this fraction was higher than that of any recognized lipoprotein class. On potassium bromide gradient centrifugation, specific [^{3}H]tamoxifen binding activity was consistently found to have pelleted at the bottom of the tube. The amount of radioactivity pelleted was decreased in the presence of excess unlabeled tamoxifen and therefore represented binding of [^{3}H]tamoxifen to the antiestrogen binding protein.

Another point of difference worth noting between the serum and tissue antiestrogen binding sites is their dissimilar specificity for this class of ligands. Rat serum low density lipoprotein is specific for triphenylethylene antiestrogens and has little affinity for structurally different antiestrogens such as benzythiophene derivatives [19]. The tissue antiestrogen binding protein, by contrast, has broader specificity and does not appear to distinguish among various nonsteroidal antiestrogens (Figs 3 and 4).

Characterization of the antiestrogen binding protein in breast cancer tissue reveals a striking resemblance to the corresponding protein in normal human tissue [13]. In normal and neoplastic human tissue, the antiestrogen binding protein is less negatively charged, more thermostable and distinctly larger than the estrogen receptor. The ligand specificity of the antiestrogen binding protein from both sources is virtually identical. These observations may reflect a highly conserved structure, as has also been suggested for steroid receptor proteins [28].

The possible significance, if any, of the finding that the high salt extract of breast cancer tissue pellets is rich in antiestrogen binding protein is unclear at present. The precise subcellular origin of the salt extracted sites is not certain and may possibly be of nuclear origin. Translocation of this protein into the nucleus has not been reported in any tissue, and its biological role is unknown. Its presence in normal breast tissue [13] suggests a physiological function, perhaps unrelated to its antiestrogen binding property, and raises interesting questions about the nature of its endogenous ligand(s).

Similarly, the widespread distribution of this novel protein in breast cancer tissue is of uncertain import. Whether measurements of the antiestrogen binding protein in breast cancer tissue can be used in the management of breast cancer is a potentially important issue that merits further study.

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