A Proteomic Microarray Approach for Exploring Ligand-initiated Nuclear Hormone Receptor Pharmacology, Receptor Selectivity, and Heterodimer Functionality*

Sung Hoon Kim, Anobel Tamrazi, Kathryn E. Carlson, and John A. Katzenellenbogen‡

Nuclear hormone receptors (NHRs) are major regulators of development and homeostasis in multiple organ systems. These proteins are ligand-modulated transcription factors that regulate gene expression in response to changes in circulating levels of their cognate hormones or hormone analogs. When NHRs bind ligands, they adopt distinct conformations that enable or disable the binding of coregulator proteins in a manner that reflects the agonist versus antagonist character of the ligand. Using the estrogen receptor ligand binding domain as a representative member of the NHR family, we show the development of functional protein microarrays and use them to explore coactivator recruitment and NHR homo- and heterodimer functionality. These NHR protein microarrays can be fabricated in either a forward mode (coactivator recruited to printed NHR) or a reversed mode (NHR recruited to printed coactivator). From these microarrays, we can predict the potency and pharmacological character of various NHR ligands through the nature of their coactivator recruitment. Additionally different coactivator proteins can be functionally classified and their affinity for NHRs can be quantified. NHR-selective antagonist ligands and small molecule coactivator mimics disrupt the coactivator-NHR complex. This novel proteomic approach was also used to assess coactivator recruitment to explore heterodimer functionality. Heterodimers of the estrogen receptor were found only to recruit coactivators when both monomers are bound with agonist ligands, an observation that provides an insight into the complex biology of hormones that act on tissues containing both NHR subtypes. We can extend this NHR proteomic approach to the analysis of multidomain full-length NHR constructs and can concurrently monitor the activation state of different classes of NHRs with a mixture of endogenous or synthetic ligands of varying NHR selectivity and pharmacology. Molecular & Cellular Proteomics 4: 267–277, 2005.

There is great interest in the complex pharmacology of agents that act through members of the nuclear hormone receptor (NHR) superfamily. The human genome project suggests that this superfamily consists of 48 different proteins, most of which are ligand-modulated transcription factors that regulate gene expression in response to changes in circulating levels of their cognate hormones or various hormone analogs (1). Alteration in activity of different NHRs leads to various pathological states from obesity and diabetes mellitus to various hormone-sensitive carcinomas. Although there are some NHRs whose activity is modulated independently of ligand binding (2), the majority of NHRs exist in a physiologically silent state when unliganded. After ligand binding, NHR transcriptional activity is mediated by receptor-ligand interaction with a series of coregulatory proteins, the most well defined of which are the p160 steroid receptor coactivators (SRC1, -2, and -3) (3, 4). The binding of agonists to NHRs stabilizes a conformation that engenders their interaction with the SRCs through the binding of specific nuclear receptor box domains on the coactivator (NR-boxes with the consensus sequence LXXLL) to a hydrophobic groove on the receptor; by contrast, the binding of antagonists stabilizes a different NHR conformation that disfavors interaction with NR-boxes and may instead lead to interaction with specific regions within corepressor proteins (5). The milieu of NHRs and the nature and levels of NHR subtypes and of coregulatory proteins are known to differ from tissue to tissue. In this way the circulating hormonal ligand of an NHR can initiate different physiological responses in different target tissues and cells.

This cascade of NHR responses is initiated by the ligand.

1 The abbreviations used are: NHR, nuclear hormone receptor; apo-ER, unliganded ER; CBI, coactivator binding inhibitor; DPN, di-arylpropionitrile; DRIP205, vitamin D receptor-interacting protein; E2, estradiol; ER, estrogen receptor; LBD, ligand binding domain; MPP, methylpiperidinopyrazole; NR-box, nuclear receptor box of coactivator proteins with the consensus sequence LXXLL; NRD, nuclear receptor domain region of coactivators that includes three NR-boxes for SRC1-NRD and SRC3-NRD and two NR-boxes for DRIP205; PPT, propylypyrazole triol; RBA, relative binding affinity; SRC, steroid receptor coactivator; T3, 3,5,3'-triiodo-1-thyronine; TOT, trans-4-hydroxytamoxifen; TR, thyroid hormone receptor; CARLA, coactivator-dependent receptor ligand assay; MTMR, tetramethylrhodamine-5-maleimide; MOPS, 3-(N-morpholino)propanesulfonic acid.
Therefore, it is easy to see the importance of fully characterizing each new ligand for the pharmacological response that it elicits from a particular NHR. Various methods developed to assay ligand-regulated coactivator binding to NRs can generically be called coactivator-dependent receptor ligand assays (CARLAs) (6). CARLAs provide information not just on ligand binding but also enable prediction of the pharmacological nature of the ligand (i.e., agonist versus antagonist) based on its ability to recruit coactivators, information that is typically obtained by cell-based assays in which the transcription of endogenous or reporter genes under the control of a specific NHR is measured. However, the result can vary with different cell types and response elements. Current genetic and proteomic approaches focus mostly on ligand pharmacology for a specific NHR; they lack the global proteomic high throughput versatility to analyze in a single setting the pharmacological effects of various endogenous, synthetic, or environmental ligands on different members of NRs.

In this report, we develop a NHR CARLA in a protein microarray format on glass slides. We exemplify these assays using both subtypes of the estrogen receptor (ER), ERα and ERβ (7), which are important regulators of estrogen action in both men and women (8), as well as the thyroid hormone receptor (TR), a key mediator of metabolic activity. We can accurately monitor the ability of various ligands to increase or decrease the activation state of ER homo- and/or heterodimer complex with different coactivators; these results are in excellent agreement with the more cumbersome assays (CARLAs) (6). CARLAs provide information not just on ligand binding but also enable prediction of the pharmacological response that it elicits from a particular NHR.

### Experimental Procedures

**Materials**—Compounds and materials were obtained from the sources indicated: cyanine dyes (Cy3 dye-maleimide and Cy5 dye-maleimide) (Amersham Biosciences) and tetramethylrhodamine-5-maleimide (MTMR) (Molecular Probes, Eugene, OR). Estradiol, estriol, trans-4-hydroxytamoxifen, genistein, diethylstilbestrol, isopropyl β-D-thiogalactopyranoside, imidazole, 30% BSA solution, MOPS, Tris(carboxyethyl)phosphine, and β-mercaptoethanol (Sigma). The pET-15b vector and competent BL21(DE3)pLysS Escherichia coli were obtained from (Novagen, Madison, WI). ICI 182,780 was from (Tocris Cookson, Baldwin, MO). Raloxifene, cyclofenil, propylpyrazole triol (PPT), methylpyderidinopryrazole (MPP), diarylpiponitirile (DPN), and coactivator binding inhibitor were synthesized in our laboratory. Nickel-nitrotriacetic acid-agarose was from Qiagen Inc. (Valencia, CA); SpotBot Microarray Robot, the microarray 384-well microplate, and SuperAldehyde microscope slides were from TeleChem International, Inc. (Sunnyvale, CA); the PAP pen was from Zymed Laboratories Inc.; full-length, purified human ERα and ERβ were from PanVera/Invitrogen (Madison, WI); and TR-LBD was a kind gift from Dr. James W. Apriletti (Metabolic Research Unit, University of California, San Francisco, CA). Data were collected using a laser scanner and GenePix Pro 4.0 program from Axon Instruments (Union City, CA) and analyzed with the GenePix Pro 4.0 and Prism 4.0 GraphPad software (San Diego, CA). Mass spectrometry was done by the personnel in the Mass Spectrometry Laboratory of the School of Chemical Sciences on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA).

**Protein Preparations**—The LBD of the human ERα (amino acids 304–554) and the corresponding LBD of the human ERβ (amino acids 256–505) were expressed from a pET-15b vector in BL21(DE3)pLysS E. coli using methods reported previously (9). ER-LBD mutants with a single reactive cysteine were constructed as previously reported (10). Constructs of p160 SRC1 (amino acids 629–831 encompassing NRD boxes 1–3), SRC3 (amino acids 627–829 encompassing NRD boxes 1–3), and vitamin D receptor-interacting protein (DRIP205) (amino acids 527–714) were prepared in a pET-15b vector by Arvin Gee (SRC1 (11)) and Ramji Rajendran (SRC3, DRIP205) and expressed in E. coli. All protein constructs were His6-tagged at the N terminus to allow purification and labeling while the protein was immobilized on a nickel resin (10). The proteins were purified to near homogeneity, as determined by SDS-PAGE, using a procedure modified from the Novagen PET Systems Manual and conducted as reported previously (9, 10). The ER-LBD mutant constructs were labeled, in a site-specific manner at a single cysteine with one fluorophore per protein, as reported previously (10). The ERs were assayed for binding activity with [3H]E2 and for coactivator recruitment by a fluorescence polarization experiment in solution. The SRC1 construct containing NRD boxes 1–3 (residues 629–831, termed SRC1-NRD) has 5 cysteines (11), and the corresponding SRC3 peptide (NR-boxes 1–3, residues 627–829, termed SRC3-NRD) has 4 cysteines. The SRC proteins were labeled in the same manner, and the extent of the labeling (~2 fluorophores/protein) was determined as described below.

**SRC-Fluorophore Characterization**—The SRC-NRD peptides have multiple cysteines and were not site-specifically labeled. The SRC1 construct has 5 cysteines, and the SRC3 peptide has 4 cysteines. The extent of the fluorophore labeling was determined by mass spectrometry on a Voyager-DE STR MALDI-TOF mass spectrometer. While the unlabeled peptides gave very nice spectra, the labeled peptides gave a low signal. As another way to determine the amount of labeling, the peptides were diluted into 4% Na2CO3 in 0.1 N NaOH with 0.02% CuSO4 and 0.04% sodium tartrate (Reagent C for the Lowry protein determination) (12). The absorbance was measured at 554 nm for Cy3-labeled peptides and at 656 nm for Cy5. The absorbance was compared with a standard curve created with the singly labeled ER-LBD proteins. The absorbance of Cy3 was 0.0937/μmol of ER, and for Cy5 at 656 nm it was 0.0808/μmol of ER. The Folin reagent was then added to the same protein solution, and the absorbance of the Lowry protein determination was measured at 750 nm. Full absorbance scans showed no overlap of the fluorophore absorbance at 750 nm. However, the absorbance of the fluorophores overlapped into the ranges of other modern protein assays, such as the Bradford assay (13).

**Printing on Slides**—The proteins were printed, as 1-nl spots, on SuperAldehyde slides as reported previously (14). Briefly the proteins were prepared in 50 mM MOPS, pH 8.0, 40% glycerol, and spotted by the SpotBot Microarray Robot using SMPS pins. The humidity in the
We have taken two approaches in constructing the NHR protein microarrays: (a) they can be fabricated in a “forward” manner (print receptor-recruit fluorophore-labeled coactivator) or (b) in a “reversed” manner (print coactivator-recruit fluorophore-labeled receptor) (Fig. 1). The NHR activation was followed by quantifying recruitment of the fluorescent partner to submicroarrays (quadruplicate spots isolated as subarrays by barriers drawn with a hydrophobic pen) prepared from unlabeled NHR in the forward or from unlabeled coactivator in the reversed microarray format. Following blocking excess reactive aldehyde sites on the glass slide with BSA, each submicroarray was incubated with ligand plus fluorophore-labeled coactivator in the forward or fluorophore-labeled ER-LBD in the reversed microarray format. Following incubation, they were washed, dried, and scanned (see “Experimental Procedures” for details). In all experiments, the NHRs were printed in the apo state and allowed to bind to the slide before introducing ligand to prevent the selective attachment to the slide seen with preliganded NHRs (14). In both the forward and reversed NHR protein microarrays, the fluorescence “background,” that is the fluorescence intensity noted outside of the array spots, reflects nonspecific binding of the fluorophore-labeled protein component (SRC in forward arrays and ER-LBD in reversed arrays). Interestingly background in reversed arrays was 10-fold lower, indicating that the fluorescent ER-LBDs show less nonspecific binding to the BSA-blocked glass slides than fluorescent SRC-NRDs. This difference likely reflects the fact that the LBDs are known to be tightly folded, whereas the SRC-NRDs are thought to be unstructured until they bind to the NHRs.

**NHR Microarrays Can Predict Ligand Biocharacter Along with the Potency and Level of Coactivator Binding**—Using forward microarrays prepared with both ERα-LBD and ERβ-LBD homodimers, we can predict the biocharacter (pharmacological nature) of subtype-nonselective estrogen agonists

---

**RESULTS**

**Forward and Reversed NHR Protein Microarrays**—LBDs of ERα and ERβ, engineered to have only 1 reactive cysteine, were expressed in *E. coli*, purified, and fluorophore-labeled in a site-specific manner as reported previously (10, 15). We have shown that these receptors maintain near-native binding affinity for estradiol (E2) and the ability to recruit coactivator proteins (10, 15). NRDs (protein fragments with three NRD boxes) from SRC1 and -3 were similarly expressed and fluorophore-labeled (see “Experimental Procedures”). The SRC-NRDs have 4 (SRC3) or 5 (SRC1) cysteines per peptide. Of these, an average of 1.8–2 were labeled with the cysteine-reactive fluorophores as determined by both MALDI-MS and the fluorophore absorbance/Lowry protein assay (see “Experimental Procedures,” data not shown). The sites of labeling on these coactivator constructs were not identified. The ER-LBD constructs were site-specifically labeled with a 1:1 fluorophore:protein stoichiometry at their corresponding single reactive cysteine residues (10). We have also used, without labeling, purified full-length ERα and ERβ from PanVera and the LBD of TR as other examples of NHR proteins.

Previously we have shown that ER-LBDs, when spotted as protein arrays on aldehyde slides, retain their ability to bind ligands as measured with an estrogen-fluoresphore conjugate ligand, EE2-Cy3 (14). We now show that protein microarrays can be used to quantitate coactivator recruitment, operating in a manner that replicates ER subtype ligand binding affinity and transactivation selectivity with high fidelity. These microarrays can be used, quantitatively and conveniently, to predict the potency and pharmacological character of various ligands through coactivator recruitment and to assess the affinity of the ER-LBD/NHR interaction.
or antagonists (Fig. 2). A list of ligands with their relative binding affinities (RBAs, compared with estradiol set at 100%) and their known biocharacters as determined through ER transactivation studies is listed in Table I. Submicroarrays containing both ERα/H9251 and ERβ/H9252 homodimers incubated with agonists (E2, DES, and estriol) were able to recruit fluorescently labeled SRC1-NRD, whereas those incubated with antagonists (TOT, raloxifene, and ICI 182,780) did not recruit coactivator (Fig. 2). Thus, we can simultaneously, on a single submicroarray panel, follow the ability of a natural or synthetic ligand to activate or deactivate several NHRs. Recruitment was specific because ER denatured by heating (80 °C for 5 min) failed to recruit coactivator proteins, and excess unlabeled SRC-NRD blocked recruitment of the labeled coactivator.

**TABLE I**

**Relative binding affinity and biocharacter of ligands for estrogen receptors α and β**

The RBA was determined by a competitive radiometric binding assay with [3H]estradiol and either the LBD or full-length constructs of human ERα and ERβ. The RBA of estradiol is 100%. Values represent the average of two or more independent determinations. The biocharacter was determined by transcription activation assays with expression vectors for human ERα and ERβ in endometrial cancer (HEC-1) cells and the estrogen-responsive gene 2ERE-pS2-Luc or by estrogenic responses following injection into immature rats.

| Ligand          | RBA (ERα) | RBA (ERβ) | Biocharacter (ERα) | Biocharacter (ERβ) |
|-----------------|-----------|-----------|--------------------|--------------------|
| **Agonist**     |           |           |                    |                    |
| E2              | 100       | 100       | Full agonist       | Full agonist       |
| Diethylstilbestrola | 219     | 278       | Full agonist       | Full agonist       |
| Estriolb        | 7.1       | 12        | Full agonist       | Full agonist       |
| **Subtype-selective** | | | | |
| Genisteinc      | 0.013     | 7.4       | Full agonist       | Full agonist       |
| PPTd            | 89        | 0.15      | Full agonist       | No activity        |
| MPPe            | 11        | 0.05      | Full antagonist    | No activity        |
| DPNf            | 0.25      | 18        | Weak antagonist    | Agonist            |
| **Antagonist**  |           |           |                    |                    |
| TOTg            | 144       | 62        | Partial antagonist | Partial antagonist |
| Raloxifeneh     | 41        | 3.5       | Full antagonist    | Full antagonist    |
| ICI 182,780i    | 79        | 25        | Full antagonist    | Full antagonist    |

a Ref. 46.
b Refs. 47 and 48.
c Ref. 42.
d Ref. 44.
e Ref. 45.
f Ref. 43.
g Ref. 49.
tor (data not shown); results with SRC3 were similar (data not shown). We find a low level of SRC recruitment to the apo-ERs, especially to ER\textsubscript{H252}, as noted by others (16, 17), consistent with the higher basal transcriptional activity of ER\textsubscript{H252} (18). Notably antagonists reduce coactivator recruitment to ER\textsubscript{H252} to levels below that of apo-ER\textsubscript{H252} (Fig. 2).

In reversed arrays, receptor titrations can be used to measure the coactivator binding potency and efficacy to specific subtypes of ER-LBD homodimers (Fig. 3). The fluorophore is attached to the ER-LBD; thus, differences in the absolute fluorescence intensity of NHR-coactivator complexes highlight varying efficacy of recruitment to the coactivator in the presence of estradiol. This reversed microarray approach simultaneously, on a single submicroarray panel, monitors the binding characteristics of a particular NHR for various coactivator proteins. SRC3 had potency (K\textsubscript{d} = 7.9 nM) and efficacy preference for ER\textsubscript{\alpha}, whereas SRC1 is recruited to ER\beta with a slightly higher efficacy and potency (6.5 nM) than was SRC3 (10.5 nM). The potencies (the K\textsubscript{d} values) obtained for these p160 coactivator proteins are comparable to those measured in solution (19, 20).

Monitoring the Selective Ligand-regulated Activation of Different NHR Subtypes—When coactivator microarray recruitment is done in a reversed microarray format as a ligand titration with fixed ER and SRC-NRD concentrations, we can characterize the pharmacology and potency of various ER ligands to activate or deactivate a particular subtype of the ER-LBD (Fig. 4). The potency, ER\textsubscript{\alpha} versus ER\beta selectivity, and pharmacological character of E\textsubscript{2} (subtype-nonselective agonist); the synthetic ligands PPT (ER\textsubscript{\alpha}-selective agonist), MPP (ER\textsubscript{\beta}-selective antagonist), and DPN (ER\beta-selective agonist); and the natural ER\beta-selective phytoestrogen genistein determined by coactivator recruitment on reversed recruitment microarrays (Fig. 4, right) agree well with the data from cell-based transactivation assays (Fig. 4, left; see Table I for information on ligands). The subtype-selective agonist ligands recruit principally one ER subtype, consistent with their selective affinities (RBAs, see Table I), the ER\textsubscript{\alpha}-selective agonist PPT recruits more ER\textsubscript{\alpha}, and the ER\beta agonists DPN and genistein recruit more ER\beta; E\textsubscript{2} shows essentially no subtype selectivity in recruitment. In the antagonist mode, where the subtype-selective ligand is titrated against 10 nM E\textsubscript{2}, the ER\textsubscript{\alpha}-selective antagonist MPP reversed the recruitment of ER\textsubscript{\alpha} but had little effect on ER\beta recruitment. Thus, these ligand concentration-dependent coactivator recruitment assays, done in a reversed microarray format, provide information that is remarkably equivalent to that obtained by genomic cell-based reporter gene transcription approaches.

NHR Microarrays to Study the Effectiveness of Coactivator Binding Inhibitors (CBIs) for Disrupting NHR-Coactivator Interactions—Elsewhere we have described the synthesis of small molecules that act as CBIs by binding directly with the hydrophobic groove in agonist-ligated ER\textsubscript{\alpha}-LBD (19). A series of triazine and pyrimidine CBI analogs were prepared and
close to that measured by fluorescence polarization (29 μM) (19). A close structural analog of this CBI with a triazine core in place of the pyrimidine was shown previously to be ~20 times weaker in its interaction with the ERα-estriadiol complex (19). It was unable to compete the SRCs but could effectively compete the lower affinity DRIP205 (Fig. 5) (19, 20).

**Dual Color Microarray Analysis to Explore NHR Heterodimer Functionality**—A number of NHRs function as heterodimers with another NHR (as is the case of the peroxisome proliferator-activated receptors and the retinoic acid receptors that heterodimerize with retinoid X receptor) or as heterodimers with another subtype of the same NHR (as is the case of ERα and ERβ). The biological consequences of homo- versus heterodimer activation of NHRs remain to be elucidated mainly due to the technical limitations of specifically evaluating the degree of NHR heterodimer activation within a complex mixture containing both receptors. An unanswered issue in ER-coactivator interaction is under what conditions ERα/ERβ heterodimers are capable of coactivator recruitment. A particular advantage of the reversed NHR microarray approach is that several NHRs labeled with different fluorophores (Cy3 or Cy5) can be used to simultaneously and selectively decipher the activation state of homo- and heterodimer populations of those NHRs.

As shown in Fig. 6, we have used a two-color analysis approach to quantify the relative level of fluorescent ERα-LBD (red) versus ERβ-LBD (green) recruitment to a reversed array of SRC1 in the presence of either E2 or the subtype-selective agonist ligands PPT (ERα-selective) or DPN (ERβ-selective). With E2, both ER subtypes are recruited as shown by the combination of green/red (yellow), whereas predominantly ERα is recruited with PPT (red) and ERβ (green) with DPN. The homo-versus heterodimer recruitment reflects the relative subtype selectivity of the two ligands, and it suggests that ERα/β heterodimers are ineffective in coactivator interaction when only one monomer is occupied by an ER agonist and that only ER dimers that are doubly liganded with agonists are competent to interact with coactivators.

Application of Forward Microarrays to Study the Pharmacology of NHRs with Multiple Domains or NHRs Belonging to Different Classes—NHRs are multidomain receptors containing domains A–F with the C domain comprising the DNA binding regions and the E domain containing the LBD (23). While we used the E domain (LBD) of both ER subtypes in most of our NHR microarrays, we were interested in using full-length ERs that contain the A–F functional regions of the receptor, including the activation function 1 region within the A/B domain of the receptor that may have a synergistic role in coactivator recruitment (24).

We found that baculovirus-expressed and purified full-length ERα and ERβ work well in forward microarrays, and we can quantify their state of activation through ligand-dependent coactivator recruitment (Fig. 7). Due to the lower concentration of the baculovirus-expressed full-length ERs, which

![Image](https://example.com/image.png)

**Fig. 4.** Genomic and proteomic approaches to characterize NHR subtype-selective ligands. Left column, dose-response curves from reporter gene cell-based transfection assays are from the following sources: genistein (42), DPN (43), PPT (44), and MPP (45). Right column, reversed arrays of SRC1-NRD were used to recruit ERα-LBD-Cy3 and ERβ-LBD-Cy5 in a ligand-dependent fashion. The ligands were added alone for agonist activity (E2, genistein, DPN, and PPT) or along with 10 nm E2 for antagonist activity (MPP). Values represent the mean ± S.E. of four replicates.
were purchased from a commercial source (PanVera), we hand-printed microliter volumes of full-length ERs (versus nanoliter volumes for the LBD) to fabricate the reversed microarrays. Nonetheless we found that the full-length ER subtypes behave much like the corresponding LBDs with agonist ligands increasing and antagonist ligands decreasing the activation state of the receptor compared with apo-ERs (Fig. 7). Once again, the apo-ER\textsubscript{H9251} shows a higher basal coactivator recruitment compared with apo-ER\textsubscript{H9252} in agreement with previously reported results from ELISA-based CARLAs (17).

We can simultaneously monitor the activation state of several NHRs belonging to different classes and do so with a mixture of ligands exhibiting selective binding affinities and pharmacological characters for either NHR. We prepared forward microarrays containing both ER\textsubscript{\alpha}-LBD and TR-LBD and screened the selective activation of these NHRs in the presence of their cognate agonist ligands (E\textsubscript{2} for ER and T\textsubscript{3} for TR) and an estrogen antagonist (TOT) (Fig. 8). There is low but measurable recruitment of fluorescent SRC1 to both apo-ER\textsubscript{\alpha}-LBD and apo-TR-LBD. In the presence of E\textsubscript{2}, however, SRC1 recruitment to ER\textsubscript{\alpha}\textsubscript{H9251}-LBD is enhanced and that to TR is actually decreased. By contrast, there is a selective marked increase in coactivator recruitment to TR in the presence of T\textsubscript{3}. Notably our results show that TOT only competes with E\textsubscript{2} recruitment of SRC1 to both apo-ER\textsubscript{\alpha}-LBD and apo-TR-LBD. In the presence of E\textsubscript{2}, however, SRC1 recruitment to ER\textsubscript{\alpha}-LBD is enhanced and that to TR is actually decreased. By contrast, there is a selective marked increase in coactivator recruitment to TR in the presence of T\textsubscript{3}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{NHR microarrays provide information on binding of CBIs. A reversed array showing the competition of ER\textsubscript{\alpha}-LBD binding to SRC1, SRC3, and DRIP205 by CBIs is shown. Representative members of two classes of CBIs were added over a range of concentrations. Left, the pyrimidine CBI is effective in blocking the coactivator-ER interaction. Right, the triazine CBI was much less effective as a competitor for the SRCs but was able to compete with the lower affinity DRIP205. Values represent the mean ± S.E. of four replicates.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Competitive recruitment of ER\textsubscript{\alpha} and ER\textsubscript{\beta} homodimers and heterodimers to SRC1 arrays. Recruitment of a mixture of ER\textsubscript{\alpha}-Cy5 (red) and ER\textsubscript{\beta}-MTMR (green) to reversed arrays of SRC1-NRD with E\textsubscript{2}, PPT (ER\textsubscript{\alpha}-selective), and DPN (ER\textsubscript{\beta}-selective) is shown. The non-selective ligand E\textsubscript{2} results in recruitment of equivalent amounts of both ER\textsubscript{\alpha} and ER\textsubscript{\beta}, resulting in yellow spots (red plus green), whereas the subtype-selective ligands effect recruitment essentially only to their preferred ER subtype (PPT, ER\textsubscript{\alpha} (red); DPN, ER\textsubscript{\beta} (green)), indicating that the ER\textsubscript{\alpha}/ER\textsubscript{\beta} heterodimers, presumed to be singly occupied with these ligands, are not effectively recruited. Values represent the mean ± S.D.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{Coactivator recruitment with full-length ER forward microarrays. Baculovirus-expressed and purified full-length ER\textsubscript{\alpha} and ER\textsubscript{\beta} were hand-printed on aldehyde slides, blocked with BSA, and probed with a 1.2 nM solution of SRC1-Cy3 and 350 nM E\textsubscript{2}, TOT, or vehicle.}
\end{figure}

\begin{thebibliography}{9}
\item
2. S. C. Lina and J. Baxter, personal communication.
\item
3. K. E. Carlson and J. A. Katzenellenbogen, unpublished.
\end{thebibliography}
NHRs are major regulators of development and homeostasis whose genomic function derives from their role as ligand-modulated transcription factors. These receptors are known to be engaged in four elemental molecular interactions: 1) the formation of NHR homo- or heterodimers, 2) the binding to DNA response elements through the DNA binding domain of the NHRs, 3) the binding of ligands to the LBD of the NHRs, and 4) the interaction of the receptor with coregulator proteins, which is a ligand-regulated process. Various in vitro radiometric and fluorometric methods have been devised to measure these molecular interactions with the interaction of receptor with a coactivator protein serving as a molecular proteomic marker of NHR activation that can be used to characterize ligand pharmacology. The current assays to monitor NHR molecular interactions vary widely in their sensitivity, convenience, and degree of quantification.

Through earlier work, we showed that we could print NHR-LBDs, both ERα and ERβ, on a standard aldehyde slide and demonstrate that the proteins retain their ability to bind ligand, the third molecular interaction listed above (14). In this report, we describe the use of protein microarray methods to study the fourth molecular interaction, the ligand-modulated interaction of NHRs with coactivator proteins, exemplifying this interaction with the LBD and full-length versions of the two estrogen receptor subtypes, ERα and ERβ, in addition to the thyroid hormone receptor LBD. The NHR microarrays can be developed in a forward or in a reversed manner (Fig. 1); both methods are equally effective for showing NHR-coactivator interactions, although in practice lower background was found in the reversed array paradigm (see Figs. 2, 7, and 8 as examples of forward and Figs. 3–6 as examples of reversed NHR microarrays).

The pharmacological nature of a synthetic, endogenous, or environmental NHR ligand can be accurately predicted based on the ability of the ligand to promote (agonists) or inhibit (antagonists) coactivator recruitment to that ligand-NHR complex. Our NHR microarray results with the thyroid receptor LBD and both subtypes of estrogen receptor strictly follow the pharmacological character of the ligands used (Figs. 2, 7, and 8 and Table I) and thus can be predictive functional parameters of NHR activation with novel or unknown ligands.

We have monitored the interaction of both ER-LBD subtypes with the nuclear receptor p160 steroid receptor coactivators SRC1 and SRC3 in a sensitive, convenient, and remarkably quantifiable manner (Figs. 2 and 3). The potency and pharmacological nature of subtype-selective ER ligands that we have developed can also be followed in a quantitative fashion (Fig. 4). Our NHR microarray results are strikingly similar to those from cell-based transactivation assays (Fig. 4), demonstrating the power of this proteomic microarray approach in following the molecular interaction that highlights the activation state of NHRs.

The manner in which various ligands affect the transactivation of the NHR-regulated genes is highly dependent on the cellular milieu of coregulator proteins, both coactivators and corepressors (3). The tissue-specific expression of the p160 coactivator proteins (SRCs) is thought to be especially important in modulating the array of physiological consequences of NHR activation through specific agonist ligands. Using a reversed NHR microarray approach, we were able to quantitate the potency and level of SRC1 and SRC3 binding to both subtypes of estrogen receptor LBDs in the presence of their cognate agonist ligand, estradiol (Fig. 3). We found a marked SRC3 efficacy preference for the E2/ERα-LBD complex that was not evident for the E2/ERβ-LBD subtype of this receptor (Fig. 3). It is of note that tissue-specific overexpression of SRC3, also known as Amplified in Breast Cancer 1, has been associated with the onset of tamoxifen resistance in breast cancer therapy (26), while the overexpression of SRC1 is thought to determine the agonistic versus antagonistic nature of tamoxifen action in certain tissues, such as the breast and the uterus (27). Thus, categorizing, at the molecular level, the binding characteristics of specific coactivators to a ligand-NHR complex could shed light onto the complex NHR-mediated signaling pathways that may be regulated by the cellular concentrations of coactivator protein.

Pharmacological intervention into NHR signaling is often implemented through the use of selective NHR antagonist ligands (28, 29), which are used in the clinical setting for the treatment of carcinomas in both men and women. The identification and characterization of novel synthetic NHR antagonists is a critical component in the discovery of new therapeutical treatments for a number of pathological states. At the molecular proteomic level, the disruption of the agonist-NHR-coactivator tripartite complex is a marker of a compound with antagonist activity for a particular NHR (19, 30). A small molecule could implement its antagonist activity for an NHR by competing at two distinct sites: (a) competing with the agonist ligand for the LBD of the receptor (i.e. an antagonist ligand) and/or (b) competing with the coactivator protein for the coactivator binding groove on the receptor (i.e. a CBI) (19, 31,

**DISCUSSION**

The manner in which various ligands affect the transactivation of the NHR-regulated genes is highly dependent on the cellular milieu of coregulator proteins, both coactivators and corepressors (3). The tissue-specific expression of the p160 coactivator proteins (SRCs) is thought to be especially important in modulating the array of physiological consequences of NHR activation through specific agonist ligands. Using a reversed NHR microarray approach, we were able to quantitate the potency and level of SRC1 and SRC3 binding to both subtypes of estrogen receptor LBDs in the presence of their cognate agonist ligand, estradiol (Fig. 3). We found a marked SRC3 efficacy preference for the E2/ERα-LBD complex that was not evident for the E2/ERβ-LBD subtype of this receptor (Fig. 3). It is of note that tissue-specific overexpression of SRC3, also known as Amplified in Breast Cancer 1, has been associated with the onset of tamoxifen resistance in breast cancer therapy (26), while the overexpression of SRC1 is thought to determine the agonistic versus antagonistic nature of tamoxifen action in certain tissues, such as the breast and the uterus (27). Thus, categorizing, at the molecular level, the binding characteristics of specific coactivators to a ligand-NHR complex could shed light onto the complex NHR-mediated signaling pathways that may be regulated by the cellular concentrations of coactivator protein.

Pharmacological intervention into NHR signaling is often implemented through the use of selective NHR antagonist ligands (28, 29), which are used in the clinical setting for the treatment of carcinomas in both men and women. The identification and characterization of novel synthetic NHR antagonists is a critical component in the discovery of new therapeutical treatments for a number of pathological states. At the molecular proteomic level, the disruption of the agonist-NHR-coactivator tripartite complex is a marker of a compound with antagonist activity for a particular NHR (19, 30). A small molecule could implement its antagonist activity for an NHR by competing at two distinct sites: (a) competing with the agonist ligand for the LBD of the receptor (i.e. an antagonist ligand) and/or (b) competing with the coactivator protein for the coactivator binding groove on the receptor (i.e. a CBI) (19, 31,
heterodimerization the presence of ER

Additionally the NHR microarrays can be used to highlight novel chemical entities with CBI activity, which compete with the coactivator at a subsequent step after agonist ligand binding to the NHR and can successfully inhibit coactivator recruitment to an agonist-NHR complex in the presence of high agonist ligand concentrations (Fig. 5). We monitored the coactivator specificity of our pyrimidine and triazine CBIs on reversed microarrays composed of two distinct classes of coactivator proteins, the SRCs and DRIP205 (Fig. 5) (19). DRIP205 belongs to a functionally distinct class of coactivators (21, 22, 33, 34). Unlike the p160 coactivators that have intrinsic histone acetyltransferase activity, DRIP205 belongs to the mediator-like class of coactivators that are believed to interact with the basal transcription machinery (35). While the pyrimidine CBI is more potent and can successfully compete with both classes of coactivator proteins, the triazine CBI shows selective inhibition of the DRIP205 coactivator and is ineffective in competing with the p160 family of coactivators (Fig. 5). A potential limitation of CBIs might be their lack of specificity for a particular NHR or coactivator protein that could lead to a myriad of side effects due to inhibition of several NHR activities. Thus, the identification of CBIs that compete with specific classes of coactivator proteins through NHR microarrays could lead to pharmaceutical leads as second line NHR antagonist agents.

An additional complexity of NHR signaling is the mixture of physiological effects observed from homo- versus heterodimer NHR signaling. The estrogen receptor provides a well-characterized example of the differential biological effects observed in the presence of both ER subtypes, which are capable of homo- and heterodimerization (7, 36, 37). Cell-based transactivation studies have shown that ERα is a more potent transcription factor than ERβ and that through heterodimerization the presence of ERβ diminished the transcriptional output of ERα (37, 38). This ERβ reduction of ERα-mediated transactivation has been described through in vivo studies in mice as a “Yin Yang” relationship of estrogen signaling (39). Furthermore homodimer signaling of ERα and that of ERβ have opposing effects at an activator protein 1 site upstream of ER regulated genes (40), while the effects of heterodimer signaling at such sites remain to be elucidated. Using a dual color reversed ER microarray approach, we found that when both ER subtypes are occupied with an agonist ligand (estradiol) they are functionally active and are capable of coactivator interaction, presumably as both homo- and heterodimers (Fig. 6). With subtype-selective ligands, however, only the homodimers of the agonist-bound ER subtype (ERβ with DNP and ERα with PPT) are functional in the sense of being capable of coactivator interaction (Fig. 6). To our knowledge, this is the first reported evidence that partially agonist-occupied ER heterodimers are functionally inactive in terms of coactivator recruitment. This finding suggests that the opposing gene expression patterns and/or the Yin Yang signaling effects of ERα and ERβ could be more effectively separated through the use of subtype-selective ligands. A recent report of heterodimers of the retinoid receptors shows a similar result: each monomer of a heterodimer complex must be occupied by an agonist for full-length SRC1 to bind to the retinoid receptors (41).

In addition to LBDs of NHRs, we can also use full-length ERα and ERβ in our NHR microarrays to explore the biology of various domains of these NHRs (Fig. 7). Protein microarrays composed of full-length NHR constructs can be used to study the effects of receptor posttranslational modifications and NHR mutations associated with certain pathological states on coregulator recruitment in a highly efficient and high throughput manner. Furthermore we can concurrently monitor single or multiple ligand effects on the activation state of NHR of different classes, such as ER and TR (Fig. 8). Such proteome profiling of NHR function can be used to identify endocrine disruptors that target specific NHRs or to discover cognate ligands for orphan NHRs.

CONCLUSIONS

Nuclear hormone receptor-coactivator recruitment was studied in a protein microarray format. As exemplified here with the NHRs ERα, ERβ, TR, and their coregulator partners SRC1, SRC3, and DRIP205, the protein microarrays provide encouraging examples of the preservation of protein function in terms of ligand-regulated receptor-coactivator interaction. They display excellent retention of subtype-selective ligand potency and efficacy through receptor-coactivator recruitment along with the potential to quantify receptor-coactivator affinity. Using this technology, we showed that both monomers of an ER heterodimer must be bound with an agonist ligand to recruit coactivators. These results were obtained using relatively simple surface chemistry involving commercially available slides and purified proteins. Given the increasing sophistication of methods for the fabrication of protein microarrays, it appears likely that high throughput NHR and coregulator protein microarrays will prove useful in the discovery and quantitative analysis of NHR ligands and the analysis of receptor-coregulator interactions.

Acknowledgments—We are grateful to Dr. Mark Band of the Keck Center for Comparative and Functional Genomics, Biotechnology Center at the University of Illinois, for helpful advice and assistance with the imaging and to Jun Sun, Shubin Sheng, and Benita Katzenellenbogen for transcription assays.

* This work was supported by National Institutes of Health Grant PHS 5R37 DK15556. The Voyager-DE STR mass spectrometer was purchased in part with Division of Research Resources, National Institutes of Health Grant RR 11966. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
43. Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2001) Estrogen receptor-β potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J. Med. Chem. 44, 4230–4251

44. Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-α-selective agonists. J. Med. Chem. 43, 4934–4947

45. Sun, J., Huang, Y. R., Harrington, W. R., Sheng, S., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (2002) Antagonists selective for estrogen receptor α. Endocrinology 143, 941–947

46. Katzenellenbogen, B. S., Iwamoto, H. S., Heiman, D. F., Lan, N. C., and Katzenellenbogen, J. A. (1978) Stilbestrols and stilbestrol derivatives: estrogenic potency and temporal relationships between estrogen receptor binding and uterine growth. Mol. Cell. Endocrinol. 10, 103–113

47. Katzenellenbogen, B. S., Bhakoo, H. S., Ferguson, E. R., Lan, N. C., Tate, T., Tsai, T. S., and Katzenellenbogen, J. A. (1979) Estrogen and antiestrogen action in reproductive tissues and tumor. Recent Prog. Horm. Res. 35, 259–300

48. Katzenellenbogen, B. S., Montano, M. M., Ediger, T. R., Sun, J., Ekena, K., Lazennec, G., Martini, P. G., McInerney, E. M., Delage-Mouroux, R., Weis, K., and Katzenellenbogen, J. A. (2000) Estrogen receptors: selective ligands, partners, and distinctive pharmacology. Recent Prog. Horm. Res. 55, 163–195

49. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95, 927–937