start site, is strictly required for expression of the Ad4BP/SF-1 gene, and that site-directed mutagenesis of the E-box sharply decreases Ad4BP/SF-1 transcriptional activity, suggesting that any mutation in this area would virtually destroy the physiological function of Ad4BP/SF-1. Fortunately, the specific sequence of the E-box, 5′CACGTG3′, is exactly the sequence recognized by the restriction enzyme *Pma*CI (*Bbr*PI), and we used this to study the E-boxes of the 30 patients with adrenal diseases and the 55 normal controls. However, as all cases were digested by PmaC, this suggests that this critical *cis*-element is highly conserved.

G146A human Ad4BP/SF-1 has impaired transactivation

To further clarify the physiological function of G146A human Ad4BP/SF-1, we first studied its transactivation capacity in CV1 cells, which do not express endogenous Ad4BP/SF-1. The wild-type and the naturally occurring mutant (G35E), which has been reported to cause X–Y sex reversal and primary adrenal failure [12], were used as controls. A reporter plasmid, pGL3-hSCCprom, in which a 0.6 kb human P450scc gene (*CYP11A*) promoter containing the Ad4 site was inserted to pGL3-basic to drive the luciferase gene, was used to study the transactivation capacity of each Ad4BP/SF-1 variant. As shown in Fig. 2A, the relative luciferase activity of mutant Ad4BP/SF-1 (G35E) was less than 0.3-fold that of the wild type, and the activity of polymorphism Ad4BP/SF-1 was also slightly (0.75-fold), but significantly, less than that of the wild type. To further test whether or not the transactivation effect is universal to different Ad4BP/SF-1 target promoters, the Ad4BP/SF-1 response of the *CYP19* armatase promoter II was also studied, a similar result was observed (Fig. 2B). The reaction pattern of pGL3-hSCCprom to increasing dosages of Ad4BP/SF-1 was also studied (Fig. 2C). The relative luciferase activity increased in a dose-dependent manner with wild-type Ad4BP/SF-1, and the same pattern could be seen with polymorphism Ad4BP/SF-1. At every dosage, polymorphism Ad4BP/SF-1-mediated luciferase activity was slightly, but sig-

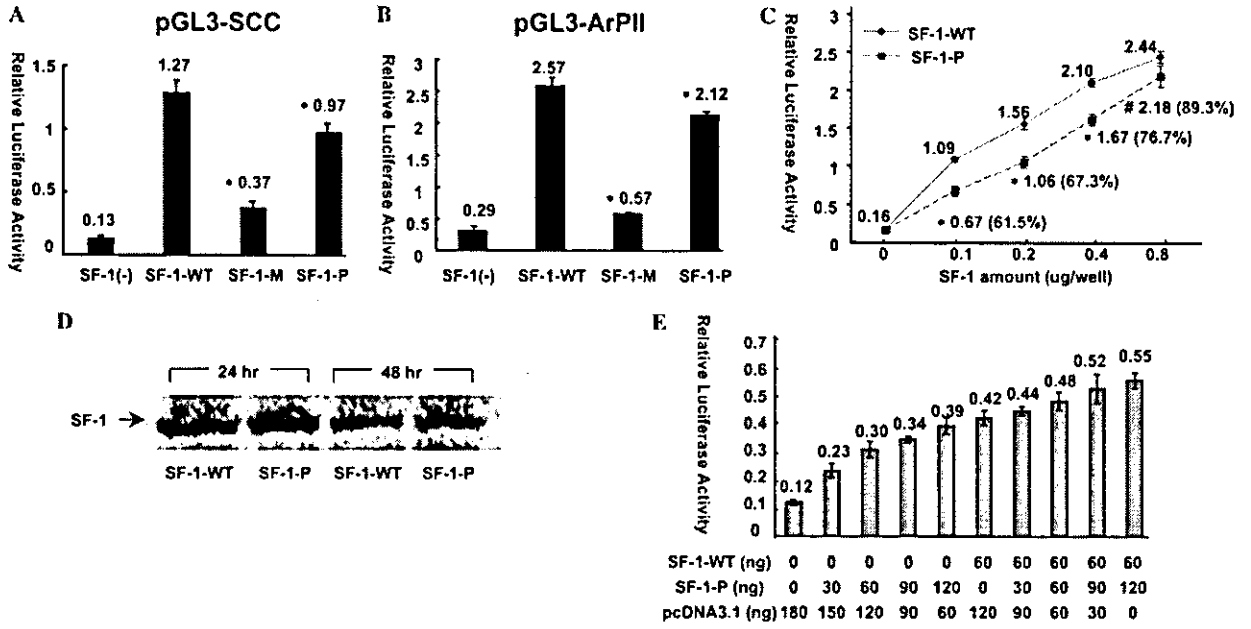

Fig. 2. G146A human Ad4BP/SF-1 bears a slightly impaired transactivation. (A) Nonsteroidogenic CV1 cells transfected with the human CYP11A promoter firefly luciferase reporter plasmid, pGL3-hSCCprom, together with the *Renilla* luciferase plasmid, phRL-CMV, as an internal control. pcDNA3.1-Ad4BP/SF-1-WT (wild-type), pcDNA3.1-Ad4BP/SF-1-M (mutant, G35E) or pcDNA3.1-Ad4BP/SF-1-P (polymorphism, G146A) was cotransfected as indicated. The relative luciferase activity was expressed as the mean ± SD. *Statistically different from SF-1-WT ($p < 0.01$). (B) The same experiment as described in (A) was carried out with the only exception that pGL3-hSCCprom is substituted for pGL3-ArPII. PGL3-ArPII responses to each variant of SF-1 are of the same pattern seen in the case of pGL3-hSCCprom. (C) CV1 cells transfected with pGL3-hSCC-prom + phRL-CMV. Increasing dosages of either pcDNA3.1-Ad4BPSF-1-WT (wild-type) or pcDNA3.1-Ad4BP/SF-1-P (polymorphism, G146A) are also cotransfected as indicated. The reporter reacts to both SF-1-WT and SF-1-P in a dose-dependent manner, at every dosage, SF-1-P mediated luciferase signal is slightly but significantly lower than that of SF-1-WT. *Statistically different from corresponding SF-1-WT group ($p < 0.01$). #Statistically different from corresponding SF-1-WT group ($p < 0.05$). (D) Western blot analysis performed to address the expression of SF-1-P as controlled by SF-1-WT, using protein extract from CV1 cells transiently transfected with SF-1-WT or SF-1-P expression vector. At both 24 and 48 h, SF-1-P expression is indistinguishable from that of SF-1-WT, suggesting that G146A variation does not interfere with protein translation or stability. (E) The SCC reporter plasmid transfected into CV1 cells with increasing amounts of SF-1-P expression vector (0, 30, 60, 90, and 120 ng/well), in the absence or presence of a constant amount of SF-1-WT (60 ng/well). The total amounts of transfected plasmids are adjusted with the empty pcDNA3.1 vector. SF-1-P presents no dominant negative effects.

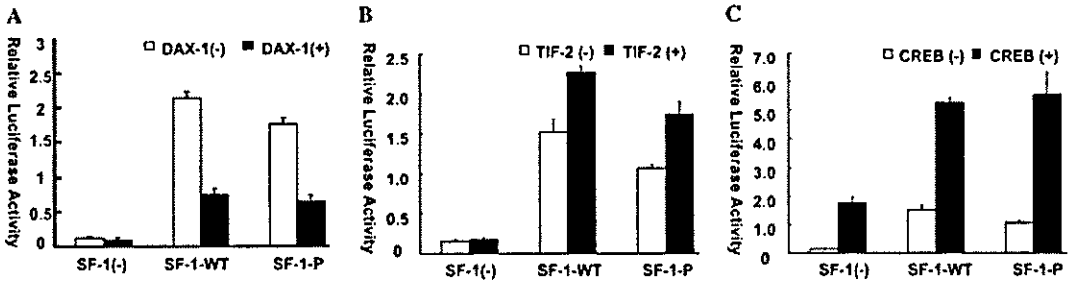

Fig. 3. Interactions with co-regulators. (A) KGN cells seeded on a 12-well plate and transfected with 0.8 μg/well pGL3-ArPII + 3 ng/well pRL-CMV + 0.15 μg/well pcDNA3.1-SF-1-WT or pcDNA3.1-SF-1-P, either an additional 0.15 μg/well DAX-1 expression vector or the empty control vectors are also cotransfected. (B) Experiment carried out in a scheme as described in (A), except that the KGN cells are substituted with CV1 cells, pGL3-ArPII is substituted with pGL3-hSCCprom, and DAX-1 expression vector is substituted with TIF2 vector. (C) Experimental strategy is the same as that in (B), except that TIF2 vector is substituted with CREB expression vector.

nificantly, lower than that mediated by the wild-type. Interestingly, the two lines became closer with increasing Ad4BP/SF-1 dosage. The percentage of luciferase signal caused by this polymorphism relative to that caused by the wild-type increased with increasing amounts of Ad4BP/SF-1, from 61.5% in the 0.1 μg/well of the Ad4BP/SF-1 group to 89.34% in the 0.8 μg/well of the Ad4BP/SF-1 group. The mild impairment of Ad4BP/SF-1 transactivation might be possibly due to different protein levels, which may have resulted from different stabilities between the wild type and the polymorphism variant. To address this possibility, the same amounts of expression plasmids (5 μg/60 mm dish) for Ad4BP/SF-1-WT or Ad4BP/SF-1-P were transfected to CV1 cells, and Western blotting was then carried out at two time spots (24 and 48 h post-transfection), using a rabbit anti-SF-1 antibody. The result in Fig. 2D, at both time spots, shows no apparent difference of protein levels between the two Ad4BP/SF-1 variants; suggesting that the protein stability of the Ad4BP/SF-1-P might not be significantly different from that of Ad4BP/SF-1-WT.

The slight impairment of polymorphism Ad4BP/SF-1 transactivation could also be seen in the steroidogenic cell lines, KGN and Y1 cells (data not shown).

To investigate a possible dominant negative interaction between Ad4BP/SF-1-P and Ad4BP/SF-1-WT, CV1 cells were transfected with an increasing amount of Ad4BP/SF-1-P (0, 30, 60, 90, and 120 ng/well) with or without the cotransfection of a constant amount (60 ng/well) of Ad4BP/SF-1-WT expression vector. In the absence of Ad4BP/SF-1-WT, Ad4BP/SF-1-P increases CYP11A promoter activity in a dose-dependent manner. Ad4BP/SF-1-WT mediated CYP11A promoter activity was further additively enhanced by Ad4BP/SF-1-P dose-dependently, suggesting that Ad4BP/SF-1-P is not a dominant negative inhibitor of wild type Ad4BP/SF-1.

Interaction with cofactors

Nuclear orphan receptor DAX-1 works as a repressor of Ad4BP/SF-1, by recruiting co-repressors like NcoR [19], or Alien [20]; and the consequence of the putative interaction between DAX-1 and Ad4BP/SF-1 determines the final fate of steroidogenic genes. To address a possible altered interaction between Ad4BP/SF-1-P and DAX-1, KGN cells were transfected with pGL3-ArPII together with Ad4BP/SF-1-WT or Ad4BP/SF-1-P, respectively. DAX-1 expression vector or a control empty vector was also cotransfected. As shown in Fig. 3A, pGL3-ArPII luciferase signals caused by both wild-type Ad4BP/SF-1 and polymorphism Ad4BP/SF-1 were decreased by around 65%.

We subsequently studied the interaction pattern of Ad4BP/SF-1-WT or Ad4BP/SF-1-P with standard coactivators. It has been reported that Ad4BP/SF-1 interacts with several transcriptional cofactors such as SRC-1, TIF2, through AF-2 domain of the receptor [25,26]. An experiment with the same strategy described in the case of DAX-1 interaction was carried out in CV1 cells, using pGL3-hSCCprom, to study the interaction of Ad4BP/SF-1-P with the coactivator TIF2. As shown in Fig. 3B, both Ad4BP/SF-1-WT and Ad4BP/SF-1-P were coactivated by TIF-2 in a similar pattern. CREB directly interacts with Ad4BP/SF-1 and the two transcriptional factors stimulate target gene in a synergistic manner [27]. A similar experiment was carried out to study how Ad4BP/SF-1 variants interact with CREB. As shown in Fig. 3C, unlike TIF-2, CREB itself stimulated CYP11A promoter activity as potent as Ad4BP/SF-1-WT, but the promoter activity was further synergistically stimulated in the co-expression of CREB and Ad4BP/SF-1-WT. The phenomenon observed in the case of Ad4BP/SF-1-P is indistinguishable from that of Ad4BP/SF-1-WT.

Subnuclear localization

As shown in Fig. 4, both GFP-SF-1-WT and GFP-SF-1-P were predominantly located inside the nucleus of KGN cells. The nucleoli demonstrated no fluorescence, suggesting that they were devoid of Ad4BP/SF-1. We recently found that, upon transactivational potentiation

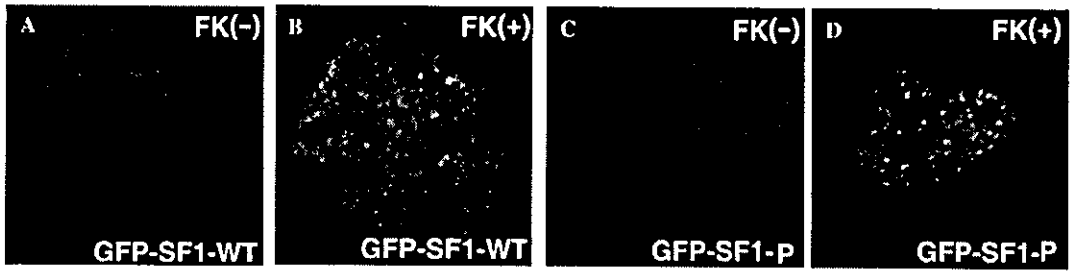

Fig. 4. Subnuclear localization of GFP-Ad4BP/SF-1-WT and GFP-Ad4BP/SF-1-P. KGN cells transfected with 0.5 mg/dish GFP-Ad4BP/SF-1-WT (A,B), or GFP-Ad4BP/SF-1-P (C,D). The fluorescent chimeric proteins expressed were observed in living cells using a Carl Zeiss LSM 510 META laser confocal microscope as described in Materials and methods. The treatment with forskolin is indicated just below each panel. Both wild-type (A) and polymorphism (C) Ad4BP/SF-1 are diffuse in the nucleus and become assembled into foci on a diffuse fluorescence background when PKA is activated (B,D).

by PKA, Ad4BP/SF-1-WT undergoes a subnuclear compartmental change and manifests foci formation, whereas a transcriptionally inactive mutant, Ad4BP/SF-1 bearing G35E, does not cause such compartmental changes [28]. In the absence of forskolin, the stimulator of adenylyl cyclase, the localization of both GFP-SF-1-WT (Fig. 4A) and GFP-SF-1-P (Fig. 4C) was quite diffuse in the nucleus. When KGN cells were treated with 10^{-6} M forskolin overnight, both GFP-Ad4BP/SF-1-WT and GFP-SF-1-P manifested clear foci formation (Figs. 4B and D). There were no distinctive differences between the reaction patterns (foci formation) of either GFP-SF-1-WT or GFPSF-1-P to forskolin.

Discussion

The orphan nuclear receptor Ad4BP/SF-1 plays essential roles at multiple levels of endocrine differentiation and function, and is the first transcriptional regulator shown to play key roles at all levels of the hypothalamic–pituitary–steroidogenic organ axis. The sequence of the human gene encoding Ad4BP/SF-1 closely resembled that of the mouse gene [21] and Ad4BP/SF-1 expression during human embryological development closely parallels that in mice [29]. Thus, it is reasonable to deduce that human Ad4BP/SF-1 mutations might cause endocrine disease. To date, only three subjects with diseases associated with Ad4BP/SF-1 mutations have been described, suggesting that Ad4BP/SF-1 mutations occur vary rarely, and thus has only a limited clinical significance for human inborn endocrine disease.

Unlikely, another orphan nuclear receptor DAX-1 (NROB1), which closely relates to Ad4BP/SF-1 functionally, and usually considered as an antagonizer of Ad4BP/SF-1, bears much more frequent pathogenic mutation in human, and is thus of greater clinical importance. Patients with clear X-linked family histories of AHC and HHG are almost always found to carry DAX-1 mutations [15,30].

In the present study, during direct sequencing Ad4BP/SF-1 gene in a group of Japanese presenting with a series of adrenal diseases, we found one base pair variation G → C, which altered the 146th amino acid from Gly to Ala (GGG to GCG) in the hinge region. Functional characterization suggests an altered transactivation function in the G146A variant protein. Unlike the R255L mutation [14] of Ad4BP/SF-1, which also happens in the hinge region, but bears a sharp impairment of transactivation ability, the G146A variant only slightly, while statistically significant, impairs the transactivation. Since the Ad4BP/SF-1 mutant of G35E has been shown to have varying effects on different Ad4BP/SF-1 responsive genes [31], we studied the transactivation ability of G146A in both the adrenal specific *cyp11A* SCC promoter and also the ovary specific *cyp19* aromatase promoter II. Similar results were observed, suggesting that the transcriptional effect is relatively universal to different Ad4BP/SF-1 target promoters/response elements. The impairment of transactivation is unlikely due to an altered protein translation or acceleration of protein degradation. Neither G146A variant shows any dominant negative activity when transiently co-expressed with wild type protein. The interaction pattern of G146A with the Ad4BP/SF-1 specific repressor DAX-1 is indistinguishable from that of wild type. Coactivation studies show that G146A also properly interacts with coactivators of both CREB and TIF2, suggesting that G146A does not alter the recruitment of standard co-regulators. G146A resides in the nucleus predominantly and undergoes a subnuclear compartmental re-organization in response to the protein kinase A signal pathway excitation, in a pattern same as that of wild type. All these data suggest that G146A variation does not remarkably alter the biological function of SF-1 protein, except the slightly impaired transactivation ability.

This G146A variation in the Ad4BP/SF-1 gene can be considered as a nonsynonymous single nucleotide polymorphism rather than a functionally significant mutation, in that it occurs in normal individuals as well as in patients with adrenal diseases.

In a randomly obtained normal control population comprised of 55 subjects, 12.7% (7 of 55) contained allele C (5 GC and 2 CC); a frequency of 8.2%. It appears that the G146A polymorphism variation occurs quite frequently in Japanese and may thus have potential clinical significance, in contrast to the very rare occurrence of the SF-1 mutation. Supporting this, in the adrenal disease group of 30 patients, 46.7% (14 of 30) of them possessed allele C (GC 10 cases, and CC 4 cases), the frequency of allele C was 30%, which was almost 4-fold that of the normal control population. On the other hand, in the allele C possessing population, 66.7% (14 of 21) were patients with adrenal diseases, while only 26.5% (17 of 64) of the GG population were patients. These data suggest that the one base variation may relate to the occurrence of adrenal disorders; and so individuals bearing the C allele might be more susceptible to adrenal diseases. However, surprisingly, the polymorphism did not correlate specifically with hypo- or hyper-function of adrenal cortex. It was observed in patients with adenomas that are hyperfunction for cortisol and for aldosterone, nonfunctioning adenomas, and also adrenal hypoplasia congenital, that G146A was seen in both hyper- and hypofunction adenocortical diseases. This suggests that this polymorphism is probably not closely associated with the capability of adrenal steroidogenesis, and together with the relatively small population of patients with adrenal diseases (especially when each disease is considered individually), we cannot at present draw a clear conclusion regarding the relationship. The polymorphism might be associated with some pathological backgrounds of the adrenal glands and thus may produce a more susceptible state to various adrenal disorders. A cooperative study with multiple centers involved to accumulate a sufficient population for each disease is definitely of great essentiality to clarify the relationship between the G146A Ad4BP/SF-1 and adrenal diseases.

Although Ad4BP/SF-1 plays such essential roles at multiple levels of endocrine differentiation and function, relatively little is known about the mechanisms that regulate the expression of this orphan nuclear receptor. The conserved E-box motif located around -80bp has been shown to be critical for Ad4BP/SF-1 expression in Sertoli cells [32], and in Y1 cells and αT3 gonadotropes [33]. However, we could not find any changes in the E-box, and the contribution of E-box variation to the pathogenesis of adrenal diseases, if any, is probably minor.

Collectively, in this study, we report the first functional characterization of the newly clarified human Ad4BP/SF-1 variation G146A. The diminished ability to transactivate Ad4BP/SF-1 target genes coupled with a relatively high frequency in Japanese, especially in patients with adrenal diseases, suggests a possibility that the G146A Ad4BP/SF-1 polymorphism might clinically relate with susceptibility to adrenal diseases.

Acknowledgment

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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

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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

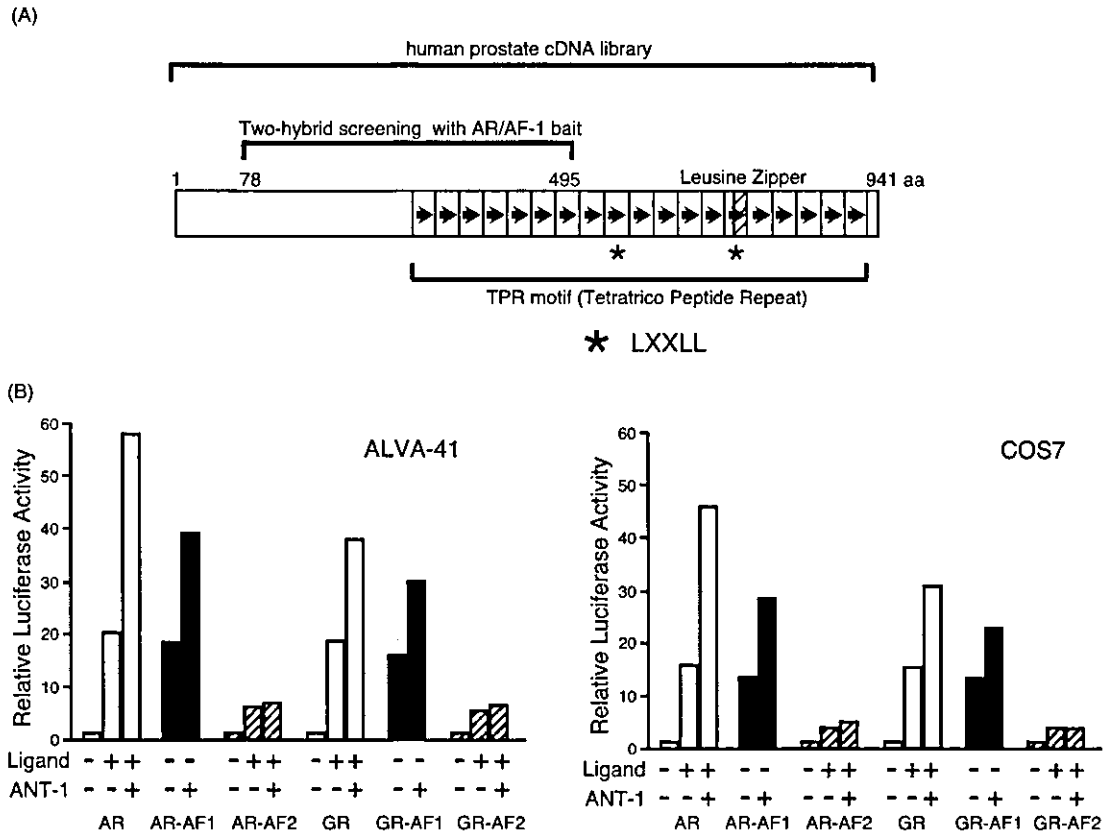

Fig. 1. (A) Schematic representation of the structure of ANT-1. ANT-1 contains 19 TPR motifs; asterisks: LXXLL motifs; dashed box: leucine zipper motif, respectively. (B) Functional analysis of ANT-1. The ANT-1 enhances the AR-AF-1 or GR-AF-1 in a ligand-independent fashion. pMMTV-luc and pCMV-ANT were 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.

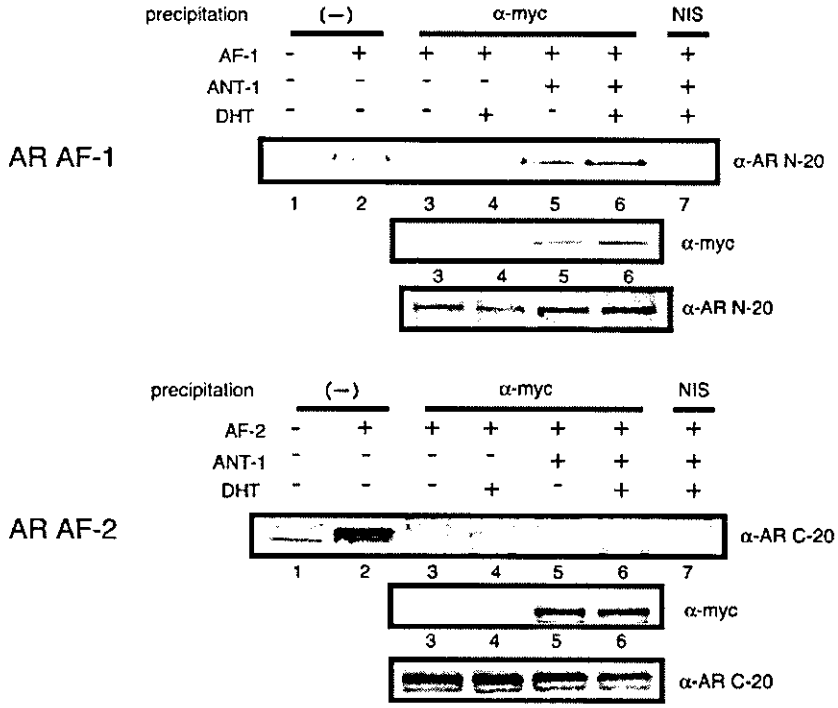

Fig. 2. Immunocoprecipitation of ANT-1 with the AR-AF-1 or AR-AF-2. The immunoprecipitaion was performed using an antibody against myc, and the precipitate was subjected to a Western blot analysis using the antibody N-20 for the AR-AF-1, and the antibody C-19 for the AR-AF-2. In each panel, the middle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

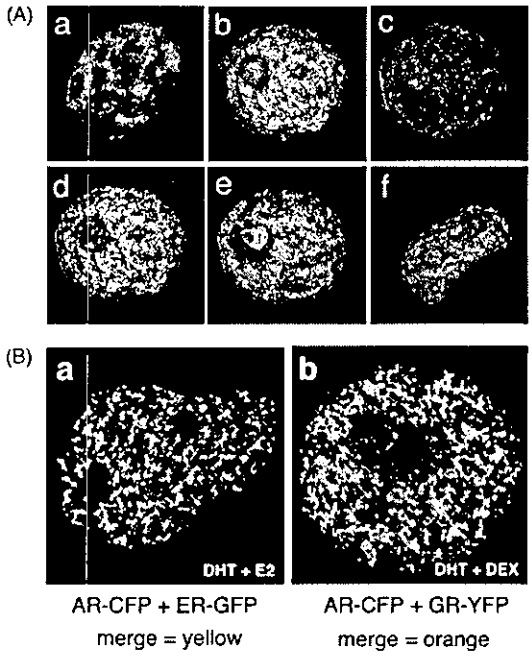

Fig. 3. (A) The three-dimensional image analysis of the intranuclear localization of the agonist- or antagonist-bound AR-GFP. COS-7 cells transfected with pAR-GFP were treated with 10^{-8} M DHT (a) or 10^{-6} M antiandrogenic chemicals (b)–(f) and were stained with Hoechst 33342, and then the confocal 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

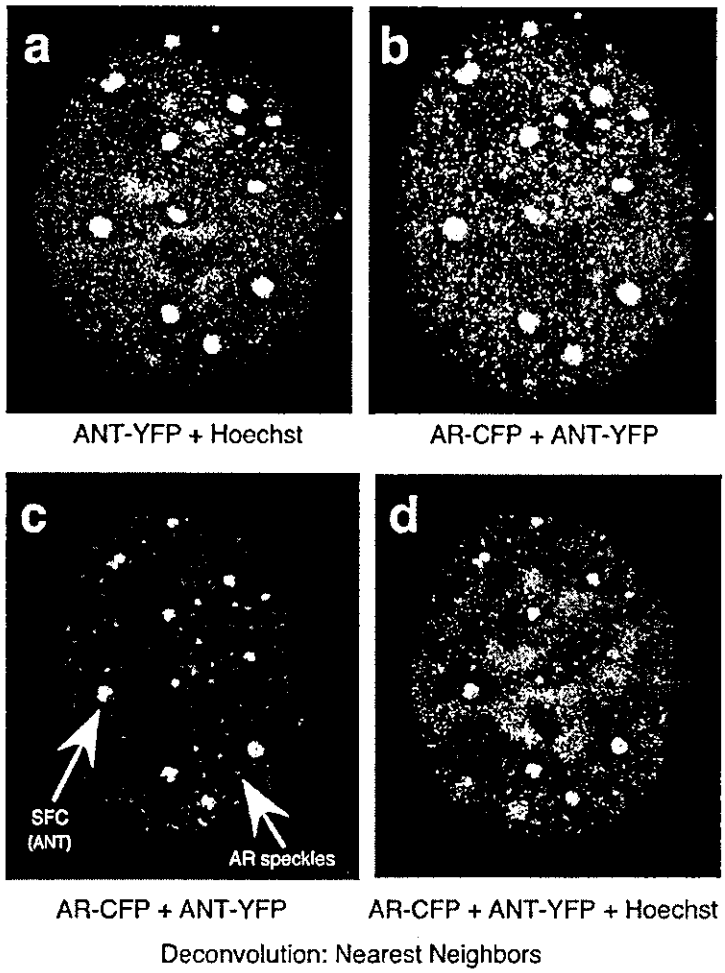

Fig. 4. The three-dimensional image analysis of the subnuclear compartmentalization of AR and ANT-1. COS-7 cells were transfected with plasmids expressing for AR-CFP fusion, ANT-1-YFP fusion, or both, treated with 10^{-8} M DHT, and then were stained with Hoechst 33342 to visualize the chromatin structures (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.

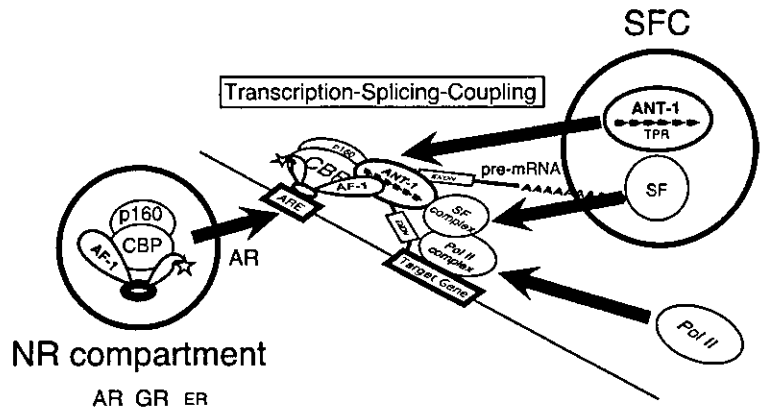

Fig. 5. The schematic representation of the possible ANT-1 function. The transcriptionally active AR is formed at the nuclear receptor (NR) compartments after the interaction with AF-2-interacting transcriptional cofactors. This AR-cofactor complex reaches the peripheral zone of SFC thus binding to basal 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.

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Both N- and C-terminal transactivation functions of DNA-bound ERα are blocked by a novel synthetic estrogen ligand

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Abstract

Estrogen receptors (ERs) play a central role in the diverse actions of estrogen. A number of synthetic ER ligands have been generated that can modulate various ER functions. Here we show that TAS-108, representing a novel class of synthetic ER ligands, blocked both ER transactivation functions without inhibiting DNA-binding activity. A transient expression assay showed that similar to ICI182,780, TAS-108 exhibited pure antagonistic activity as it blocked both the N-terminal AF-1 and C-terminal AF-2 transactivation functions. However, unlike ICI182,780, TAS-108 promoted the recruitment of the SMRT co-repressor that abolished ER transactivation function without inhibition of the ability of ERα to bind to its target DNA. Both TAS-108 and ICI182,780 acted as antagonists for the transactivation functions of the D351Y mutant, derived from tamoxifen-resistant breast cancer cells, while estrogen and known selective estrogen receptor modulators (SERMs), 4-OH tamoxifen and raloxifene, stimulated D351Y-mediated transcription. Thus, our findings indicated that TAS-108 acts as a novel estrogen antagonist that recruits co-repressors to ERs without AF-1 activation or prevention of DNA binding. Therefore, TAS-108 may be effective against tamoxifen-resistant breast cancer via a different mechanism than that for ICI182,780.

Keywords: Breast cancer; Antiestrogen; ER; Co-repressor; DNA binding

Estrogen exerts a wide variety of biological actions in many tissues in both females and males. However, estrogen can also act as an inducer during breast cancer development and enormous efforts have been devoted to the blocking of estrogen-dependent tumor growth. This growth-stimulatory action of estrogen is believed to be mediated via the nuclear estrogen receptor (ER) [1]. ER is a member of the nuclear receptor (NR) gene superfamily and exists as two subtypes, ERα and ERβ, that both act as ligand-inducible transcriptional factors [2,3]. ERs exhibit two activation functions (AFs) that are situated in the N-terminal A/B domain and the C-terminal E domain, also known as the ligand-binding domain (LBD). The AF-1 transactivation function in the A/B domain is ligand-independent, while AF-2 function, mapped to the LBD region, is induced in a ligand-dependent manner [4,5]. Like other NRs, ligand binding to ER induces structural alterations, including shifting of the C-terminal-most α-helix (H12) located in the LBD. Estrogen-induced ER transactivation functions are characterized by the presence of a number of co-regulators that associate with ERs through direct ligand-dependent interaction with H12 [6,7]. At least three complexes have been identified that co-activate agonist-bound ER [8,9]. ER ligands with antagonistic activity have been reported to prevent both direct and agonist-induced co-activator interactions, but also appear to induce non-physiological interactions with NR co-repressors, such as N-CoR and SMRT, presumably through co-repressor complexes that contain histone deacetylase (HDAC) and/or improper shifting of H12 [10–13].

Of the current therapeutic treatments for breast cancer, inhibition of ER function using synthetic ER

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ligands has been shown to be one of the most effective in terms of growth suppression [14]. The representative ER synthetic ligand tamoxifen has been widely used in clinical treatment and has proved successful as an ER antagonist. While its use has led to significant tumor-remission in female reproductive organs and mammary tissue [1], synthetic ER ligands, including tamoxifen and raloxifene, can act clinically as partial estrogen agonists in tissues other than female-specific organs. Based on these findings, these ER ligands are now considered to be selective estrogen receptor modulators (SERMs) [13]. While the tissue-specific agonistic activities of SERMs are of benefit to the skeletal and cardiovascular systems, it is suspected that SERMs can lead to an increased risk of endometrial carcinoma [15] and support the development of hormone-resistant breast cancers. As the agonistic activities of SERMs are thought to be due to their induction of ER AF-1 activity [16], the partial agonistic property of SERMs limits their therapeutic use in breast cancer for fear of growth stimulation via ER AF-1 activation [17,18]. Of the 7α-substituted derivatives of estradiol (E2), ICI182,780 has been developed as a pure-antiestrogen completely devoid of estrogenic activity and is able to block both ER AF-1 and AF-2 functions by inhibiting the DNA-binding activity of ER [19]. Recently, ICI182,780 (Fulvestrant; Faslodex) was reported to be clinically effective in patients for whom tamoxifen therapy had failed [20,21]. However, it appears that the beneficial agonistic effects of synthetic ER ligands were also abolished by the inhibition of ER DNA binding.

In an effort to develop new and more effective synthetic ER ligands for use against tamoxifen-resistant tumors that nevertheless retain some beneficial activities, we recently generated a novel steroidal compound with a high oral bioavailability designated TAS-108 (Fig. 1). While TAS-108 was as potent as 17β-estradiol in terms of ER binding (RBA = 64.3 ± 23.7%), TAS-108 largely failed to stimulate uterine tissue growth in ovariectomized rats. In the present study, we found that TAS-108 fully suppressed both the AF-1 and AF-2 functions of DNA-bound ERα by attenuating the recruitment of p160 family co-activators TIF2 and p300, but inducing SMRT co-repressor interaction. In contrast, neither SMRT recruitment nor DNA binding of ERα was induced by treatment with the pure antagonist ICI182,780. Thus, TAS-108 appeared to represent a novel class of pure estrogen antagonists that blocked both AF-1 and AF-2, abolished the recruitment of co-activators, but promoted the recruitment of co-repressors and allowed normal DNA binding.

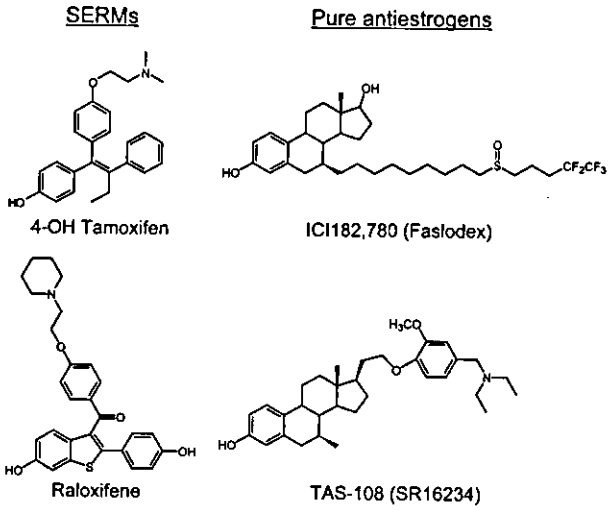

Fig. 1. Chemical structures of the antiestrogens.

Materials and methods

Chemicals. 17β-Estradiol (E2) and 4-hydroxy tamoxifen (OHT) were purchased from Sigma–Aldrich (St. Louis, MO). TAS-108 (TAS), Raloxifene (RAL), and ICI182,780 (ICI) were synthesized by Taiho Pharmaceutical. The chemical structures of TAS-108 and the other antiestrogens used in this study are presented in Fig. 1.

Plasmids. A cDNA fragment encoding the LBD region of ERα was cloned into the pM vector (Clontech, Palo Alto, CA) to generate GAL-ERα DEF (GAL-DEF). cDNA fragments encompassing the C-terminal region of TIF2 (including the NR interaction domains AD1, AD2, and AD3) and SMRT (including the NR interaction domains ID1 and ID2) and the N-terminal region of p300 (including the NR interaction domain) were cloned into the pVP16 vector (Clontech) to generate VP-TIF2, VP-SMRT, and VP-p300, respectively. ERα expression vectors (HEG0, HEG19, and ERα (D351Y)) and reporter constructs (17m8-luc and EREx3-Luc) have been described previously [22–25].

Transfection, luciferase assay, mammalian two-hybrid assay, and mammalian one-hybrid assay. For transfections, 293T or COS-1 cells were seeded in 12-well plates in phenol red-free DMEM (Invitrogen) supplemented with 10% charcoal dextran-treated FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin, and transfected at 50–60% confluence. For mammalian two-hybrid assays, 1 μg of 17m8-luc vector was cotransfected with 250 ng GAL-DEF in combination with 250 ng VP-SMRT or VP-TIF2 plasmids. For luciferase assays, 100 ng EREx3-Luc plasmid was cotransfected with 25 ng full-length ERα expression vector (HEG0) or A/B region-deleted ERα expression vector (HEG19). For one-hybrid assays, 250 ng EREx3-Luc plasmid was cotransfected with 25 ng full-length ERα expression vector (HEG0) or A/B region-deleted ERα expression vector (HEG19) in combination with 250 ng VP-p300 plasmid. As a reference plasmid to normalize for transfection efficiency, 5 ng pRL-CMV vector (Promega) was cotransfected in all experiments. Six hours after transfection, media were replaced with fresh medium containing 10% FBS. At this time, the ligands E2, TAS-108, OHT, RAL, ICI182,780, or ethanolic vehicle only were added and cells were incubated for additional 24 h. Preparation of cell extracts and dual luciferase assays were performed following the manufacturer's protocols (Promega). Individual transfections, each consisting of triplicate wells, were repeated at least three times [18,24–26].

Protease digestion assay. ^{35}S-Radiolabeled proteins were synthesized from 1 μg HEG0 by in vitro translation using the TNT-coupled transcription–translation system (Promega). From the 40 μl labeled in vitro translated protein reaction, 15 μl was preincubated for 30 min at 37 °C in 40 μl binding buffer (10 mM Tris–HCl, pH 8.0, 80 mM KCl, 0.1% NP40, 7% glycerol, and 1 mM dithiothreitol (DTT)) containing

either E2 (100 nM), OHT (1 μM), TAS-108 (1 μM), ICI (1 μM), or RAL (1 μM). Protease digestion was initiated by the addition of 2 μl of 5× stock solution of trypsin to 8 μl translation products and incubated for 10 min at 37 °C. Reactions were stopped by the addition of 10 μl of 2× loading buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 5 mg/ml bromophenol blue). Samples were electrophoresed on 12% acrylamide–SDS gels. Gels were dried under vacuum for 2 h and analyzed using an image analyzer (BAS1500, Fuji Film, Tokyo, Japan).

Electrophoretic mobility shift assay. Recombinant human ERα (Affinity BioReagents, Golden, CO) and double-stranded vitellogenin-A2 ERE DNA (5′-TCGAGCAAAGTCAGGTCACAGTGACCTGATC AAT-3′) were used. ERα and ligands (100 nM E2) or antiestrogens (1 μM) were incubated for 30 min on ice in binding buffer (5 mM Tris–HCl, pH 8.0, 100 mM KCl, 6 % glycerol, and 1 mM DTT), 1 mg poly(deoxyinosinic–deoxycytidylic) acid, and 0.3 mg BSA in a final volume of 20 μl. ERE probe labeled with ^{32}P was then added and the mixtures were incubated for 30 min at room temperature. Entire reaction mixtures were loaded onto 3.5% polyacrylamide gels in TBE buffer and electrophoresed. Gels were dried under vacuum for 2 h and analyzed using an image analyzer (BAS1500, Fuji Film) [18,24,25].

Results

TAS-108 is an estrogen antagonist

Antagonistic activity in ligand-induced ERα transactivation of TAS-108 and known ER synthetic ligands (Fig. 1) compared using a transient expression assay. A reporter plasmid containing three copies of vitellogenin A2-ERE (EREx3-Luc) and an ERα expression vector were cotransfected into 293T cells, and the cells were incubated in the presence of 17β-estradiol with and without synthetic ligands. Dose-dependent inhibition of E2-induced ERα transactivation by the synthetic ligands was observed (Fig. 2). OHT exhibited partial antagonist activity at concentrations above 10^{-7} M, presumably through inhibition of the AF-2 function of the OHT-bound ERα but activation of the tissue-specific AF-1 function [4,16,27–29]. In contrast, ICI and TAS-108 effectively antagonized E2-induced transactivation function. TAS-108 appeared to show stronger antagonistic effects at lower concentrations compared to ICI and RAL.

SERMs and TAS-108 inhibit co-activator recruitment and induce co-repressor recruitment to ERα without preventing ERα-DNA binding

Recent analyses of ligand-bound ER structures revealed that the shifting of H12 in the ERαLBD was dependent on the ligand used, and that estrogen antagonists induced improper shifting of H12, which led to antagonism through the abrogation of co-activator recruitment, along with pharmacologically induced recruitment of co-repressors [6,7,13]. To test if TAS-108 binding caused improper H12 shifting in a similar manner to known antagonists, structural alterations in ligand-bound ERα were examined by protease digestion assay (Fig. 3A). The addition of increasing concentrations of trypsin in the presence or absence of ligand (100 nM 17β-estradiol or 1 μM synthetic ligands) resulted in the appearance of several ^{35}S-labeled ERα fragments protected from digestion. Compared to the higher molecular weight bands, the low molecular weight digest bands were more abundant in ERα bound to TAS-108 or OHT compared to ERα bound to E2 and ICI182,780. This confirmed that the structural alterations induced by ligand binding were dependent on ligand type. To further verify this hypothesis, we performed a band shift assay using vitellogenin A2-ERE as a DNA probe for ligand-bound ERα. The binding of ERα to ERE DNA was independent of the bound ligand (compare lanes 2 and 3, Fig. 3B), and ICI182,780 appeared to inhibit the DNA binding of ERα, as previously reported [30–33]. While OHT-bound ERα exhibited a slower migration rate, TAS-108 was similar

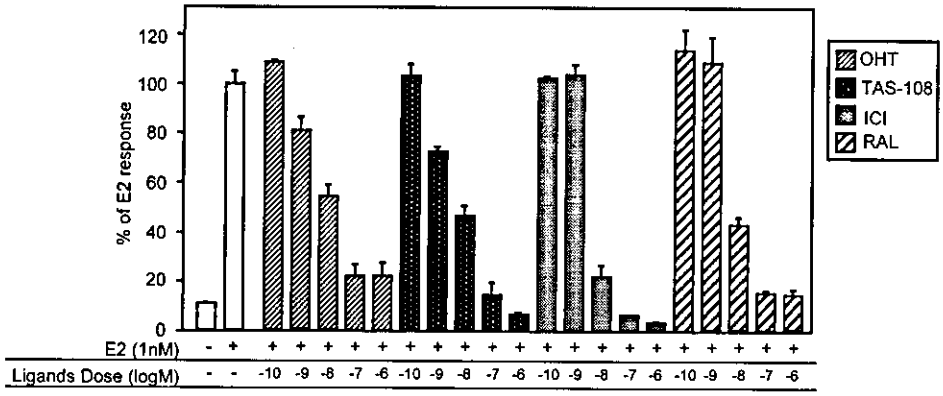

Fig. 2. Antagonist dose responses in ERα-mediated transactivation. Dose responses of the antagonists on ERα transcriptional activity in the presence of 1 nM E2 were studied using the EREx3-Luc reporter plasmid in 293T cells. Bars show the percentages of transcriptional activity with respect to E2 alone (set at 100%). The basal level of untreated ERα is also shown. Results represent the average of at least three independent experiments; error bars indicate ±SD.

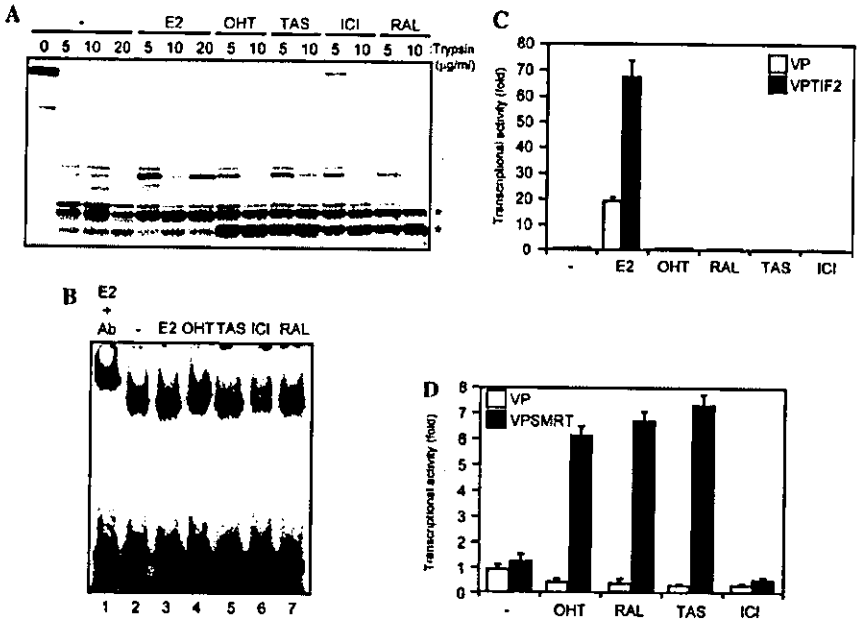

Fig. 3. Conformational changes of ERα in response to binding by different ligands. (A) Radiolabeled ERα was digested with different concentrations of trypsin (as indicated) in the presence or absence (–) of E2 (100 nM), OHT (1 μM), TAS-108 (1 μM), ICI182,780 (1 μM) or, RAL (1 μM). Digestion products were electrophoresed on 12.5% SDS–polyacrylamide gels. Asterisks indicate the distinctive bands. (B) DNA binding of ERα is not impaired by antiestrogens. Recombinant ERα was incubated with radiolabeled ERE in the absence (lane 2) or presence of 100 nM E2 (lane 3), OHT (lane 4), TAS-108 (lane 5), ICI182,780 (lane 6) or RAL (lane 7). Anti-ERα (B10 clone) supershifts the band-shift due to E2 (lane 1). (C) Binding between ERα and TIF2 was examined using the mammalian two-hybrid system. 293T cells were transfected with 1 μg luciferase reporter plasmid bearing GAL4-binding elements (17m8-luc) and 0.5 μg GAL4-fused ERα DEF (GAL-DEF) along with either 0.5 μg VP16-fused TIF2 (VP-TIF2) or empty VP16 vector (VP) in the presence or absence (–) of E2 (10 nM), OHT (100 nM), RAL (100 nM), TAS-108 (100 nM) or ICI182,780 (100 nM). Bars show fold-change in luciferase activity relative to VP vector in the absence of ligand. Results represent the average of at least three independent experiments; error bars indicate ±SD. (D) Binding between ERα and SMRT was examined using the mammalian two-hybrid system. 293T cells were transfected with 1 μg luciferase reporter plasmid bearing GAL4-binding elements (17m8-luc) and 0.5 μg GAL4-fused ERαDEF (GAL-DEF) along with either 0.5 μg VP16-fused SMRT (VP-SMRT) or empty VP16 vector (VP) in the presence or absence (–) of OHT (100 nM), RAL (100 nM), TAS-108 (100 nM) or ICI182,780 (100 nM). Bars show fold-change in luciferase activity relative to VP vector in the absence of ligand. Results represent the average of at least three independent experiments; error bars indicate ±SD.

to E2 in terms of its effect on ERα migration and DNA-binding activity (Fig. 3B).

Several reports have indicated that tamoxifen inhibits the recruitment of co-activators to ERα, while inducing interaction with co-repressors such as N-CoR and SMRT [10–12,34,35]. Therefore, we examined whether TAS-108 modulated the interaction between these co-regulators and ERα by mammalian two-hybrid assay using a GAL4DBD-fused ERαLBD mutant (Fig. 3C). An expression vector containing the GAL4 DNA-binding domain fused with the DEF ligand-binding domain (LBD) of an ERα mutant (GAL-DEF) was cotransfected with expression vectors for either the VP16 transactivation domain fused with the co-repressor SMRT (VP-SMRT) or the co-activator TIF2 (VP-TIF2) into 293T cells. While E2-dependent recruitment of TIF2 was detected, no TIF2 recruitment in the presence of synthetic ligands was observed (Fig. 3C). A strong interaction between the ERαDEF (AF-2) region and SMRT was induced by TAS-108, OHT, and RAL binding, but not by E2 (Fig. 3D). In contrast, no co-repressor interaction was observed using ICI182,780 (Fig. 3D). These results indicated that the inhibitory action of TAS-108 was similar to that of SERMs, but distinct from ICI182,780 in terms of co-repressor recruitment.

Both the AF-1 and AF-2 functions of ERα were blocked by TAS-108

It has been reported that OHT suppresses the function of AF-2 but not AF-1 in ERα, thereby acting as a partial agonist/antagonist, which is characteristic of SERMs. To investigate whether TAS-108 efficiently blocked ERα AF-1 as well as AF-2, TAS-108 activity was examined using ERα deletion mutants. While OHT acted as a weak antagonist for full-length ERα (HEG0), it appeared to act as a pure antagonist for ERα AF-2 function as apparent from results using a mutant that lacked the AF-1 A/B domain (HEG19) (Fig. 4A). In contrast, TAS-108 and ICI182,780 blocked transactivation of full-length ERα (HEG0), which indicated that TAS-108 effectively blocked the AF-1 activity of ERα, irrespective of normal DNA binding. Thus, it appeared that the mode of antagonistic activity of TAS-108 was distinct from that of

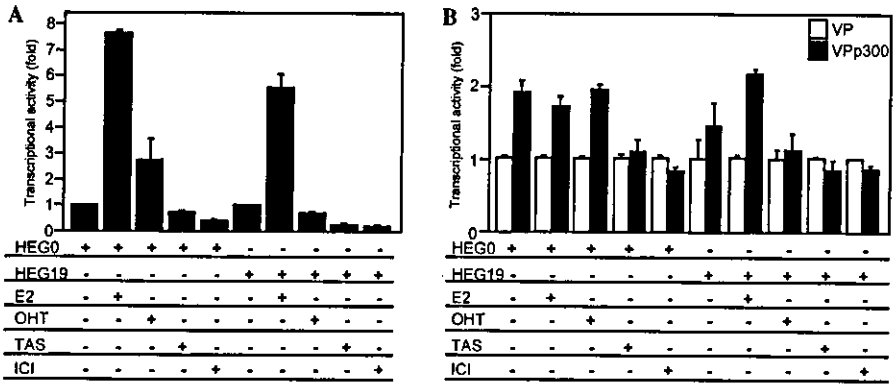

Fig. 4. Effects of antagonists on ERα-mediated transactivation. (A) 293T cells were cotransfected with 250 ng EREx3-Luc reporter and 25 ng ERα (HEG0) or ERαΔA/B (HEG19) expression plasmid and incubated for 24 h in the presence (+) or absence (–) of E2 (10 nM), OHT (100 nM), TAS-108 (100 nM) or ICI182,780 (100 nM) before being assayed for luciferase activity. Results represent means ± SD of three separate experiments and are expressed as the fold response over basal level of ER without E2 or antagonists, which was arbitrarily set at 1. (B) Binding between ERα and p300 was examined using the mammalian one-hybrid system. COS-1 cells were cotransfected with 250 ng EREx3-Luc reporter and 25 ng ERα (HEG0) or ERαΔA/B (HEG19) expression plasmid with either 0.5 μg VP16-fused SMRT (VP-SMRT) or empty VP16 vector (VP) in the presence (+) or absence (–) of E2 (10 nM), OHT (100 nM), TAS-108 (100 nM) or ICI182,780 (100 nM) before being assayed for luciferase activity. Bars show fold-change in luciferase activity relative to VP vector in the absence of ligand. Results represent the average of at least three independent experiments; error bars indicate ±SD.

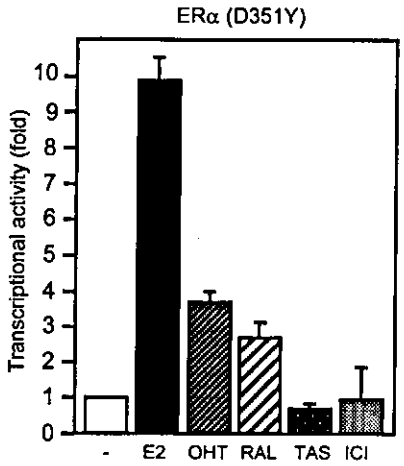

Fig. 5. Effects of antagonists on tamoxifen-resistant mutant ERα (D351Y)-mediated transactivation. 293T cells were cotransfected with 250 ng EREx3-Luc reporter and 25 ng ERα (D351Y) expression plasmid and incubated for 24 h in the presence or absence (–) of E2 (10 nM), OHT (100 nM), RAL (100 nM) TAS-108 (100 nM) or ICI182,780 (100 nM) before being assayed for luciferase activity. Results represent means ± SD of three separate experiments and are expressed as the fold response over the basal level of ER without E2 or antagonists.

ICI182,780. Consistent with the antagonistic activity of TAS-108, the recruitment of the p300 co-activator to ERα was not induced by TAS-108 (Fig. 4B) [18].

TAS-108 and ICI182,780 act as pure antagonists for the ERα (D351Y) mutant derived from a tamoxifen-resistant breast cancer cell line

The D351Y mutation in ERα-LBD was thought to underlie the tamoxifen-resistance acquired during hormone therapy of the original breast cancer [36]. We have previously reported that the ERα D351Y mutation results in the loss of interaction between ERα and its co-repressors induced by tamoxifen binding [37]. Thus, it is possible that the antagonistic actions of synthetic ligands can be abrogated by the loss of co-repressor recruitment. SERMs such as OHT and RAL activate transcription in the D351Y mutant, but to a lesser extent than E2 (Fig. 5). Like ICI182,780, TAS-108 induced no transactivation in the D351Y mutant, which suggested that TAS-108 might act as a pure antagonist for mutant ERα in tamoxifen-resistant breast cancers, and may therefore be useful as a treatment for ERα-ligand-dependent breast cancer.

Discussion

ERα and ERβ each harbor two transactivation domains, the N-terminal AF-1 and the C-terminal AF-2 [5]. The activity of both AFs is dependent on cell type and the state of the particular cell or tissue [38]. As tamoxifen, which exerts a mixed agonist-antagonist effect on ER function, blocks AF-2 but not AF-1 activity in ERα, it is thought that tamoxifen exerts its agonistic effects in tissues with high AF-1 activity, and its antagonist effects in high-AF-2 tissues [16,39]. Thus, it is possible that tamoxifen can serve as an agonist in breast cancer where ERα AF-1 function is up-regulated by growth factor stimulation or by some other unknown reason [17,40]. Indeed, resistance to tamoxifen does occur, probably due to its intrinsic agonist properties, and can lead to severe tumor progression in affected patients [41]. The agonistic activity of tamoxifen has

also been reported to increase the risk of endometrial cancer, although tamoxifen still has a relatively good clinical record in inducing the remission of ER-positive metastatic breast cancer. Thus, the ideal estrogen antagonist for use in breast cancer therapy would block both ERα AF activities. As TAS-108 potently inhibited both AF-1 and AF-2, like ICI182,780, it can be considered as a pure estrogen antagonist.

While ERα and β expression in mammary glands has been shown [42], it is thought that mammary tumor progression is promoted mainly via ERα [43]. Thus, it was noteworthy that TAS-108 potently blocked E2-mediated ERα responses. Preliminary data showed that like ERα, ERβ activity was also strongly blocked by TAS-108 (Y.Y. et al., unpublished results). This is in agreement with the finding of strong competition of TAS-108 against the binding of E2 to both ERs. Competitive inhibition of estrogen binding is thought to underlie the antiestrogen-mediated functional impairment of ER.

Crystallographic studies have revealed that the binding of synthetic ER ligands causes ligand-type-dependent structural alterations, such as abnormal shifting of ERα H12, which are not observed upon E2 binding [6,7]. The improper shifting of ERα H12 appears to prevent the normal recruitment of co-activators and induce non-physiological interactions with co-repressors, leading to modified ER function [7,44]. To investigate the structural alterations of ERα induced by TAS-108 binding, the ligand-dependent interaction between ERα and a number of known co-regulators was examined in the presence of TAS-108 and other ligands. As expected from the antagonistic activity of TAS-108 demonstrated in animal studies and cell growth analyses, TAS-108 induced co-repressor interaction via SMRT, similar to tamoxifen and raloxifene, while TIF2 co-activator recruitment was not observed. Thus, the co-regulator interaction profile was consistent with the observed antagonistic activity of TAS-108. Differences in H12 position between TAS-108-bound ERα and E2-bound ERα were also inferred from a protease digestion assay of liganded ERs (Fig. 3A). Although ICI182,780 showed similar antagonistic activity as TAS-108, TAS-108 did not affect ERα DNA binding, in contrast to ICI182,780. These observations suggested that TAS-108 may represent a novel class of pure ERα antagonists that induce a unique structural alteration, thereby preventing co-regulator recruitment without affecting DNA-binding ability.

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