The Subnuclear Three-dimensional Image Analysis of Androgen Receptor Fused to Green Fluorescence Protein*

Received for publication, February 26, 2001
Published, JBC Papers in Press, May 21, 2001, DOI 10.1074/jbc.M101755200

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To establish the novel approach in order to distinguish the transcriptionally active androgen receptor (AR) from the transcriptionally inactive AR, we performed the three-dimensional construction of confocal microscopic images of intranuclear AR. This method clearly distinguished the subnuclear localization of transcriptionally active AR tagged with green fluorescent protein (AR-GFP) from the transcriptionally inactive AR-GFP. Transcriptionally active AR-GFP mainly produced 250–400 fluorescence foci in the boundary region between euchromatin and heterochromatin. Although the AR-GFP bound to such antiandrogens as hydroxyflutamide or bicalutamide translocated to the nucleus, they homogeneously spread throughout the nucleus without producing any fluorescence foci. Antiandrogenic environmental disrupting chemicals, such as 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, vinclozolin, or nitrofen, also disrupted the intranuclear fluorescence foci. A point mutation (T877A) resulted in the loss of ligand specificity in AR-GFP. Even in this mutant receptor, agonists, such as dihydrotestosterone, hydroxyflutamide, or progesterone, produced the fluorescence foci in the nucleus, whereas the transcriptionally inactive mutant binding bicalutamide was observed to be spread homogeneously in the nucleus. Taken together, our findings suggest that, after nuclear translocation, AR is possibly located in the specific region in the nucleus while demonstrating clustering tightly depending on the agonist-induced transactivation competence.

Androgen receptor (AR) is a member of the steroid hormone receptor family and is known to be ligand-specifically activated. The primary subcellular localization of ligand-unbound steroid hormone receptors differs among the various family members. For instance, estrogen receptor is located in the nucleus both in ligand-bound forms and in ligand-unbound forms, whereas unliganded AR (2, 3) as well as glucocorticoid receptor (4, 5) are both primarily located in the cytoplasm. After dihydrotestosterone (DHT) binding, AR translocates into the nucleus and then binds to the specific DNA sequences; as a result DHT-bound AR activates the transcription of its target genes. AR plays an essential role during the differentiation of male gonadal tissues, and as a result, mutations in the AR gene may cause infertility in men, the severest form of which is called testicular feminization (6, 7). In addition, recent evidence suggests that some abnormalities in the male reproductive system are also mediated via AR (8). Chemicals synthesized for herbicides or insecticides, such as vinclozolin or 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE), are known to act as antiandrogenic endocrine-disrupting chemicals and thus have been suspected to be a cause of infertility in wildlife (9).

AR is also involved in the proliferation of some androgen-dependent tumors, the most well known of which is prostatic cancer (10, 11). Many synthetic compounds have been made which either stimulate or antagonize the transactivation functions of nuclear receptors. Among these, nonsteroidal hydroxyflutamide (OHF) and bicalutamide (CAS) specifically suppress the androgen-dependent transactivation function of AR by the competitive binding to the hormone binding domain of AR (12). They are called pure antiandrogens and are currently widely used for the treatment of patients with the advanced prostatic cancers (13, 14). AR found in the advanced or metastasized prostatic cancer cells have sometimes been shown to harbor mutations that result in the amino acid substitutions in the receptor (15). Among these, the AR in LNCaP cells, established from the metastatic prostate cancer cells, harbors a point mutation that causes a substitution of threonine residue at codon 877 to alanine (T877A), which thus results in the loss of ligand specificity. The mutated AR (AR(T877A)) binds to OHF paradoxically stimulates the proliferation of the LNCaP cells (16).

We established a three-dimensional image analysis to characterize the images of the cells treated with chemicals possessing either an androgenic or antiandrogenic action. The images were collected using confocal laser scanning microscopy, from the living cells treated with natural steroids, pure antiandrogens for the treatment of prostatic cancers, and possible antiandrogenic chemicals. This approach allowed us to visualize the spatial interrelations of AR with the chromatin structures stained with DNA dye, and has proven to be a useful approach for screening antiandrogenic chemicals devoid of disadvantages promoting the antiandrogen withdrawal syndrome.

EXPERIMENTAL PROCEDURES

Chemicals—Natural steroids and all chemicals except for OHF and CAS were purchased from Wako Pure Chemical Industries, Ltd.
transfected with pCMVAR-H, transfected with pEGFP-N2, cells transfected with pCMVAR-H, cells transfected with pCMVAR-H, cells transfected with pAR-GFP, and cells transfected with pAR(T877A)-GFP, lane 5, respectively.

In each panel, the cellular AR or Western blotting was performed using the PAGE (7% separation gel), and then pAR(T877A)-GFP were subjected to SDS-AR(T877A)-GFP in the COS-7 cells.

Reduced activities, macia Biotech) and were expressed as values normalized by pRL-in-

using a Wallac ARVO.SX 1420 Multilabel Counter (Amersham Phar-

The firefly luciferase reporter plasmid (pGL3-MMTV) was constructed by inserting the MMTV-long terminal repeat promoter sequence, obtained from pMAM vector (CLONTECH), into a pGL3-basic vector (Promega). The construction of a pCMVAR-H, which expressed full-length human AR, has been reported previously by us (21). With the polymerase chain reaction technique using pCMVAR-H as a template, the NotI restriction site was created at the authentic stop codon, and then the site was blunt-ended. The Nhel/blunt-ended NotI fragment was ligated in frame to the Nhel/Smal site in the pEGFP-N2 (CLONTECH) and thus generated a vector expressing for AR-GFP in which GFP sequence was fused to the C terminus of the AR sequence. Mutated AR(T877A) sequence was amplified by reverse transcriptase-polymerase chain reaction using RNAs from LNCaP cells. The vector expressing AR(T877A)-GFP was created using the same methods as those mentioned above. The validity of the plasmid constructs was confirmed by both nucleotide sequencing and Western blot. Western blotting was performed using rabbit polyclonal antibody, AR(N-20), raised against mouse AR (Santa Cruz Biotechnology), or a rabbit polyclonal antibody against GFP (CLONTECH).

Functional Reporter Assay—COS-7 cells, which lack endogenous AR, were used for the functional assays. 2 x 10⁵ cells/well were transfected using 3 μg/well LipofectAMINE (Life Technologies, Inc.) with 0.5 μg/well pGL3-MMTV (Promega), 3 ng/well pRL (Renilla luciferase)-CMV (Promega) as an internal control, and 0.1 μg/well expression vector for AR- or AR(T877A)-GFP. Five hours posttransfection, 0.5 ml of DMEM, containing 10% dextran/charcoal-treated FBS, with or without DHT, 17β-estradiol, progesterone, OHE, CAS, or 49 chemicals candidates for the endocrine disrupters, at the indicated concentrations, was added. At 48 h posttransfection, the cells were lysed in Lysis Buffer for a luciferase assay (Promega). The reporter gene activities were measured using a Wallac 1420 Multilabel Counter (Amersham Pharmacia Biotech) and were expressed as values normalized by pRL-induced activities, i.e. (firefly luciferase activity)/(Renilla luciferase activity). All experiments were repeated at least twice. The data are presented as the means ± S.D.

Microscopy and Imaging Analysis—The cells were divided into 35-mm glass-bottom dishes (MatTek Corporation) and then were transfected with 0.5 μg of the plasmids using 2.5 μl/dish of Superfect reagents (Qiagen). Six to 18 h posttransfection, the culture medium was replaced with a fresh DMEM. At first, the cells were scanned without any hormone treatment, and then the hormones or chemicals were added. One hour after adding the chemicals, they were scanned using confocal laser scanning microscopy (Leica TCS-SP system, Leica Microsystems, Heidelberg, Germany). The cells were imaged for green fluorescence by excitation with the 488 nm line from an argon laser, and the emission was viewed through a 496–505 nm band pass filter. A series of 30–50 images were collected for each single nucleus. The nuclei were stained with Hoechst 33342 (2 μg/ml) and were imaged by excitation with the 350 nm line from a UV laser, and the emission was viewed through a 400–450 nm band pass filter. The two-dimensional tomographic images taken by the confocal microscope were reconstructed using the three-dimensional analysis TRI Graphics Program software package (Ratoc System Engineering, Tokyo) on a Dell computer.

RESULTS

Construction of Plasmids Expressing for AR-GFP and AR(T877A)-GFP—cDNA fragment containing the entire sequence of human AR, either wild-type or the mutant in LNCaP cells, was ligated to the 5′-end of pEGFP for generating pAR-GFP or pAR(T877A)-GFP. These plasmids were transiently transfected into COS-7 cells to express the fusion proteins, AR-GFP and AR(T877A)-GFP, respectively. Within 3 h posttransfection, the fluorescence was detected in the cytoplasm. A Western blot analysis using the transfected cellular lysates probed with antiserum against GFP (Fig. 1a) or AR (Fig. 1b) revealed the single band corresponding to the AR-GFP or AR(T877A)-GFP, which migrated at the expected molecular weight (128 kDa). In a separate experiment, we also created a plasmid construct encoding the 5′-GFP-AR-3′ fusion protein, in which AR was fused to at 3′-end of GFP. The subcellular distribution of 5′-GFP-AR-3′ was not consistent, i.e. the fluorescence of the unliganded receptor was either cytoplasmic, nuclear, or both. When the COS-7 cellular lysates containing the 5′-GFP-AR-3′ was probed with anti-GFP antisera, several signals migrating faster than the expected molecular weight were observed (data not shown). To rule out the possibility that the observed fluorescence signals from the expressed 5′-GFP-AR-3′ indeed represented the prematurely terminated proteins, we used pAR-GFP or pAR(T877A)-GFP for the subsequent experiments.

Transactivation Function and Intranuclear Localization of Androgen Receptor—COS-7 cells, transiently transfected with pAR-GFP together with pGL3MMTV-luc, were treated with various concentrations of DHT (10⁻¹¹ to 10⁻⁷ M) for 48 h. The DHT-dependent transactivation capacity of AR-GFP compared with that of AR was ~50% at 10⁻⁹ M DHT (Fig. 2a). This DHT-dependent transactivation capacity of the AR-GFP was detected in other cell lines, including LNCaP, ALVA-41, ALVA-101, Du145, and PC3 (data not shown). Pure antiandrogen, OHE or CAS, acted as a potent antagonist for AR-GFP, and they thus did not induce any such transactivation (data not shown). Furthermore, the treatment of cells with OHE or CAS in the presence of 10⁻⁸ M DHT suppressed the transactivation function in a dose-dependent fashion (Fig. 2B).
The point mutation, T877A, has been known to result in the loss of the ligand specificity, namely AR(T877A) binds to progesterone, 17β-estradiol, or OHP, as well as DHT, and thereby activates the AR target genes (16, 22). The profiles of ligand-bound transactivation function of AR(T877A)-GFP was assessed using COS-7 cells. The DHT-bound AR(T877A)-GFP activated the reporter gene in a concentration-dependent fashion. Progesterone or OHP acted as an agonist, whereas CAS acted as an antagonist. In contrast, 17β-estradiol did not exert any AR(T877A)-GFP-dependent transactivation (Fig. 2C).

By using two-dimensional confocal laser scanning microscopy, the intranuclear distribution of AR-GFP or AR(T877A)-GFP was visualized in COS-7 cells or in LNCaP, ALVA-41, ALVA-101, DU145, and PC3 (data not shown). In each cell line, unliganded AR-GFP was primarily located in the cytoplasm (Fig. 3, A, C, and E) as described previously (2), whereas after 10^{-8} \text{M} DHT binding, AR-GFP translocated into the nucleus within 1 h, and thereafter produced fluorescence foci (Fig. 3, B, D, and F). In all experiments, the nucleoli demonstrated no fluorescence. When transfected cells were treated with 10^{-6} \text{M} OHP or CAS, AR-GFP translocated to the nucleus as well as DHT-bound AR-GFP. However, AR-GFP bound to OHP or CAS did not produce any fluorescence foci, but it was homogeneously distributed in the nucleus as described recently (23) (Fig. 3, G and H). Although the unliganded AR(T877A)-GFP was located in both the cytoplasm and in the nucleus (Fig. 4A), AR(T877A)-GFP was exclusively located in the nucleus and thus produced fluorescence foci when transfected cells were treated with DHT, progesterone, or OHP (Fig. 4, B, D, and E). CAS- or 17β-estradiol-bound AR(T877A)-GFP did not make any foci, but it was spread homogeneously in the nucleus (Fig. 4, C and F). As a result, the fluorescence focus formation and the transactivation capacities were closely linked even in AR(T877A)-GFP which had lost the ligand specificity. However, the resolving power of regular confocal images was not sufficient to observe further the spatial interrelation of GFP clusters with the chromatin image

Screening of Chemicals Acting as Possible Antiandrogenic Chemicals Using the Functional Reporter Assay—We obtained 49 chemicals known to possibly act as environmental endocrine disrupters, and we examined the antiandrogenic actions in COS-7 cells, using a luciferase gene as a reporter (data not shown). At 10^{-8} \text{M}, 6 chemicals, such as p, p‘-DDE, which is a major and persistent metabolite of DDT, vinclozolin, alachlor, metribuzin, bisphenol A (BPA), and nitrofen, suppressed the 10^{-8} \text{M} DHT-dependent transactivation by about 50–70% (Fig. 2D). Similar results were observed in ALVA-41 cells established from human prostatic cancer. Among these 6 chemicals, vinclozolin (24), p,p‘-DDE (25), and BPA (26) were previously reported to be antiandrogenic EDC. However, alachlor, metribuzin, and nitrofen are not known to be antiandrogens.

Construction of the Three-dimensional Image of the Intranu-
Distribution of Active or Inactive AR-GFP—Much evidence has been accumulated suggesting that the chromatin structure is dynamic and tightly linked to the transcriptional activity. Nuclear staining with DNA dyes, such as 4,6-diamidino-2-phenylindole or Hoechst 33342, has been used to discriminate the heterochromatin region from the euchromatin region. To clarify further the difference in the intranuclear distribution between the agonist-bound AR and the antagonist-bound AR, we used a novel approach by performing a three-dimensional reconstruction of the confocal images of GFP fluorescence in the nucleus. COS-7 cells transiently transfected with pAR-GFP were treated with 10^{-8} M DHT or 10^{-6} M OHF, and then the nuclei were simultaneously stained with Hoechst 33342. One hour after adding the chemicals and Hoechst 33342, the confocal images were collected using the Leica TCS-SP system. For each confocal image, low brightness noise rejection and median filter processing were carried out in the blue (chromatin) and green (GFP) channels, respectively, then the chromatin and the GFP images were extracted and constructed in three dimensions. For the chromatin images that were stained with Hoechst 33342, less dense areas (namely euchromatin region) were cut off and thus were shown as blank images. With our procedures to construct the three-dimensional images of the nucleus, the GFP images could be observed in high resolution, thus allowing us to observe any spatial interrelations with chromatin structures. The three-dimensional images were constructed for several cells to make sure that closely equivalent green or blue fluorescence volumes were obtained for each cell in each treated group. The final images were then displayed after carrying out the permeability compensation, and brightness, contrast enhancements.

The images of GFP and chromatin in the DHT-treated cells (Fig. 5, a and b, respectively) were viewed from the surface, and then they were spatially merged (Fig. 5c). The DHT-bound AR-GFP was distributed almost exclusively in the euchromatin region which is shown as the blank region. In the single nucleus of the DHT-treated cells, about 250–400 bright GFP

![Fig. 3. Confocal laser microscopy images of AR-GFP with or without ligand binding.](#)
P4AR-GFP was transfected into COS-7 (A, B, G, and H), LNCaP (C and D), or ALVA-41 (E and F). A, C, and E, cells not treated with ligands; B, D, and F, cells treated with 10^{-8} M of DHT; G, cells treated with 10^{-6} M of OHF; H, cells treated with 10^{-6} M of CAS. Bar, 10 μm.

![Fig. 4. Confocal laser microscopy images of AR(T877A)-GFP in the COS-7 cells, with or without ligand bindings.](#)A, cells without any ligands. Two cells adjacent to each other are shown. B, cells treated with 10^{-8} M DHT; C, cells treated with 10^{-6} M 17β-estradiol; D, cells treated with 10^{-6} M progesterone; E, cells treated with 10^{-6} M OHF; F, cells treated with 10^{-6} M CAS. Bar, 10 μm.
spots existed as a distinct volume. The tomographic views of the three-dimensional image in DHT-treated cells were displayed in the center on the z axis (Fig. 5d). In this view, it became clear that most of the GFP spots were located in the peripheral zone of the euchromatin region adjacent to the heterochromatin region in which Hoechst 33342 staining was more dominant.

In strong contrast to the number of the bright GFP spots (250–400) in the single nucleus of the DHT-treated cell, the number of GFP spots in the single nucleus of OHF-treated cell was 1 (that is a whole nuclear space is the single volume of GFP after performing low brightness noise rejection), thus further showing GFP to homogeneously distribute in the nucleus without any clustering (Fig. 5e). Next, to reveal the spatial interrelationship with the chromatin structures in the nucleus of OHF-treated cells, images were displayed as tomographic (Fig. 5f for GFP, Fig. 5g for Hoechst 33342, and Fig. 5h for the merge, respectively). The antagonist-bound AR-GFP was diffusely distributed, except in the nucleus, thus making a cyan space in which the two fluorescences were mixed. Therefore, the three-dimensional image more clearly distinguished the difference in the intranuclear localization of AR between the agonist-bound and antagonist-bound form than the regular two-dimensional confocal images (compare Fig. 3 and Fig. 5). We thus concluded that it can be applied to the screening of the chemicals possessing the antiandrogenic activities.

AR-GFP bound to vinclozolin, p,p'-DDE, or nitrofen translocated to the nucleus, and the intranuclear distribution of such AR-GFPs was homogeneous as observed in the cells treated with OHF or CAS (Fig. 6b–f, Fig. 6a as a control). Furthermore, when the cells were treated with both 10^{-6} M DHT and 10^{-6} M vinclozolin, p,p'-DDE, or nitrofen, the intranuclear GFP cluster formation was strongly disrupted and was also observed against the homogeneous GFP fluorescence background that thus demonstrated diffusely distributed AR-GFP (Fig. 6, g–i). In contrast, the profiles of AR-GFP bound to alachlor, metribuzin, or BPA were different. They did not translocate to the nucleus, and the treatment of cells with 10^{-6} M of these chemicals in the presence of 10^{-9} M DHT revealed preserved DHT-induced fluorescence focus formations in the nucleus (data not shown).

DISCUSSION

In this report, we showed that a three-dimensional image construction approach is sensitive enough to dramatically discriminate the intranuclear distribution of AR between DHT-bound and antiandrogenic chemical-bound forms. The transcriptionally active wild-type glucocorticoid receptor (5), vitamin D receptor (27), estrogen receptor-α (1), and mineralocorticoid receptor (28) that are fused to GFP, regardless of the predominant subcellular localization of ligand-unbound forms, have been found to be distributed in the nuclei that produce the GFP fluorescence foci. Antagonists such as ICI 182780 or hydroxytamoxifen evoke the intranuclear cluster formation of GFP-fused estrogen receptor-α (1). Although Tyagi et al. (23) reported that 10^{-6} M 17β-estradiol produced a punctate subnuclear distribution of AR, we showed that, at least in the AR the ligand binding of which triggered the nuclear translocation, the intranuclear fluorescence focus formation closely depends on whether the receptor is transcriptionally active or inactive (Fig. 2C and Fig. 4). As described recently, the profile of the intranuclear distribution of the AR closely agreed with that of glucocorticoid receptor or mineralocorticoid receptor, in that the distribution of the inactive receptor is homogeneous (5, 23, 28). This is the first report to demonstrate that the correlation between the intranuclear cluster formation and the transactivation capacities remains even when ligand specificity has been lost. The activation of AR(T877A) by OHF has been attributed to the pathogenesis of “antiandrogen withdrawal syndrome,” in which prostatic tumor cells paradoxically proliferate after treatment with antiandrogens, but the growth is suppressed after the treatment is stopped (29, 30). Unexpectedly, unliganded AR(T877A)-GFP was both cytoplasmic and nuclear. This subcellular localization pattern of the unliganded steroid hormone receptor was reported for vitamin D receptor or mineralocorticoid receptor (27, 28). The exact mechanism of the subcellular localization of the unliganded AR(T877A)-GFP remains to be elucidated.

The exact nature of the fluorescent foci in the nucleus remains to be elucidated. The intranuclear GFP clusters are shown to be detected even in the nuclear matrix preparations after the DNase treatment; therefore, it has been suggested that the clusters are closely related to the nuclear matrix structure itself (23, 28, 31–33). Recent studies revealed the colocalization of the transcriptionally active nuclear receptor, such as estrogen receptor-α or peroxisome proliferator-activated receptor, with the transcriptional cofactor, SRC-1 (34, 35). In addition, a negative correlation would be expected between the spatial distribution of GFP-fused mineralocorticoid receptor and 4,6-diamidino-2-phenylindole using the fluoress-
cance microscope (28). To our knowledge, this is the first detailed report on the spatial distribution of the transcriptionally active steroid hormone receptor tagged with GFP. With the novel approach using the high resolution three-dimensional imaging analysis, we demonstrated from 250 to 400 clusters of transcriptionally active AR to be mainly localized in the peripheral region (adjacent to the heterochromatin) of the transcriptionally active euchromatin. In the nucleus, the chromatin structures are dynamic, and this dynamic structural change is essential for the transcriptional activation mechanisms (36). Recently, a transcriptional cofactor, exclusively localized in the euchromatin area, has been identified (37). The cofactor, TIF-1a, is preferentially localized at borders between euchromatin and heterochromatin and is thus suggested to act as a “docking protein” to which liganded nuclear receptors can bind to enhance the efficiency of the transcription by selectively scanning the euchromatin. We have found that a putative cofactor specific to the N-terminal region (AF-1) of AR is crucial to the androgen-specific transactivation mechanism (38). The dynamic change in such chromatin structures may involve the interaction of nuclear matrices with the transcriptional machinery composed of steroid hormone receptor–transcriptional cofactor complex, and the active basal transcriptional machinery, which is bridged by the molecule like a docking protein.

The antiandrogenic action of p,p'-DDE is suggested to be mediated through the competitive inhibition of androgen binding to AR and subsequent inhibition of transcription activity (25) and that vinclozolin decreases the DNA binding of the bound AR (24). We showed that the profiles of the AR binding to such chemicals as p,p'-DDE, vinclozolin, or nitrofen were very similar to those of OHF- or CAS-bound AR. The AR bound to those chemicals translocated into the nucleus but was distributed homogeneously without producing any foci in the nucleus. Furthermore, a high resolution three-dimensional image analysis clearly showed that when those antiandrogenic chemicals were added in the presence of DHT, the intranuclear GFP cluster formation was strongly disrupted even for nitrofen. Nitrofen was originally synthesized as a herbicide; however, it is no longer on the market now because of its suspected carcinogenicity (39). It is also suspected that nitrofen contamination during pregnancy may cause the congenital diaphragmatic hernia or anomalies of the great vessels in newborns (40). Recent studies revealed lung hypoplasia, caused by nitrofen, also to be mediated by the down-regulation of the thyroid transcription factor TTF-1 mRNA (41). Although its antiandrogenic action was relatively weak in comparison to either p,p'-DDE or vinclozolin, the three-dimensional imaging techniques clearly showed the images characteristic to the pure antiandrogens such as OHF or CAS. Limitations of this high resolution three-dimensional image analysis were found for such antiandrogenic chemicals as alachlor, metribuzin, and BPA. These chemicals neither caused the nuclear translocation of AR nor disrupted the DHT-induced intranuclear cluster formation of the AR. Further studies are called for to clarify the mechanisms of the actions for these chemicals, since they might affect the posttranscriptional levels such as mRNA stability.

In summary, we suggest that the mechanism of the action of antiandrogenic chemicals is a more complex action than the simple competitive binding to AR. The high resolution three-dimensional image analysis of the intranuclear cluster formation of AR in the living cell is a very sensitive and a useful method for the screening of the antiandrogenic chemicals.

Acknowledgment—We thank Mitoshi Toki for valuable technical assistance in performing the three-dimensional imaging analysis.

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J. Biol. Chem. 2001, 276:28395-28401.
doi: 10.1074/jbc.M101755200 originally published online May 21, 2001

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