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Importance of A/B and C Domains of the Estrogen Receptor for Its Adsorption to Hydroxylapatite

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Regulatory properties of estrogen receptor (ER) result from the existence of functional domains within its primary structure. Thus, A/B and C domains which are rich in tyrosyl residues control gene expression while the E domain confers estrogen binding capacity. Hydroxylapatite (HAP) is known to adsorb ER. Scatchard plot analysis of [^{3}H]estradiol binding patterns of HAP batches to which cytosolic ER had been adsorbed revealed that AB and/or C domains are mainly responsible for this property. Thus, treatment of these batches with the tyrosine reagent tetranitromethane (TNM) led to a dramatic release of adsorbed receptors. This did not occur with ER preparations devoid of exposed ABC domains obtained by selective immunoextraction with H-226 anti-ER monoclonal antibody prior to HAP assay. KCl treatment (500 mM) of HAP batches also led to a release of bound receptors especially those devoid of exposed ABC domains. Such binding characteristics were also found with full length and truncated ERs produced in yeast: the full length receptor strongly interacted with HAP while the truncated receptor devoid of AB and C domains displayed only a weak adsorption. Additional investigation revealed that estradiol binding to cytosolic ER does not modify its reactivity towards TNM.

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INTRODUCTION

The estrogen receptor (ER) is a member of the steroid–thyroid receptor superfamily, a class of regulatory proteins governing the expression of genes involved in growth control and/or differentiation [1]. Regulatory properties of ER result from the existence within its primary structure of various functional domains denoted from N- to C-terminal by the letters A to F [2, 3]. Thus, A/B and C domains are involved in gene expression while domain E confers estrogen binding capacity to the protein; domain D is a hinge between these two functional regions. Domain C contains two zinc fingers with very high binding affinity for specific palindromic sequences of nucleotides; on estradiol (E_2) binding, various changes occur in the tridimensional structure of ER leading to the emergence of this domain as demonstrated by DNA–cellulose chromatography. This transition is usually referred to as the "activation" of the receptor.

Among E_2 binding assays developed for the measurement of ER, the so-called "dextran-coated charcoal assay" (DCC assay) has progressively become the method of choice for many investigators. However, this procedure has limitations since it cannot distinguish activated from inactivated receptors or native 67 kDa ER peptides from degradation products or variants [4] containing no operative A/B and C domains. This led us to investigate whether the hydroxylapatite (HAP) adsorption assay, which has been widely used for the measurement of the nuclear (activated) form of the receptor [5–7], might be more efficient for the characterization of the estrogen sensitivity of samples. We speculated that various ER forms present in the preparations would differ in regard to their adsorption to this matrix. To verify this assumption, we analysed which of the main domains of the cytosolic ER are involved in its interaction with HAP.

The data reported here clearly show that the A/B and C domains of ER largely contribute to its adsorption to the HAP matrix especially in buffer containing 500 mM KCl, the concentration usually used for the extraction of the activated receptor from the nucleus. Moreover, they show that tyrosyl residues located within these three domains play a prominent role in this regard. Thus, it seems that HAP assays run in the presence of 500 mM KCl may quantify the amount of receptors containing operative A/B and C domains

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and, therefore, may help to distinguish activated from unactivated receptors present in cytosol and nuclear preparations. This approach may also be helpful to identify alterated receptors lacking ABC domains.

EXPERIMENTAL

Materials

[^{3}H]E_2 (±100 Ci/mmol) was obtained from Amersham (Bucks., England), [^{125}I]E_2 (±2000 Ci/mol) from NEN (Dreieich, Germany) and unlabeled E_2 from Sigma (St Louis, MO). Tetranitromethane (TNM) was purchased from Aldrich Europe (Belgium). All other reagents were of analytical grade. HAP was obtained from Bio-Rad (Richmond, CA).

Rat monoclonal antibodies (H-222 and H-226) were kindly provided by Dr C. Nolan (Abbott Labs, North Chicago, IL). Anti-rat IgG-agarose was obtained from Sigma.

Full length receptor (*yER*) and truncated receptor expressing solely the hormone binding domain (*yHBD*), both expressed in yeast [8], were kindly provided by Dr M. Ericsson (KARO BIO AB, Huddinge, Sweden).

Cytosolic ER preparations

All uterine tissues were from selected origins (calf without any hormonal treatment from a local slaughterhouse, mouse and immature rat from IFFA CREDO, France); MCF-7 cells were from our culture unit. Breast cancer samples were obtained from our surgery department (samples for steroid hormone receptor measurements).

Calf uterine tissue was homogenized in 10 mM Tris–HCl buffer pH 8 (T10) at 0°C by successive grindings with an ultraturrax (Janke and Kunkel) and a whole glass Potter while mouse and rat uterine tissue as well as human breast cancer samples were homogenized solely with a glass Potter. MCF-7 cells were homogenized using a Teflon–glass Potter. All homogenates were centrifuged for 1 h at 100,000 *g* to obtain the cytosol preparations. These

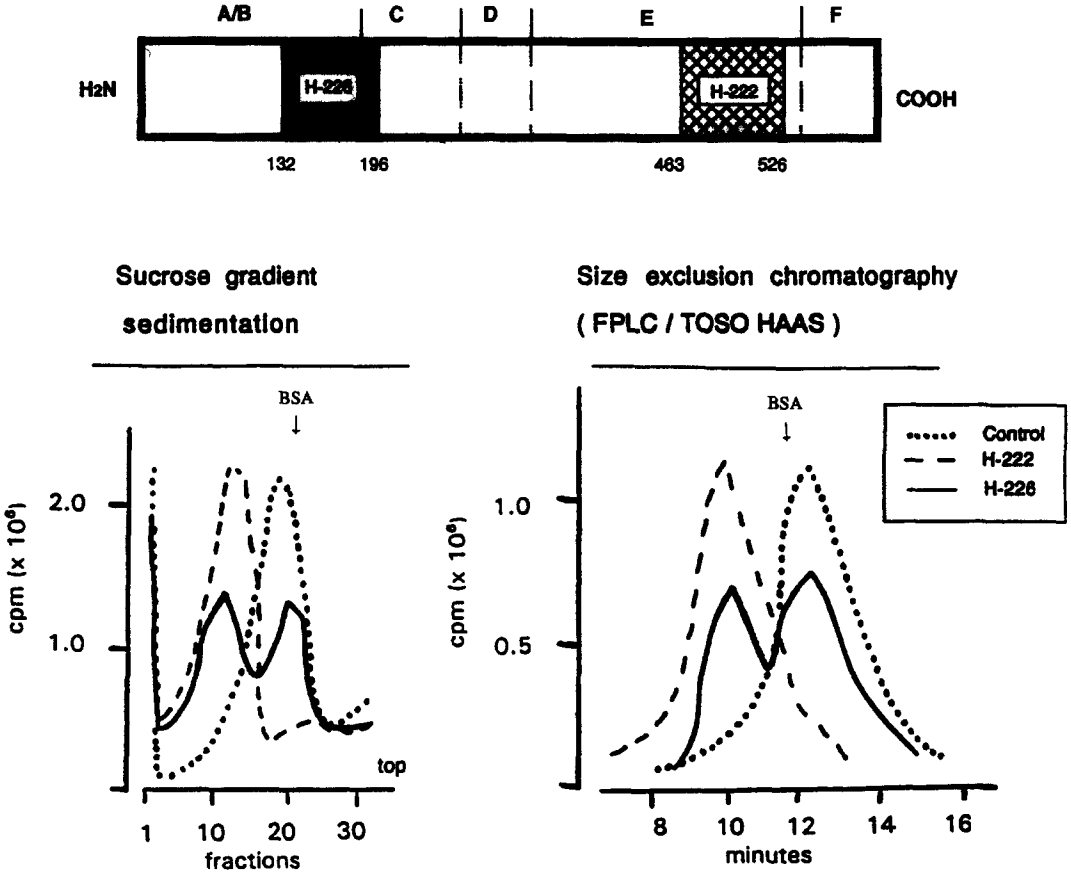


Fig. 1. Influence of addition of H-226 or H-222 anti-ER monoclonal antibodies on sucrose gradient sedimentation (SGS) or FPLC size-exclusion chromatography profiles of cytosolic ER. The upper part of the figure refers to the epitopes of these monoclonal antibodies on ER: H-226 is located just upstream of the C domain (DNA binding domain), H-222 within the E domain (E_2-binding domain). Cytosolic ER samples labelled with [^{3}H]E_2 (SGS) or [^{125}I]E_2 (FPLC) were treated with H-226 or H-222 just before fractionation. The shift of only a part of the whole ER population in the presence of H-226 reveals the interaction of this antibody with about 50% of the receptor molecules; H-222 produces a total shift indicating its interaction with all receptor molecules. BSA: bovine serum albumin standard run in parallel.

preparations were stored in liquid nitrogen until assay (1–2 weeks).

ER expressed in yeast

Full length receptor (*yER*) and truncated receptor devoid of AB and C domains (*yHBD*) were sampled to a final concentration of about ~2000 fmol/ml in a buffer [20 mM Tris, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1.5 mM EDTA, and 10% glycerol; pH 7.8] and stored at −70°C as recommended by the manufacturer. For experiments, samples were diluted twice with T10 containing 4 mg/ml of bovine serum albumin (BSA) to obtain a protein concentration as in cytosolic preparations.

Immunoadsorption procedure

H-226 and H-222 anti-ER monoclonal antibodies were incubated with the cytosol for 2 h at 4°C (1 μl per ml of cytosol of an antibody preparation at 1 mg/ml). Immune complexes were adsorbed on anti-rat IgG-agarose by overnight exposure at 4°C (100 μl suspension/ml cytosol). Adsorbed ERs were then removed from the cytosol by centrifugation (10 min, 800 *g*).

HAP assay

HAP slurry was washed with T10 until pH 8 was reached in the washing supernate; final HAP:buffer ratio amounted to approx. 0.7.

For ER assay, a set of 12 tubes were filled with 200 μl of cytosol (or immunoadsorption supernatant) at a protein concentration between 1 and 2 mg/ml (6 tubes for [^{3}H]E_2, 6 tubes for [^{3}H]E_2 with excess of unlabelled E_2; see below). 250 μl of HAP suspension were added to each tube and incubated at 0°C for 30 min with occasional vortexing. Tubes were then centrifuged at 2000 *g* for 10 min to remove unbound material. Bound ER was subsequently assayed by overnight incubation of the tubes at 0°C with 200 μl of buffer containing increasing amounts of [^{3}H]E_2 (range: 0.25 to 5 nM) in the absence or presence of 1 μM unlabelled E_2. After 2 successive washes of the tubes with 1 ml T10 containing 1% Tween 80 (Sigma), adsorbed [^{3}H]E_2 was extracted from the HAP matrix with 500 μl ethanol (30 min at room temperature) and measured by liquid scintillation counting with an efficiency of ~50%(Ecoscint H scintillation fluid, Atlanta, GA). Data were analysed according to Scatchard.

Assessment of HAP adsorption of full length and truncated ERs expressed in yeast was carried out according to the same protocol.

Sucrose gradient sedimentation

300 μl of [^{3}H]E_2 labelled cytosol (1 h incubation at 0°C with 1 nM [^{3}H]E_2 followed by a DCC treatment; 0.5% charcoal, 0.05% dextran) were layered onto the top of a 10–30% sucrose gradient containing 500 mM KCl in T10. After centrifugation at 50,000 rpm for 16 h (Beckman SW 60 Rotor), gradients were divided in 100 μl fractions and their radioactivity measured by liquid scintillation. Sedimentation patterns of radioactive peaks were compared with the migration of BSA run in parallel (4.4 S).

Influence of H-226 and H-222 monoclonal antibodies on ER migration was assessed by adding 1 μl of antibody to the [^{3}H]E_2-labelled cytosol before centrifugation.

Fast pressure liquid chromatography (FPLC), size-exclusion chromatography

50 μl of [^{125}I]E_2 labelled cytosol (1 h incubation at 0°C with 0.1 nM [^{125}I]E_2 followed by a DCC treatment) were injected in a FPLC system (Pharmacia), in line fitted with a Radiomatic A-200 Flow Beta-one detector (Canberra). The size-exclusion column was a TSK-GEL G3000 SW (TOSO HAAS Corp., Philadelphia, NJ) equilibrated with T10 containing 500 mM KCl. ER retention time was determined using BSA as standard.

Influence of H-226 and H-222 monoclonal antibodies on ER retention time was assessed by adding 1 μl of antibody to the [^{125}I]E_2-labelled cytosol before injection.

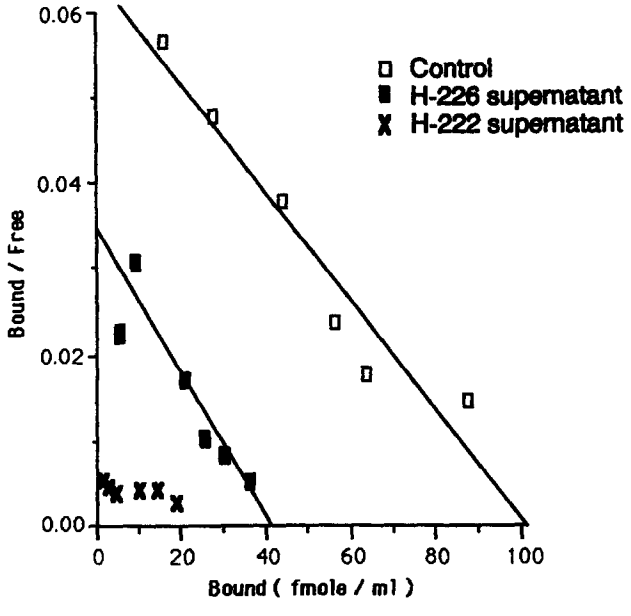
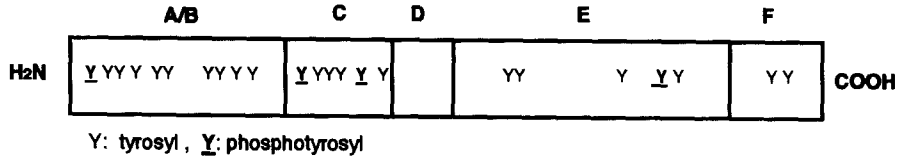
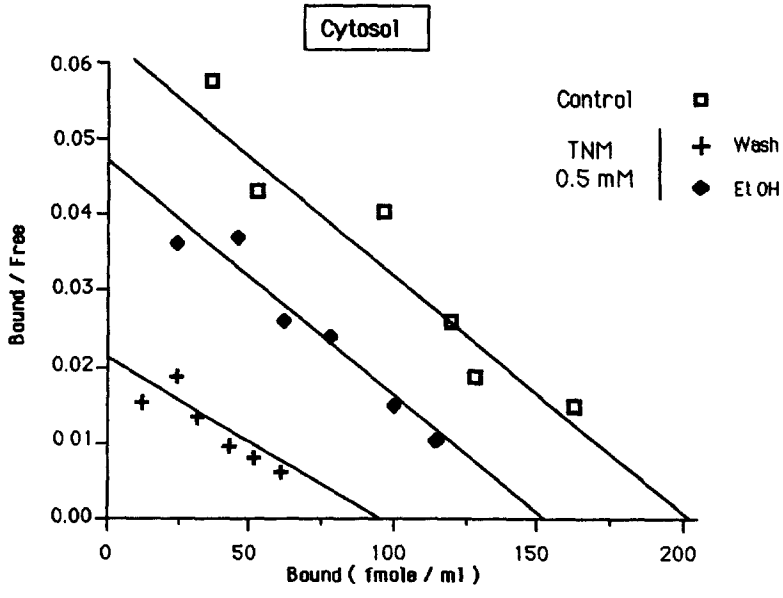


Fig. 2. Adsorption to HAP of remaining ERs after immunoextraction with H-226 or H-222 monoclonal antibodies. Cytosolic ER samples were immunoadsorbed with one of these two monoclonal antibodies and anti-rat agarose, non-immunoprecipitated receptors (supernatants) were then adsorbed on HAP; untreated (control) samples were run in parallel. Adsorbed proteins on HAP were incubated with increasing amounts of [^{3}H]E_2 in the absence or presence of an excess of unlabelled E_2 for ER measurement. Bound steroids were finally extracted with ethanol. Radioactivity of each extract was measured and the data analysed according to Scatchard. The figure shows about 50% of the ER content remained after immunoextraction with H-226 while no receptor could be detected after immunoextraction with H-222 (K_d values of the binding reactions: Control: 0.7 nM; H-226 supernatant: 0.5 nM; H-222 supernatant: non-specific binding).





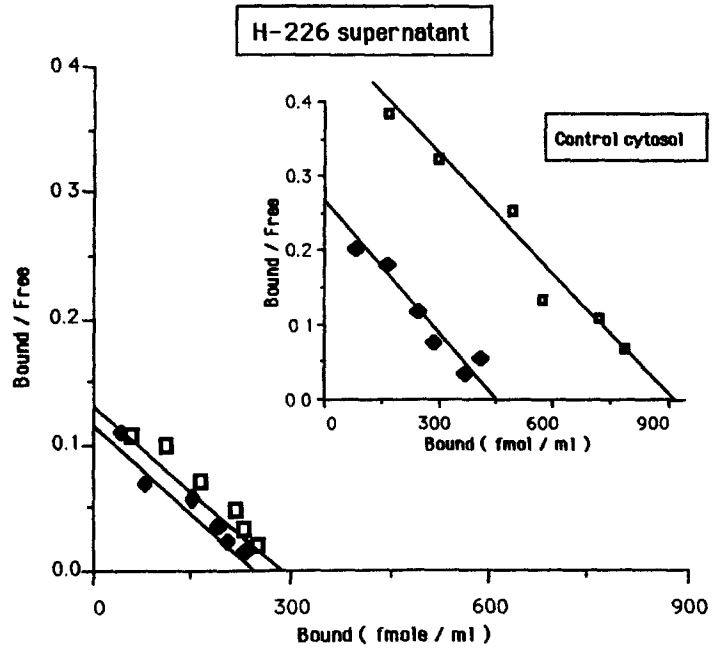


Fig. 3. Effect of the tyrosine reagent TNM on the adsorption properties of cytosolic ER to HAP. The upper scheme shows the location of tyrosyl residues (Y) within the ER molecule. *Upper panel.* Cytosolic ER samples were adsorbed on HAP and incubated with increasing amounts of [^{3}H]E_2 in the absence or presence of an excess of unlabelled E_2 for ER measurement. Bound labelled proteins were then treated with 0.5 mM TNM; untreated (control) samples were run in parallel. Radioactivity of proteins released within the wash preceding the ethanolic extraction of bound steroids as well as of these ethanolic extracts was measured and the data were analysed according to Scatchard. The figure shows that TNM produces a release of about 30% of bound ER (K_d values of the binding reactions: Control: 1.4 nM; TNM: 1.4 nM, wash: 1.7 nM). *Lower panel.* Cytosolic ER was partially immunoextracted with H-226 and labelled with [^{3}H]E_2 as described in Fig. 2. Unextracted ER (supernatant) was then adsorbed to HAP and treated with TNM as described above. The figure shows about 95% of the ER content of this fraction was still present (ethanolic extraction) after TNM treatment (K_d values of the binding reactions: Control: 1.0 nM; TNM: 1.1 nM); control untreated cytosol (inset) behaved as described above (48% of original concentration).

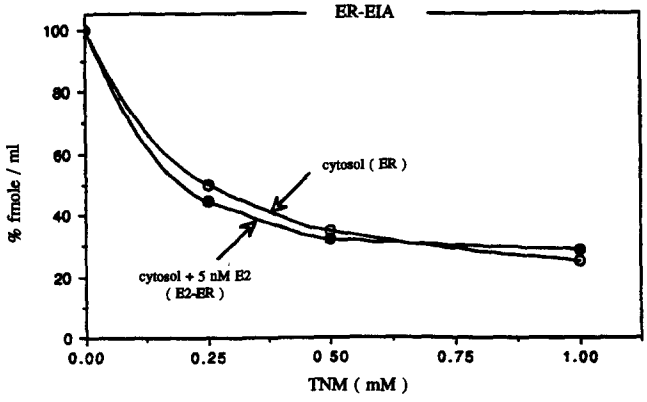


Fig. 4. Effect of TNM on immunoreactivity of cytosolic ER. E_2 labelled or unlabelled cytosolic ER samples (labelling with 5 nM E_2) were treated with increasing amounts of TNM and then assessed by the Abbott ER enzyme immunoassay (ER-EIA) for their receptor content. The figure shows that TNM reduces the immunoreactivity of both E_2-labelled and unlabelled ER.

RESULTS

Cytosolic ER

Numerous experiments revealed that cytosolic ER from uterus (calf, mouse and rat), human breast cancers and MCF-7 cells did not differ qualitatively in regard to HAP adsorption. Therefore, the graphs given in the next sections should be considered as relevant to ER from all origins.

Interaction of cytosolic ER with H-226 and H-222 monoclonal antibodies. H-226, the epitope of which is localized in the A/B domain just upstream of the C domain, has been reported to be especially effective for the identification of the receptor population expressing the C domain [9]. Therefore, we postulated that an immunoextraction of ER with H-226 before HAP assay may be an appropriate method for the identification of such receptors. In agreement with this postulate, we found that addition of H-226 to radiolabelled E_2 cytosol samples before sucrose gradient sedimentation (Fig. 1, left) or size-exclusion FPLC (right) led to only a *partial* shift (~50%) of the labelled receptors. This phenomenon, due to either the masking (no ER activation) [9]or the absence (ER cleavage) of the H-226 epitope was not observed with the control H-222 monoclonal antibody which interacts with an epitope of the estrogen binding domain (E): H-222 led to the *total* displacement of bound [^{3}H]E_2. Confirming this observation, immunoextraction of ER with H-226 before HAP assay maintained about 50% of the original [^{3}H]E_2 binding capacity of the cytosol while the control immunoadsorption with H-222 led to its total disappearance (Fig. 2).

Effect of TNM on ER adsorption to HAP. The A/B and C domains of ER are rich in tyrosyl residues (Fig. 3) and are therefore targets for the tyrosine reagent TNM under our experimental conditions (pH 8). Cysteine, the other potentially TNM reactive residue, should not be taken into account since it is oxidized only at pH 6 [10–12].

As shown in the upper panel of Fig. 3, treatment with 0.5 mM TNM of HAP batches, to which [^{3}H]E_2-labelled ER preparations had been previously adsorbed, led to a dramatic release of bound receptors ($\overline{X}$ = 30%, range = 21–51). Released [^{3}H]E_2-labelled ERs were totally recovered in the wash. This phenomenon did not modify the estrogen binding affinity as evidenced by the absence of significant variations in K_d values (parallel lines on Scatchard plots) suggesting that the tyrosyl residues of the A/B and C domains contribute extensively to the interaction of the receptor with HAP. In support of this conclusion we found that TNM did not influence the adsorption to the matrix of ER forms remaining after immunoprecipitation with H-226 which are mainly devoid of exposed A/B and C domains (Fig. 3, lower panel).

Interestingly, TNM treatment of the cytosol before HAP adsorption and labelling as well as treatment of unlabelled ER already adsorbed to the matrix gave identical desorption patterns (data not shown) indicating that E_2 binding to the receptor does not markedly modify its reactivity towards TNM. Confirming this statement we found that TNM produced the same reduction of ER immunoreactivity in the Abbott enzyme immunoassay for both free and E_2-labelled receptors (Fig. 4).

KCl extraction of the ER from the matrix. KCl treatment (500 mM) of HAP batches to which [^{3}H]E_2-labelled ER preparations had previously been adsorbed led to partial desorption of the receptors (~30%) (Fig. 5, upper panel). Interestingly, the efficiency of release differed with the origin of the samples: ERs from MCF-7 were slightly sensitive to KCl treatment ($\overline{X}$ = 2.3%) while receptors from uterus (mouse: $\overline{X}$ = 12.6%; calf: $\overline{X}$ = 41.6%) and human breast cancer ($\overline{X}$ = 25.6%) were significantly extracted.

These ER forms released by KCl corresponded mainly to the peptides without exposed A/B and C domains in view of the fact that receptors remaining after H-226 immunoadsorption were hardly extracted under such conditions (~82%) (Fig. 5, lower panel). In agreement with this hypothesis, TNM treatment, while reducing the amount of adsorbed ER to HAP,

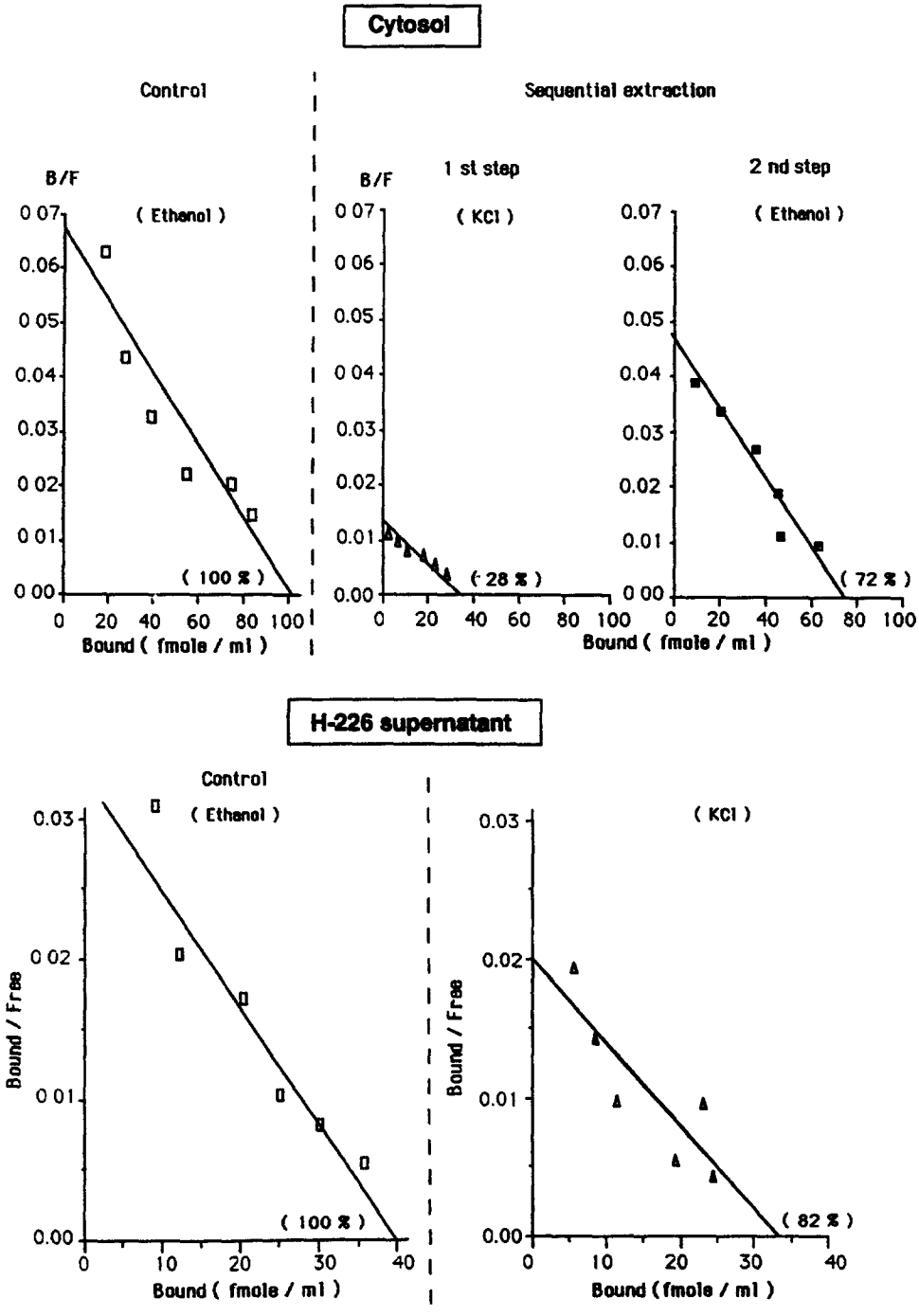


Fig. 5. KCl extraction of ER adsorbed on HAP. *Upper panel.* Cytosolic ER samples were adsorbed on HAP and incubated with increasing amounts of [^{3}H]E_2 in the absence or presence of an excess of unlabelled E_2 for ER measurement. Part of the labelled receptors was then released with 500 mM KCl, bound steroids from unreleased receptors were subsequently extracted with ethanol. Radioactivity of all fractions was then measured and the data were analysed according to Scatchard. The figure shows that KCl treatment led to the extraction of about 28% of the ER content (K_d values of the binding reactions: Control: 0.7 nM; sequential extraction: 1st step =1.5 nM, 2nd step =0.8 nM). *Lower panel.* Cytosolic ER was partially immunoextracted with H-226 and labelled with ^{3}H E_2 as described in Fig. 2. Unextracted ER (supernatant) was then successively treated with 500 mM KCl and ethanol as above. The figure shows that almost all ERs of this fraction were extracted with KCl.

also significantly increased the extraction potency of KCl (~3-fold increase).

ERs expressed in yeast

On the basis of a DCC assay, the same amounts of full length (*A–F domains*) and truncated (*E–F domains*) ER preparations were incubated with HAP. Figure 6 shows that, in comparison to the former preparation, only about half of the latter adsorbed to the matrix indicating a higher binding stability of the full length ER. This property was especially evident when HAP was eluted with 500 mM KCl: under such

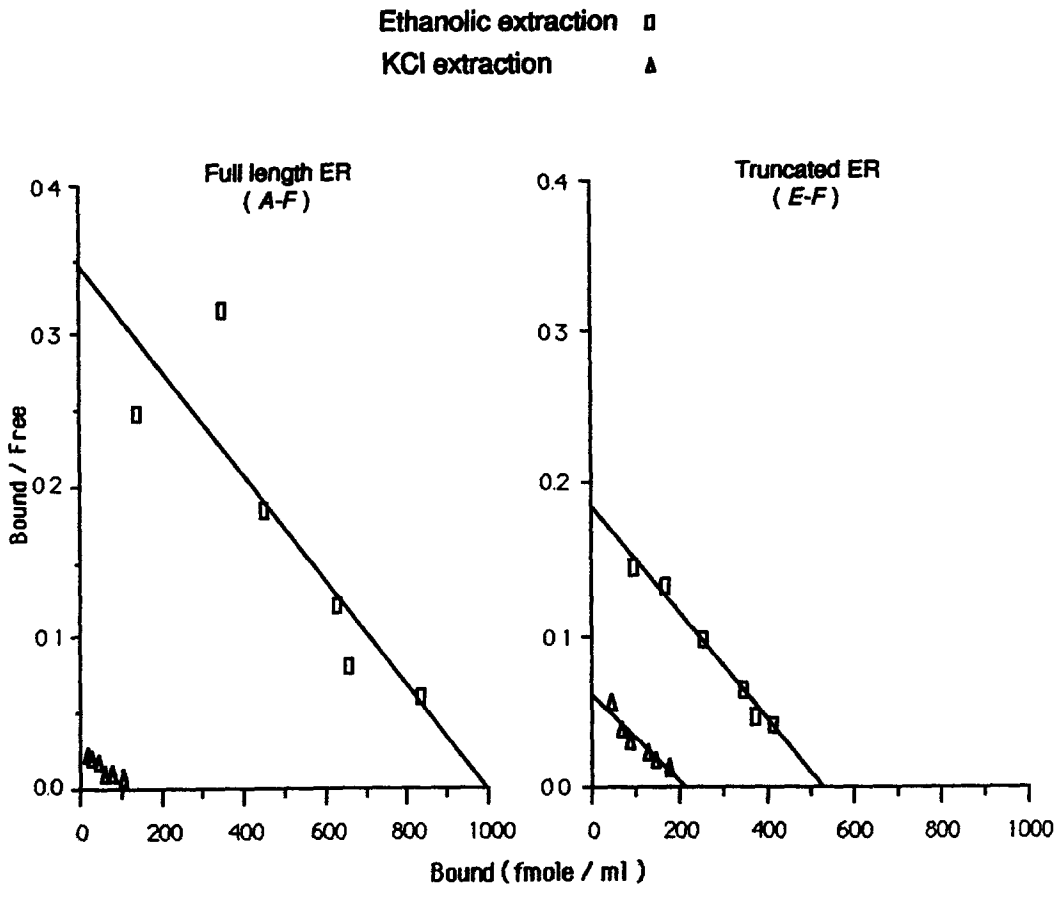


Fig. 6. Adsorption to HAP of full length and truncated ERs expressed in yeast. Samples of both preparations containing same levels of ER on the basis of DCC assay were adsorbed on HAP and incubated with increasing amounts of [^{3}H] E_2 in absence or presence of an excess of unlabelled E_2 for ER measurement. Bound steroids or labelled receptors were then extracted with either ethanol or 500 mM KCl, respectively. Radioactivity of each extract was measured and the data analysed according to Scatchard. The figure shows that in comparison to the full length ER (left), solely ~50% of the truncated ER devoid ABC domains (right) was adsorbed to HAP (ethanolic extraction). The figure also reveals that the full length ER was largely more resistant to the KCl extraction than the truncated receptor (~10 vs 45%) (K_d values of the binding reactions: full length ER: 1.1 nM, ethanolic extraction; truncated ER: 1.0 and 1.2 nM, ethanolic and KCl extraction, respectively).

conditions, almost no full length ER was recovered in the elution buffer while about 30% of the truncated ER was released from the matrix.

TNM treatment of both ER preparations confirmed the importance of the tyrosyl residues of the A/B and C domains for a strong interaction with HAP. Thus, 0.5 mM TNM did not markedly release adsorbed truncated ER while it significantly released (37.3%) its full length form.

DISCUSSION

Data reported here reveal that A/B and/or C domains of ER contribute to its property to strongly adsorb to HAP. This is especially evident in buffer containing 500 mM KCl which markedly reduce the adsorption of receptor devoid of such domains. In fact, our observations are not surprising if we refer to the hydropaticity of the various domains of the receptor [3]. Indeed, ABC domains are hydrophilic while E is hydrophobic. ABC domains, being rich in positively charged amino acids, may interact with the net negative charge of the HAP matrix [13].

HAP in small batches adsorbs ER allowing its measurement by [^{3}H]E_2 labelling in a semi-solid phase without any major interferences from receptor degradative activities present in cytosolic and nuclear preparations [5]. Although this property confers the method an advantage over the conventional DCC assay, HAP has been used mainly for nuclear receptor assay. Our data showing that receptors expressing A/B and/or C domains are strongly adsorbed on HAP, provide an explanation for this restrictive use. The additional observation that measurement of nuclear receptors by enzyme immunoassay (ER-EIA Abbott) and HAP assays always gave higher EIA values [6, 7] is therefore logical since the former procedure which detects the DEF domains [14, 15], measures all estrogen binding forms whether or not they contain exposed ABC domains (D-547 and H-222 antibodies of ER-EIA recognize epitopes located, respectively within the D and E domains).

The lower nuclear receptors values measured by HAP assay in comparison to those established by ER-EIA may also result from the antagonistic effect of the KCl content of the nuclear extracts on the adsorption of ER to HAP. This antagonistic effect should be especially effective with truncated receptors produced by tissue grindings and other manipulations.

Assessment of various cytosolic ER preparations revealed that the efficiency of this antagonistic effect of KCl varied with the tissular origin of the samples. This phenomenon is most probably related to ER populations differing in regard to their sizes and/or configurations. In agreement with this view, we found that cytosols from MCF-7 cells, which in our hands [16] always contain high amounts of native receptors (67 kDa ER), were characterized by the lowest extraction potency. In support of this conclusion, we found that the full length ER produced in yeast was almost not desorbed from the HAP matrix with KCl while the truncated receptor devoid of AB and C domains was significantly released.

TNM, at concentrations up to 0.5 mM, decreased the adsorption of the receptor to HAP without modifying its E_2 binding characteristics (affinity and capacity). This observation is partly at variance with those of other investigators who suggest a reduction in hormone binding capacity [17, 18]. However, these authors did not measure the radioactivity levels of the proteins released from the HAP matrix by TNM treatment. If we also limit the analysis to adsorbed receptors only, the results are identical. Nevertheless we cannot exclude an alteration of the hormone binding domain of the receptor at higher TNM concentrations which we believe, however, would have markedly modified E_2 binding affinity (an increase in K_d values would most probably occur). Diethylpyrocarbonate, another reagent of tyrosyl residues, modifies the binding affinity of the receptor for estrogens but not for antiestrogens [19].

Tyrosyl residues of the A/B and/or C domains are directly involved in these degradative effects of TNM. Interestingly, such TNM-induced alterations modify the spatial configuration of DEF domains as demonstrated by a reduction in the immunoreactivity of the receptor in the Abbott enzyme immunoassay which detects solely the latters. This observation suggests that the ABC domains involved in gene expression may influence the tridimensional structure of domain E. Whether or not such a property may have some physiological relevance is unknown. It is, however, in agreement with a recent report indicating that the interaction of ER with the DNA reduces the stability of the E_2–ER complexes [20].

Finally, breast cancer cytosols were recently reported to contain ER mutants as well as altered receptors devoid of operative ABC domains which may play a prominent role in the development of the disease [4, 21]. In view of the present data, it seems that HAP assays complementary to conventional DCC assays would be extremely useful for the quantification of such variant receptors. In the presence of 500 mM KCl, HAP assays would be limited to ER containing exposed ABC domains and the HAP/DCC ratio would provide an estimate of the amount of alterated receptors. Such results may be compared with those established by gel shift assays which were shown to be very sensitive to the detection of DNA binding forms of the receptor [22, 23]. Immunoblotting studies (Western blots) with monolonal antibodies raised against epitopes of the A/B or C domains may also be considered in such a correlation study. This approach has now been undertaken in our laboratory to evaluate the efficiency of the proposed HAP/DCC test which, at the present time, could easily be introduced into routine practice.

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