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Estrogen Receptor Microarrays: Subtype-Selective Ligand Binding

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The two estrogen receptors, ERα and ERβ, are ligand-regulated transcription factors of the nuclear receptor (NR) class whose activity is modulated by the binding of ligands and interaction with coregulators.[1,2] Various methods have been developed to assay these interactions,[3,4] but only some of these are sufficiently convenient for high-throughput analysis. Here, we describe the preparation of protein microarrays of ERα and ERβ ligand-binding domains (LBDs) on glass slides; the ERs on these arrays remain active and can be used to assay the specific binding of different ligands in a rapid and convenient manner. Although DNA microarrays are well known, there are only a few examples of protein microarrays,[5−7] because proteins are structurally diverse and often sensitive to denaturation.[6] The NRs are considered to be very sensitive proteins.

To follow the tethering of the ERs to glass, we used engineered ERα and ERβ LBDs labeled site specifically at a singly reactive cysteine residue between helices 7 and 8 (C417 in ERα and C369 in ERβ) with thiol-reactive fluorophores (tetramethylrhodamine (MTMR) or Cy3, and Cy5) in a manner that preserves their binding activity.[8] We evaluated various commercially available slides (amine, aldehyde, epoxide, nickel) and found attachment to aldehyde slides to be most efficient (Figure 1A). Attachment was rapid (2−5 h), reproducible (CV < 5%), and linear over an extended concentration range. Curiously, the level of ER attachment depended on the liganded state of the ER-LBD, with more apo-ER and antagonist-liganded ERα and ERβ (i.e., hydroxytamoxifen, TOT, or ICI 182,780) becoming attached than the agonist-liganded ERs (i.e., either estradiol, E_2, or diethylstilbestrol, DES; Figure 1A and B, left).[9] The attachment of the ERs was irreversible, and the level of attached receptor was unaffected by subsequent ligand treatment.

Most of the 11 lysines in ERα-LBD are folded deeply within the protein,[2,4] but two, K529 and K531, appear exposed and are the most likely attachment sites to the aldehyde surface (Figure 1C). These lysines are in a region where conformation is markedly ligand regulated: In agonist structures, both are tightly folded back onto the protein surface (Figure 1C, green), whereas in antagonist structures, K531 projects outward toward solvent and K529 remains exposed but is somewhat closer to the protein surface (Figure 1C, red).[2,4] The exposure of K531 in ER-antagonist structures is supported by its high sensitivity to trypsin proteolysis,[10] and apo-ER is believed to resemble antagonist-bound ER.[10] Thus, the attachment of ER to aldehyde slides appears to be specific, with K531 in ERα and the corresponding K482 in ERβ the most likely sites of attachment.

Good attachment was evident with apo ER or [^{3}H]TOT-liganded ER, but very little activity was recovered when [^{3}H]E_2-prebound ER was used (Figure 1B, right). The greater attachment of apo and antagonist-bound ER than agonist-bound ER, determined here radiometrically, is consistent with the results using fluorophore-tagged ERs (Figure 1B, left) and indicates that the attached ER retains ligand-binding activity.

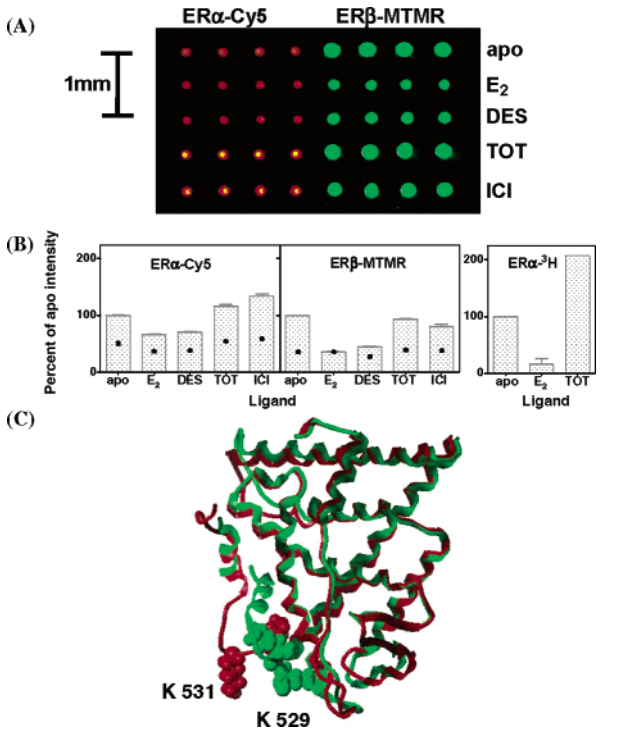
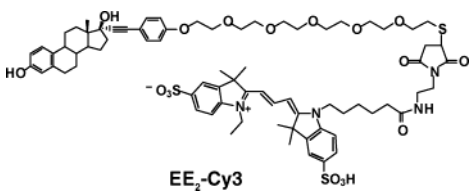


Figure 1. Attachment of the ER-LBD-ligand complexes to an aldehyde slide. Panel A: A microarray showing the differential attachment of ERα-Cy5 (red) and ERβ-MTMR (green) bound with various ligands. Agonist-bound ERs (E_2 and DES) show less attachment as compared to antagonist-liganded (TOT and ICI) or apo-ER. Panel B: Quantification of the attachment of ERα-Cy5 and ERβ-MTMR, and of nonfluorescent ERα-pre-bound with [^{3}H]E_2 or [^{3}H]TOT. Dots show the level of attachment to plain glass. Panel C: A ribbon structure of the ERα-LBD, showing the position of the two important lysines (K529 and K531 in ERα) when the ligand is the agonist DES (green) or the antagonist TOT (red).

The ligand-binding activity of ER-LBDs, tethered to aldehyde slides, can be measured using a fluorophore-estradiol conjugate in which a Cy3 fluorophore has been attached through an oligoethylene glycol spacer to a 17α-ethynylestradiol derivative (**EE_2-Cy3**). **EE_2-Cy3** binds to both ERα and ERβ in a manner that shows good linearity with the concentration of spotted ER (Figure 2).



The specificity of this binding is evident from Figure 2, in which **EE_2-Cy3** binding to both ERα and ERβ is blocked by either

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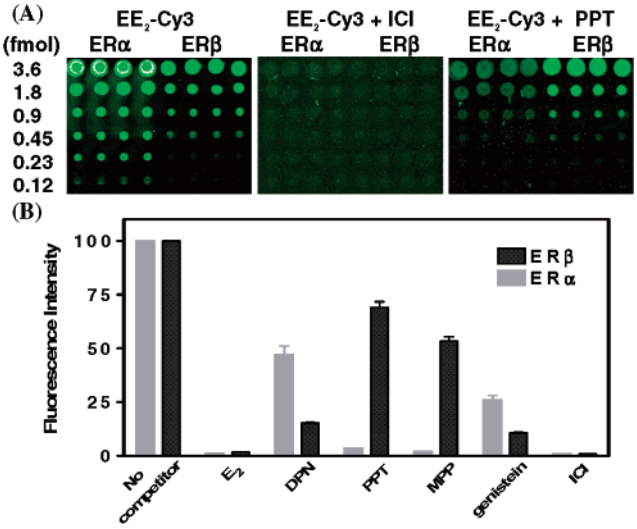

Figure 2. Microarrays showing **EE_2-Cy3** binding to ERα and ERβ. The ERs were printed in quadruplicate at six concentrations, denoted by the fmol values that correspond to the amount of ER-LBD applied to each spot. Panel A, left: Microarray showing the concentration-dependent binding of **EE_2-Cy3** to ERα and ERβ. The fluorescence from the highest concentration of ERα saturates the detector. Panel A, middle: Microarray showing the blocking of 700 nM **EE_2-Cy3** binding to both ERα and ERβ by 7 μM ICI. Panel A, right: The selective blocking of only ERα by the ERα-selective ligand PPT. Panel B: Quantification of the binding of 700 nM **EE_2-Cy3** to 3.6 fmol of ERs with no competitor, or when blocked by 7 μM of E_2, ICI, or subtype-selective ligands, DPN, PPT, and MPP, or by the ERβ-selective phytoestrogen genistein.

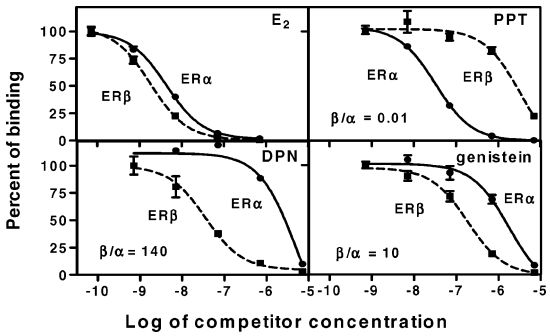

Figure 3. Plot of the fluorescence intensity of **EE_2-Cy3** bound to ERα and ERβ on a microarray, showing competition by four ligands. E_2 is the strongest competitor but is not subtype selective; competition by PPT is strongly ERα selective, and DPN and genistein are ERβ selective.

unlabeled ICI or E_2, and **EE_2-Cy3** binding to ERα is blocked selectively by the ERα-selective agonist propylpyrazole triol (PPT)[11] and methyl piperidinopyrazole (MPP),[12] whereas the binding to ERβ is selectively blocked by the ERβ-selective agonist diarylpropionitrile (DPN)[13] and genistein.

By adding varying amounts of competitors, we can use these fluorometric competition experiments to quantify ligand-binding affinity, making them operationally the equivalent of a relative binding assay (RBA).[14] The percent of the remaining fluorescence intensity can be plotted against the competitor concentration to generate a displacement curve (Figure 3).

The nonselective ligand, E_2, competes with the binding of **EE_2-Cy3** to both ERα and ERβ with a similar affinity, whereas the ERα-selective ligand, PPT, competes ca. 100-fold more effectively with **EE_2-Cy3** for binding to ERα than to ERβ. Conversely, both ERβ-selective ligands, DPN and genistein, compete better for binding to ERβ than to ERα. The ER subtype selectivity of these compounds mirrors quite closely their selectivity measured in radiometric assays, as does the affinity of various antiestrogens (not shown).[11,13]

Thus, microarrays of ERα- and ERβ-LBDs printed on aldehyde slides retain good binding activity that can be assessed using a fluorescent ligand, and the specific and ER subtype-selective binding of ligands can be determined conveniently in a competitive binding assay. Elsewhere, we will describe the use of NR-LBD microarrays in coactivator recruitment assays.

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Supporting Information Available: Details of conditions for printing ERα- and ERβ-LBD protein arrays on aldehyde slides and for ligand-binding assays on these arrays. Synthesis and characterization of 17α-ethynylestradiol-Cy3 (**EE_2-Cy3**) and table of ligand structures and receptor binding affinities (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) We printed the ER LBDs onto the aldehyde slides (TeleChem International Inc, Sunnyvale, CA) using a SpotBot Protein Edition Microarray Robot (TeleChem International Inc.), under conditions of controlled humidity (65−70%) and temperature (15 °C). Each slide was then blocked by placing it carefully, face down, in a solution of 3% bovine serum albumin (BSA) in 50 mM MOPS (3-[*N*-morpholino]propanoic sulfonic acid) buffer, pH 8.0, 0.01% sodium azide, and then incubated in 50 mL of fresh BSA-MOPS buffer at room temperature, with gentle agitation for 1 h. The slide was dried by a brief centrifugation (1 min) at 1500 rpms (400*g*). Printed slides can be stored for at least 3 months in a solution of 3% BSA at 4 °C before probing. In ligand-binding experiments, separate subarrays on the slide were isolated by streaking with a hydrophobic pen (Pap Pen) and were then incubated with the fluorescent ligand, **EE_2-Cy3** alone (700 nM in 3% BSA solution at room temperature) or together with different concentrations of nonlabeled ligands. After incubation (1 h in humidity chamber, at room temperature), arrays were washed as noted above, dried, and scanned using a GenePix 4000 dual-laser at 535 and 635 nm with 33% power and PMT gain to give a signal slightly below saturation (Axon Instruments, Union City, CA), and the arrays were quantified using the GenePix Pro 4.0 software (Axon Instruments). Typically, the fluorescence intensity was considered to be the mean value of a constant diameter circle. Gain adjustments can usually be made so that the highest signal corresponds to ∼45 000 fluorescence intensity units (intensity per pixel); background intensity is typically 500 units, giving a 90:1 signal to background.
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