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Original article

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

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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-1e. 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 $D_3$ (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

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

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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; Rupprecht 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, DH5α, was used as a host strain to amplify plasmid DNA. *Saccharomyces cerevisiae* strains are listed in Table S-1. All *S. cerevisiae* strains used in this study were isogenic with W303**a** (*MAT*a, *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), 17β-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–*Sal*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).