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The Human Estrogen Receptor Hormone Binding Domain Dimerizes Independently of Ligand Activation

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High level expression of biochemically active human estrogen receptor hormone binding domain (hER-HBD) was achieved using a *Saccharomyces cerevisae* expression system. Using dissociation kinetic analysis, density gradient centrifugation and cross-linking studies, a spontaneous dimerization activity of hER-HBD independent of the presence of the DNA binding domain, ligand, and of elevated temperature is demonstrated.

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INTRODUCTION

Many transcription factors have been suggested to form homo- or heterodimers as a prerequisite for activation of their target genes [1–3]. One such class of transcription factors is the steroid hormone receptor superfamily. In the presence of ligand, steroid receptors form dimers at their cognate steroid responsive elements and activate transcription [4, 5].

Studies examining the dissociation of [^{3}H]estradiol from calf uterus estrogen receptor (ER), have revealed two dissociation rates. A fast and a slower dissociation rate have been shown, which are purported to correspond to estradiol dissociating from the nonactive ER (monomer form) and the active ER (dimer form), respectively [6]. It has also been shown that activation of calf uterus ER is temperature and ligand dependent [7]. Using gel-shift analysis, sequences essential for dimerization have been identified in the C-terminal part of the hormone binding domain (HBD) of the mouse ER [8–10], and sequence comparison shows that equivalent residues are conserved in all members of the nuclear hormone receptor superfamily. On the other hand, previous studies using HBD fragments obtained following trypsinization of calf uterus ER from tissue homogenates [11,12] did not conclusively show whether or not ER-HBD itself may dimerize without the presence of the DNA-binding region. Expression of defined receptor domains in heterologous systems would offer an alternative to enzymatic digestions to obtain sufficient material for such dimerization studies.

In a recent publication, it was shown that the full-length hER and the hER-HBD expressed in yeast *Saccharomyces cerevisiae* are as stable as full-length MCF-7 ER [13]. The hER was very similar to the MCF-7 ER in its affinity ($K_d = 0.35 \pm 0.05$ nM), dissociation rate, and structural specificity for ligands. The hER-HBD showed a slightly reduced affinity for estradiol ($K_d = 1.00 \pm 0.17$ nM) while it was similar to MCF-7 ER in dissociation rate and structural specificity for ligands.

Here we present kinetics of dissociation for estradiol complexed with hER or with hER-HBD expressed in yeast, demonstrating a spontaneous dimerization activity independent of the presence of ligand and of temperature. The results are supported by biochemical studies.

EXPERIMENTAL

Materials

17β-[2,4,6,7-^{3}H]estradiol (90–109 Ci/mmol) was obtained from New England Nuclear (Massachusetts, U.S.A.) and Protein A, 3-(4-hydroxy, 5-[^{125}I]iodophenyl) propionamid was from Amersham (England). 17β-Estradiol, diethylstilbestrol and 2-iminothiolane-hydrochloride were purchased from Sigma (Missouri, U.S.A.). DEAE-ion exchange paperdiscs (DE 81, 23 mm) were purchased from Whatman International Ltd (England). Hydroxylapatite (Bio-gel HTP) and BIO-RAD protein assay dye reagent concentrate were purchased from BIO-RAD (California, U.S.A.).

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Scintillation cocktail was OptiPhase Hisafe 3 from Pharmacia (Uppsala, Sweden). The monoclonal antibody D75 was kindly provided by Dr G. L. Greene (University of Chicago, U.S.A.).

Buffers

Buffer A; 20 mM Tris, 1 mM EDTA, 50 mM NaCl, 10 mM sodium molybdate, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.8. TEDG; 10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.8. ETG; 1 mM EDTA, 20 mM Tris, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.8. PBS; 0.14 M NaCl, 0.01 M potassium phosphate buffer, 0.02 M KCl, pH 7.4.

Expression of hER and hER-HBD in S. cerevisiae

Expression and preparation of recombinant hER (aa 1–595) and hER-HBD (aa 302–595) was performed essentially as described previously [13, 14].

Ligand binding assays

In equilibrium binding experiments, yeast extracts containing hER or hER-HBD were incubated with [^{3}H]estradiol in buffer A overnight at + 4°C in a total volume of 200 μl. The incubation was terminated by addition of 250 μl of a hydroxylapatite (HAP)–ETG slurry, prepared by washing HAP three times in ETG buffer and addition of fresh ETG buffer (1:1) before use. Samples were put on ice for 15 min to allow adsorption of receptor material, vortexing every 5 min. Following centrifugation at 6000 *g*, supernatants were discarded while pellets were washed three times by centrifugation and resuspension in ice-cold ETG. Receptor–[^{3}H]estradiol complexes were quantitated by extracting the HAP pellets with 200 μl 0.5 M potassium phosphate solution (pH 7.8) for 15 min at room temperature with vortexing every 5 min. After a final centrifugation, radioactivity in the supernatant was counted in a Beckman β-counter. Specific binding was determined by subtracting the [^{3}H]estradiol bound at a 300-fold excess of unlabeled estradiol.

Alternatively, yeast extracts containing hER or hER-HBD were equilibrated with a 10-fold excess of [^{3}H]estradiol in TEDG overnight at + 4°C. The free ligand was removed by adding an equal volume of DCC solution (1% charcoal, 0.01% dextran in TEDG). The samples were then incubated for 10 min at + 4°C with gentle shaking followed by subsequent centrifugation. The supernatant was removed and used for cross-linking and sedimentation analysis ("prelabeled" receptor; see below).

Dissociation kinetics of [^{3}H]estradiol from hER and hER-HBD

The dissociation of [^{3}H]estradiol from the receptor was measured by the exchange of [^{3}H]estradiol with an excess of unlabeled estradiol. In brief, yeast extracts containing 2 nM of receptor were incubated with 6 nM [^{3}H]estradiol in polypropylene test tubes at + 4°C for 2 h. The samples were then placed in a waterbath (29°C) and a 1000-fold excess of unlabeled estradiol was added ($=t_0$) directly or after 30 min of preincubation at 29°C. DEAE filtration was performed to terminate the dissociation-rate experiments.

DEAE paper discs were put in a ten-position filter manifold. A 50 μl sample was applied to each of the dry filter discs. Receptor–ligand complexes were allowed to bind to the filter discs for 2 min and then vacuum was applied. The filters were washed with six 10 ml portions of ice-cold 20 mM Tris buffer, pH 7.8. Following transfer to scintillation vials, radioactivity was allowed to dissolve in the scintillation cocktail for 4 h before quantitation in a β-counter.

Control experiments confirmed that both hER and hER-HBD were stable for at least 70 min, i.e. there was no significant receptor loss due to degradation or inactivation during the course of the assay. Rate constants were calculated assuming first order kinetics. The rate constant of the first component was estimated after subtracting the contribution of the second component. The slope of the second component was extrapolated to zero time and its value at each time point was subtracted from the experimentally measured values. The difference was plotted semilogarithmically to obtain the dissociation rate constant of the first component (k_{-1}). Linear, least squares regression analyses were performed to obtain the best fit.

Sucrose gradient analysis

"Prelabeled" hER-HBD was analyzed on 5–20% linear sucrose gradients in TEDG with or without 0.4 M NaCl and "prelabeled" hER was analyzed on 10–30% linear sucrose gradients in TEDG containing 0.4 M NaCl. Samples, 100–150 μl, were layered onto 5-ml gradients and centrifuged at 100,000 *g* for 23 h in a Beckman SW55 Ti rotor. Fractions were collected from the bottom of the tube. The fraction volume was measured and the radioactivity was counted in a β-counter. "Postlabeled" hER-HBD was obtained by labeling the collected fractions after the centrifugation. The fractions were incubated with 60 nM [^{3}H]estradiol in TEDG overnight at + 4°C. The free ligand was removed using the DEAE-filter assay described above. The relative mobility of the fractions were determined from the ratio (collected volume)/(total volume). Standard proteins used were: cytochrome C (1.2S), ovalbumin (3.5S), BSA (4.5S) and aldolase (7.9S). Gradients run in the presence of 3 M urea and/or 0.4 M NaCl were analyzed using separate standard curves.

Chemical cross-linking

The yeast extract estradiol–hER-HBD complexes were cross-linked with the cross-linker 2-iminothiolane by incubating 187.5 μl (12 pmol hER-HBD) of yeast extract with 7.5 μl 1 M triethanolamine buffer (pH 8.5), 10 μl 50 mM magnesium acetate and 42.5 μl freshly made 2-iminothiolane (1 mg/100 μl in 20 mM triethanolamine buffer) for 1 h at 0°C. The unreacted 2-iminothiolane was removed by dialysis for 1.5 h against 20 mM triethanolamine and 1 mM EGTA with

0.6 M NaCl, pH 8.5. The samples were then incubated with 1.5 μl 30% H_2O_2 for 20 min at 0°C [15]. Reversal of the 2-iminothiolane cross-linking was achieved by adding 0.13 M β-mercaptoethanol to the sample before heating. Control samples were treated as the cross-linked samples except that H_2O was added instead of 2-iminothiolane. The samples were separated on a 10% SDS-polyacrylamide gel [16]. Procedures for protein transfer were as described for the Bio-Rad Mini Trans Blot equipment used. The nitrocellulose membrane was stained by 0.2% Ponceau R for 5 min, washed in H_2O and photographed. The membrane was thereafter blocked with 1% Tween 20 in PBS for 1 h and incubated sequentially with anti-hER-HBD monoclonal antibody D75 [17], rabbit-anti-mouse IgG, [^{125}I]protein A and visualized by autoradiography.

RESULTS

Optimization of a DEAE filter ligand binding assay

In order to study the dissociation kinetics of estradiol from yeast expressed hER and hER-HBD, a DEAE-filter assay was developed and optimized with respect to number of washes, incubation time and total protein applied (not shown). hER-HBD extract equilibrated with [^{3}H]estradiol was efficiently retained on the filters. The amount of radioactivity retained was reduced by addition of a 300-fold excess of unlabeled estradiol or diethylstilbestrol to the binding buffer, and likewise, the specific binding was lost when the extract was incubated with proteinase K. Binding of [^{3}H]estradiol to the filter discs was <0.9%. The amount of bound receptor increased when the sample was incubated on the filter before vacuum was applied, and binding was optimal after 2 min of incubation. With respect to maximal binding (B_{max}), the filter binding assay was shown to be linear in the 0.025 to 0.4 mg range of total protein or 0.26 to 4.2 pmol hER-HBD. In addition, B_{max} was 1.5 times higher in the filter assay, when compared to the hydroxylapatite-assay.

Dissociation of [^{3}H]estradiol from hER and hER-HBD

When unlabeled estradiol was added to [^{3}H]estradiol liganded hER at 29°C, directly following the [^{3}H]-estradiol incubation at 4°C, two first order dissociation rates were revealed by analysis of the obtained dissociation curve [Fig. 1(A)]. The dissociation rate constant of the first rapid component, k_{-1} (after subtraction of the second, slower component, k_{-2}) was determined to $(8.3 \pm 2.5) \times 10^{-2}\,min^{-1}$ ($n = 4$, $\pm$ SD). The second (slower component) dissociation rate constant, k_{-2}, was $(1.9 \pm 0.5) \times 10^{-2}\,min^{-1}$ ($n = 4$), [Fig. 1(A)]. In contrast, when hER was preincubated with labeled ligand for 30 min at 29°C before the unlabeled ligand was added, the first (rapid) component disappeared [Fig. 1(B)] and only one dissociation rate, $(1.7 \pm 0.4) \times 10^{-2}\,min^{-1}$ ($n = 3$), similar in magnitude to k_{-2}, was observed.

Unlike the experiments performed with hER, similar experiments using hER-HBD, allowing dimerization to occur during the actual dissociation of [^{3}H]-estradiol, did not reveal biphasic dissociation kinetics [Fig. 2(A)]. Instead, only one dissociation rate, $(2.1 \pm 0.1) \times 10^{-2}\,min^{-1}$ ($n = 2$), comparable to k_{-2} for hER was observed. The same slow dissociation rate, $(2.2 \pm 0.1) \times 10^{-2}\,min^{-1}$ ($n = 2$), was observed following preincubation for either 7 or 30 min at 29°C, i.e. under conditions allowing dimerization to occur prior to the dissociation experiment [Fig. 2(B)].

Dimeric–monomeric states of hER-HBD as analyzed by sucrose gradient centrifugation

Sucrose gradient sedimentation analysis of hER-HBD prelabeled with [^{3}H]estradiol showed one single peak at 4S [Fig. 3(A)]. Upon preincubation of prelabeled hER-HBD at 29°C for 30 min, no change in

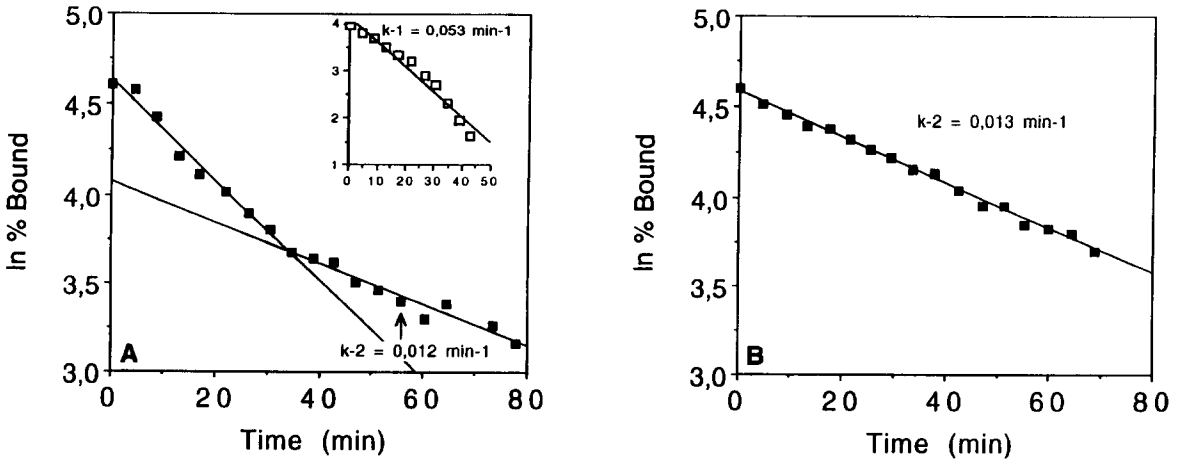

Fig. 1. Dissociation of [^{3}H]estradiol bound to hER in yeast extracts. (A) 2 nM hER was incubated with 6 nM [^{3}H]estradiol at 4°C for 2 h before shifting to 29°C and addition of a 1000-fold excess of unlabeled estradiol. Insert: The fast phase of [^{3}H]estradiol dissociation as obtained following subtraction from the biphasic plot. (B) Dissociation of [^{3}H]estradiol from hER following incubation for 30 min at 29°C prior to addition of unlabeled estradiol. Representative data from experiments performed in quadruplicate.

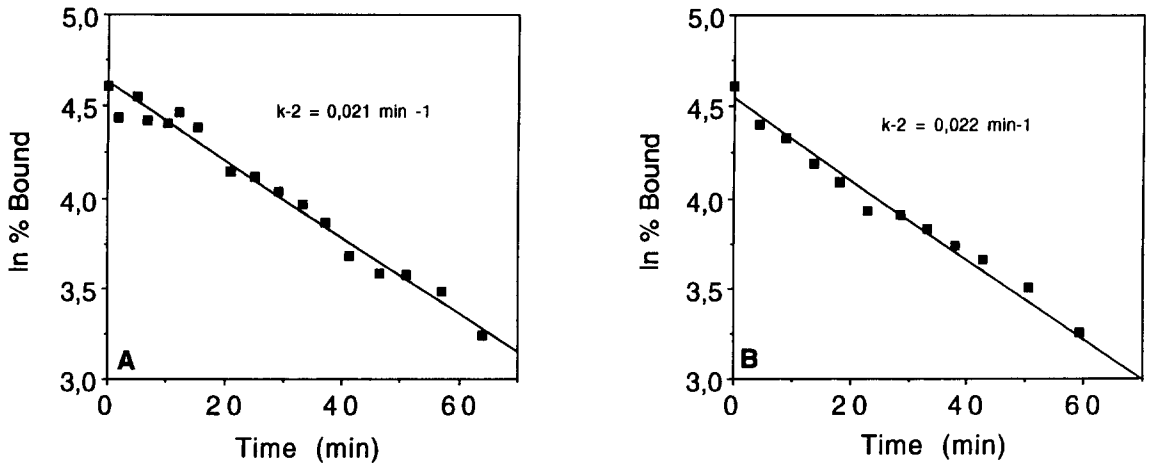

Fig. 2. Dissociation of [^{3}H]estradiol bound to hER-HBD in yeast extracts. (A) 2 nM hER-HBD was incubated with 6 nM [^{3}H]estradiol at 4°C for 2 h before shifting to 29°C and addition of a 1000-fold excess of unlabeled estradiol. (B) Dissociation of [^{3}H]estradiol from hER-HBD following incubation for 30 min at 29°C prior to addition of unlabeled estradiol. Representative data from experiments performed in quadruplicate.

the sedimentation coefficient was observed [Fig. 3(B)], whereas control experiments using hER showed a shift from a 4S to a 5S peak (not shown) corresponding to the monomer (4S) and the dimer (5S) form of hER [6, 12, 15]. To examine whether the presence of ligand induced the formation of the presumtively dimeric 4S form of hER-HBD, yeast extract was sedimented on a sucrose gradient and the collected fractions were labeled with [^{3}H]estradiol. The hER-HBD again migrated as a 4S form [Fig. 3(C)]. The effect of 3M urea in the sucrose gradient was investigated, in this case the hER-HBD sedimented at 2S and 4S if the sample was treated with or without urea, respectively [Fig. 3(D and E)]. Taken together, these results

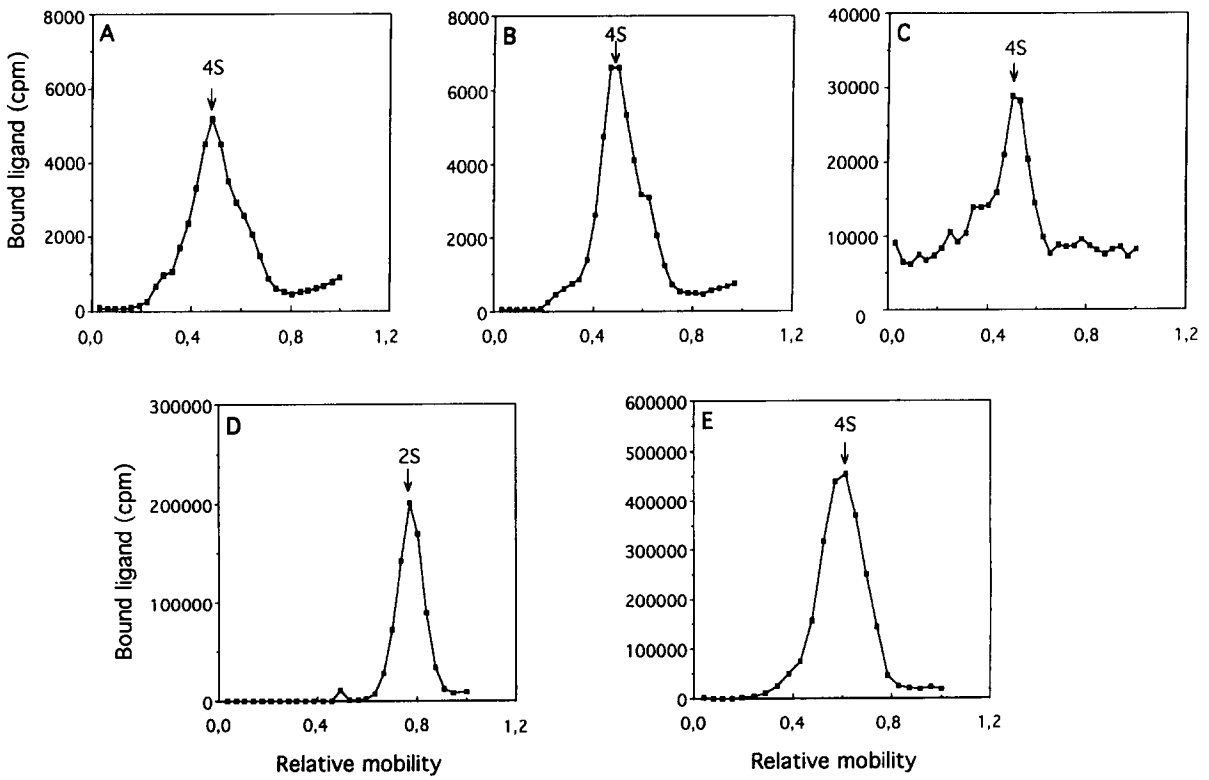

Fig. 3. Sedimentation analysis of hER-HBD in sucrose gradients. Yeast extracts were incubated as indicated, before layering onto 5–20 % linear sucrose gradients and centrifugation for 23 h at 100,000 *g*. (A) hER-HBD prelabeled with [^{3}H]estradiol at 4°C. (B) hER-HBD prelabeled with [^{3}H]estradiol at 4°C followed by 30 min incubation at 29°C. (C) hER-HBD labeled with [^{3}H]estradiol after centrifugation. Fractions were individually labeled and assayed with the DEAE-filter assay. (D) hER-HBD prelabeled as in (A) and centrifuged in a gradient containing 0.4 M NaCl and 3 M urea. Note the shift from 4S to 2S. (E) hER-HBD prelabeled as in (A) and centrifuged in a gradient containing 0.4 M NaCl.

indicate that the hER-HBD 4S form is a dimer, and that the dimerization is not dependent on elevated temperature and presence of ligand.

Dimeric–monomeric state of hER-HBD as analyzed by chemical cross-linking

To verify the interrelationships between the 4S and 2S hER-HBD forms, ligand–receptor complexes were chemically cross-linked with the mercaptan-reversible cross-linker 2-iminothiolane and separated by SDS–PAGE [15]. The noncross-linked receptor had the expected molecular weight of 33 kDa (Fig. 4, lane a), whereas the 2-iminothiolane cross-linked sample was shifted to a molecular weight corresponding to 66 kDa (Fig. 4, lane b). The reaction was reversible, as addition of β-mercaptoethanol to the cross-linked sample caused a reversal to a faster migrating species with an apparent molecular weight of 33 kDa (Fig. 4, lane c).

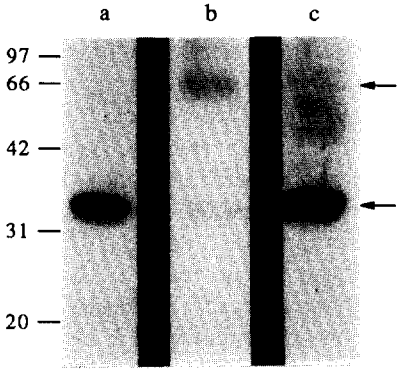

Fig. 4. SDS–PAGE of untreated hER-HBD yeast extract (lane a), 2-iminothiolane cross-linked extract (lane b), 2-imminothiolane cross-linked extract, subsequently reduced with β-mercaptoethanol (lane c). hER-HBD was detected by an immunoassay using ^{125}I labeled protein A and subsequent autoradiography.

DISCUSSION

Several groups have shown that ER binds to its response element as a dimer and that the hormone binding domain of the receptor constitutes an important interactive region in receptor dimerization [4, 8, 18]. By selectively producing the hormone binding domain of the hER, we have been able to study the dimerization process in the absence of other receptor domains that may modulate dimer formation.

The DEAE filter ligand binding assay confers a rapid separation of free and ER-bound estradiol, which makes it suitable for kinetic studies. Compared to a similar assay for the glucocorticoid receptor and dexamethasone [19], less between-sample variation was obtained if the samples were applied to dry filter discs and washed with a larger volume of buffer.

In the binding experiments we were able to confirm that hER produced in yeast shows a temperature dependent shift in the dissociation rate for estradiol (Fig. 1). The obtained results agree well with previously published data on hER derived from calf uterus [6].

In contrast, we found that hER-HBD has only *one* dissociation rate for estradiol (Fig. 2). The apparent rate for release of estradiol from the hER-HBD is of the same order of magnitude as the slower, k_{-2} rate constant of the full-length receptor. This suggests that the hER-HBD exists as a dimer in the yeast extract, and therefore also possesses two estradiol binding sites. These data are in contrast to those obtained by Notides *et al.* [11], who showed that a 33–35 kDa HBD-like C-terminal fragment obtained by trypsin treatment of calf uterus ER, lost its ability to dimerize and thereby its positive cooperativity for estradiol binding. Whether or not the two ligand binding sites in the hER-HBD dimer interact to yield a cooperativity in binding of estradiol needs to be further investigated. If such cooperativity exists within the hER-HBD dimer, it is likely to reside in differences in the association rates between mono- and unliganded dimer, since only one dissociation rate for estradiol unbinding was observed for the hER-HBD molecule (Fig. 2).

The sedimentation coefficient of hER-HBD in sucrose gradients was 4S regardless of whether incubations were performed under conditions that prevent or induce dimerization (Fig. 3). A shift to a 2S mobility was observed only when urea was added to the gradients. Thus, disruption of hydrophobic interactions yielded hER-HBD monomers. This is in line with Sabbah *et al.* [12], who showed that limited proteolysis by trypsin of the calf uterus ER yields a 4S species with an apparent molecular weight of 66 kDa, and that the 4S form could be reversibly transformed into a 3S form by treatment with NaSCN.

The capacity of hER-HBD to form homodimers is supported by the cross-linking experiments, showing an apparent dimerization of the 33 kDa monomer into a 66 kDa species (Fig. 4). A similar result might be obtained for heterodimeric complexes containing only a single molecule of hER-HBD, although at present we favor the possibility of homodimerization since the dissociation k_{-2} rates for hER and hER-HBD are nearly identical (Figs 1 and 2).

It is well known that yeast hsp 90 can bind to steroid receptors [20, 21]. The hER-HBD was extracted from yeast cells in a low ionic strength buffer in order to allow this interaction, provided the hER-HBD molecule possesses hsp interaction sites. However, we consider this possibility unlikely since a 4S truncated ER form (aa 275–595), transiently expressed in Cos 7-cells did not complex with hsp:s, as determined by density gradient centrifugation experiments [22]. Thus, the interaction of hER-HBD with hsp:s is probably insignificant in yeast; alternatively, binding of hER-HBD to yeast hsp:s is not sufficiently stable to allow association during sucrose gradient centrifugations.

In conclusion, using kinetic analysis of ligand binding, density gradient centrifugation and cross-linking studies, we have shown that recombinant hER-HBD is spontaneously dimerized in a ligand and temperature

independent manner, revealing the strong hydrophobic forces mediating hER dimerization *in vivo*. This would be in accordance with works using mouse ER mutants, showing that mutants lacking estrogen binding activity retain their ability to dimerize [8]. As the monomeric 4S form of authentic hER does not dimerize in the absence of ligand and elevated temperature, we suggest that the dimerization sites in the HBD portion of the full-length receptor are normally masked in the non-activated ER. Since the hER-HBD molecules strongly attract each other, there must be a structural portion of the full-length receptor or an associated inhibitor (eg., hsp:s etc.) that prevents the hydrophobic interaction. The precise localization or structure of such a functional dimerization suppressor is unknown. By its identification, studies on the regulation of the dimerization process would be facilitated.

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