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Single Plasmids Expressing Human Steroid Hormone Receptors and a Reporter Gene for Use in Yeast Signaling Assays

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Abstract

Single plasmids designed to express the six human type I steroid hormone receptors and detect signaling activity are described in this report. These stably replicating plasmids reported ligand-induced transcriptional activation via lacZ assays in Baker's yeast (*Saccharomyces cerevisiae*). The ligand concentrations needed to activate signaling in yeast expressing these plasmids spanned five orders of magnitude as based on comparisons of EC₅₀ values. Radicicol, a direct inhibitor of heat shock protein 90 (Hsp90) and an indirect inhibitor of steroid hormone receptor signaling, was used to determine the functional utility of this yeast reporter system. The inhibitory effect of radicicol was similar on the signaling of all six steroid hormone receptors and was distinguishable from cytotoxic effects that occurred with higher concentrations. These yeast plasmids provide a high throughput system for comparative assessment of steroid hormone receptor signaling and may be useful in screening for pharmacological or xenobiotic activities.

Keywords

steroid hormone receptor; yeast; radicicol; Hsp90; reporter assay

1. Introduction

1.1 The yeast *Saccharomyces cerevisiae* provides a relatively simple and well-defined eukaryotic system for the expression of genes from other organisms [1,2]. Protein kinases, tumor suppressor genes, receptors, transcription factors, and other metazoan proteins are functional and can be studied when expressed in yeast [3-7]. Ease of genetic manipulation and proficient expression of genes as single exons help make this yeast amenable to studying foreign proteins [8]. Additionally, *S. cerevisiae* expresses endogenous Hsp70 and Hsp90 proteins that are structurally and functionally similar to their counterparts in vertebrates [9]. These chaperones help to correctly fold and regulate foreign proteins expressed in yeast.

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1.2 Numerous investigators have utilized yeast to study vertebrate steroid hormone receptors (SHRs¹). The type I SHRs display ligand dependent activation of transcription as detected through genetic signaling assays. The glucocorticoid (GR), progesterone (PR), androgen (AR) and estrogen receptor alpha (ER α) receptors are the most studied in yeast. For instance, genetic studies expressing mammalian GR, AR, ER α and PR in yeast have revealed a need for Hsp90 and its co-chaperones and have been used to identify transcriptional co-activators [9-12]. Yeast strains expressing AR, ER α estrogen receptor beta (ER β), and PR have been frequently used in screens to identify endocrine disrupting agonist and antagonist ligands [13-19]. Expression and function of the ligand-binding domain of mineralocorticoid receptor (MR) has been minimally explored in yeast, but suggests behavior similar to other SHRs, and it signals strongly when co-expressed with a transcriptional co-activator [11,20].

1.3 When multiple plasmids are used to conduct SHR signaling assays, the potential for plasmid copy number imbalances arises [21-23]. Copy number differences may occur after plasmid transformation or may evolve over time as cells are cultured. Plasmid copy number imbalances numerically alter the elements responsible for producing the signals that are being measured in reporter assays. To eliminate the potential for plasmid imbalances, both the SHR expression and reporter constructs were placed on single plasmids to create the pRR series. The function of these singular receptor-reporter plasmids is described in this report. Utility of this plasmid series was demonstrated by comparing the effects of radicicol, an indirect-acting SHR inhibitor that directly blocks Hsp90 function, on signaling by the type I class of SHRs.

2. Materials and Methods

2.1 Chemicals, hormones, and other reagents used in these experiments were obtained from Fisher Scientific, Inc. and the Sigma-Aldrich Company. Restriction enzymes, Antarctic phosphatase, Klenow DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Amplitaq DNA polymerase for PCR was purchased from Invitrogen, Inc. Plasmid cloning and expansion were done in DH5 α and NEB10 β strains of *E. coli*, and the yeast studies were conducted with the W303a strain of *S. cerevisiae* (obtained from American Type Culture Collection).

2.2 The pRW95-3 plasmid was used as the backbone for our receptor-reporter (pRR) constructs [24]. This is a low copy *CEN/ARS* plasmid that contains multiple cloning sites adjacent to a minimal *S. cerevisiae* cytochrome C promoter sequence that is positioned next to the coding DNA of the *E. coli lacZ* gene. The plasmid has genes encoding β -lactamase (ampicillin resistance gene, AMP) and *TRP1* (phosphoribosylanthranilate isomerase) that confer selective growth in *E. coli* and *trp1* yeast strains, respectively. Single stranded ERE5 and HRE5 sequences were designed and then purchased from Integrated DNA Technologies. ERE and HRE consensus sequences incorporated into these synthetic DNAs were defined previously [25]. The longer single stranded DNAs were annealed to a short 5'-primer and extended with Klenow DNA polymerase and deoxynucleotides (Table 1). The resulting double stranded ERE5 sequence contains five consensus sequences for estrogen receptor binding (EREs, underlined). The synthetic double stranded HRE5 sequence has five hormone receptor response elements (HREs, underlined) that interact with androgen, glucocorticoid, mineralocorticoid, and progesterone receptors. *Bam*HI and *Xma*I restriction sites (restriction sites are indicated as bold sequence, Table 1) engineered into the ends of the two response element sequences were used for directional insertion between the *Bg*/III and *Xma*I sites of pRW95-3. Plasmid and inserts were digested with restriction endonucleases and then joined using T4 DNA ligase to create

¹**Abbreviations:** steroid hormone receptor (SHR), androgen receptor (AR), estrogen receptor alpha (ER α), estrogen receptor beta (ER β), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), β -galactosidase gene (*lacZ*), β -lactamase gene (AMP), polymerase chain reaction (PCR), heat shock protein 90 (Hsp90)

pERE5Z and pHRE5Z, respectively. Single fragment insertion was verified by digestion of the *SaII* site engineered into the central region of the 5ERE and 5HRE sequences along with digestion patterns generated by various diagnostic restriction enzymes. These reporter plasmids were transformed into yeast and shown to lack intrinsic expression of the *lacZ* gene when no steroid hormone receptor was present. Various steroid hormone receptors were co-expressed from separate plasmids along with these pERE5Z or pHRE5Z plasmids. LacZ expression required both receptor and ligand for appreciable activity in signaling assays (data not shown).

2.3 SHR expression constructs were generated by PCR and inserted into the pERE5Z and pHRE5Z plasmids. The pHRE5-Z and pERE5-Z reporter plasmids were first digested to a linear form using *SnaBI* and then dephosphorylated using Antarctic phosphatase. The synthetic ER α or β receptor constructs were ligated into the pERE5Z plasmid, and the other four steroid hormone receptor expression constructs were inserted into pHRE5Z using T4 DNA ligase. Orientation of the inserted receptor expression sequence was determined by restriction digestion patterns. Receptor DNA sequencing was performed by SeqWright, Inc. Thus, six plasmids were made to express and report the activity of the human steroid receptors. Specific details regarding the individual plasmids are listed in Table 2. Figure 1 illustrates the general features of this plasmid set. The pRR plasmids have been submitted to the Addgene repository (www.addgene.com), which provides them to investigators for noncommercial uses.

2.4 Methods for evaluation of SHR ligand activation by *lacZ* reporter assays have been described in detail and are essentially the same as methods used here [26]. Four independent yeast clones containing each pRR plasmid were assessed to insure the generality of the phenotypes being observed. Ligands and other chemicals used to treat the yeast were dissolved in dimethylsulfoxide. Total dimethylsulfoxide vehicle concentration did not exceed 1% in any experiment. Results shown are normalized (percent) average responses of the clones that were analyzed in three or more independent trials. Cells were treated with vehicle alone (control) and a range of ligand concentrations spanning seven orders of magnitude in a minimal synthetic galactose medium (Table 2). Treated yeast cultures were grown overnight in 96 well plates at 30 degrees C. Approximately 18 h later the cell density was read on a plate spectrophotometer at a wavelength of 600 nm. Twenty-five μ l of each suspended culture was removed and placed in a separate 96 well plate and lysed by Z buffer. Z buffer with o-nitrophenol- β -D-galactopyranoside (ONPG) substrate (225 μ l) was added and reactions were incubated at 37 degrees C for 5-30 min., depending on the activity as detected by appearance of the yellow colored nitrophenol product of the *lacZ* assay. Reactions were stopped by addition of 100 μ l of a 1 M sodium carbonate solution. The *lacZ* assay plates were read at 405 nm on a plate spectrophotometer. LacZ units (or Miller units) were calculated as described previously and then normalized to 100% percent response for purposes of comparison. Curve fitting and interpolation of the doses giving a 50% signaling response (EC₅₀) or 50% inhibition of signaling or growth (IC₅₀) were performed using Prism 4 software (Graphpad Software). Analysis of variance (ANOVA) was performed to determine statistical significance of the data.

Results

3.1 A family of pRR plasmids that both express individual SHRs and report their activity was constructed. A general diagram of the pRR plasmid family is shown in Figure 1. These plasmids can be transformed into any *trp1* strain of *S. cerevisiae* to confer the ability to sense hormonal activation or inhibition. Notable features of these plasmids include the autonomous replicating sequence (ARS) and the centromeric (*CEN*) DNA sequence that confer efficient episome replication and segregation functions, respectively, in yeast. The *CEN* element also helps to maintain a very low and stable plasmid copy number, as accumulation of multiple *CEN*-containing plasmids may be toxic to yeast [22]. Two different types of yeast promoter elements were used to direct SHR synthesis from the pRR plasmids. The *GPD* promoter of the yeast

glyceraldehyde phosphate dehydrogenase gene is highly expressive and active both in glucose- and galactose-containing media. The *GALI,10* promoter from the divergently transcribed *GALI* and *10* genes is tightly repressed in glucose medium and is highly induced when galactose is the carbon source. Construction of all the SHRs under control of the *GALI,10* promoter was initially planned. We had no trouble expressing four functional SHRs from the *GALI,10* promoter, but for some unknown reason the human AR and ER β cDNAs subcloned in this context were non-functional when assessed in signaling assays (data not shown). However, the same AR and ER β cDNAs expressed from the *GPD* promoter of different plasmids worked well. Thus, the *GPD* promoter versions of the AR and ER β expression constructs were used to complete the pRR plasmid set.

3.2 A graph of the ligand-induced receptor signaling assays in yeast containing the six pRR plasmids is shown in Figure 2. No treatment or vehicle treatment typically produced no or very low background signal. Addition of optimal ligand concentrations robustly generated hundreds or thousands of lacZ units, depending on the pRR plasmid being tested. Approximately ten-fold or greater induction levels of LacZ signal were observed after optimal ligand treatments, with each pRR plasmid displaying a good signal to background ratio. For purposes of graphical comparison we normalized these six sets of signaling assay data by setting the maximal point of each dose-response curve to 100%. The dose-response curves for the reporter assays shown in Figure 2 were used to determine the EC₅₀ values. Ligand concentrations corresponding to the EC₅₀ values shown in Table 3 provide for assessment of signaling among the receptors and for comparison of our assay system to those of others. The six yeast strains range over about five orders of magnitude in sensitivity to their respective ligands. The EC₅₀ values generally correspond to the relative sensitivities previously reported for each receptor type (see references cited in Introduction). Signaling in yeast containing the pRR-ER β 5Z plasmid was activated by sub-nanomolar 17 β -estradiol concentrations. At the other end of the spectrum, the yeast with pRR-GR-5Z required micromolar dexamethasone concentrations for signaling activation. Surprisingly, yeast transformed with the ER β plasmid detected 17 β -estradiol at lower concentrations than did the ER α reporter plasmid (pRR-ER α 5Z). Yeast transformed with pRR-AR-5Z, pRR-PR-5Z, and pRR-MR-5Z plasmids signaled with intermediate sensitivity to their cognate ligands.

3.3 Agents that inhibit steroid hormone receptor signaling can be used for medicinal purposes or, if exposure is unintentional, can act as endocrine disrupting agents. Radicicol is a bacterial antibiotic that inhibits the Hsp90 protein, which is needed to chaperone steroid hormone receptors to a functional state. Blocking Hsp90 function should in turn inhibit SHR signaling as well as other cellular processes that require Hsp90 proteins. The cytotoxicity of radicicol in the W303a yeast strain is shown in Figure 3. The concentration of radicicol needed to inhibit cell density by 50% (IC₅₀) was approximately 3 μ M. Clones of the six yeast strains were treated with increasing radicicol concentrations alone or in the presence of an activating concentration of the relevant ligands listed in Table 3. Radicicol treatment alone did not stimulate signaling, indicating that it is not an agonist for any of the SHRs. When given in combination with each receptor's cognate ligand, equivalent radicicol concentrations produced similar levels of signaling inhibition. Graphical data (not shown) similar to that in Fig. 2 were obtained, and the Prism program was used to determine the IC₅₀ values for radicicol (Table 4). Radicicol preferentially inhibited SHR signaling at approximately three-fold to ten-fold lower concentrations than those required to cause cytotoxicity as seen by comparison of data in Fig. 3 and Table 4. The IC₅₀ concentrations of radicicol needed to inhibit signaling ranged by a factor of three when comparing the different SHRs. These values were not significantly different from one another when compared by ANOVA. Thus, within the context of a comparable cellular environment, the effect of the Hsp90 inhibitor was relatively uniform on all six SHRs.

Discussion

4.1 We created a group of plasmids designed to express individual SHRs and to indirectly report their activity through the production of β -galactosidase activity. These plasmids are novel in comparison to SHR signaling systems developed by others (reviewed in Introduction) that typically employ receptor expression and reporter sequences as separate plasmids or genome-integrated constructs. The possibility of having copy level imbalances is reduced for the pRR plasmid series since the receptor expression and reporter genes are contained on a single plasmid. Since the pRR plasmids have *CEN* and *ARS* elements they partition and replicate with similar efficiency to strains with integrated constructs, and can easily be transformed into any *trp1* strain. Signaling systems that are integrated into the yeast genome provide for very stable reporter assays if they provide sufficient activity. One disadvantage of using integrated reporters and receptors is that it is laborious to introduce them into multiple strains, such as when study signaling in a panel of mutants. Having a portable receptor-reporter system on a single stable plasmid is advantageous in instances when one wishes to compare receptor function among several different strains or genetic backgrounds.

4.2 The SHRs naturally display a broad range of ligand sensitivities. The estrogen receptors are the most sensitive to hormones, with 17β -estradiol being active at subnanomolar concentrations in some cell types and genetically engineered yeast [15,17]. ER α typically shows a small but significantly greater affinity and activation potential for 17β -estradiol than does ER β in most systems [27]. In our system the yeast expressing pRR-ER β -5Z was approximately 30-fold more sensitive to 17β -estradiol than the yeast expressing the pRR-ER α -5Z plasmid (comparing EC₅₀ values, Table 3). The simplest explanation for this difference was that an alteration in the ER α gene occurred during cloning, but this was not the case. Sequence analysis did not reveal any mutations that deviated from the standard human ER α sequences in Genbank. Repeating the cloning process with an independent ER α cDNA source yielded the same result when signaling from independently obtained plasmids was assessed in yeast. The fact that ER β signaling occurred at much lower ligand concentrations rules out explanations related to 17β -estradiol permeability or the inability to achieve sufficient intracellular ligand concentrations. Further analysis using immunoblotting revealed that ER α protein is expressed well from the pRR-ER α -5Z (data not shown). Presently we do not have a good explanation for the signaling discrepancies between the two estrogen receptor plasmids. One possibility is that the positioning of the ligand activated ER α protein on the EREs is not optimal in this plasmid context. This notion is supported by data for GR that suggests response elements direct receptor interactions with co-activators and other transcriptional factors to modify output of a target gene [28]. Other reasons for the relative insensitivity of ER α signaling may reside in the W303a strain or with other nuances in plasmid construction that somehow influence signaling efficiency. We are currently trying to identify the basis for this effect. Nevertheless, pRR-ER α -5Z is similar in ligand sensitivity to ER α expression systems described by others and is useful for screening and other purposes as illustrated by the radicicol inhibition experiments (Table 4).

4.3 The system we describe here can be used to rapidly assess agonist or antagonist SHR ligands. Additionally, monitoring effects on SHR signaling in this system can assess the action of indirect-acting agents, such as Hsp90 inhibitors. Radicicol and other agents that interfere with the N-terminal ATP binding site of Hsp90 are being intensely investigated as potential anti-cancer agents and for non-cancer diseases as well [29-31]. Data from Fig. 3 and Table 4 demonstrate this system can be used to identify off target effects of radicicol, which in a clinical setting might appear as an adverse effect. Note that concentrations of radicicol needed to interfere with cell growth or viability (Fig. 3) are greater than doses needed to interfere with SHR signaling. This result suggests that SHR function is especially dependent on Hsp90. The power of this system is illustrated by comparison of the effects of radicicol on signaling of six

different human SHRs within a constant cellular background. It would be difficult to perform a similar series of experiments in mammalian cells. This would require identification of a mammalian cell line that lacked all these receptors and then introduction and assessment of genetically stable SHR expression and reporter gene constructs. Radicicol caused a similar effect across all the SHRs (Table 4). Thus, in a constant cellular (Hsp90) environment, the drug would be expected to be a general inhibitor of SHR function. However, tumor cells and perhaps other cell types that might be targeted by Hsp90 inhibitors differ in the levels and isoforms of Hsp90 proteins and co-chaperones they express [32]. These expression differences provide the basis for use of Hsp90 inhibitors as therapeutic agents.

4.4 It is notable that ER β signaling was affected by radicicol. This SHR has not been extensively characterized for its interactions with the Hsp90 system. Some genetic and pharmacological evidence for a role of Hsp90 in ER β signaling have been presented [33]. Our finding that similar radicicol concentrations affected all the SHRs suggests a singular target, namely Hsp90, is involved. Radicicol-mediated inhibition of signaling supports the notion of a common regulatory effect of Hsp90 across the type I SHRs, including ER β . Interestingly, radicicol preferentially interfered with the Hsc82 isoform of Hsp90 over the Hsp82 isoform in a previous study in which aryl hydrocarbon receptor was expressed in yeast [34]. Thus, when used in combination with genetic approaches, these humanized yeast strains may be especially useful for drug or xenobiotic screening. Future studies with these yeast lines will entail coupling of genetic and pharmacological approaches to dissect the common from unique features of the SHRs and their interactions with chaperone proteins.

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Figure 1. General Structure of pRR Plasmids

The pRR series of plasmids is designed to express the individual type I human steroid hormone receptors, as depicted by the receptor cDNA sequence (labeled RECEPTOR cDNA). The different response elements (5-RE) upstream of a minimal cytochrome C (CYC) promoter that direct β -galactosidase reporter gene (LACZ) regulation are described in Table 2. Selective plasmid maintenance in yeast is conferred by the *TRP1* gene. The autonomously replicating sequence (ARS) and centromere sequence (CEN) provide replication and segregation functions, respectively, in yeast. Replication and selective propagation in *E. coli* are conferred by the *Col E1* or *f1* origins and the β -lactamase gene (AMP), respectively.

Figure 2. Dose Response Curves for Yeast with pRR Plasmids

Yeast containing pRR plasmids were treated with ligands for 18 hr and then assessed for signaling using lacZ assays. The scale on the x-axis is logarithmic, and data were normalized to a maximal 100% ligand response to facilitate graphical comparisons. Symbols representing each receptor signaling profile are noted in the panel, and the error bars indicate the standard error of the mean. The ligands used to activate receptors were testosterone (AR), 17 β -estradiol (ER α and β), dexamethasone (GR), aldosterone (MR), and progesterone (PR).

Figure 3. Cytotoxicity of Radicicol

Yeast cultures were treated with a range of radicicol concentrations and assessed after 18 h in culture. The curve reflects radicicol cytotoxicity as measured by relative culture density, with error bars indicating the standard error of the means. The scale of the x-axis is logarithmic, and asterisks indicate points that are significantly different from the control (100%).

Table 2Relevant Features of the Receptor-Reporter (pRR) Plasmids.^b

Plasmid	Promoter	Human Receptor, cDNA Source	SHR Response Elements
pRR-AR-5Z	<i>GPD</i>	Androgen, pARH, [10]	5 HREs
pRR-ER α -5Z	<i>GAL1,10</i>	Estrogen- α , p2HG/ER, [12]	5 EREs
pRR-ER β -5Z	<i>GPD</i>	Estrogen- β (Origene, SC119216)	5 EREs
pRR-GR-5Z	<i>GAL1,10</i>	Glucocorticoid (Origene, SC302164)	5 HREs
pRR-MR-5Z	<i>GAL1,10</i>	Mineralocorticoid, phMR3750, [35]	5 HREs
pRR-PR-5Z	<i>GAL1,10</i>	Progesterone, pSG5-hPR1, [36]	5 HREs

^b The plasmid names, the yeast promoter used to direct receptor expression, the sources of the receptor cDNAs used, and the response elements (EREs or HREs) that direct reporter gene expression are shown. Two cDNAs were obtained from Origene, Inc., and their product numbers are listed.

Table 3EC₅₀ Values for Ligand-Induced Signaling Activation in Yeast With pRR Plasmids.

<u>RECEPTOR</u>	<u>LIGAND</u>	<u>EC₅₀ (nM)</u>	<u>95% C.I. (nM)</u>
AR	TESTOSTERONE	45	26-68
ER α	17 β -ESTRADIOL	1.1	0.7-1.7
ER β	17 β -ESTRADIOL	0.034	0.024-0.048
GR	DEXAMETHASONE	10,000	7,800-13,400
MR	ALDOSTERONE	1,000	800-1,300
PR	PROGESTERONE	130	104-161

Table 4IC₅₀ Values for Radicicol-Induced Inhibition of SHR Signaling in Yeast.^c

<u>RECEPTOR</u>	<u>IC₅₀ (μM)</u>	<u>95% C.I. (μM)</u>
AR	0.39	0.22-0.72
ERα	0.54	0.31-0.93
ERβ	0.89	0.48-1.62
GR	0.59	0.34-1.03
MR	1.23	0.62-2.47
PR	0.97	0.48-1.98

^cThe IC₅₀ values were not statistically different from one another when assessed by ANOVA.