Fluorescent Cellular Sensors of Steroid Receptor Ligands

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Steroid hormone receptors comprise a major class of therapeutic drug targets that control gene expression by binding steroid hormone ligands. These small molecule – protein interactions are typically characterized in living cells by quantification of ligand-mediated reporter gene expression. As an alternative, non-transcriptional approach, we constructed fluorescent cellular sensors by expressing yellow fluorescent protein (YFP) fused to the ligand binding domains (LBDs) of estrogen receptor-alpha (ERα), estrogen receptor-beta (ERβ), androgen receptor (AR), and the glucocorticoid receptor (GR). These proteins were tethered through a short two amino acid linker and expressed in S. cerevisiae *yeast. Recombinant yeast treated with cognate steroid receptor ligands exhibited dose-dependent fluorescence enhancements that were correlated with known relative receptor binding affinity values. These effects generally paralleled ligand-mediated receptor dimerization quantified with analogous yeast two-hybrid transcriptional assays, suggesting that the majority of the observed fluorescence enhancements were conferred by conformational changes coupled with receptor dimerization, such as ligand-mediated stabilization of protein folding. Remarkably, certain interactions such as the binding of cortisol, progesterone, and dexamethasone to the GR were undetectable with yeast two-hybrid assays. However, these interactions were detected with the fluorescent cellular sensors, indicating the sensitivity of this system to subtle ligand-induced conformational effects. These sensors provide a novel, non-transcriptional, and high-throughput method to identify and analyze ligands of nuclear hormone receptors.*

KEYWORDS:

biological activity · biosensors · green fluorescent protein · high-throughput screening · medicinal chemistry

Introduction

Specific interactions between small molecules and proteins control numerous biological processes and provide a basis for the pharmacological treatment of disease. A typical consequence of molecular recognition between small molecules and enzyme active sites or protein ligand binding domains (LBDs) is alteration of protein conformational states. Conformational effects resulting from binding of ligands to steroid hormone receptors such as the estrogen, androgen, and glucocorticoid receptors promote dissociation of bound heat shock proteins, receptor homodimerization, and interactions with components of the transcriptional machinery.[1–3] Ligand binding can also affect the stability of cellular proteins. These effects can result from alterations of the volume of the hydrophobic protein core or from changes in secondary, tertiary, or quaternary protein structure.

Protein structure and stability can be affected by small molecules of diverse structure. These compounds include enzyme substrates, enzyme inhibitors, receptor agonists, and receptor antagonists.[4–6] Because ligand binding affects the conformations of steroid receptor LBDs, the activity of proteins fused to these LBDs can often be controlled by steroid receptor ligands.[7] For example, ligand-mediated protein stabilization was recently employed in yeast assays to couple cellular growth to ligand binding of estrogen receptor proteins fused to the essential metabolic enzyme dihydrofolate reductase.[5] Spectral variants of the green fluorescent protein (GFP) from *Aequorea victoria*[8] have also been fused to steroid hormone receptors in order to detect ligand-mediated interactions with coactivator proteins by use of fluorescence resonance energy transfer (FRET).[9–11] Insertion of proteins into loops on the surface of GFP has yielded related biosensors that detect protein oligomerization and calcium – calmodulin interactions.[12–14] GFP can also function as a fluorescent reporter that is sensitive to the folded state of fusion partners.[15] Although only a few examples of yeast-based biosensors have been reported, most yeast-derived cellular sensors of small molecule – protein interactions are based on the relatively complex yeast two-hybrid system,[16] in which ligand binding is used to trigger protein oligomerization to activate expression of a reporter gene.[17–19]

Here we report the construction of simple yeast-based biosensors that detect interactions between steroid hormone receptor LBDs and cell-permeable ligands. Steroid hormone

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receptors were chosen for investigation because these members of the nuclear hormone receptor superfamily of proteins represent major drug targets involved in the progression of numerous diseases including breast cancer, prostate cancer, and inflammation. As shown in Figure 1, our method involves coupling of the fluorescence of the red-shifted mutant of GFP

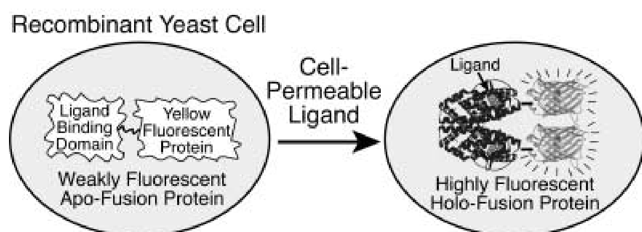
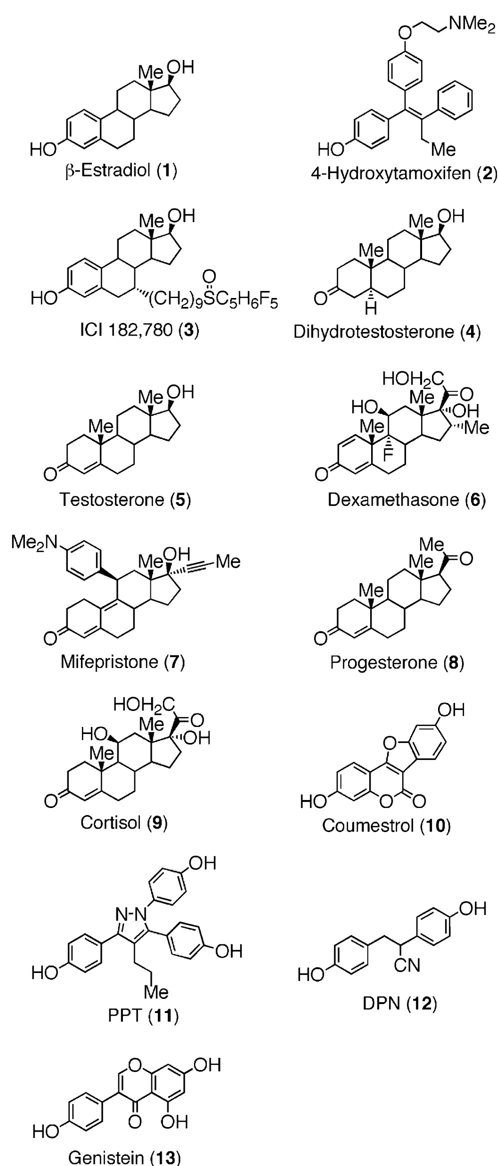


Figure 1. *Fluorescent cellular sensor design. Steroid hormone receptor ligand binding domains (LBDs) were fused to yellow fluorescent protein (YFP) through a short linker to detect ligands that affect protein conformation by influencing the folding of the tightly coupled YFP.*

known as yellow fluorescent protein (YFP)[20] to fused steroid hormone receptors expressed in living *Saccharomyces cerevisiae* yeast cells. We hypothesized that, because the fluorescence of GFP is sensitive to the folded state of proteins fused to its N terminus,[15] the fluorescence of steroid hormone receptor LBDs fused to YFP might be sensitive to changes in protein conformation resulting from the binding of small molecules such as compounds **1** – **13**. We report here that yeast expressing appropriately engineered LBD-YFP fusion proteins can be used as a sensor for cell-permeable agonists and antagonists of cognate receptors, and that these recombinant yeasts provide novel non-transcriptional and high-throughput assays of small molecule ligands.



Results

Fusion proteins were designed from X-ray crystal structures of YFP (PDB 1YFP)[20] and ligand-bound estrogen receptor-alpha (ERα, PDB 3ERT),[21] estrogen receptor-beta (ERβ, PDB 1HJ1),[22] androgen receptor (AR, PDB 1I37),[23] and glucocorticoid receptor (GR)[24] proteins. As shown in Figure 2, these fusion proteins directly link the N terminus of YFP to the C terminus of the steroid receptor LBD through a short two amino acid (Val-Glu) linker to couple conformational effects resulting from ligand binding closely to the fluorescence of YFP. This design approach was based on the previously reported observation that GFP expressed in *E. coli* is sensitive to the folded state of proteins fused to its N terminus.[15] The ER fusion proteins were designed on the basis of the structure of the compact ERβ LBD bound to the antiestrogen ICI 164384 (structurally similar to ligand **3**). Because helix-12 at the C terminus of ERβ is unstructured when bound to ICI 164384,[22] the ERβ and ERα LBDs were truncated at the beginning of this helix and fused to YFP through a two amino acid (Val-Glu) linker to couple this fluorescent protein closely to the LBDs. In contrast, the C termini of the structurally related AR and GR fusion proteins are close to their LBDs. These proteins were not truncated, and their C-terminal amino acids were directly linked to YFP through the Val-Glu linker.

To assess the effects of ligands on LBD-YFP proteins expressed in yeast qualitatively, cellular fluorescence and protein subcellular localization was examined by epifluorescence microscopy. As shown in Figure 3, the LBD-YFP fusion proteins were co-expressed in yeast with cyan fluorescent protein (CFP) fused to a nuclear localization signal (NLS) simultaneously to label the yeast cell nucleus with an orthogonal cyan fluorescent probe. Control experiments with yeast expressing only YFP or CFP confirmed that these fluorescent proteins were spectrally orthogonal with the fluorescence filter sets employed (data not shown). In the absence of ligand, the yellow fluorescence of the LBD-YFP proteins was dim and localized adjacent to the nucleus, possibly associated with part of the endoplasmic reticulum contiguous with the nuclear membrane (Figure 3). In contrast, addition of cognate steroidal ligands (**1**, **4**, or **7**) was found to enhance cellular yellow fluorescence dramatically and to shift the subcellular localization of these proteins to the yeast

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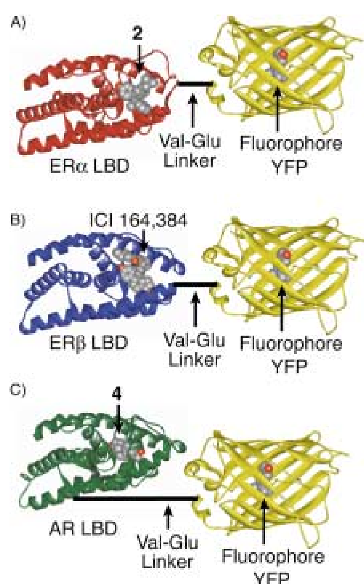

Figure 2. Models of LBD-YFP fusion proteins. A) ERα LBD-YFP; B) ERβ LBD-YFP; C) AR LBD-YFP.

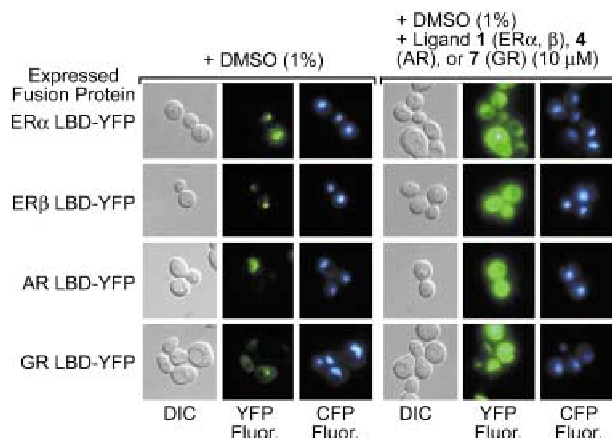

Figure 3. Differential interference contrast (DIC) and epifluorescence micrographs of recombinant yeast. Cyan fluorescent protein (CFP) was co-expressed fused to the SV40 nuclear localization signal as a nuclear marker. All epifluorescence micrographs were captured with identical exposure times. YFP: Yellow fluorescent protein. ERα LBD: residues 305 – 532. ERβ LBD: residues 255 – 482. AR LBD: residues 668 – 919. GR LBD: residues 497 – 795. Cells were grown at 30 °C for 16 h prior to analysis.

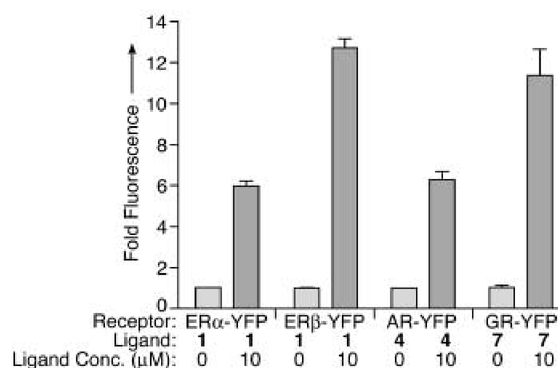

Figure 4. Quantification of LBD-YFP fluorescence on a 96-well microtiterplate. YFP fusion proteins were extracted from cells that were grown in media containing the ligand + DMSO (1%) or DMSO (1 %) alone for 16 h at 30 °C. Fold fluorescence = observed fluorescence/fluorescence without ligand.

cytoplasm. The short linker between the ER proteins and YFP was critical for this effect. No ligand-dependent fluorescence enhancement was observed with longer linkers comprising ERα(1 – 595)-YFP, ERα(305 – 552)-YFP, or ERβ(255 – 509)-YFP (data not shown). The short linker between the LBD and YFP proteins may disrupt protein folding in the absence of ligand, potentially resulting in association with the endoplasmic reticulum.[25] Hence, the addition of ligand may promote folding of these proteins, enabling the shift of protein localization to the cytoplasm.

The fluorescence of YFP fusion proteins extracted from ligand-treated cells was analyzed by fluorescence measurements on 96-well microtiterplates. As shown in Figure 4, these experiments confirmed that the ligand-mediated fluorescence enhancements detected by microscopy could be quantified in a high-throughput microtiterplate format. Although significant differences in the fluorescence of protein extracts were observed when living cells were treated with ligands, the addition of ligands to proteins extracted from yeast did not substantially affect protein fluorescence (data not shown). This difference presumably resulted from instability of the apo-LBD-YFP proteins under the protein extraction conditions.

To examine whether addition of ligand might have influenced the expression or proteolytic stability of the LBD-YFP proteins, cellular extracts were analyzed by immunoblotting against HA-tag peptides fused to the N termini of these proteins. As shown in Figure 5, no ligand-dependent effects on protein expression

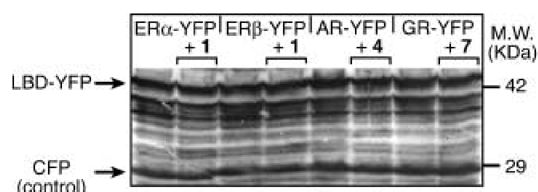

Figure 5. Analysis of protein expression by immunoblotting against fused HA epitope tags. The concentration of added ligands was 10 μM. The steroid receptor LBD-YFP protein was co-expressed with cyan fluorescent protein (CFP) as a control for differences in protein loading.

were observed. In these experiments, CFP was simultaneously co-expressed with the LBD-YFP fusion proteins as a control for variations in the amount of protein loaded on the SDS PAGE gel. These results indicated that the observed ligand-mediated fluorescence enhancements were not the result of significant changes in protein expression or of protection of the protein from intracellular proteolysis.

To investigate whether receptor dimerization might be correlated with the observed ligand-mediated fluorescence enhancements, yeast two-hybrid assays were constructed by use of these short steroid hormone receptor LBD proteins. The "interaction trap" yeast two-hybrid system[26] was employed to

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measure ligand-mediated changes in reporter gene expression. In this system, the bacterial LexA protein was fused to the N terminus of the steroid receptor LBD to anchor this protein on DNA sites that control expression of a β-galactosidase (*lacZ*) reporter gene. The bacterial B42 activation domain (AD) was similarly fused to the N terminus of the steroid receptor LBD to activate gene expression upon dimerization of the B42-LBD with LexA-LBD fusion protein. As shown in Figure 6, co-expression of

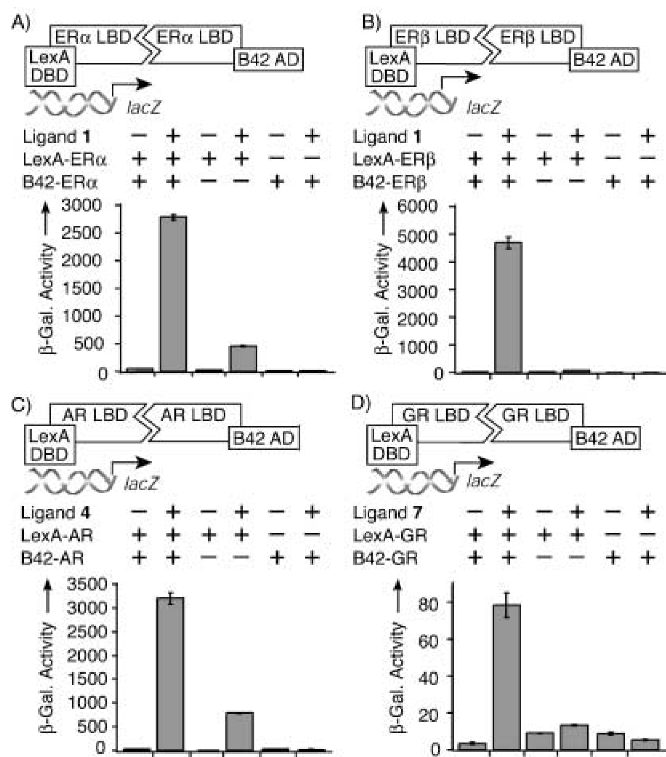

Figure 6. Validation of yeast two-hybrid assays quantifying ligand-mediated dimerization of steroid receptor LBDs and corresponding omission control experiments. The LBDs are expressed as two fusion proteins: a LexA DNA-binding domain (DBD) fusion and a B42 activation domain (AD) fusion. Ligand-induced dimerization of the LBDs activates transcription of a lacZ *reporter gene by reconstituting a functional transcriptional activator. Concentration of ligands = 10 μM. A) ERα assays; B) ERβ assays; C) AR assays; D) GR assays.*

these fusion proteins in yeast transformed with a *lacZ* reporter plasmid activated gene expression only in the presence of the cognate ligand (**1**, **4**, or **7**). Although some ligand-mediated "one-hybrid" activity was observed with the short LexA-ERα and LexA-AR LBDs in the absence of the corresponding B42 fusion protein (Figure 6 A and C), expression of both B42-LBD and LexA-LBD provided substantially higher levels of reporter gene expression, consistent with protein dimerization conferring maximal activity.

The specificity of ligands **1**–**9** was examined by flow cytometry analysis of the effects of all of these compounds on the fluorescence of each LBD-YFP protein expressed in living yeast cells. These effects were directly compared with the transcriptional yeast two-hybrid assays that measure ligand-induced dimerization of receptor LBDs.[27, 28] As shown in Figure 7,

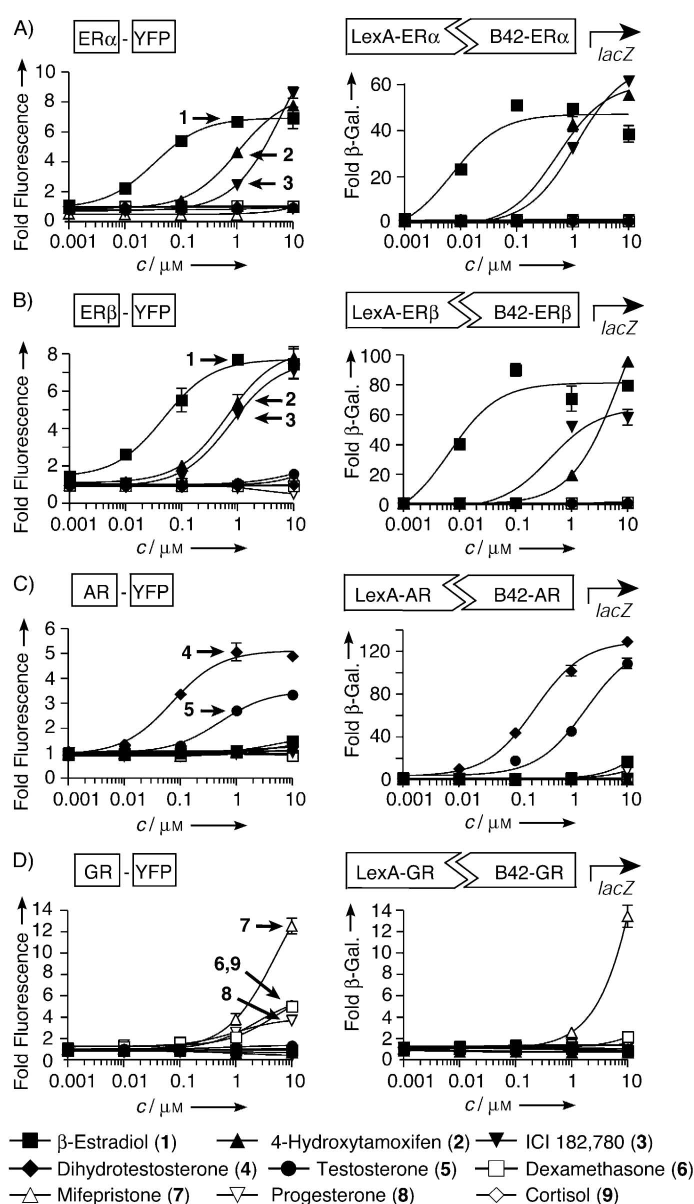

Figure 7. Whole-cell dose response curves of ligands 1–9. Steroid receptor LBD-YFP fluorescence measured by flow cytometry (left-hand panels) is compared with transcriptional two-hybrid assays (right-hand panels). A) ERα assays; B) ERβ assays; C) AR assays; D) GR assays. Fold fluorescence = observed fluorescence/fluorescence without ligand. Fold β-Gal = observed β-galactosidase activity/β-galactosidase activity in the absence of ligand. c *= ligand concentration.*

ligand-induced LBD-YFP fluorescence generally paralleled the analogous yeast two-hybrid assays. In some cases, the fluorescent cellular sensors were significantly more sensitive in terms of overall response to ligand than analogous two-hybrid assays, even though the two-hybrid assays produce an enzymatic product that provides an amplified signal. For example, the GR ligands dexamethasone (**6**), progesterone (**8**), and cortisol (**9**) provided significant four- to fivefold fluorescence enhancements when tested against GR-YFP, but these ligands were not detected (effects less than twofold) with the corresponding transcriptional assay. The low activity of compounds in the GR

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two-hybrid assay is consistent with previous studies of the relatively weak dimerization affinity of the GR LBD ($K_d \approx$ 1.5 μM).[24] Importantly, the fluorescent sensors could discriminate between structurally highly similar molecules, such as detection of the 17β-hydroxyl group of testosterone (**5**) but not the analogous 17β-methyl ketone of progesterone (**8**, Figure 7 C).

The estrogenic compounds **10**–**13** exhibit selectivity for the ERα or ERβ isoforms. To examine the potential for selectivity in yeast-based ER assays, these compounds were investigated with fluorescent cellular sensors and analogous yeast two-hybrid assays as shown in Figure 8. These experiments revealed that the ERβ-selective ligands coumesterol (**10**), 2,3-*bis*(4-hydroxyphenyl)propionitrile (DPN, **12**), and genistein (**13**) were significantly more potent in yeast expressing ERβ-YFP than ERα-YFP. In general, these effects paralleled the yeast two-hybrid assays. Surprisingly, the ERα-selective ligand 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT, **11**) was only detected at high concentrations in the ERα yeast two-hybrid assay. This result illustrates the complementary nature of the fluorescent cellular sensors and analogous yeast two-hybrid assays for identification of steroid receptor ligands.

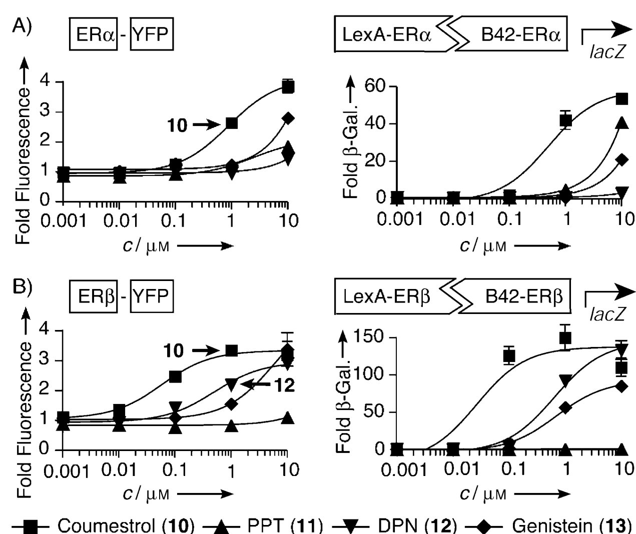

*Figure 8. Whole-cell dose response curves with the selective estrogen receptor ligands **10**–**13**. ER-YFP fluorescence measured by flow cytometry (left panels) is compared with ER LBD two-hybrid assays (right panels). A) ERα assays; B) ERβ assays.* c = *ligand concentration.*

Cognate ligands were found to confer dose-dependent enhancements of LBD-YFP fluorescence consistent with literature values for in vitro receptor binding affinities (RBA, Table 1),[29–44] particularly with structurally similar compounds likely to be of similar cellular permeability. For example, testosterone (**5**, RBA = 19) binds the AR more weakly in vitro than structurally similar dihydrotestosterone (**4**, RBA = 100), and these compounds are likely to exhibit very similar cellular permeability characteristics in whole-cell assays. As shown in Table 2, calculated ligand EC_{50} values were consistent with this observation. Comparison of these EC_{50} values revealed that in the AR-YFP assay, testosterone was ≈14% as potent as dihydrotestosterone. Similarly, in the AR two-hybrid assay, testosterone was ≈12% as potent as dihydrotestosterone. These results are similar to known RBA values from literature sources, which report testosterone to be ≈19% as potent as dihydrotestosterone in vitro.[30, 33] Surprisingly, comparison of measured EC_{50} values revealed that these ligands were three to four times more potent in the AR-YFP assays than in the AR two-hybrid assays. This difference in potency may in part relate to the formation of nonproductive homodimeric complexes competing with formation of the productive heterodimeric protein complexes that provide the transcriptional response in the yeast two-hybrid system. Alternatively, the AR-YFP assays may detect ligand-mediated changes in protein conformation at ligand concentrations below those required to promote protein dimerization. Differences between in vitro RBA rankings and intracellular potencies probably reflect differences in the cellular permeabilities of the compounds under investigation. The construction of analogous biosensors from more permeable yeast strains such as those lacking the *ERG6* gene[45, 46] might further enhance the sensitivity of these assays.

***Table 1.** Reported literature values for relative binding affinities (RBAs) of ligands for receptors in vitro.*[a]

Ligand, RBA	ERα	ERβ	AR	GR
β-Estradiol (**1**)[29]	100	100	Neg.	3
4-Hydroxytamoxifen (**2**)[29]	149	62	N.A.	N.A.
ICI 182,780 (**3**)[29]	32	25	N.A.	N.A.
Dihydrotestosterone (**4**)[30, 33, 40, 41]	Neg.	Neg.	100	3
Testosterone (**5**)[30, 31, 33]	Neg.	Neg.	19	Neg.
Dexamethasone (**6**)[30–32, 35, 36]	Neg.	Neg.	Neg.	100
Mifepristone (**7**)[32, 37, 60]	Neg.	Neg.	5	340
Progesterone (**8**)[30–33]	Neg.	Neg.	Neg.	115
Cortisol (**9**)[31, 35, 36, 38]	Neg.	Neg.	Neg.	92
Coumestrol (**10**)[42]	34	100	N.A.	N.A,
PPT (**11**)[43]	49	Neg.	N.A.	N.A.
DPN (**12**)[44]	Neg.	18	N.A.	N.A.
Genistein(**13**)[42, 44]	Neg.	13	N.A.	N.A.

[a] These values are based on the ratio of the concentration of a high-affinity ligand (RBA = 100) to the concentration of a competitor ligand required to displace 50% of a specific radiolabeled probe. Neg. = Negligible affinity (RBA < 1.0). N.A. = Data not available.

***Table 2.** Ligand EC_{50} values (nM) calculated from yeast LBD-YFP and yeast two-hybrid (Y2H) assays.*[a]

Ligand, EC_{50}	ERα-YFP	ERα-Y2H	ERβ-YFP	ERβ-Y2H	AR-YFP	AR-Y2H
β-Estradiol (**1**)	30	10	50	10	Neg.	Neg.
4-Hydroxytamoxifen (**2**)	1050	540	N.C.	N.C.	Neg.	Neg.
ICI 182780 (**3**)	N.C.	N.C.	760	420	Neg.	Neg.
Dihydrotestosterone (**4**)	Neg.	Neg.	Neg.	Neg.	70	230
Testosterone (**5**)	Neg.	Neg.	Neg.	Neg.	520	1850
Coumestrol (**10**)	880	510	60	30	N.D.	N.D.
DPN (**12**)	Neg.	Neg.	500	650	N.D.	N.D.

[a] N.D.: Not determined. N.C.: Not calculated, due to insufficient data. Neg.: Negligible affinity.

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Discussion

Binding of ligands to steroid hormone receptors alters the conformations of these proteins and can affect receptor stability.[6] As an alternative to transcriptional assays, one method used to investigate these effects is analysis of the sensitivity of proteins to proteases in the presence or absence of ligand. For example, interactions of agonistic or antagonistic ligands of the estrogen receptor alpha and beta isoforms have previously been detected by analysis of trypsin digestion patterns.[43] However, this method is not generally amenable to high-throughput screening for the identification of small molecule ligands. In an effort to overcome this limitation, we fused yellow fluorescent protein to nuclear hormone receptor fragments and expressed these proteins in yeast in order to construct fluorescent cellular sensors. To validate the generality of this approach, four steroid hormone receptors (ERα, ERβ, AR, and GR) were investigated. The ligand-binding domains of these receptors were fused to YFP through short two amino acid linkers to couple fluorescence closely to conformational effects resulting from ligand binding. To validate the effectiveness of these cellular sensors, the fluorescence of cell extracts was quantified on 96-well plates to establish that this assay could be executed in a high-throughput format. Moreover, ligand-mediated effects on fluorescence in living yeast cells were analyzed in more detail by epifluorescence microscopy and flow cytometry and compared with analogous transcriptional yeast two-hybrid assays that quantify ligand-mediated protein dimerization.

The effects of ligands on the fluorescence of cells expressing LBD-YFP proteins was generally found to parallel activation of gene expression in analogous yeast two-hybrid assays. Hence, changes in protein conformation that are coupled to protein dimerization presumably contribute significantly to the enhanced protein fluorescence. However, simple intermolecular association (dimerization) of the YFP moieties themselves is presumably not responsible for this fluorescence enhancement, because dimerization of GFP under these conditions should not alter its intrinsic fluorescence properties.[47]

Remarkably, the fluorescent cellular sensors could detect therapeutically relevant compounds—such as the GR ligands dexamethasone (**6**), progesterone (**8**), and cortisol (**9**)—that were undetectable in the GR-based yeast two-hybrid assays. Furthermore, the AR ligands dihydrotestosterone (**4**) and testosterone (**5**) were three to four times more potent in the AR-YFP assays than in the two-hybrid assays. These results suggest that conformational effects not involved in protein dimerization may also play an important role in influencing protein fluorescence. Previous studies of GFP fusion proteins have demonstrated that the folded state of proteins fused to the GFP N terminus is directly related to the fluorescence of GFP.[15] Hence, ligand binding may stabilize folding of the LBD, which may in turn be tightly coupled to folding of YFP protein, resulting in enhanced fluorescence.[4, 6, 15] Consistently with this mechanism, a short two amino acid Val-Glu linker was required for the ligand-mediated fluorescence enhancement. Furthermore, intracellular protein expression or proteolysis was not significantly affected by cognate ligands.

Addition of ligands resulted in a shift of the localization of LBD-YFP proteins from a site near the nucleus (possibly associated with part of the endoplasmic reticulum) to the cytoplasm. This difference in localization could potentially influence YFP fluorescence as a result of changes in protein microenvironment. For example, YFP is sensitive to environmental factors and exhibits enhanced fluorescence at elevated pH values.[48] Furthermore, the pH values in certain organelles such as the nucleus differ from that in the cytoplasm,[49] so changes in sub-cellular localization may have directly contributed to the enhanced protein fluorescence. Alternatively, we propose that the apo-LBD-YFP proteins may become localized in part of the endoplasmic reticulum contiguous with the nuclear membrane as a consequence of protein misfolding. The misfolding of other proteins is known to promote retention in the endoplasmic reticulum.[50] Addition of ligand may facilitate folding of the LBD, enhance the fluorescence of the closely fused YFP, and enable translocation of the LBD-YFP proteins to the cytoplasm. Other mechanisms that might have contributed to the effects of ligands on the fluorescence of these proteins could include the recruitment or dissociation of other proteins such as the heat shock protein chaperones known to associate with steroid hormone receptors.[51, 52] The approach described here provides a novel high-throughput method for non-transcriptional analysis of small molecule-protein interactions in living cells.

Experimental Section

Reagents: β-Estradiol, testosterone, and cortisol were purchased from Steraloids (Newport, RI). Dexamethasone was obtained from RBI (Natick, MA), and ICI 182780, DPN, and PPT were purchased from Tocris (Ellisville, MO). Restriction enzymes were from New England Biolabs (Beverly, MA). Other reagents were from Sigma (St. Louis, MO).

Construction of steroid hormone receptor genes: The polymerase chain reaction (PCR), with use of *Pfu* polymerase (Stratagene), was employed to add in-frame 5′ – *Eco*RI – Receptor LBD – *Sal*I – Stop – *Xho*I – 3′ restriction sites to genes encoding ligand-binding domains of human estrogen receptor-α (residues 305 – 532), human estrogen receptor-β (residues 255 – 482), human androgen receptor residues (668 – 919), and rat glucocorticoid receptor (residues 497 – 795). The GR LBDs included the following known mutations: L600P/L602F (K. Yamamoto, personal communication), F620S,[53] and C656G.[54] The last two mutations are known[17] to improve the affinity of yeast-expressed GR for dexamethasone. Plasmid pCMV5hER (a gift from Prof. B. Katzenellenbogen, U. Illinois) provided the template for ERα. The gene encoding ERβ was amplified from pMT-hERβ 530 (a gift from Dr. S. Nilsson, KaroBio). The gene encoding AR was amplified from pNLVP-hAR (a gift from Dr. E. Wilson, UNC Chapel Hill). The gene encoding rat GR was amplified from pEGHBD7 (a gift from Dr. J. Liu, Johns Hopkins). Any internal *Eco*RI, *Sal*I, or *Xho*I restriction sites within the receptor genes were removed by introduction of silent mutations by megaprimer PCR mutagenesis.[55]

Construction of plasmids for fluorescent cellular sensors: The following in-frame restriction sites were added to the EYFP gene by PCR: 5′ – *Eco*RI – *Xho*I – EYFP – *Sal*I – 3′. The commercial vector pEYFP-C1 (Clontech) provided the gene template. This gene product was

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digested with *Eco*RI/*Sal*I and subcloned into the *Eco*RI/*Xho*I-digested yeast vector pRF4–6 (a 2 μm yeast shuttle vector with the *TRP1* marker and *GAL1* promoter from pJG4–5,[26] but substituting the B42 activation domain/SV40 NLS with an HA epitope tag; R. Brent, personal communication) to afford pRF4–6 *Eco*RI-*Xho*I-EYFP. This vector added an HA epitope tag to the N terminus of expressed genes. The ERα, ERβ, AR, and GR receptor gene sequences flanked with in-frame 5′–*Eco*RI–Receptor LBD–*Sal*I–Stop–*Xho*I–3′ restriction sites were digested with *Eco*RI/*Sal*I and subcloned into *Eco*RI/*Xho*I-digested pRF4–6 *Eco*RI-*Xho*I-EYFP. Nuclear-localized cyan fluorescent protein (ECFP, Clontech) was expressed in yeast from the vector pAM423.[56] All new constructs were confirmed by automated dideoxynucleotide sequencing at the Penn State University Biotechnology Institute.

Construction of plasmids for yeast two-hybrid assays: The receptor genes sequences flanked with in-frame 5′–*Eco*RI–Receptor LBD–*Sal*I–Stop–*Xho*I–3′ restriction sites were digested with *Eco*RI/*Xho*I and inserted into vector pJG4–5 (digested with *Eco*RI/*Xho*I, Invitrogen), which fused the B42 activation domain to the receptor N terminus. These genes were similarly subcloned into vector pAM423-LexA (digested with *Eco*RI/*Xho*I, HIS3 marker, 2 μm origin, essentially identical to pEG202 (Clontech),[57] but containing a *GAL1* promoter), which fused the bacterial LexA protein to the receptor N terminus. Plasmid pSH18–34 (Invitrogen), containing four dimeric LexA DNA sites driving *lacZ* (β-galactosidase) expression, was employed as the reporter gene.

Microtiterplate β-galactosidase reporter gene assays: *S. cerevisiae* FY250 (*MATα*, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*) was used to assay LexA-driven β-galactosidase reporter gene expression. Yeasts were transformed by the lithium acetate method,[58] and yeast transformants, which were derived from multiple combined colonies, were grown to saturation at 30 °C in selection media (yeast nitrogen base without amino acids (Difco), appropriate dropout powder (QBiogene), penicillin (Gibco, 100 units mL^{-1}), streptomycin (Gibco, 100 mg mL^{-1}), 2% galactose (Sigma), and 1% raffinose (Sigma)). Aliquots (50 μL) of saturated yeast cultures were diluted in selection media (175 μL) on a sterile 96-well plate. A solution of the compound under investigation in DMSO/selection medium (1:10, 25 μL) was then added to afford a final well volume of 250 μL (1% DMSO). The plate was shaken at 30 °C for 16 h and centrifuged (4300 rpm, 10 min), and the supernatant was removed by aspiration. Z-Lysis buffer (Z-buffer[59] containing 2% EtOH, 1% $CHCl_3$, and 0.3% β-mercaptoethanol, 200 μL) was added, and the plate was shaken for 5 min. Aliquots of suspended cells (10 μL or 50 μL for high or low levels of β-galactosidase activity) were transferred to wells containing sufficient Z-lysis buffer to provide a final volume of 150 μL. The absorbance at 590 nm (OD_{590}) was measured to determine cell density, followed by addition of the substrate chlorophenol red-β-D-galactopyranoside (Calbiochem, 15 mM, 30 μL) in sodium phosphate buffer (0.1 M, pH 7.5). The plate was shaken at 23 °C with periodic (5, 10, 15, 30, 60, and 120 min) absorbance measurements at 570 nm (OD_{570}). β-Galactosidase activity was calculated as follows: activity = 1000*(OD_{570}-BLANK-OD_{590})/(TIME*(OD_{590})). The BLANK value corresponded to the absorbance (570 nm) of wells containing substrate (30 μL) and Z-lysis buffer (150 μL) only. The TIME value was expressed in minutes. Values typically represent the mean of two independent experiments. Error bars represent standard errors of the mean. Dose-response curves and EC_{50} values were calculated by nonlinear regression with a one-site competition model (GraphPad Prism 3.0 software).

Fluorescent cellular sensor assays: *S. cerevisiae* FY250 was used for fluorescence measurements by flow cytometry. Yeasts were transformed with the LBD-YFP expression vector by the lithium acetate method, and yeast transformants derived from multiple combined colonies were grown to saturation at 30 °C in selection media. Aliquots (50 μL) of saturated yeast cultures were diluted in selection media (175 μL) on a sterile 96-well plate. Ligands in DMSO/selection media (1:10, 25 μL) were added to afford a final well volume of 250 μL (1% DMSO). The plate was shaken at 30 °C for 16 h, and aliquots of suspended cells (250 μL) were transferred to tubes containing sufficient sterile deionized water to provide a final volume of 500 μL for analysis by flow cytometry. Flow cytometry measurements employed an XL-MCL bench top cytometer (Beckman Coulter, Miami, FL) equipped with a 15 mW, air-cooled argon ion laser. Fluorescence was quantified by excitation at 488 nm and optical filtering of fluorescence emission through a 530 ± 30 nm band-pass filter. Forward-scatter (FS) and side-scatter (SC) dot plots afforded cellular physical properties of size and granularity that allowed gating of live cells. After cell gating, 10000 events (cells) were counted, and median fluorescence intensity was quantified. Values typically represent the mean of two independent experiments. Error bars represent standard errors of the mean.

Microscopy: Epifluorescence and Differential interference contrast (DIC) micrographs were obtained with a 100X Zeiss Fluar objective on a Zeiss Axiovert S100TV microscope fitted with a Zeiss Axiocam digital camera. Fluorescence filter sets for YFP (Yellow GFP BP) and CFP (Cyan GFP) were obtained from Chroma. Images were processed with Adobe Photoshop 5.0.

Fluorescence measurements of cell extracts on 96-well microtiterplates: Recombinant FY250 yeast expressing LBD-YFP fusion proteins (2 mL cultures) was grown at 30 °C in the presence or absence of ligand for 16 h. Cell pellets were isolated by centrifugation (4300 rpm, 10 min), 100 μL of Y-PER (Yeast Protein Extraction Reagent, Pierce) was added, and samples were shaken for 30 min. Cellular debris was removed by centrifugation (14000 rpm, 2 min) and the fluorescence of the supernatant was analyzed on a 96-well black plate (Corning). Fluorescence measurements employed a Perkin–Elmer HTS-7000 microtiterplate reader (band pass filter excitation: 485 ± 20 nm; emission: 535 ± 25 nm). Fluorescence values represent the mean of two independent experiments. Error bars represent standard errors of the mean.

Immunoblotting: The immunoblotting experiments employed yeast expressing LBD-YFP fusion proteins grown in selection media (2 mL cultures) containing ligands (10 μM, 1% final DMSO concentration) or DMSO (1% final concentration) alone for 16 h at 30 °C. These cultures were centrifuged (4300 rpm, 10 min), 2X SDS loading buffer (20 mL) was added to the pellet, and these samples were boiled at 100 °C for 10 min. Cellular debris was removed by centrifugation (14000 rpm, 2 min) and the supernatant was analyzed on a 15% Tris-Glycine-SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, followed by immunoblotting with monoclonal rabbit anti-HA IgG, which was probed with an alkaline phosphatase(AP)-conjugated goat anti-rabbit antibody. This bound AP conjugate was visualized by treatment with Western Blue stabilized substrate (Promega).

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