PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 85 (2003) 201–208

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Activation function-1 domain of androgen receptor contributes to the interaction between two distinct subnuclear compartments☆

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Abstract

The nucleus contains different sets of functional compartments often called “speckles”. The splicing factor compartment (SFC) has been speculated to consist of SFs and transcription factors, which thus make transcription-splicing coupling possible at the periphery of SFC. Androgen receptor (AR), as well as glucocorticoid receptor (GR), is unique since most, if not all, of its activities are mediated via the constitutive activity of the activation function-1 (AF-1) function. Transcriptionally active AR produces 250–400 subnuclear fine speckles11 shared with GR or estrogen receptor (ER), which colocalize with chiefly activation function-2 (AF-2)-interacting p160 family- or CBP-related speckles. We herein report the isolation of ANT-1 (AR N-terminal domain (NTD) transactivating protein-1) enhancing autonomous AF-1 transactivation function of AR or GR, but not of estrogen receptor α (ERα). The ANT-1 was identical to a binding protein of human splicing factor U5 snRNP (U5 snRNP-associated protein). ANT-1 was compartmentalized into 15–20 coarse SFC speckles which were spatially distinct from but surrounded by the AR compartments. Our results suggest that ANT-1 may play a key role in the molecular interaction between two spatially distinct subnuclear compartments in a receptor-specific fashion, and thereby induce the strong autonomous transactivation functions either of AR- or GR-AF-1.

Keywords: Activation function-1; Androgen receptor; Splicing factor compartment

1. Introduction

The androgen receptor (AR) belongs to a nuclear receptor (NR) superfamily, and the ligand-AR complex acts as a transcription factor on the target genes, which then promote the genital virilization. The AR, similarly to other nuclear receptors, has two major transactivation domains called activation function-1 (AF-1) in the N-terminal domain (NTD) and activation function-2 (AF-2) in the C-terminal ligand binding domain. AF-1 acts as a constitutional activator in a ligand-independent fashion, in contrast to AF-2 which functions in a ligand-dependent fashion. In AR, communication between AF-1 and AF-2 is essential to full transactivation function. In AR, most, if not all, of its activities are mediated via the constitutive strong activity of the AF-1 function. This is clinically supported by our recent finding in which the absence of a specific transcription coactivator binding to an AF-1 fragment of the AR resulted in androgen insensitivity syndrome (AIS) [1]. The transmission of a transactivating signal from AF-1 of the AR to the basal transcriptional machinery is disrupted in the AIS patient. Interestingly, cyclin E is known to interact with U2 snRNP [2]. To identify the cDNAs encoding proteins binding to the AR-AF-1 sequence, we performed yeast two-hybrid screening.

2. Materials and methods

2.1. Plasmids

pEYFP-ANT-1, and pANT-myc were constructed by inserting the ANT-1 cDNA into Kpn I and Sma I sites of pEYFP-C2 (Clontech), and Kpn I and Not I sites of pcDNA3-myc-his, respectively. The reporter plasmid pMMTV-luc, containing the luciferase gene driven by

☆ Presented at the 11th International Congress on Hormonal Steroids and Hormones and Cancer, ICHS & ICHC, Fukuoka, Japan, 21–25 October 2002.

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doi:10.1016/S0960-0760(03)00196-1

mouse mammary tumor virus LTR harboring hormone response element for both AR and glucocorticoid receptor (GR), have been described previously. Expression plasmids for human estrogen receptor α (ERα) (pSG5-ER, and a reporter plasmid for ERα harboring three copies of estrogen response elements, and pGEX-4T-ER(29–180 aa), pGEX-4T-ER(AF-2) were kindly provided by Dr. Shigeaki Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan).

2.2. Isolation of ANT-1 by swapped yeast two-hybrid screening, and mRNA analysis

MatchMaker Plus (Clontech) was used for the yeast two-hybrid screening. Total RNA was isolated from primary-cultured human skin fibroblasts, followed by a poly(A) RNA fractionation. cDNA library was constructed using TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech) with random primers (Amersham Pharmacia Biotech), and was inserted into pLexA-BD included in the kit. A cDNA fragment, for bait, encoding NTD (1–532 aa residue) of human AR was ligated in frame into pB42-AD, thus creating the pB42-AD-AF-1 expressing for AR-AF-1 fused to GAL1 activation domain. Yeast EGY48 strain was transformed with the pLexA-BD carrying cDNA libraries and with the pB42-AD-AF-1 according to the manufacturer's protocol, then transformants were selected for growth on an appropriate nutrition medium. Positive candidate plasmids for AR-AF-1 binding proteins were recovered from the yeast, and the nucleotide sequences were determined using the ABI PRISM 377 DNA Sequencer (Perkin-Elmer). The specificity of interaction was further confirmed by a liquid galactosidase assay. To obtain full-length ANT-1 cDNA, the partial cDNA fragment encoding 78–495 aa residues of ANT-1, obtained by the two-hybrid screening, was ^{32}P-labeled as a probe for the screening of human prostate cDNA library carried by a λgt10 phage vector (Clontech). The full-length ANT-1 cDNA fragment was ligated into pcDNA3 (Invitrogen) to create pcDNA3-ANT. For the Northern blot analysis, MTN blots were purchased from Clontech.

2.3. Cell culture, transient transfection, reporter assay

COS-7 and the prostatic cancer cells ALVA-41 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were transiently transfected using a Superfect Transfection Kit (Life Technologies, Inc.). The total amounts of transfected plasmid DNA were kept constant by adding pcDNA3 vector plasmid. At 16 h post-transfection, the cells were rinsed, and then were fed with medium containing 10% charcoal-stripped fetal calf serum with or without various steroid hormones. After an additional 18 h, the cells were harvested and assayed for luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

2.4. Protein–protein interaction

For immunocoprecipitation analysis, COS-7 cells were transfected with plasmids expressing for myc-tagged ANT-1 and full-length or truncated AR, and were maintained with or without 10^{-8} M of dihydrotestosterone (DHT). Whole cell lysates were prepared by lysing cells in a buffer (1.0% Nonidet P-40, 50 mM Tris–Cl, 150 mM NaCl, 1 mM dithiothreitol, one tablet of protease inhibitor cocktail). In one experiment, nuclear lysates were prepared. The lysates were incubated at 4 °C for 1 h with the antibody raised against c-myc (Santa Cruz Biotechnology) in immunoprecipitation (IP) buffer (0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris–Cl, 200 mM NaCl, 1 mM dithiothreitol, one tablet of protease inhibitor cocktail), and then were further incubated with protein-A Sepharose beads (Pharmacia) at 4 °C for 2 h. After being washed, the pellets were suspended in an SDS-PAGE sample buffer. The proteins were separated on SDS-PAGE, transferred to nitrocellulose filter, and then were subjected to a Western blot analysis using antibody against AR (N-20) (Santa Cruz Biotechnology) for the detection of full-length or NTD fragment of AR, or using antibody C-19 (Santa Cruz Biotechnology) for the detection of C-terminal fragment of AR.

2.5. 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 pAR-CFP and pANT-1-YFP using 2.5 μl per dish of Superfect reagents (QIAGEN). Six to eighteen hours post-transfection, the culture medium was replaced with a fresh DMEM containing 10^{-8} M DHT. Confocal microscopy was performed essentially as previously described. In brief, 1 h after adding DHT, the cells were scanned using Leica TCS-SP system (Leica Microsystems, Heidelberg, Germany). The cells were imaged for yellow or cyan fluorescence by excitation with the 514 and 450 nm line, respectively, from an argon laser. The emissions were viewed through either a 530–590 nm band pass filter for YFP or a 470–500 nm band pass filter for CFP. 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. A series of 30–50 images were collected for each single nucleus. In each plane, the cyan, yellow, and ultraviolet fluorescence were consecutively collected using the serial scanning methods equipped in Leica TCS-SP system. Three-dimensional image reconstruction was performed by either using the 3D analysis TRI Graphics Program software package (Ratoc System Engineering, Tokyo),or using the deconvolution method (nearest neighbors).

3. Results and discussion

To identify the cDNA encoding proteins binding to the AR-AF-1 sequence, we performed swapped yeast two-hybrid screening, since AR-AF-1 possesses a strong autonomous transactivation capacity. We identified a clone containing approximately 1.3 kb of an open reading frame, and then this fragment was used to probe the human prostate cDNA library to obtain the full-length cDNA. Translation of the coding sequence within the full-length cDNA fragment revealed that the putative protein consisted of 941 aa residues with a predicted molecular weight of 102 kDa, which was named ANT-1 (AR N-terminal domain transactivating protein-1) (Fig. 1A) [3]. A Northern blot analysis revealed the ANT-1 sequence to be ubiquitously expressed among the tissues examined (data not shown). Surprisingly, the homology search to the known nucleotide and amino acid sequences revealed that ANT-1 was identical to a nucleoprotein, which is a binding protein to the human splicing factor U5 snRNP (GenBank Accession AF221842) [4,5]. U5 snRNP binding protein has been shown to be a member of a unique protein family possessing tetratricopeptide repeat (TPR) elements. ANT-1 contains 19 TPR elements, two LXXLL motifs, and one leucine zipper motif. Typically, the TPR motif, which has been speculated to form helix-turn structures, appears in a tandem array as found in ANT-1, and thus provides the scaffolds to mediate protein–protein interactions. In view of interaction between AR and pre-mRNA splicing factors, myocardium-specific coactivator FHL2 specifically binding to the AR, was shown to interact with the polypyrimidine tract binding protein-associated splicing factor (PSF) [6]. However, the binding is in an AF-2-dependent fashion.

Immunocoprecipitation experiments, using either whole cell extracts or nuclear extracts, were performed to test whether or not ANT-1 binds to AR in living cells. A plasmid expressing myc-tagged ANT-1 was cotransfected into COS-7 cells together with expression plasmid for the full-length or truncated mutants of AR, and then the cells were maintained with or without 10^{-8} M DHT. AR-AF-1 as well as full-length AR was specifically precipitated with myc-tagged ANT-1 in a ligand-independent fashion, while ANT-1 did not bind to AR-AF-2 in living cells (Fig. 2). Two fragments covering ER(29–180) for the activation

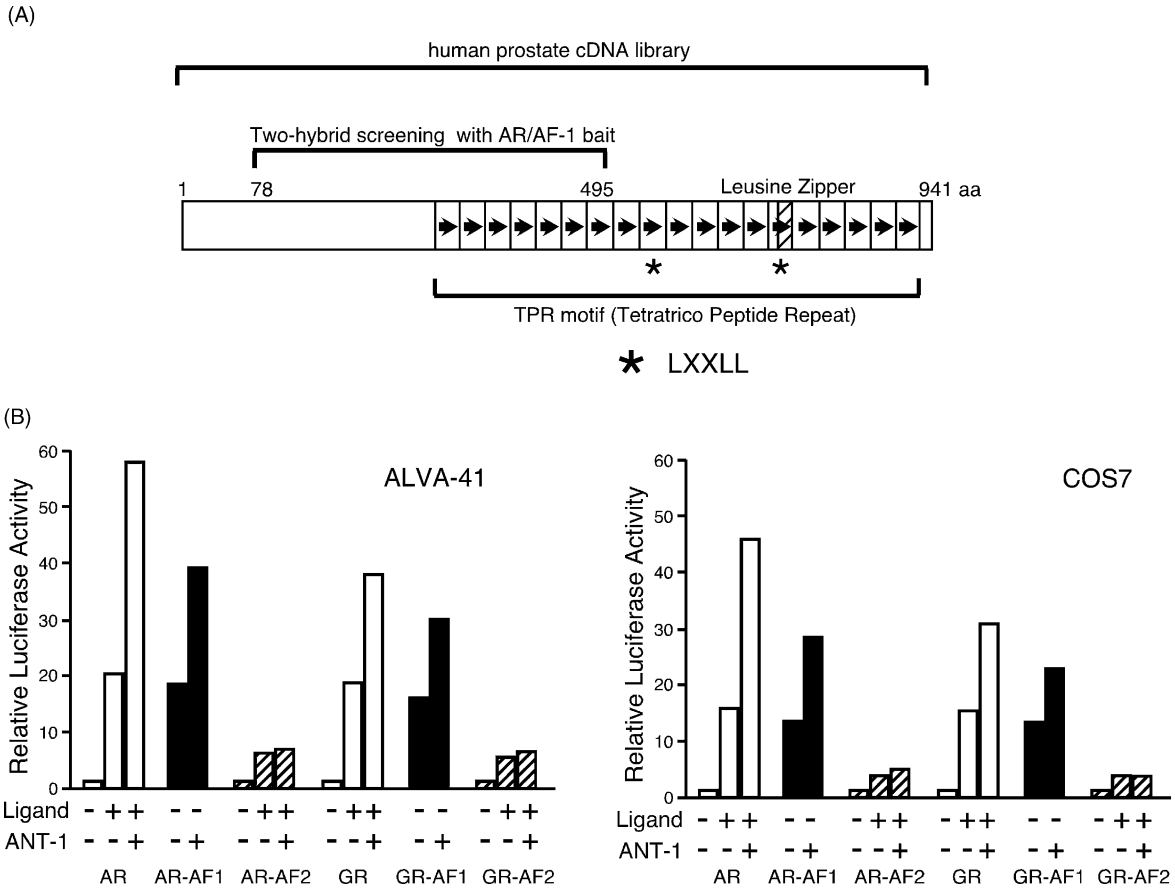

Fig. 1. (A) Schematic representation of the structure of ANT-1. ANT-1 contains 19 TPR motifs; asterisks: LXXLL motifs; dashed box: leucine zipper motif, respectively. (B) Functional analysis of ANT-1. The ANT-1 enhances the AR-AF-1 or GR-AF-1 in a ligand-independent fashion. pMMTV-luc and pCMV-ANT were transfected into ALVA-41 prostate cancer cells (left) or into COS-7 cells (right), and the cells were treated with or without 10^{-8} M DHT or 10^{-7} M dexamethasone.

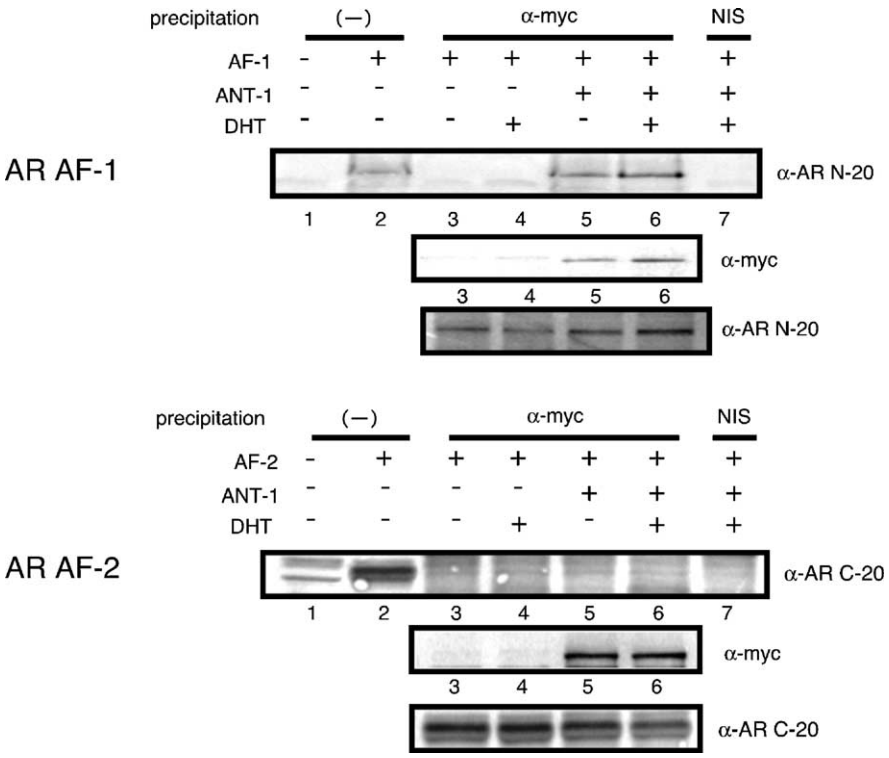

Fig. 2. Immunocoprecipitation of ANT-1 with the AR-AF-1 or AR-AF-2. The immunoprecipitaion was performed using an antibody against myc, and the precipitate was subjected to a Western blot analysis using the antibody N-20 for the AR-AF-1, and the antibody C-19 for the AR-AF-2. In each panel, the middle and the bottom blot represent the Western blot of the lysates used for the immunocoprecipitation, as controls.

domain of ERα or ER(282–595) for the AF-2 region failed to bind to ANT-1, and the addition of 17α-estradiol did not promote such binding (data not shown). To examine the effect of ANT-1 on the transactivation function of AR, GR, or ERα, we cotransfected an expression plasmid for each receptor together with the plasmids expressing ANT-1 and an appropriate reporter plasmid (pMMTV-luc for AR or GR, pERE2-tk109-luc for ERα) into ALVA-41 cells or COS-7 cells. The reporter gene luc+ (Clontech) harbored in pMMTV-luc does not contain any intronic sequences. In each cell line, ANT-1 further enhanced the ligand-induced transactivation function of full-length AR and GR (three-fold for AR in the presence of 10^{-8} M of DHT, and two-fold for GR in the presence of 10^{-7} M of dexamethasone, respectively). This enhancement was exerted by the enhancement of ligand-independent autonomous transcriptional enhancement of AF-1 of either AR or GR. When the plasmid expressing the truncated mutant of AR, such as AR-AF-1 (aa residues 1-622) or AR-AF-2 (aa residues 563–919), were cotransfected, with the expression plasmid for ANT-1 and a reporter plasmid, ANT-1 enhanced the constitutive transactivation function mediated by AR-AF-1 by 2–2.5-fold, as well as the ligand-dependent overall transactivation function of full-length AR (Fig. 1B). However, no enhancement of the ligand-dependent transactivation mediated by AR-AF-2 was observed. A similar profile of the domain-specific transactivation was also observed for GR, namely ANT-1 enhanced the transactivation mediated by GR via enhancing the autonomous AF-1 transactivation function. In contrast, ANT-1 did not enhance ERα-dependent transactivation. Together with the findings in the immunocoprecipitation experiments, we concluded that ANT-1 is primarily AF-1-interacting transcriptional coactivator for AR or GR, but not for ERα in which AF-1 transactivation is weak. This is in a strong contrast to that peroxisome proliferator-activated receptor-μ (PPARμ)-coactivator-1 (PGC-1), possessing the pre-mRNA splicing activity in itself, can bind to ERα [7].

To establish the novel approach in order to distinguish the transcriptionally active AR from the transcriptionally inactive AR, we previously reported the establishment of the three-dimensional construction of confocal microscopic images of intranuclear AR [8]. This method clearly distinguished the subnuclear localization of transcriptionally active AR tagged with green fluorescent protein (AR-GFP) from the transcriptionally inactive AR-GFP (Fig. 3A). 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

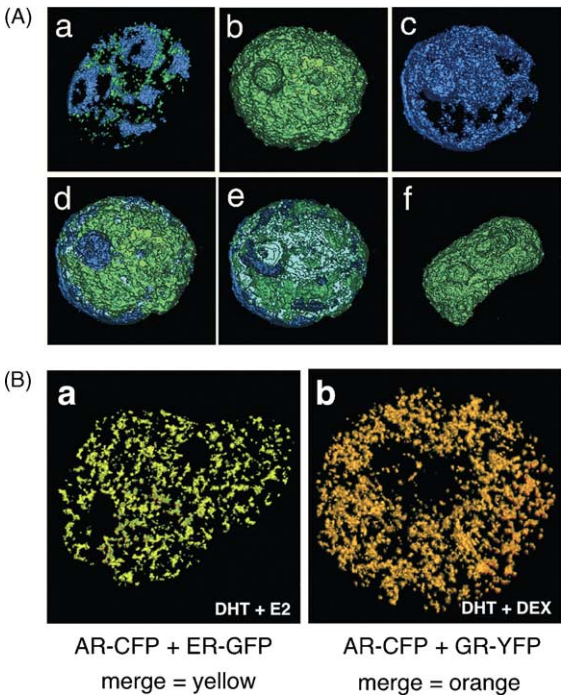

Fig. 3. (A) The three-dimensional image analysis of the intranuclear localization of the agonist- or antagonist-bound AR-GFP. COS-7 cells transfected with pAR-GFP were treated with 10^{-8} M DHT (a) or 10^{-6} M antiandrogenic chemicals (b)–(f) and were stained with Hoechst 33342, and then the confocal images of the nucleus were collected to reconstruct the three-dimensional images. The images were displayed as a surface view (b)–(d) and (f) or the tomographic sectional view (a) and (e). (a) Tomographic sectional image of the nucleus treated with 10^{-8} M DHT as a control; (b) the surface view of the distribution of AR-GFP in the nucleus of 10^{-6} M nitrofen-treated cells; (c) the surface view of the chromatin structure of the same nucleus as shown in (b); (d) the spatial merge of (b) and (c); (e) the tomographic sectional image of (d); (f) the surface view of the distribution of AR-GFP in the nucleus of 10^{-6} M vinclozolin-treated cell. The image was displayed as in (b). (B) The three-dimensional image analysis of the intranuclear localization of liganded AR-CFP, GR-YFP and ER-GFP in COS-7 cells. COS-7 cells were transfected with the expression plasmids indicated in each panel. The cells were then scanned after treatment with 10^{-8} M ligand for each receptor as indicated. The images are displayed as surface views. The GFP, YFP, and CFP signals are represented as green, yellow and red, respectively. (a) Yellow-colored foci formation by AR-CFP and ER-GFP; (b) orange-colored foci formation by AR-CFP and GR-YFP. The numbers of spots identified as a distinct volume were quantified as 312 ± 30 (a), and 303 ± 33 (b) (mean $\pm$ S.D.) from four independent experiments.

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 spread homogeneously in the nucleus. Altogether, these 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.

Furthermore, the foci formation of DHT-bound AR-GFP in COS-7 cells was abolished by the cotransfection of a CBP (118–2393 aa) fragment eliciting a dominant negative effect on the transactivation capacity of the AR. The N-terminal AR fragment (AR-AF-1-YFP), which has a strong constitutive transactivation function, formed foci without DHT, whereas the C-terminal AR fragment (AR-AF-2-CFP), which has a quite low transactivation function, was distributed homogeneously even in the presence of DHT. The reporter gene assay showed a synergism between the transactivation functions of AR-AF-1 and AR-AF-2. The DHT-bound wild-type AR-GFP alone or AR-AF-1-YFP plus DHT-bound AR-AF-2-CFP was distributed as approximately 300 discrete spots in one nucleus, whereas AR-AF-1-YFP alone was distributed as one volume in a reticular pattern. Furthermore, not only AR but also the glucocorticoid receptor-YFP, ER-GFP, and YFP-tagged SRC-1, TIF2, and CBP were found to be accumulated in identical spots in the presence of ligand (Fig. 3B). The above results indicate that CBP is one of the factors essential for foci formation of the AR, and may propose the hypothesis that transcriptionally-activated steroid receptors, regardless of the type of receptor, are transferred to common compartments and form a complex with coactivators, and this process is essential to full transactivation [9].

We were interested in the subnuclear spatial interrelation between nuclear receptor compartment, colocalizing with p160 members and p300/CBP, and splicing factor compartment (SFC). Therefore, the spatial interrelation of AR-CFP with ANT-1-YFP was explored in detail using a three-dimensional image analysis. A volume method in three-dimensional reconstruction showed the nuclear receptor speckles (fluorescence foci) and revealed many small spatial “pockets” where no cyan fluorescence was observed as in the nucleolus (cyan fluorescence was digitally converted into red as pseudocolor). The subnuclear localization of ANT-1 was clearly distinct from that of AR. In a good agreement with the subnuclear distribution of prp6p (yeast homologue of ANT-1) [10], the ANT-1 distribution was identical to the known distribution pattern of splicing factors. The transfected ANT-1-YFP distributed in the nucleus in two distinct patterns as follows: a diffuse fine reticular distribution throughout nucleus, devoid of a nucleolus, and a coarsely clustered distribution (speckles) known as the splicing factor compartment, both of which were exclusively in the euchromatin region where the Hoechst 33342 staining was less dense (Fig. 4a). When images of AR-CFP and ANT-1-YFP were spatially merged, the CFP and YFP fluorescence was colocalized only in the diffuse fine distribution (Fig. 4b, merged area is represented in orange). To focus on the spatial interrelation between ANT-1 speckles (SFC) and AR-CFP (nuclear receptor speckles), the diffuse fine reticular distribution of ANT-1-YFP was cut off and expressed as a blank image. As a result, it became clear that the ANT-1 speckles fall into the small spatial pockets of the cyan fluorescence

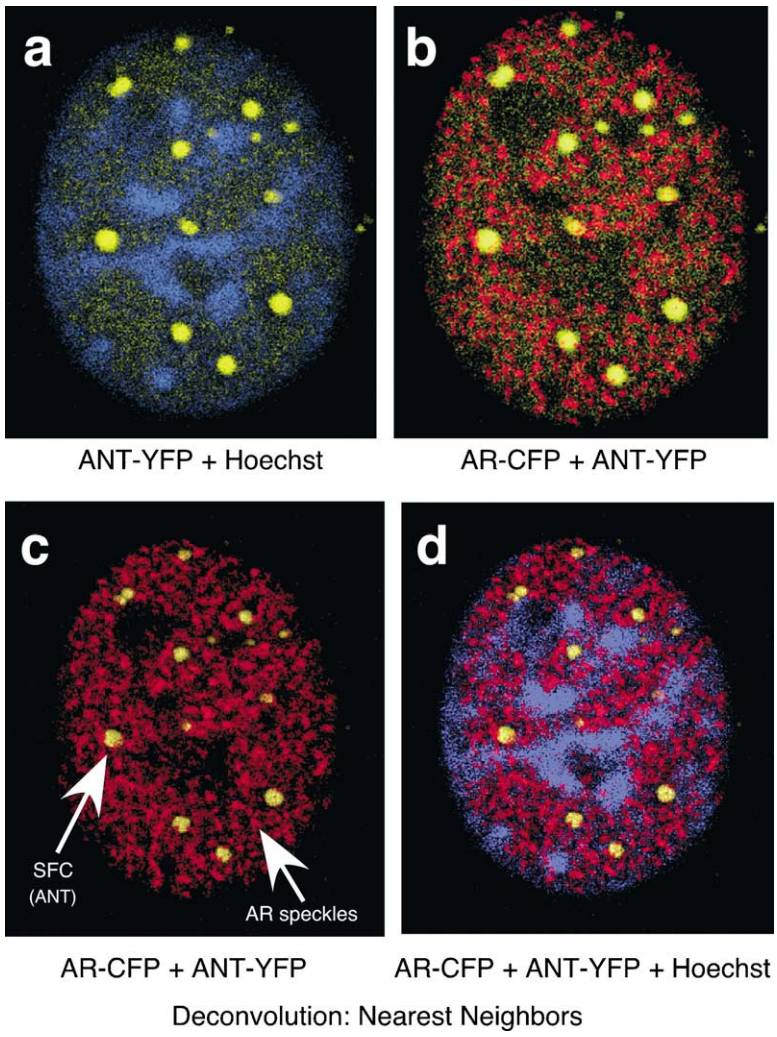

Fig. 4. The three-dimensional image analysis of the subnuclear compartmentalization of AR and ANT-1. COS-7 cells were transfected with plasmids expressing for AR-CFP fusion, ANT-1-YFP fusion, or both, treated with 10^{-8} M DHT, and then were stained with Hoechst 33342 to visualize the chromatin structures (blue in (a) and (d). The three-dimensional reconstruction was performed using deconvolution methods (nearest neighbors). The AR-CFP is visualized in red as pseudocolor for (b)–(d). For the chromatin images, less densely stained areas (namely euchromatin region) were shown as blank images, and densely stained areas (heterochromatin region) were shown as blue. (a) The surface view of spatial merge of ANT-1-YFP with chromatin structures; (b) the surface view of spatial merge of AR-CFP with ANT-1-YFP; (c) the surface view of the spatial merge of AR-CFP with ANT-1-YFP. To highlight the ANT-1 speckles, the diffusely distributed fine reticular network found in c was cut off and is shown as blank image; (d) the surface view of the spatial merge of AR-CFP, ANT-1-YFP, and chromatin images.

volume (Fig. 4c and d). When the image was digitally magnified, the ANT-1 volume was shown to possess a rough surface, which was surrounded by a spatial mass representing transcriptionally active ARs without merging with each other [3].

In the nucleus, there exist different sets of functional compartments often called “foci” or “speckles”, including SFC which demonstrate nearly 20 large speckles, and nuclear receptor speckles possibly associated with the nuclear matrix structures [11]. Since these subnuclear compartments are not defined by membranes, the nuclear protein, including hormone-occupied receptor, undergoes rapid exchange between chromatin and the nucleoplasmic compartment. SFCs consist of many protein complexes including snRNPs, pre-mRNA processing factor. The active gene transcription simultaneously proceeding with pre-mRNA processing has been speculated to occur at the periphery of SFC, which is called “transcription-splicing coupling” [12]. We first visualize the spatial relationship of the subnuclear compartment between steroid hormone receptor and SFC. Recent studies have shown that the SFC may represent the site for the storage and/or assembly of the splicing factors and that splicing factors can be rapidly recruited from the SFC into the active sites of transcription. In this regard, the merging of the diffuse ANT-1 distribution with AR speckles near the SFC may represent where the ANT-1 or ANT-1-snRNP complex meets the active AR-cofactor complex.

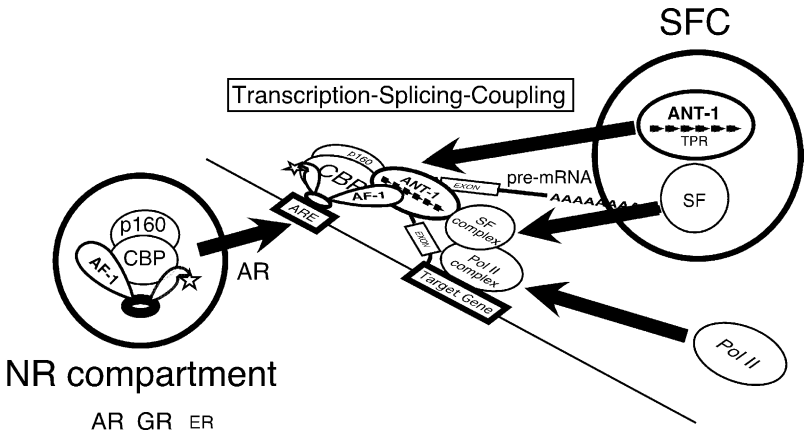

Fig. 5. The schematic representation of the possible ANT-1 function. The transcriptionally active AR is formed at the nuclear receptor (NR) compartments after the interaction with AF-2-interacting transcriptional cofactors. This AR-cofactor complex reaches the peripheral zone of SFC thus binding to basal transcription/splicing machinery complex, formed at on site (actively transcribed gene at periphery of SFC), or possibly formed in SFC. This binding is mediated by ANT-1.

The interaction of AR-AF-1 with ANT-1 may play a key role in the interaction between two distinct sets of the transcription factors located at the distinct subnuclear compartments (Fig. 5). One is a steroid hormone receptor compartment colocalizing with transcriptional cofactors chiefly interacting with AF-2, while another is the SFC at the periphery in which the cotranscriptional splicing takes place. We therefore speculate that the transcriptionally active AR is formed at the nuclear receptor compartments (speckles) after the interaction with AF-2-interacting transcriptional cofactors. This ready-to-promote-transactivation AR, after roaming in nucleoplasm, reaches the peripheral zone of SFC thus binding to basal transcription/splicing machinery complex, formed at on site (actively transcribed gene at periphery of SFC), or possibly formed in SFC. This binding is mediated by ANT-1. Furthermore, ANT-1 may selectively recruit AR or GR, while ER, in which AF-1 transactivation is much weaker than that of AR or GR, is not recruited. In this model, a strong autonomous activity of AF-1 truncated fragment of AR or GR is the direct recruitment of transcription-splicing couplings.

Acknowledgements

We thank Mr. Mitoshi Toki for his assistance and excellent technical advice for creating three-dimensional figures. We also thank Drs. Shigeaki Kato and Jun Yanagisawa for their kind gift of the plasmid construct. Part of this work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan. Furthermore, part of this work was performed at Station for Collaboration, Kyushu University.

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