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ScienceDirect
Analytical Biochemistry 373 (2008) 263–271
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Androgen receptor transactivation assay using green fluorescent protein as a reporter

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Received 1 August 2007
Available online 12 September 2007

Abstract

For screening of a large number of samples for androgenic activity, a robust system with minimal handling is required. The coding sequence for human androgen receptor (AR) was inserted into expression plasmid YEpBUbi–FLAG1, resulting in the plasmid YEpBUbiFLAG–AR, and the estrogen response element (ERE) on the reporter vector YRpE2 was replaced by an androgen response element (ARE), resulting in the plasmid YRpE2–ARE. Thus, a fully functional transactivation assay system with β-galactosidase as a reporter gene could be created. Furthermore, green fluorescent protein (GFP) was introduced as an alternative reporter gene that resulted in a simplification of the whole assay procedure. For evaluation of both reporter systems, seven steroidal compounds with known AR agonistic properties (5α-dihydrotestosterone, testosterone, androstenedione, 17α-methyltestosterone, progesterone, epitestosterone, and D-norgestrel) were tested, and their potencies obtained in the different assays were compared. Furthermore, potencies from the transactivation assays were compared with IC_{50} values obtained in radioligand binding assays. The newly developed androgen receptor transactivation assay is a useful tool for characterizing compounds with androgenic activity.

Keywords: Androgen receptor; Green fluorescent protein (GFP); Transactivation; Radioligand binding; 5α-Dihydrotestosterone (5α-DHT)

The androgen receptor (AR)[1] is involved in numerous physiological regulation and differentiation processes in a wide variety of organs [1], especially in embryonic development and sex differentiation [2]. In addition to the naturally occurring steroid hormones, such as testosterone and 5α-dihydrotestosterone (5α-DHT), ligands with a similar chemical structure can bind to the AR and modulate its function. These ligands are either of anthropogenic origin, such as herbicides, pesticides, fungicides, and industrial chemicals [3,4], or phytochemicals [5]. These ligands can interfere with normal androgen action by binding to AR [2]. Due to the large number of environmental chemicals that interact with the AR and might lead to disorders in male reproduction in wildlife and humans [4,6,7], it is important to establish rapid in vitro test systems for the assessment of a compound's activity on the AR. The development of in vitro tests also becomes increasingly important because regulatory authorities require an increasing number of animal tests necessary for toxicity studies of chemicals [8]. In vitro assays are a useful tool for screening a large number of substances before analyzing selected ones in more expensive and time-consuming in vivo tests; thus, the number of tests can be reduced. According to Soto and coworkers [9], various in vitro assay principles are in use for the assessment of endocrine active substances on the AR; ligand binding assays determine binding affinities of compounds to a specific receptor [10,11], cell

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[1] *Abbreviations used:* AR, androgen receptor; 5α-DHT, 5α-dihydrotestosterone; ER, estrogen receptor; PR, progesterone receptor; ARE, androgen response element; GFP, green fluorescent protein; DMSO, dimethyl sulfoxide; ONPG, *o*-nitrophenyl-β-galactopyranoside; cDNA, complementary DNA; ERE, estrogen response element; CYC1, iso-1-cytochrome *c;* DTT, dithiothreitol; IgG, immunoglobulin G.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.09.006

proliferation assays determine inhibition of cell proliferation in response to AR agonists [12], and reporter gene assays determine the transactivation potential of a compound [13,14].

The AR, together with other sex steroid receptors such as the estrogen receptor (ER) and progesterone receptor (PR), belongs to the steroid–thyroid superfamily of nuclear receptors [15,16]. They all act as ligand-activated transcription factors, have a common domain structure [17], and in their active state are located in the nucleus of the cell. In its inactive state, AR is bound to chaperone molecules such as heat shock proteins [18] in the cytoplasma. On ligand binding, the conformation of the receptor changes; it dissociates from the heat shock proteins and dimerizes [17,18]. The activated ligand-bound AR dimer translocates to the nucleus, where it can bind to specific androgen response elements (AREs) in the promoter region of its target genes and, thus, can activate gene transcription [19,20].

As shown previously by Purvis and coworkers [21], the AR can be expressed in yeast in a form that can activate transcription of a reporter gene in an androgen-dependent manner, meaning that the genomic pathway of AR action can be mimicked in yeast transactivation assays. Such biological screening assays enable us to quantify the androgenic activity of a compound or sum of compounds and relate it to the activity of a natural potent AR ligand (e.g., 5α-DHT). Cells expressing the AR are transformed with a reporter plasmid containing an ARE in the promoter region of the reporter gene. Mammalian cell lines stably or transiently [13,14,22] expressing AR are used mostly as a host for transactivation assays, but there also exist test systems that use yeast with heterologously expressed AR protein [21,23–25]. Yeast cells have the advantages of fast growth, easy handling, cheap media components, and robustness toward toxic effects of test chemicals or solvents. In yeast, the activity of substances toward the AR can be determined without the presence of any other mammalian proteins influencing the AR pathway [26]. Thus, the yeast androgen screen is a fast and easy tool for monitoring the androgenic activity of large sample numbers prior to subjecting them to more complex and expensive test systems such as animal tests.

Various reporter genes are in use for transactivation assays. Transcription of a reporter gene leads to expression of a protein that can be easily quantified over a background of endogenous proteins [27]. β-Galactosidase [26] or luciferase [13,14,24] is used mostly as reporter gene that can be easily quantified by photometric or luminescent measurement, respectively. Nowadays, alternatives such as green fluorescent protein (GFP) are frequently used as reporters. GFP is an autofluorescent protein derived from the jellyfish *Aequorea victoria* that can be used for many biotechnological applications [28], and a variety of GFP mutations for optimized expression in different organisms have been created [29]. GFP emits green fluorescing light that can be measured directly from the culture without disintegration of the cells. The chromophore forms without the need for cofactors by intramolecular cyclization with subsequent dehydrogenation [30].

A large number of transactivation assays in yeast exist for the determination of the estrogenic activity of compounds due to the interest in the ER as a drug target [26,31–34]. Studies using the AR in yeast transactivation assays are not so abundant and use mostly β-galactosidase as a reporter [23,25,26]. In addition to the yeast estrogen screen that has been in use in our lab for several years and has been applied for a great variety of samples and sample matrices [35–40], we created a similar test system for the assessment of the androgenic activity of compounds. Furthermore, we replaced the commonly used reporter gene lacZ for expression of β-galactosidase with the GFP gene. Thus, it was able to quantify androgenic activity of steroids and related compounds by simple fluorescence measurement of whole cells without previous yeast disintegration. This is an effective method to shorten the whole assay procedure of the yeast androgen screen and to make it less susceptible to inaccuracies caused by multiple pipetting and handling steps. In addition, ligand binding assays of the test compounds were performed and related to the results of transactivation assays. This gives a more complete overview of a ligand's function on the AR.

Materials and methods

Chemicals and consumables

Buffer reagents, dimethyl sulfoxide (DMSO), *o*-nitrophenyl-β-galactopyranoside (ONPG), and dextran-coated charcoal were purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA). For yeast media preparation, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ, USA) and amino acid supplement mix was obtained from MP Biomedicals (Illkirch, France). Enzymes for molecular biology were purchased from Fermentas (St. Leon-Rot, Germany), and electrocompetent *Escherichia coli* was obtained from Invitrogen (Carlsbad, CA, USA). Protease inhibitor cocktail for fungal and yeast cells was purchased from Sigma–Aldrich. 5α-DHT, testosterone, 4-androstenedione, 17α-methyltestosterone, progesterone, epitestosterone, and D-norgestrel were obtained from Sigma–Aldrich. Glass beads were purchased from Merck. The solvents used were HPLC grade. Black microplates were obtained from Corning (Schiphol-Rijk, The Netherlands).

Construction of plasmids

The yeast expression plasmid YEpBUbi–FLAG1 [41] carrying the CUP promoter and a tryptophan selection marker was used as a backbone for cloning of the AR expression plasmid YEpBUbiFLAG–AR (Fig. 1). Plasmid pSVAR0 containing the full-length complementary DNA (cDNA) of human AR was a kind gift from A. O. Brink-