Construction of sensitive reporter assay yeasts for comprehensive detection of ligand activities of human corticosteroid receptors through inactivation of *CWP* and *PDR* genes

Sayoko Ito-Harashima^a, Kazuhiro Shiizaki^{a,1}, Masanobu Kawanishi^a, Koji Kakiuchi^a, Kana Onishi^a, Ryoichi Yamaji^b, and Takashi Yagi^{a,c,*}

^aDepartment of Biological Science, Graduate School of Science,

^bDepartment of Applied Life Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka, Japan, and ^cDepartment of Life Science, Dongguk University, Seoul, Korea

*Corresponding author at: Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan. Tel.: +81 72 254 9862; fax: +81 72 254 9938.

E-mail address: yagi-t@riast.osakafu-u.ac.jp (T. Yagi).

¹ Present address: Division of Cancer Development System, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan.

Keywords: glucocorticoid receptors, mineralocorticoid receptors, steroid hormone, *Saccharomyces cerevisiae*, reporter assay, agonist, antagonist

Abbreviations: GR, glucocorticoid receptor; MR, mineralocorticoid receptor; GRE, glucocorticoid response element

ABSTRACT

Introduction: The glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) are members of the nuclear receptor superfamily and ligand-dependent transcription factors, whose major ligands are glucocorticoid and mineralocorticoid, so-called corticosteroids. The corticosteroids are a class of substances that include steroid hormones naturally produced in the adrenal cortex of vertebrates and analogues of these hormones that are synthesized in industry. They are involved in a wide range of physiological processes including stress and immune responses, and the regulation of carbohydrate metabolism, protein catabolism, sodium homeostasis, and inflammation. These substances are potential environmental contaminants because they are clinically consumed in large amounts worldwide. To develop a simple and sensitive bioassay to detect corticosteroids, we newly established reporter assay yeasts expressing human GR and MR. **Methods:** Ligand responses of the established assay yeasts were improved by forced expression of a human transcription coactivator SRC-1 ϵ . Further enhancement of the responses was achieved by inactivating the *CWP* and *PDR* genes that encode cell wall mannoproteins and plasma membrane efflux pumps, respectively, which may be attributable to an increased intracellular concentration of ligands. **Results:** These new assay yeasts were more responsive to both natural and synthetic agonist ligands than the conventional assay yeasts. They detected both agonistic and antagonistic activities of mifepristone, spironolactone, and eplerenone in a receptor-selective manner. They also detected ligand activities contained in oral pharmaceutical tablets and human urine.

Discussion: This assay system will be a valuable tool to detect agonists as well as antagonists of corticosteroid receptors, in the fields of drug discovery and the assessment of environmental pollutants.

1. Introduction

Endogenous corticosteroids are hormones secreted by the adrenal cortex that mediate various physiological processes including metabolism, immune responses, and electrolyte homeostasis (Rhen & Cidlowski, 2005). The corticosteroid receptors, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), are members of the nuclear receptor superfamily, which includes receptors for steroid hormones, thyroid hormones, retinoic acid, and vitamin D₃ (Mangelsdorf, et al., 1995; Schena & Yamamoto, 1988). All members of this superfamily function as ligand-dependent transcription factors and share structural similarities, which are characterized by three distinct domains: an N-terminal transcriptional activation domain (AD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The activation function 1 (AF-1) region within AD is essential for full transcriptional activity of the GR (Freedman, Yoshinaga, Vanderbilt, & Yamamoto, 1989; Tsai, et al., 1990; Wright, McEwan, Dahlman-Wright, & Gustafsson, 1991). AF-2, another region within LBD, requires a ligand to manifest its activity. The binding of ligands, corticosteroids, to the GR and MR causes their translocation into the nucleus, and the receptors bind as homodimers to a specific DNA element, termed the glucocorticoid response element (GRE), which is present upstream of corticosteroid-regulated gene promoters. Upon binding to the GRE, the receptors regulate the temporal and spatial expression of target genes by recruiting a wide spectrum of coregulator proteins that activate or repress transcription (Beato, Herrlich, & Schutz, 1995; Mangelsdorf, et al., 1995).

The GR and MR are particularly closely related, and the majority of the sequence variability and probable receptor specificity resides in the N-terminus, whereas the DBD and LBD are relatively homologous, sharing 94% and 57% identity, respectively (Arriza, et al., 1987). This sequence conservation is reflected in their overlapping functions, both *in vitro* and *in vivo*. *In vitro*, the GR and MR are poorly differentiated in terms of the specificity of their ligands and hormone response elements. Although the mineralocorticoid aldosterone binds to MR with relatively high specificity, the physiological glucocorticoid cortisol binds to both GR and MR. While the maximal level of transactivation by GR is 10-fold greater than by MR (this difference is mediated by their N-terminus sequences), ligand binding and transactivation by MR occur at 10-fold-lower cortisol concentration than by GR (reviewed by (Lim-Tio, Keightley, & Fuller, 1997). The DNA binding and transactivation by simple GREs, such as those within the mouse mammary tumor virus (MMTV) long terminal repeat promoter, reflect the high level of identity of DBD of GR and MR. Domain swap experiments in which the DBD of both receptors are exchanged did not exhibit any difference in the transactivation of simple promoters, which implies functional equivalence of the DBD, at least at these response elements. On the basis of these studies, it has been suggested that GR and MR could be referred to as low- and high-affinity corticosteroid receptors, rather than as specific receptors for glucocorticoids and mineralocorticoids (Evans & Arriza, 1989; Rupperecht, et al., 1993).

In vivo, glucocorticoids and mineralocorticoids have both distinct and overlapping physiological effects. Both steroids indirectly affect blood pressure by regulating

sodium and water absorption across epithelial tissues such as kidney and colon (Evans, 1988; Fuller, 1991; Grotjohann, Schulzke, & Fromm, 1999; Naray-Fejes-Toth & Fejes-Toth, 1990). However, the mechanisms by which they regulate sodium and potassium transport, at least in the distal colon, differ (Bastl, Schulman, & Cragoe, 1992; Lomax & Sandle, 1994), indicating that the MR and GR play distinct roles at nonepithelial tissues. Glucocorticoids act independently of mineralocorticoids on diverse processes such as inflammation, bone turnover, and glucose metabolism. The MR-specific functions include central blood pressure control (Gomez Sanchez, 1995), modulation of neuronal excitability (Joels & de Kloet, 1990), and stimulation of cardiac fibrosis (Weber, Sun, & Guarda, 1994). With respect to neuronal excitation, MR and GR have opposite effects (Joels & de Kloet, 1990).

Reflecting this wide array of functions, synthetic corticosteroids with glucocorticoid activities in particular constitute a large class of drugs that are indispensable in treating inflammation, autoimmune disorders, cancer, organ transplant rejection, and brain edema (Rhen & Cidlowski, 2005). Besides their glucocorticoid properties, these synthetic steroids possess mineralocorticoid properties causing unexpected side effects such as fluid-electrolyte imbalance and hypertension (Fried & Borman, 1958). Therefore, a simple and convenient assay system that can distinguish specific ligands for the GR and MR may facilitate novel drug development and the evasion of adverse side effects. Moreover, both natural and synthetic corticosteroids have been emerging as environmental contaminants (Bovee, Helsdingen, Hamers, Brouwer, & Nielen, 2011; Kugathas, Williams, & Sumpter, 2012; Tolgyesi, Verebey,

Sharma, Kovacsics, & Fekete, 2010). Hence, such assay systems may be applicable for the assessment of environmental pollutants.

Several *in vitro* reporter assays have been developed to screen nuclear receptor ligands. These bioassays are based on the quantification of reporter enzymes that reflect nuclear receptor activation in mammalian or yeast cells. Yeast-based bioassays are more cost-effective and convenient than mammalian cell-based ones. Moreover, yeasts do not possess any endogenous mammalian nuclear receptor homologs; therefore, they can be used to detect ligands that are specific to individual receptors. In this study, we developed yeast reporter assay systems that express human GR and MR in mutant yeast strains besides the wild-type strain. Yeast genes involved in cell wall and membrane biosynthesis were modified genetically to alter the substance permeability of the cells. In addition to the physical barrier provided by the cell wall and membrane, budding yeasts also possess a dynamic biochemical defense system against xenobiotics called pleiotropic drug response or pleiotropic drug resistance (PDR) (Balzi & Goffeau, 1995). PDR is mediated by a network of ATP-binding cassette (ABC) transporter proteins that constitute active efflux pumps for a broad spectrum of unrelated chemicals. Therefore, the deletions of these genes may improve the sensitivities of the yeast reporter assay by increasing the intracellular concentration of ligands. We examined whether the GR and MR assay yeast strains that lack cell wall mannoproteins (Cwp1p, Cwp2p) and/or plasma membrane-localized ABC transporters (Pdr5p, Pdr10p) are highly sensitive to both natural and synthetic corticosteroids, and applicable to detect ligand activities from various samples.

2. Materials and methods

2.1. Strains and media

An *Escherichia coli* strain, DH5 α , was used as a host strain to amplify plasmid DNA. *S. cerevisiae* strains are listed in Table S-1. All *Saccharomyces cerevisiae* strains used in this study were isogenic with W303a (*MATa*, *ura3-1*, *ade2-1*, *trp1-1*, *leu2-3*, *his3-11, 15*, *can1-100*). Yeast extract peptone dextrose (YPD) and synthetic dextrose complete dropout (SDC-X) media were prepared as previously described (Burke, Dawson, & Stearns, 2000). YEP(Gal) and synthetic galactose complete dropout (SGC-X) media contained 2% (w/v) galactose instead of dextrose. YPD containing G418 (Geneticin, Gibco BRL) has been described previously (Wach, Brachat, Pohlmann, & Philippsen, 1994). All solid media contained 2% (w/v) agar in plates.

2.2. Chemicals

Dimethyl sulfoxide (DMSO), cortisone, corticosterone, dexamethasone, hydrocortisone, betamethasone, prednisolone, and testosterone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dithiothreitol (DTT), 17 β -estradiol (E2), and progesterone were obtained from Nacalai Tesque (Kyoto, Japan). Aldosterone, eplerenone, mifepristone, spironolactone, and *o*-nitrophenyl- β -D-galactopyranoside (ONPG) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Fludrocortisone acetate was obtained from MP Biomedicals, Inc. (Solon, OH, USA). Triamcinolone was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Restriction enzymes, DNA modification enzymes, and other chemicals

were obtained from Wako Pure Chemical Industries, Ltd., TaKaRa Bio Inc. (Otsu, Japan), or TOYOBO Co. (Osaka, Japan).

2.3. Plasmid construction

The GR and MR expression plasmids, and the reporter plasmid were constructed for the development of GR and MR ligand reporter assays. The primer sequences used in this study were synthesized by Sigma-Aldrich Japan (Tokyo, Japan) and are listed in Table S-2.

DNA fragments containing the human GR open reading frame (ORF) were obtained by polymerase chain reaction (PCR) from a plasmid, pcDNA3.1-GR (Harada, et al., 2007), with primers GRfRbBm and GRrXh, which contain a restriction site and/or a yeast ribosomal binding consensus sequence near the initiation codon. PCR was carried out with high-fidelity PCR polymerase KOD plus (TOYOBO Co. Ltd., Osaka, Japan), according to the manufacturer's instructions. The amplified fragment was digested with *Bam*HI and *Xho*I, and cloned into *Bam*HI-*Sa*I sites of the expression vector pUdp6 (Shiizaki, Asai, Ebata, Kawanishi, & Yagi, 2010). The resultant plasmid was designated as pUdp6GR. The plasmids were isolated and purified using QIAGEN Mini Prep Kit (Valencia, CA).

The human MR ORF was amplified from human kidney total RNA (TaKaRa Bio Inc., Otsu, Japan) by reverse-transcription polymerase chain reaction (RT-PCR). The cDNA was obtained using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), and then MR cDNA was amplified by PCR using the KOD-plus PCR kit

with two sets of primers. The first PCR was performed with hMR1f and hMR3006r, and the second nested PCR was carried out with hMRfBg and hMTrHi. The amplified fragment was digested with *Afl*III and blunt-ended using KOD polymerase, followed by *Bgl*III digestion. The pUdp6 vector was digested with *Hind*III, blunt-ended using KOD polymerase, and digested with *Bam*HI. The digested MR cDNA and pUdp6 were ligated to construct pUdp6MR. Nucleotide sequences of GR and MR ORFs were confirmed using the ABI DNA sequencer.

The reporter plasmid pYT β -GRE-R8CS was constructed by inserting eight copies of glucocorticoid responsive element (GRE)-containing oligonucleotides into pRW95-3 vector (Wolf, Roder, & Schweizer, 1996) upstream of the CYC1 minimal promoter. The oligonucleotides GREcsfBg and GREcsrBc (Table S-2) were annealed, phosphorylated with T4 polynucleotide kinase, ligated, and inserted into the *Bgl*III site of pRW95-3. The copy number and the orientation of GRE in pYT β -GRE-R8CS were confirmed by sequencing. The control reporter plasmid for constitutive expression of β -galactosidase was constructed by ligation of two *Nae*I-*Sca*I fragments from pYT-cyc containing *CYC* promoter-*lacZ* fusion (Shiizaki, et al., 2010) and pESC-LEU containing yeast 2 μ origin and the *LEU2* gene (Agilent Technologies, Inc., Santa Clara, CA, USA). The resultant plasmid was designated as pESC-LEU-CYCp.

2.4. PCR-mediated gene deletion to construct yeast mutants

To construct W303a mutant yeasts, deletion of the *CWP1*, *CWP2*, *PDR5*, and/or *PDR10* genes was carried out by homologous recombination of the *loxP*-kanMX-*loxP*

target gene cassette and subsequent *Cre*-induced excision of kanMX (Guldener, Heck, Fielder, Beinhauer, & Hegemann, 1996). The *loxP*-kanMX-*loxP* cassette was amplified by PCR with the primers shown in Table S-2 using Platinum *Taq* DNA polymerase (Invitrogen, CA, USA). Transformation of the yeast strains with the cassettes and *Cre*-expression plasmid was performed by the lithium acetate procedure (Gietz, Schiestl, Willems, & Woods, 1995). The *Cre*-mediated excision of marker ORF from the *loxP*-kanMX-*loxP* cassette was performed using the plasmid pSH47 (Guldener, et al., 1996) under culture conditions described previously (Ito-Harashima & McCusker, 2004). Colony PCR was used to confirm insertion-deletion mutations as described previously (Niedenthal, Riles, Johnston, & Hegemann, 1996).

2.5. Construction of GR and MR reporter assay yeasts

To construct the GR and MR assay yeasts, the reporter plasmid pYT β -GRE-R8CS and the expression plasmid for human transcriptional coactivator SRC1-e, pESC-Leu-SRC1e (Chu, Shiizaki, Kawanishi, Kondo, & Yagi, 2009), were introduced into wild-type and mutant W303a strains. A transformant grown on SDC-TRP/LEU agar medium was isolated and used as a host for subsequent transformation. The GR expression plasmid pUdp6GR and MR expression plasmid pUdp6MR were linearized by *EcoRV* digestion and integrated into the *ura3* locus in the yeast genome by homologous recombination. Transformants were selected on SCD-TRP/LEU/URA agar plates. The CYC yeast, a control strain for the antagonist assays, was constructed by introducing pESC-LEU-CYCp into wild-type W303a, and transformants were selected

on an SDC-LEU agar plate.

2.6. Corticosteroid activity assay using GR and MR reporter yeasts

The assays were conducted as described previously (Shiizaki, et al., 2010) with minor modification. The yeast strains were grown overnight at 30 °C in SDC-TRP/LEU/URA medium, and optical density (OD) at 595 nm of each culture was adjusted to 1.0 with the same medium. A 1 µl aliquot of the test chemicals dissolved in 10% DMSO, 10 µl of the overnight culture yeast, and 90 µl of SGC-TRP/LEU/URA was mixed in a 96-well polystyrene microplate with subsequent incubation for 18 hours at 30 °C. Each cell suspension (10 µl) was transferred to a new 96-well microplate and 100 µl of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM KCl, 2 mM DTT, and 0.2% sarcosyl, adjusted to pH 7.0), containing 1 mg/ml ONPG, was added to the plates with subsequent incubation for 60 min at 37 °C. Absorbances at wavelengths of 405 and 595 nm were measured by using Micro Plate Reader Model 680 (BioRad Laboratories, Inc.) to estimate the β-galactosidase activity as the amount of *o*-nitrophenol produced and the yeast cell density, respectively. The agonist-dependent *lacZ* reporter induction was demonstrated as “increase of induction”, which was calculated using the following formula: {OD₄₀₅ (sample)/OD₅₉₅ (sample)}-{OD₄₀₅ (DMSO)/OD₅₉₅ (DMSO)}.

The antagonistic activities of chemicals were examined by competition between antagonist and agonist added at the concentration of EC₅₀. In the GR antagonist assay, dexamethasone was mixed with test ligands (8.3 µM and 550 nM for the wild type and

the *pdr5Δpdr10Δ* strain, respectively). In the MR antagonist assay, aldosterone was mixed with test ligands (374 nM and 31 nM for the wild type and the *cwp1Δpdr5Δ* strain, respectively). The dose-dependent repression of *lacZ* reporter expression by antagonists is shown as “relative activity” (%), which was calculated using the following formula: $\{OD_{405} \text{ (sample)}/OD_{595} \text{ (sample)}\}/\{OD_{405} \text{ (DMSO)}/OD_{595} \text{ (DMSO)}\} \times 100$. To distinguish antagonist activity from non-specific inactivation of gene expression, the CYC yeast constitutively expressing the *lacZ* reporter was used for comparison.

2.7. Extraction and concentration of synthetic corticosteroids from oral tablets

To test GR and MR ligand activities of pharmaceutical products, ten oral tablets for each of decadron, florinef, ledercort, and rinderon were ground into fine powder in a mortar and suspended in methanol (1 g/12 ml) in polycarbonate tubes. These tubes were shaken for five minutes to extract organic substances. After centrifugation at 4000 rpm for five minutes, the supernatants were transferred to 1.5 ml microtubes (1 ml/tube) and dried using a vacuum concentrator. The extracts were redissolved in 15 µl of DMSO and used for yeast reporter assays.

2.8. Extraction and concentration of organic substances from urine

To detect GR and MR ligand activities of human samples, 50 ml of urine from an adult female was filtered using a GF/C 47 mmφ membrane (Whatman International Ltd., Maidstone, England) and acidified by adding H₂SO₄ at a final concentration of 0.5 M

for deconjugation. After incubation at 60 °C for 1 hr, urine samples were neutralized using 10 N NaOH and flowed through a Waters Sep-pak Plus C18 Environmental cartridge (Waters Corporation, MA, USA). The bound substances were eluted from the cartridge with 2 ml of DMSO at a flow rate of 1 ml/min and then dried using a vacuum concentrator. The extract was redissolved in 500 µl of DMSO (i.e., concentration factor was 100) and used as test samples in the yeast reporter assays.

3. Results

3.1. Construction of GR and MR ligand assay yeasts, and enhancement of their responses by inactivation of cell wall mannoprotein and/or ABC transporter genes

When human GR and MR were expressed in the wild-type yeast strain W303a, dose-dependent induction of the *lacZ* reporter gene was observed in response to human intrinsic ligands, corticosterone and aldosterone, respectively (Fig. 1). The EC₅₀ values of GR and MR were 6.5 μ M and 990 nM, respectively, which were less sensitive than a reporter assay with mammalian cells (Wilkinson, Hayes, Thompson, Whitney, & Bi, 2008). To improve the yeast reporter assay for GR and MR, we established deletion mutants of cell wall mannoproteins and/or efflux pumps on the plasma membrane because disruption of these classes of genes could enhance the sensitivities of DNA-damage-sensing yeasts to test chemicals (M. Zhang, et al., 2010; M. Zhang, et al., 2008); single, double, and triple deletion mutants of *CWP1*, *CWP2*, *PDR5*, and/or *PDR10* genes were established by the *Cre-loxP* method (Table S-1). The GR and MR assay yeasts were established by using each mutant as well as wild-type W303a. Fig. 1 represents the dose-dependent induction of *lacZ* reporter against corticosterone and aldosterone in the GR and MR assay yeasts, respectively. For GR, the assay yeasts established in six mutants, *pdr5* Δ , *pdr10* Δ , *cwp1* Δ *pdr5* Δ , *cwp1* Δ *pdr10* Δ , *pdr5* Δ *pdr10* Δ , and *cwp1* Δ *pdr5* Δ *pdr10* Δ , showed an increased response to corticosterone (Fig. 1A). The GR yeast with *pdr5* Δ *pdr10* Δ was the most responsive: relative reporter activity was 20-fold higher, the EC₅₀ value was lowered to 1/20, and the minimum detection limit was also decreased to 1/10, in comparison with those for wild-type W303a (Table 1).

For the MR assay yeasts, the response to aldosterone was improved in the mutants, except for *cwp1Δ* (Fig. 1B). The most responsive MR assay yeast was obtained in the *cwp1Δpdr5Δ* mutant that has 13-fold-higher relative reporter activity, 1/70-lower EC₅₀ value, and 1/100-lower minimum detection limit, in comparison with those for wild-type W303a (Table 1).

3.2. Cross-reactivity of newly established GR and MR assay yeasts against endogenous and synthetic corticosteroids, and sex steroid hormones

We chose the most responsive mutant yeast strains *pdr5Δpdr10Δ* and *cwp1Δpdr5Δ* for the GR and MR reporter assays, respectively, and compared responses against endogenous and synthetic corticosteroids with that of wild-type W303a. Three glucocorticoids, hydrocortisone (cortisol), corticosterone, and cortisone, and a mineralocorticoid, aldosterone, were used as endogenous ligands for the reporter assay (Fig. 2A). Dexamethasone, prednisolone, triamcinolone, and betamethasone were used as synthetic glucocorticoids, and fludrocortisone acetate as a synthetic mineralocorticoid (Fig. 2B). Both GR and MR mutant assay yeasts responded to these agonistic ligands more sensitively than the wild-type yeasts (Fig. 2 and Table 2). It is known that GR and MR share ligand specificities, and our results are consistent with the ligand responses shown *in vivo* because GR and MR assay yeasts were able to detect these ligands in common, but also possess specific responses unique to each of them (Fig. 2A, B and Table 2). Consistent with the findings that mineralocorticoid aldosterone as well as physiological glucocorticoids bind to MR with a higher affinity

than to GR (Arriza, et al., 1987), the mutant MR assay yeast detected these endogenous corticosteroids more effectively than the mutant GR assay yeast (Fig. 2A). In the assay of the synthetic corticosteroids, the mutant MR assay yeast detected dexamethasone, prednisolone, and fludrocortisone acetate more sensitively, while triamcinolone and betamethasone showed more potent agonistic activities toward the mutant GR assay yeast (Fig. 2B and Table 3).

We also examined the cross-reactivity of the GR and MR assay yeasts to sex steroid hormones, 17 β -estradiol (E2), testosterone, and progesterone, which are ligands for estrogen receptors (ER), androgen receptor (AR), and progesterone receptor (PR), respectively. The GR mutant assay yeast induced slight *lacZ* reporter activity in response to E2 and testosterone at high doses. The MR mutant assay yeast barely detected E2, while significantly high reporter activity was induced against testosterone. Both wild-type and mutant GR assay yeasts, and the mutant MR assay yeast responded to progesterone in a dose-dependent manner, with markedly higher sensitivity in mutant MR assay yeast (Fig. 3 and Table 4).

3.3. Response to mifepristone, spironolactone, and eplerenone in GR and MR assay yeasts

We investigated the antagonistic activities of three chemicals, mifepristone, spironolactone, and eplerenone, by simultaneous exposure to dexamethasone and aldosterone for GR and MR assay yeasts, respectively. As a reference, we used the control yeast strain CYC that expresses β -galactosidase constitutively.

In GR assay yeasts, the transactivation by dexamethasone was significantly inhibited by mifepristone, which possesses antiprogesterone and antiglucocorticoid activity, compared with the MR assay yeasts. Spironolactone, which is an antimineralocorticoid with progestogenic and antiandrogenic activities (de Gasparo, et al., 1987), showed antagonist activity in the mutant GR assay yeast, and both wild-type and mutant MR assay yeasts. Eplerenone, an MR-selective antagonist (de Gasparo, et al., 1987), showed a significant inhibitory effect on both wild-type and mutant MR yeasts, especially mutant MR yeast, while the GR assay yeasts were barely affected by this chemical (Fig. 4A and Table 5). At concentrations up to 100 μ M, no transcriptional repression of constitutive β -galactosidase activity was found in the control strain CYC yeast (Fig. 4A), indicating that the GR and MR assay yeasts detected antagonist activities of these ligands in a receptor-selective manner.

It was previously reported that mifepristone and spironolactone possess agonist activities to mouse GR (S. Zhang, Jonklaas, & Danielsen, 2007) and human MR (Massaad, Lombes, Aggerbeck, Rafestin-Oblin, & Barouki, 1997), respectively. Therefore, we also carried out a reporter assay to detect agonistic activities of these three chemicals. Mifepristone showed higher agonistic activity in mutant GR assay yeast than in the wild-type GR assay yeast. Mutant MR assay yeast showed a higher response to spironolactone than the mutant GR assay yeast. The induction of reporter gene expression by eplerenone was detected only in mutant MR assay yeast (Fig. 4B and Table 6). These data indicate that the GR and MR assay yeasts detected agonist activities of these ligands, as well as antagonist activities, in a receptor-selective

manner.

3.4. Validation of the GR and MR reporter assay yeasts

To validate the established GR and MR assay yeasts, we tested whether corticosteroid activities were detectable from the oral pharmaceutical tablets and human urine samples. We extracted and concentrated the organic compounds from urine and the oral tablets containing endogenous or synthetic corticosteroids. These samples were used as ligands in reporter assays. In the assays of oral tablets, we used four different tablets, decadron, florinef, ledercort, and rinderon, which contain dexamethasone, fludrocortisone acetate, triamcinolone, and betamethasone, respectively, as pharmaceutically active ingredients. As shown in Fig. 5, the wild-type GR assay yeast detected corticosteroid activities from decadron and rinderon only in highly concentrated samples. The wild-type MR assay yeast barely detected ligand activities from any of the tablets. The mutant GR and MR assay yeasts responded more sensitively and detected ligand activities from all of the tablets, with a more than 10-fold-lower detection limit.

We next examined whether corticosteroid activities were detectable from human urine. Both wild-type GR and MR assay yeasts barely detected *lacZ* reporter activities in urine, while the mutant assay yeasts clearly showed these activities (Fig. 6). The MR assay yeast was highly responsive compared with the GR assay yeast. These results indicate that these GR and MR assay yeasts established in the mutants are valuable to detect trace amounts of corticosteroid activities in urine.

4. Discussion

To understand the regulatory mechanisms and ligand specificities of mammalian GR and MR, reconstitution of corticosteroid-dependent transcriptional regulation has been undertaken in yeast cells (Bohen & Yamamoto, 1993; Hong, Kohli, Garabedian, & Stallcup, 1997; Schena & Yamamoto, 1988; Wright, Carlstedt-Duke, & Gustafsson, 1990; Wright & Gustafsson, 1992). Several yeast assay systems to detect nuclear receptor ligands have been developed. Most assays adopt the yeast two-hybrid system by using a fusion protein consisting of the ligand-binding domain of the nuclear receptor and the Gal4 DNA binding domain. We attempted to construct a yeast reporter assay that was as similar as possible to the human cellular process with an intact nuclear receptor. In our laboratory, several yeast reporter assay systems with intact mammalian nuclear receptors have been developed (Chu, et al., 2009; Kawanishi, et al., 2008; Kawanishi, Sakamoto, Shimohara, & Yagi, 2006; Shiizaki, et al., 2010; Shiizaki, et al., 2014). In our present advanced systems, we used three classes of plasmids with different replication origins to establish and optimize recombinant assay yeasts. First, the nuclear receptor expression vector does not possess a yeast replication origin, so this is integrated into the *ura3* locus on chromosome V by homologous recombination, which enables stable expression of the nuclear receptor at an appropriate level. Second, a reporter plasmid carrying *lacZ* containing a *CEN6-ARS4* origin is maintained at low copy numbers in yeast cells, and expresses the reporter gene product at a low level. Third, the expression plasmid for transcriptional coactivator SRC-1e, the major transcript of SRC-1 (Hayashi, Ohmori, Ito, & Seo, 1997) containing the yeast 2 μ origin,

exists as an episomal plasmid, and is maintained at high copy numbers in yeast cells. According to this strategy, we successfully established reporter assay yeasts expressing human corticosteroid receptors GR and MR, and enhanced their responses to ligands by inactivating the *CWP* and *PDR* genes.

Construction of yeast reporter assay systems by expressing intact human GR to detect glucocorticoids were previously reported (Bovee, et al., 2011; Wright, et al., 1990; Wright & Gustafsson, 1992). Compared with their GR assay yeast, the response of our wild type GR assay strain was four- to twenty-fold more intense with respect to the EC₅₀ (Bovee, et al., 2011). There are some differences among these systems, in terms of the expression vector and reporter gene constructs and the detection method. In addition, the presence of the transcriptional coactivator SRC-1 in our yeast strains should be a notable distinction from the other yeast systems. There are similar reports showing that mouse glucocorticoid receptor-interacting protein 1 (GRIP1) that is ortholog of human SRC-2, another member of p160 coactivator family (Xu & Li, 2003) potentiated transactivation of rodents GR and MR in yeast (Ding, et al., 1998; Hong, et al., 1997; Hong, Kohli, Trivedi, Johnson, & Stallcup, 1996). These results indicate that the introduction of SRC-1e is the basis of the establishment of highly sensitive reporter assay yeasts for GR and MR in our system.

It has been previously reported that mannoproteins form an external cell wall layer, which determines wall porosity (Klis, Boorsma, & De Groot, 2006; Klis, Mol, Hellingwerf, & Brul, 2002), and *CWP1* and *CWP2* are two main genes encoding the cell wall mannoproteins (van der Vaart, Caro, Chapman, Klis, & Verrips, 1995). *PDR5*

is one of the three major ABC transporters, which have substrate specificities as well as overlapping functions for some chemicals in yeast (Kolaczkowski, Kolaczowska, Luczynski, Witek, & Goffeau, 1998; Kolaczkowski, et al., 1996). Mutation in the *PDR5* gene was previously found to potentiate the responses of rat GR and MR to ligands in yeast (Kralli, Bohen, & Yamamoto, 1995). Although the physiological role of the *PDR10* gene in PDR is not clear, the findings that Pdr10p is closely related to Pdr5p (67% identity) and is localized on the plasma membrane suggest that the deletion of this gene affects the responses of GR and MR to corticosteroids. As expected, single deletion of the *PDR10* gene resulted in improvement of the responses to ligands in both GR and MR assay yeasts. Moreover, simultaneous deletion of the *PDR5* and *PDR10* genes had an additive effect to improve the sensitivity of GR assay yeast. In contrast, deletion of the *CWP* genes did not show a significant effect. The MR assay yeasts established in *cwp2Δ* and *cwp1Δcwp2Δ* mutants showed a slight increase of their responses to aldosterone at a high concentration. However, simultaneous deletion of *CWP1* and *PDR5* greatly enhanced the sensitivity of MR assay yeast compared with *pdr5Δ* (Fig. 1). Examination of the agonist activities of endogenous and synthetic ligands revealed that the responses of *pdr5Δpdr10Δ* and *cwp1Δpdr5Δ* mutant assay yeasts for GR and MR, respectively, were greatly improved. Their EC₅₀ values were over 10-fold lower than those of the wild-type assay yeasts (Fig. 2-3 and Table 1-3). These data suggest that Pdr10p as well as Pdr5p act as plasma membrane-localized efflux pumps for corticosteroids, and *cwp1Δ* also contributes to improvement of the cell permeability for corticosteroids.

In experiments examining the cross-reactivity of the our GR and MR assay yeasts, we observed that both wild-type and mutant MR assay yeasts responded to aldosterone and hydrocortisone (cortisol) more sensitively than the GR assay yeasts (Fig. 2A and Table 2). This agrees with previous reports showing that MR and GR are high- and low-affinity corticosteroid receptors, respectively, in mammalian cells (Evans & Arriza, 1989; Rupprecht, et al., 1993). On the other hand, these strains, especially mutant MR yeasts, showed abnormal ligand specificities: cortisone, an inactive glucocorticoid interconvertible with hydrocortisone *in vivo* (Edwards, et al., 1988; Funder, Pearce, Smith, & Smith, 1988) showed apparent agonist activity for the first time in reporter assay systems including mammalian cells (Fig 2A). In addition, progesterone showed strong agonist activity that is comparable to corticosteroids in our GR and MR assay yeasts (Fig. 3). Although progesterone has been known to act as an agonist in both yeast and mammalian cells expressing human and rodent corticosteroid receptors (Berger, Parandoosh, Perry, & Stein, 1992; Bovee, et al., 2011; Grossmann, et al., 2004; S. Zhang, et al., 2007), the efficacies were quite different between both systems. Progesterone was more potent agonist than glucocorticoids in yeasts (Garabedian & Yamamoto, 1992; Wright & Gustafsson, 1992), which is consistent with our result. Similarly, 11-deoxycorticosterone, which is precursor of corticosterone and aldosterone with weak mineralocorticoid activity *in vivo*, significantly stimulated transactivation via GR (human and rat) and MR (rat) in yeasts (Bohen & Yamamoto, 1993; Wright & Gustafsson, 1992). Corticosteroids and progesterone are compounds consisting of 21 carbons, therefore, the newly established reporter assay yeasts for GR and MR would be

suitable to explore new steroidal compounds with closely related structure that can be their ligands. Moreover, it might be possible to turn this as an advantage to reveal undesirable side effects of synthetic steroids including sex hormones on corticosteroid receptors.

We also tested whether these assay yeasts can measure antagonist activity. Antagonist ligands block the binding of agonist to the nuclear receptor by competition. Agonist ligands promote coactivator binding while antagonists block it by inducing a conformational change of the receptor. The antagonistic effects can be seen in the presence of coactivator and a moderate concentration of agonist ligand. Our assay yeasts were expected to detect antagonist because a general coactivator, SRC-1, had been introduced. In the yeast reporter assay of simultaneous exposure to chemicals with agonist (dexamethasone and aldosterone for GR and MR, respectively), three chemicals: mifepristone, spironolactone, and eplerenone, repressed agonist-induced reporter expression in a receptor-selective manner (Fig. 4A). In order to discern whether this repression is due to antagonist activities or nonspecific transcriptional repression of the test chemicals, we used a reporter assay yeast strain that constitutively expresses β -galactosidase under the control of the 5'-flanking region of the *CYCI* gene. In this control assay yeast strain, none of these chemicals repressed constitutive reporter gene expression, indicating that GR and MR assay yeasts indeed detected antagonist activities rather than nonspecific transcriptional repression. Mifepristone is clinically used for early pregnancy termination by its antiprogesterone activity, but also for the treatment of Cushing syndrome due to its antigluocorticoid activity (reviewed by

(Cadepond, Ulmann, & Baulieu, 1997). Both spironolactone and eplerenone have been used for the treatment of heart failure and hypertension by their antimineralocorticoid activities. Spironolactone, which was developed in the 1950s, possesses structural elements of the progesterone molecule; thus, it is associated with progestogenic and antiandrogenic adverse effects. Spironolactone is also known to activate GR (de Gasparo, et al., 1987). Eplerenone is a spironolactone derivative designed to enhance selective binding to MR while minimizing the effects on GR, PR, and AR, although spironolactone was approximately 40-fold more potent than eplerenone in blocking aldosterone activation of MR (de Gasparo, et al., 1987). The receptor selectivity and difference in efficacy of these antagonists in our yeast reporter assays (Fig. 4A and Table 6) were in good agreement with previous observations described above, as well as human cell-based reporter assay systems for steroid hormone receptors (Wilkinson, et al., 2008). Furthermore, agonistic activities of these chemicals were detected using GR and MR assay yeasts (Fig. 4B), which is also consistent with previous reports in mammalian systems (Massaad, et al., 1997; S. Zhang, et al., 2007). These results indicate that our assay system enabled, for the first time in yeast, the detection of both agonistic and antagonistic activities of mifepristone, spironolactone, and eplerenone, in a receptor-selective manner, with higher sensitivities using the mutant assay yeasts.

Finally, the GR and MR assay yeasts were applied to evaluate ligand activities in mixtures of organic compounds extracted from oral pharmaceutical tablets and human urine. This is the first example showing that a trace amount of corticosteroid activities from these samples are detectable in yeast reporter assay for human GR and MR (Fig. 5

and 6). Corticosteroids are metabolized to tetrahydrocorticosteroids and glucuronidized and excreted in urine (Ikegawa, et al., 2009), and some compounds are known to have agonist activities against rat GR and MR (Koshida, et al., 1990; McInnes, et al., 2004). Our GR and MR assay yeasts could detect such corticosteroid metabolites, in addition to active synthetic compounds tested in this study.

Although the yeast reporter assays are unable to identify particular chemicals, they will be powerful tools to evaluate total corticosteroid activities in a variety of samples, especially environmental samples. Our assay system will be a valuable tool to detect agonists as well as antagonists of corticosteroid receptors, in the fields of drug discovery and assessment of environmental pollutants.

Contributors

Sayoko Ito-Harashima designed the study and conducted most experiments, data analysis and draft manuscript preparation. Kazuhiro Shiizaki and Kana Onishi contributed to construction of plasmids and establishment of the yeast reporter assay. Koji Kakiuchi contributed to improvement of reporter assay yeasts, and validated them with various ligands. Masanobu Kawanishi contributed to preparation of essential materials and resources from the grants. Ryoichi Yamaji provided materials for the plasmid construction. Takashi Yagi organized the research project and arranged necessary resources from the grants.

Acknowledgements

We thank Shingo Ebata for construction of the plasmid pESC-LEU-CYCp. This work was supported by the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) (M.K.) in 2005-2007, and by the Japan Science and Technology Agency (JST) (T.Y. and M.K.) in 2008-2010, and also supported in part by Grants-in-Aid for Scientific Research (#18651028 and #80182301) (T.Y. and M.K.) from the Japan Society for the Promotion of Science (JSPS).

References

- Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., & Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science*, 237, 268-275.
- Balzi, E., & Goffeau, A. (1995). Yeast multidrug resistance: the PDR network. *J Bioenerg Biomembr*, 27, 71-76.
- Bastl, C. P., Schulman, G., & Cragoe, E. J., Jr. (1992). Glucocorticoids inhibit colonic aldosterone-induced conductive Na⁺ absorption in adrenalectomized rat. *Am J Physiol*, 263, F443-452.
- Beato, M., Herrlich, P., & Schutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. *Cell*, 83, 851-857.
- Berger, T. S., Parandoosh, Z., Perry, B. W., & Stein, R. B. (1992). Interaction of glucocorticoid analogues with the human glucocorticoid receptor. *J Steroid Biochem Mol Biol*, 41, 733-738.
- Bohen, S. P., & Yamamoto, K. R. (1993). Isolation of Hsp90 mutants by screening for decreased steroid receptor function. *Proc Natl Acad Sci U S A*, 90, 11424-11428.
- Bovee, T. F., Helsdingen, R. J., Hamers, A. R., Brouwer, B. A., & Nielen, M. W. (2011). Recombinant cell bioassays for the detection of (gluco)corticosteroids and endocrine-disrupting potencies of several environmental PCB contaminants. *Anal Bioanal Chem*, 401, 873-882.
- Burke, D., Dawson, D., & Stearns, T. (2000). *Methods in Yeast Genetics-A Laboratory Course Manual, Cold Spring Harbor (2000 edition)*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Cadepond, F., Ulmann, A., & Baulieu, E. E. (1997). RU486 (mifepristone): mechanisms of action and clinical uses. *Annu Rev Med*, 48, 129-156.
- Chu, W. L., Shiizaki, K., Kawanishi, M., Kondo, M., & Yagi, T. (2009). Validation of a new yeast-based reporter assay consisting of human estrogen receptors alpha/beta and coactivator SRC-1: application for detection of estrogenic activity in environmental samples. *Environ Toxicol*, 24, 513-521.
- de Gasparo, M., Joss, U., Ramjoue, H. P., Whitebread, S. E., Haenni, H., Schenkel, L., Kraehenbuehl, C., Biollaz, M., Grob, J., Schmidlin, J., & et al. (1987). Three new epoxy-spirolactone derivatives: characterization in vivo and in vitro. *J*

Pharmacol Exp Ther, 240, 650-656.

- Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., & Stallcup, M. R. (1998). Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. *Mol Endocrinol*, 12, 302-313.
- Edwards, C. R., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R., & Monder, C. (1988). Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. *Lancet*, 2, 986-989.
- Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. *Science*, 240, 889-895.
- Evans, R. M., & Arriza, J. L. (1989). A molecular framework for the actions of glucocorticoid hormones in the nervous system. *Neuron*, 2, 1105-1112.
- Freedman, L. P., Yoshinaga, S. K., Vanderbilt, J. N., & Yamamoto, K. R. (1989). In vitro transcription enhancement by purified derivatives of the glucocorticoid receptor. *Science*, 245, 298-301.
- Fried, J., & Borman, A. (1958). Synthetic derivatives of cortical hormones. *Vitam Horm*, 16, 303-374.
- Fuller, P. J. (1991). The steroid receptor superfamily: mechanisms of diversity. *FASEB J*, 5, 3092-3099.
- Funder, J. W., Pearce, P. T., Smith, R., & Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science*, 242, 583-585.
- Garabedian, M. J., & Yamamoto, K. R. (1992). Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast. *Mol Biol Cell*, 3, 1245-1257.
- Gietz, R. D., Schiestl, R. H., Willems, A. R., & Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS- DNA/PEG procedure. *Yeast*, 11, 355-360.
- Gomez Sanchez, E. P. (1995). Mineralocorticoid modulation of central control of blood pressure. *Steroids*, 60, 69-72.
- Grossmann, C., Scholz, T., Rochel, M., Bumke-Vogt, C., Oelkers, W., Pfeiffer, A. F., Diederich, S., & Bahr, V. (2004). Transactivation via the human glucocorticoid

- and mineralocorticoid receptor by therapeutically used steroids in CV-1 cells: a comparison of their glucocorticoid and mineralocorticoid properties. *Eur J Endocrinol*, 151, 397-406.
- Grotjohann, I., Schulzke, J. D., & Fromm, M. (1999). Electrogenic Na⁺ transport in rat late distal colon by natural and synthetic glucocorticosteroids. *Am J Physiol*, 276, G491-498.
- Guldener, U., Heck, S., Fielder, T., Beinhauer, J., & Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res*, 24, 2519-2524.
- Harada, N., Yasunaga, R., Higashimura, Y., Yamaji, R., Fujimoto, K., Moss, J., Inui, H., & Nakano, Y. (2007). Glyceraldehyde-3-phosphate dehydrogenase enhances transcriptional activity of androgen receptor in prostate cancer cells. *J Biol Chem*, 282, 22651-22661.
- Hayashi, Y., Ohmori, S., Ito, T., & Seo, H. (1997). A splicing variant of Steroid Receptor Coactivator-1 (SRC-1E): the major isoform of SRC-1 to mediate thyroid hormone action. *Biochem Biophys Res Commun*, 236, 83-87.
- Hong, H., Kohli, K., Garabedian, M. J., & Stallcup, M. R. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol Cell Biol*, 17, 2735-2744.
- Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., & Stallcup, M. R. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci U S A*, 93, 4948-4952.
- Ikegawa, S., Hasegawa, M., Okihara, R., Shimidzu, C., Chiba, H., Iida, T., & Mitamura, K. (2009). Simultaneous determination of twelve tetrahydrocorticosteroid glucuronides in human urine by liquid chromatography/electrospray ionization-linear ion trap mass spectrometry. *Anal Chem*, 81, 10124-10135.
- Ito-Harashima, S., & McCusker, J. H. (2004). Positive and negative selection LYS5MX gene replacement cassettes for use in *Saccharomyces cerevisiae*. *Yeast*, 21, 53-61.
- Joels, M., & de Kloet, E. R. (1990). Mineralocorticoid receptor-mediated changes in membrane properties of rat CA1 pyramidal neurons in vitro. *Proc Natl Acad Sci U S A*, 87, 4495-4498.

- Kawanishi, M., Kondo, M., Shiizaki, K., Chu, W. L., Terasoma, Y., & Yagi, T. (2008). Construction of a reporter yeast strain to detect estrogen receptor signaling through aryl hydrocarbon receptor activation. *Environ Sci Technol*, 42, 6897-6902.
- Kawanishi, M., Sakamoto, M., Shimohara, C., & Yagi, T. (2006). Establishment of reporter yeasts for guinea pig and syrian hamster aryl hydrocarbon receptor ligand activity. *Gene & Environ.*, 28, 167-172.
- Klis, F. M., Boorsma, A., & De Groot, P. W. (2006). Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*, 23, 185-202.
- Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev*, 26, 239-256.
- Kolaczkowski, M., Kolaczowska, A., Luczynski, J., Witek, S., & Goffeau, A. (1998). In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. *Microb Drug Resist*, 4, 143-158.
- Kolaczkowski, M., van der Rest, M., Cybularz-Kolaczowska, A., Soumilion, J. P., Konings, W. N., & Goffeau, A. (1996). Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. *J Biol Chem*, 271, 31543-31548.
- Koshida, H., Miyamori, I., Soma, R., Matsubara, T., Ikeda, M., Takeda, R., Nakamura, S., Kiuchi, F., & Tsuda, Y. (1990). Mineralocorticoid and renal receptor binding activity of 21-deoxyaldosterone. *Endocrinology*, 126, 1410-1415.
- Kralli, A., Bohen, S. P., & Yamamoto, K. R. (1995). LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc Natl Acad Sci U S A*, 92, 4701-4705.
- Kugathas, S., Williams, R. J., & Sumpter, J. P. (2012). Prediction of environmental concentrations of glucocorticoids: the River Thames, UK, as an example. *Environ Int*, 40, 15-23.
- Lim-Tio, S. S., Keightley, M. C., & Fuller, P. J. (1997). Determinants of specificity of transactivation by the mineralocorticoid or glucocorticoid receptor. *Endocrinology*, 138, 2537-2543.
- Lomax, R. B., & Sandle, G. I. (1994). Comparison of aldosterone- and RU-28362-induced apical Na⁺ and K⁺ conductances in rat distal colon. *Am J*

Physiol, 267, G485-493.

- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., & Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell*, 83, 835-839.
- Massaad, C., Lombes, M., Aggerbeck, M., Rafestin-Oblin, M. E., & Barouki, R. (1997). Cell-specific, promoter-dependent mineralocorticoid agonist activity of spironolactone. *Mol Pharmacol*, 51, 285-292.
- McInnes, K. J., Kenyon, C. J., Chapman, K. E., Livingstone, D. E., Macdonald, L. J., Walker, B. R., & Andrew, R. (2004). 5alpha-reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor. *J Biol Chem*, 279, 22908-22912.
- Naray-Fejes-Toth, A., & Fejes-Toth, G. (1990). Glucocorticoid receptors mediate mineralocorticoid-like effects in cultured collecting duct cells. *Am J Physiol*, 259, F672-678.
- Niedenthal, R. K., Riles, L., Johnston, M., & Hegemann, J. H. (1996). Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. *Yeast*, 12, 773-786.
- Rhen, T., & Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med*, 353, 1711-1723.
- Rupprecht, R., Arriza, J. L., Spengler, D., Reul, J. M., Evans, R. M., Holsboer, F., & Damm, K. (1993). Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. *Mol Endocrinol*, 7, 597-603.
- Schena, M., & Yamamoto, K. R. (1988). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. *Science*, 241, 965-967.
- Shiizaki, K., Asai, S., Ebata, S., Kawanishi, M., & Yagi, T. (2010). Establishment of yeast reporter assay systems to detect ligands of thyroid hormone receptors alpha and beta. *Toxicol In Vitro*, 24, 638-644.
- Shiizaki, K., Yoshikawa, T., Takada, E., Hirose, S., Ito-Harashima, S., Kawanishi, M., & Yagi, T. (2014). Development of yeast reporter assay for screening specific ligands of retinoic acid and retinoid X receptor subtypes. *J Pharmacol Toxicol Methods*, 69, 245-252.
- Tolgyesi, A., Verebey, Z., Sharma, V. K., Kovacsics, L., & Fekete, J. (2010).

- Simultaneous determination of corticosteroids, androgens, and progesterone in river water by liquid chromatography-tandem mass spectrometry. *Chemosphere*, 78, 972-979.
- Tsai, S. Y., Srinivasan, G., Allan, G. F., Thompson, E. B., O'Malley, B. W., & Tsai, M. J. (1990). Recombinant human glucocorticoid receptor induces transcription of hormone response genes in vitro. *J Biol Chem*, 265, 17055-17061.
- van der Vaart, J. M., Caro, L. H., Chapman, J. W., Klis, F. M., & Verrips, C. T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *J Bacteriol*, 177, 3104-3110.
- Wach, A., Brachat, A., Pohlmann, R., & Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, 10, 1793-1808.
- Weber, K. T., Sun, Y., & Guarda, E. (1994). Structural remodeling in hypertensive heart disease and the role of hormones. *Hypertension*, 23, 869-877.
- Wilkinson, J. M., Hayes, S., Thompson, D., Whitney, P., & Bi, K. (2008). Compound profiling using a panel of steroid hormone receptor cell-based assays. *J Biomol Screen*, 13, 755-765.
- Wolf, S. S., Roder, K., & Schweizer, M. (1996). Construction of a reporter plasmid that allows expression libraries to be exploited for the one-hybrid system. *Biotechniques*, 20, 568-574.
- Wright, A. P., Carlstedt-Duke, J., & Gustafsson, J. A. (1990). Ligand-specific transactivation of gene expression by a derivative of the human glucocorticoid receptor expressed in yeast. *J Biol Chem*, 265, 14763-14769.
- Wright, A. P., & Gustafsson, J. A. (1992). Glucocorticoid-specific gene activation by the intact human glucocorticoid receptor expressed in yeast. Glucocorticoid specificity depends on low level receptor expression. *J Biol Chem*, 267, 11191-11195.
- Wright, A. P., McEwan, I. J., Dahlman-Wright, K., & Gustafsson, J. A. (1991). High level expression of the major transactivation domain of the human glucocorticoid receptor in yeast cells inhibits endogenous gene expression and cell growth. *Mol Endocrinol*, 5, 1366-1372.
- Xu, J., & Li, Q. (2003). Review of the in vivo functions of the p160 steroid receptor coactivator family. *Mol Endocrinol*, 17, 1681-1692.

- Zhang, M., Hanna, M., Li, J., Butcher, S., Dai, H., & Xiao, W. (2010). Creation of a hyperpermeable yeast strain to genotoxic agents through combined inactivation of PDR and CWP genes. *Toxicol Sci*, *113*, 401-411.
- Zhang, M., Liang, Y., Zhang, X., Xu, Y., Dai, H., & Xiao, W. (2008). Deletion of yeast CWP genes enhances cell permeability to genotoxic agents. *Toxicol Sci*, *103*, 68-76.
- Zhang, S., Jonklaas, J., & Danielsen, M. (2007). The glucocorticoid agonist activities of mifepristone (RU486) and progesterone are dependent on glucocorticoid receptor levels but not on EC50 values. *Steroids*, *72*, 600-608.

Figure legend

Fig. 1. Dose-dependent response to corticosteroids in GR and MR assay yeasts established in wild-type W303a and mutants.

The reporter assay yeasts expressing human GR (A) and MR (B) were established in wild-type yeast W303a and deletion mutants for cell wall mannoproteins (*CWP1*, *CWP2*) and/or plasma membrane efflux pumps (*PDR5*, *PDR10*). The yeasts were exposed to 0.01 – 100 μ M of corticosterone (A) or 0.001 – 100 μ M of aldosterone (B), and ligand-dependent induction of β -galactosidase activities was measured.

Fig. 2. Cross-reactivity of GR and MR assay yeasts against endogenous (A) and synthetic (B) corticosteroids.

The GR assay yeasts (wild-type W303a and *pdr5 Δ pdr10 Δ*) and MR assay yeasts (W303a and *cwp1 Δ pdr5 Δ*) were exposed to 0.0001 – 100 μ M of endogenous ligands (hydrocortisone, corticosterone, cortisone and aldosterone) (A), or 0.01 – 100 μ M of synthetic ligands (dexamethasone, prednisolone, triamcinolone, betamethasone and fludrocortisone acetate) (B). Ligand-dependent induction of β -galactosidase activities was measured.

Fig. 3. Cross-reactivity of GR and MR assay yeasts against sex steroids.

The GR assay yeasts (wild-type W303a and *pdr5 Δ pdr10 Δ*) and MR assay yeasts (W303a and *cwp1 Δ pdr5 Δ*) were exposed to each of E2, testosterone and progesterone. 0.01 – 100 μ M (GR) or 0.0001 – 100 μ M of ligands (MR) were used, and

ligand-dependent induction of β -galactosidase activities were measured.

Fig. 4. Response of GR and MR assay yeasts against mifepristone, spironolactone and eplerenone.

The GR assay yeasts (wild-type W303a and *pdr5 Δ pdr10 Δ*) and MR assay yeasts (W303a and *cwp1 Δ pdr5 Δ*) were exposed to mifepristone, spironolactone, and eplerenone, and antagonistic activities (A) and agonist activities (B) of these ligands were examined. Antagonistic activities of these chemicals were examined by competition between antagonist and agonist (dexamethasone for GR and aldosterone for MR) that was added at the concentration of EC₅₀. The dose-dependent repression of *lacZ* reporter expression by antagonists was shown as “relative activity (%)”. The CYC yeast, constitutively expressing *lacZ* was used to distinguish antagonist activity from non-specific inactivation of gene expression (A). In agonist assay, ligand-dependent induction of β -galactosidase activities was measured as shown in Fig. 1- 3 (B).

Fig. 5. Detection of corticosteroid activities from oral tablets.

The organic compounds were extracted and concentrated from oral tablets, decadron, florinef, ledercort, and rinderon. The samples were serially diluted and used as ligands in yeast reporter assay using GR assay yeasts (wild-type W303a and *pdr5 Δ pdr10 Δ*) and MR assay yeasts (W303a and *cwp1 Δ pdr5 Δ*).

Fig. 6. Detection of corticosteroid activities from human urine.

The organic compounds were extracted and concentrated from human urine. The concentration factor of 100 was used in yeast reporter assay using GR assay yeasts (wild-type W303a and *pdr5Δpdr10Δ*) and MR assay yeasts (W303a and *cwp1Δpdr5Δ*), and β -galactosidase activities were measured.

3. Figure
Fig. 1

Fig. 2A

Fig. 2B

Fig. 3

Fig. 4A

Fig. 4B

Fig. 5

Fig. 6

Table 1. Comparison of responses to agonist ligands among wild-type and various mutant yeasts expressing GR and MR

	GR (corticosterone)			MR (aldosterone)		
	Fold difference	Detection limit	EC ₅₀	Fold difference	Detection limit	EC ₅₀
	(at 1 μM)	(μM)	(μM)	(at 1 μM)	(μM)	(μM)
W303a	1	1	6.5	1	0.1	0.99
<i>pdr5Δ</i>	13.7	0.2	0.65	9.8	0.001	0.017
<i>cwp1Δpdr5Δ</i>	13.6	0.3	0.79	12.7	0.001	0.014
<i>pdr5Δpdr10Δ</i>	19.8	0.1	0.32	11.6	0.01	0.017

EC₅₀, 50% effective concentration

Table 2. EC₅₀ values of endogenous corticosteroids in GR and MR in agonist assays

Receptor	Yeast strain	Hydrocortisone	Corticosterone	Cortisone	Aldosterone
GR	W303a	31.6	6.5 ^a	n.d.	31.6
	<i>pdr5Δpdr10Δ</i>	0.78	0.32 ^a	31.6	1.76
MR	W303a	11.5	0.4	n.d.	0.99 ^a
	<i>cwp1Δpdr5Δ</i>	0.1	0.001	0.97	0.014 ^a

EC₅₀, 50% effective concentration (μM), n.d. not detectable

Table 3. EC₅₀ values of synthetic corticosteroids in GR and MR agonist assays

Receptor	Yeast strain	Dexamethasone	Prednisolone	Triamcinolone	Betamethasone	Fludrocortisone Acetate
GR	W303a	16.0	31.6	31.6	16.7	31.6
	<i>pdr5Δpdr10Δ</i>	0.28	1.3	3.16	0.32	9.04
MR	W303a	n.d.	n.d.	n.d.	n.d.	31.6
	<i>cwp1Δpdr5Δ</i>	1.2	0.59	3.16	3.16	0.17

EC₅₀, 50% effective concentration (μM), n.d. not determined

Table 4. EC₅₀ values of sex steroid hormones in GR and MR agonist assays

Receptor	Yeast strain	E2	testosterone	progesterone
GR	W303a	n.d.	31.6	31.6
	<i>pdr5Δpdr10Δ</i>	31.6	10.0	3.16
MR	W303a	n.d.	n.d.	n.d.
	<i>cwp1Δpdr5Δ</i>	10.0	10.0	0.003

EC₅₀, 50% effective concentration (μM), n.d. not determined

Table 5. Comparison of responses of GR and MR assay yeasts to antagonist ligands

Receptor	Yeast strain	Mifepristone		Spironolactone		Eplerenone	
		IC ₅₀	Maximum inhibition (%)	IC ₅₀	Maximum inhibition (%)	IC ₅₀	Maximum inhibition (%)
GR	W303a	1.89	76	2.69	22	n.d.	1
	<i>pdr5Δpdr10Δ</i>	6.09	74	0.39	52	n.d.	5
MR	W303a	11.2	24	0.31	49	8.16	45
	<i>cwp1Δpdr5Δ</i>	9.85	12	0.31	49	2.65	68

IC₅₀, 50% effective concentration for inhibition (μM), n.d. not determined

Table 6. EC₅₀ values of mifepristone, spironolactone, and eplerenone in GR and MR agonist assays

Receptor	Yeast strain	Mifepristone	Spironolactone	Eplerenone
GR	W303a	0.32	n.d.	n.d.
	<i>pdr5Δpdr10Δ</i>	0.03	3.16	n.d.
MR	W303a	n.d.	n.d.	n.d.
	<i>cwp1Δpdr5Δ</i>	0.32	0.03	1.0

EC₅₀, 50% effective concentration (μM), n.d. not determined