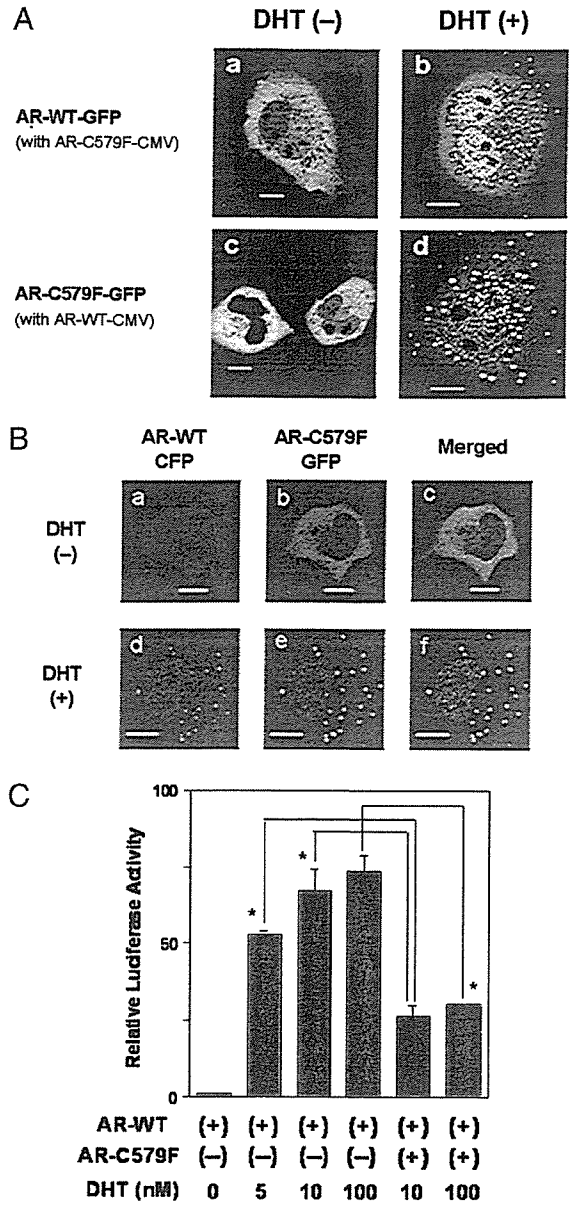

FIG. 6. Coexpression of wild-type and mutant ARs. A, a and b, Equal amounts of AR-WT-GFP and AR-C579F-CMV (without GFP) were cotransfected into COS-7 cells and images were collected before (a) and after (b) the addition of DHT. c and d, COS-7 cells expressing both AR-C579F-GFP and AR-CMV (without GFP) were observed before (c) and after (d) treatment with DHT. If AR WT and mutant proteins have equal chances to form a dimer and formed heterodimer show WT and mutant pattern of signals at an equal rate, the ratio of the intact AR signal *vs.* the abnormal one is expected to be 7:4 (b) and 4:7 (d). Signal patterns of WT and mutant in b and d were consistent with the expected ratios. *Scale bar*, 10 μm. B, Colocalization of wild-type and mutant ARs. AR-WT-CFP and AR-C579F-GFP were coexpressed in COS-7 cells in the absence or presence of DHT. Fluorescent signals were collected using the confocal microscope in the absence (a–c) or presence (d–f) of 10 nM DHT. Signals for AR-WT-CFP (d, *red*) and AR-C579F-GFP (e, *green*) were colocalized (f, merged signals). *Scale bar*, 10 μm. C, AR-C579F inhibited the transactivation mediated by the wild-type AR. COS-7 cells were cotransfected with pGL3-PSA reporter, pRL-CMV, and pAR-CMV with or without pAR-C579F-CMV. After the treatment with or without various concentrations of DHT, the cells were subjected to the luciferase assay. The *bars* show the luciferase activity relative to that of the wild-type AR without DHT. The means ± SD of three independent experiments are shown. *, $P < 0.05$.

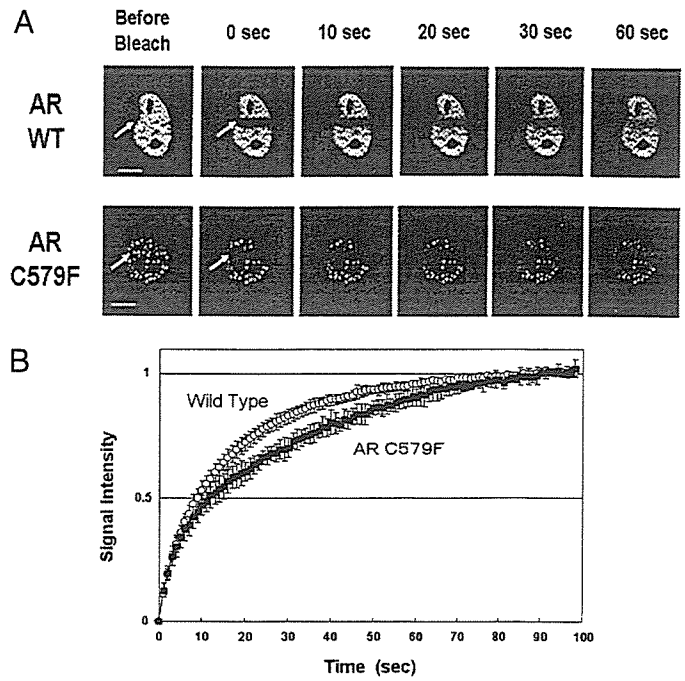

FIG. 7. Comparison of the intranuclear mobilities of the wild-type (WT) and mutant ARs by FRAP analysis. A, FRAP analysis of the intranuclear foci of the wild-type and mutant ARs. COS-7 cells were transfected with a wild-type or mutant AR expression vector. Twenty-four hours after the transfection, the cells were treated with DHT. Five hours after the addition of DHT, a region of interest in the nucleus was photobleached, and images were then taken at the indicated time points using a laser confocal microscope. *Scale bar*, 10 μm. B, Quantification of the fluorescence recovery in the FRAP analysis. The relative fluorescence intensities in the bleached areas of the wild-type and mutant AR foci were plotted. *Open circles*, intensity of the wild-type AR; *closed squares*, intensity of AR-C579F. The means ± SD of 10 cells are shown.

steroid hormone receptors with their transactivation function has been widely accepted (18–20).

There is a significant bias in the distribution of mutations of the AR gene in AIS patients, although the mutations are spread throughout the whole gene (9). Although exon 1 of the AR gene encodes more than half of the AR protein, including the transcription activation domain of the amino terminus, the number of mutations found in this exon is only about 10% of the total number of mutations, and most of the exon 1 mutations are nonsense or frameshift mutations. The mutation hot spots in AIS patients are part of the LBD constituting the pocket for androgen binding. In the DBD, 32 different mutations have been reported and 15 of these were detected in the first zinc finger motif. Most of the mutations in the DBD or LBD are single-base substitutions (3, 9). Some AIS patients do not carry any AR mutations. In such cases, there must be some abnormality in the signal transduction between the AR and the basic transcription machinery, and an impediment in a cofactor interacting with the N-terminal transcription activation domain of the AR has been suggested (14).

The amino acid substitution in the CAIS patient (subject 1) occurred at the cysteine residue contained in the first zinc finger motif of the AR. This AR-C579F mutant would not be able to coordinate the zinc ion, resulting in complete loss of the ligand-dependent DNA-binding ability. In the PAIS patient,

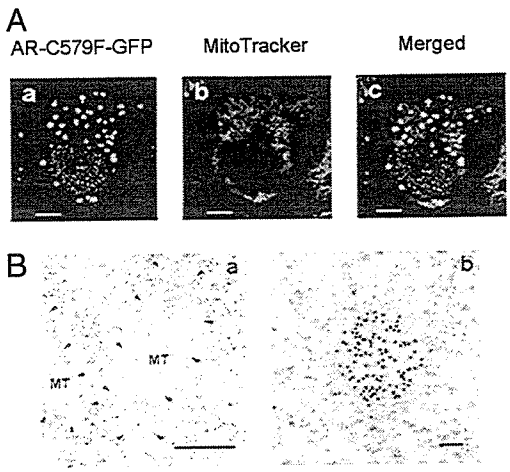

FIG. 8. Mitochondrial localization of cytoplasmic AR-C579F. A, COS-7 cells were transfected with 0.5 μg of AR-C579F-GFP. Twenty-four hours after the transfection, the cells were treated with 10^{-8} M DHT. After a 1-h incubation with DHT, 200 nM MitoTracker-Orange was added to the medium, and the cells were incubated for 30 min. After washing with PBS, the cells were observed under a laser confocal microscope. *Green signals*, AR-C579F-GFP; *red signals*, mitochondria. *Scale bar*, 10 μm. B, Immunoelectron microscopy of the AR-C579F mutant. After treatment with DHT, COS-7 cells expressing AR-C579F-GFP were fixed, embedded, and incubated with an anti-GFP antibody at 4 C overnight, followed by incubation with colloidal gold-conjugated goat antirabbit IgG. Sections were stained and observed with an electron microscope as described in *Subjects and Methods*. a, Low resolution (*bar*, 1 μm); b, high resolution (*bar*, 0.1 μm). MT, Mitochondria. The *arrowheads* show the edges of the mitochondria.

the amino acid substitution (F582Y) occurred next to the P-box and may cause a conformational change of the zinc finger motif. The impaired DNA-binding of these mutant ARs was confirmed by gel mobility shift assays, as we previously reported (11). Before the present imaging analysis, it was anticipated that these mutants would be able to enter the nucleus after ligand treatment but be unable to form foci like the wild-type AR due to the loss of the DNA-binding capacity. However, these mutant ARs initially formed cytoplasmic dots instead of the nuclear foci and nuclear dots subsequently appeared. Because there were no differences among the localizations of the wild-type and mutant ARs in the absence of the ligand, the conformations of the ligand-bound mutant ARs are considered to differ from that of the wild-type AR.

In the present study, ligand-induced formation of cytoplasmic dots of the mutant ARs was observed close to the mitochondria. However, the mutant AR signals were not detected inside the mitochondria by immunoelectron microscopy. Similar findings of an association between cytoplasmic protein aggregates and mitochondria have been observed for other mutant proteins, including the polyQ AR mutants (21–24). The mechanism for such aggregation close to mitochondria has been speculated to be that the ubiquitin-proteasome system tries to degrade a large amount of aggregated proteins and therefore requires an increased amount of ATP around the protein inclusions. The mechanism of localization close to mitochondria for the polyQ AR mutant might also occur for our AR-DBD mutants, although pathophysiology is quite different between our AIS and polyQ diseases. However, further study is necessary for elucidation of abnormal dot formation of our AR mutants.

The C579F mutation in the AR-DBD showed lower mobility than the wild-type AR. FRAP analysis has recently been used to examine the intranuclear dynamics of nuclear receptors (25–27). In the presence of ligands, the mobility of the nuclear receptors was reduced. It has been reported that ligand-induced intranuclear foci formation of steroid hormone receptors is associated with the nuclear matrix in which coactivators are also recruited (28). This nuclear matrix binding induced by ligand treatment is suggested to cause the decreased mobility of the receptors. A glucocorticoid receptor (GR) mutant carrying a deletion of the N-terminal region showed a much lower mobility (26). This mutant was deprived of all the putative phosphorylation sites of the GR. In ATP-deprived cells, GRs are dephosphorylated and tightly bound to the nuclear matrix (29, 30). Therefore, it has been speculated that appropriate reduction of the mobility of steroid hormone receptors, namely nuclear matrix binding with coactivators (6, 31–33), is an essential process for the normal transactivation functions of steroid hormone receptors. Lower or much increased mobility of ligand-bound steroid hormone receptors, as shown for the present AR C579F mutant, may indicate an impaired transactivation process.

The present reanalysis of our mutant ARs unexpectedly revealed two kinds of functional defects, *i.e.* lack of DNA-binding ability reported previously (11) and impairment of translocation from the cytoplasm to the nucleus. The finding that mutations in DNA-binding domain of AR impair nuclear translocation is novel and suggests the existence of an important intramolecular domain for nuclear translocation except for the hinge region. This finding is expected to contribute to the study of translocation mechanism. A mutant AR, K632A/K633A, which has mutations in the hinge region, has an intact DNA-binding domain, but its nuclear translocation is impaired (34). The pattern of the translocation impairment of this AR-K632A/K633A mutant was quite similar to that of our AR mutants, namely the AR-K632A/K633A mutant formed cytoplasmic aggregates (large dots) and its transactivation function examined by a reporter luciferase assay was markedly low (34, 35).

These reported results clearly indicate that impairment of nuclear translocation such as cytoplasmic dot formation can be responsible for the suppression of transactivation function of AR. As is well known, for transactivation function of AR, AR first must be translocated into the nucleus, secondly form a complex with coactivators, and then bind to target genes. Therefore, it would be reasonable that impairment of nuclear translocation in AR-C579F and AR-F582Y is largely responsible for AIS. Abnormal dot formation and decreased mobility of liganded AR-DBD mutants in the nucleus might be due to lack of DNA-binding ability, and thus, a defect in DNA-binding also would be responsible for AIS to some extent. Recent studies have revealed dynamic movement of nuclear receptors during a transactivation process within the nucleus (25, 36, 37). Liganded steroid hormone receptors including AR are transferred to subnuclear compartments (foci) and form a complex with coactivators. These receptor-coactivator complexes are mobilized to the target genes. The

steroid hormone receptors and coactivators show multiphasic on and off for binding to promoter elements of genes. The receptor-coactivator complexes also undergo a rapid exchange between target genes and the compartments. The present AR-DBD mutants are not able to access target genes. This may disturb the dynamic movement (mobility), resulting in prolonged stay at nuclear matrix and abnormal dot formation. This speculation may be supported by the reported observation that the AR-K632A/K633A mutant did not show abnormal intranuclear dot formation, although the authors did not touch on it.

In conclusion, the AR-DBD mutations, C579F and F582Y, found in our AIS patients showed abnormalities in ligand-dependent nuclear translocation, nuclear matrix targeting, and intranuclear mobility of the receptor, which may cause AIS in these patients.

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Tob proteins suppress steroid hormone receptor-mediated transcriptional activation

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Abstract

Although sex steroid hormones have significant effects on bone metabolism, the molecular mechanisms of these actions have not been fully elucidated yet. We examined the functional relationship between steroid hormone receptors and Tob, a member of an anti-prolifelative protein family and a negative regulator of osteoblast proliferation and differentiation. Luciferase assay using promoters carrying hormone-responsive elements revealed that both Tob1 and Tob2 proteins but not PC3 suppressed steroid hormone receptor-dependent transcriptional activation in MC3T3-E1 osteoblastic cells. Mutated Tob proteins carrying amino acid substitutions at an LXXLL motif also showed the same degree of inhibition of the transcriptional activation as the wild type. By observation of androgen receptor (AR)-tagged with green fluorescent protein under a confocal laser scanning microscope, we found that Tob1 inhibits the nuclear foci formation of dihydrotestosterone-bound AR. These results indicate that Tob family proteins may negatively regulate sex steroid hormone action in bone formation.

Keywords: Steroid hormone receptor; Osteoblast; GFP; LXXLL motif; Androgen

1. Introduction

Sex steroid hormones play important roles not only in the reproductive system but also in bone metabolism. It is well known that estrogen deficiency after menopause is the most important factor in osteoporosis development among women. Estrogen replacement is one of the major therapies for female postmenoposal osteoporosis. On the other hand, loss of androgen function also causes various abnormalities in bone metabolism (Hofbauer and Khosla, 1999; Manolagas et al., 2002). As well as ovariectomy, orchidectomized mice exhibited marked bone loss due to excessive bone resorption (Onoe et al., 2000). More recently, androgen receptor-deficient mice were generated and showed decreased bone volume with increased bone resorption (Yeh et al., 2002; Kawano et al., 2003).

In the last few years, the mechanism of steroid hormone action in a cell has been extensively studied at molecular level. After steroid hormones enter their target cells, these hormones bind to their specific receptors. Ligand-bound receptors change their conformation and regulate transcription by binding to hormone responsive elements located in regulatory regions of target genes. The effects of nuclear receptors on transcription are modulated by coregulator proteins, which enhance (coactivators) or reduce (corepressors) transactivation of target genes. Many of these coactivators possess histone acetylase activity, whereas corepressor complexes often contain histone deacetylase activity. Reversible acetylation of core histones modulates chromatin structure and regulates transcription. These coactivators commonly possess an LXXLL motif in their amino acid sequences through which they can interact with steroid hormone receptors. Introducing mutations in this motif abolished the function of these coactivators (Leo and Chen, 2000; Aranda and Pascual, 2001; Cheng et al., 2002; Heinlein and Chang, 2002).

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Tob, which was originally discovered as a protein interacting with a membrane receptor c-erbB2, is a member of a functionally and structurally related protein family involved in negative control of the cell cycle (Matsuda et al., 1996; Ikematsu et al., 1999; Matsuda et al., 2001; Tirone, 2001). This family comprises six proteins; Tob1, Tob2, BTG1, BTG2/PC3/TIS21, ANA/BTG3 and PC3B. These proteins share a homology at N-terminal 120 amino acid residues. Anti-proliferating activity of Tob protein is regulated by phosphorylation of three serine residues (Ser152, 154 and 164) that are modified by Erk MAP Kinase. In quiescent cells, Tob1 blocks their proliferation by suppression of cyclin D1 expression. Growth signals activate Erk that phosphorylates Tob protein to lose its anti-proliferating activity, resulting in continuous expression of cyclin D1 and cell cycle progression (Maekawa et al., 2002; Suzuki et al., 2002; Yoshida et al., 2003).

It has been indicated that Tob member proteins would mainly act on transcription. These proteins have been shown to physically interact with Caf1, whose yeast homologue is involved in a transcription complex (Rouault et al., 1998; Ikematsu et al., 1999; Prevot et al., 2001). Furthermore, four of the Tob family proteins carry an LXXLL motif being common in coactivators for nuclear receptors. In fact, BTG1 and BTG2 modulate the estrogen receptor (ER)-mediated transcriptional activation through this LXXLL motif (Prevot et al., 2001).

Mice carrying a targeted deletion of the *Tob* gene have a greater bone mass resulting from increased numbers of osteoblasts. Bone morphogenetic protein-2 (BMP-2), a member of the transforming growth factor-beta (TGF-β) superfamily, has been shown to control osteoblast proliferation and differentiation. BMP signals are mainly mediated by Smad proteins. In these Tob-deficient mice, BMP-2-induced osteoblast proliferation and differentiation was enhanced. Tob was shown to associate with receptor-regulated Smads (Smad1, 5 and 8), which are the mediators of BMP signals, and inhibit the signal transduction. These results indicate that Tob protein negatively regulates bone formation in vivo by repressing BMP-2-induced, Smad-mediated transcriptional activation (Yoshida et al., 2000).

In the present study, we investigated whether Tob expression influences steroid hormone receptor-dependent transcriptional activation in osteoblasts. Tob proteins suppressed the ligand-dependent transcriptional activation of steroid hormone receptors such as androgen receptor (AR) and estrogen receptor (ER) in osteoblastic cells. These results indicate that Tob proteins could regulate the action of steroid hormone in bone formation.

2. Materials and methods

2.1. Cells

MC3T3-E1 osteoblastic cells were obtained from Riken Cell Bank (Tokyo, Japan). Cells were maintained in α-MEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., Canada). COS-7 monkey kidney cells were maintained in DMEM (Invitrogen) with 10% FBS and PC3 prostatic carcinoma cells were maintained in RPMI-160 medium (Sigma-Aldrich Co., St. Louis, MO).

2.2. Plasmid constructs

The firefly luciferase reporter plasmid, pGL3-MMTV (Tomura et al., 2001) and the expression vector for AR (pCMV-hAR) were prepared as previously described (Nakao et al., 1992; Adachi et al., 2000). The expression plasmid for human ERα (pSG5-ERα) and a reporter plasmid (pERE2-tk109-luc) were kindly provided by Dr. Shigeaki Kato (University of Tokyo, Japan). Plasmid vector expressing AR-GFP fusion protein, named pAR-GFP, was constructed as described previously (Tomura et al., 2001). A 644-bp fragment of 5′-flanking region of the prostate specific antigen (PSA) gene was amplified by KOD DNA polymerase (Toyobo, Osaka, Japan) using a set of primers: PSAP-N (5′-aggtaccgaattccacattgtttgctgc-3′) and PSAP-C (5′-tccgggtgcaggtggtaagcttgg-3′). The PCR-amplified fragment was digested with *Kpn*I and *Hind*III and cloned into pGL3-Basic vector (Promega Co., Madison, WI) and the resulting construct was named pGL3-PSA. Mouse CREB binding protein (CBP) expression vector (pcDNA/mCBP) was prepared as previously described (Miyagishi et al., 2000). TIF2 expression plasmid, pYFP-TIF2, was constructed as previously described (Saitoh et al., 2002).

The expression vectors for Tob proteins were prepared using the RT-PCR technique. As previously described (Ohnaka et al., 2001), cDNA was prepared from human osteoblasts using Superscript First-Strand Synthesis System (Invitrogen). Tob1, Tob2 and PC3 cDNAs were amplified by Pfx DNA polymerase (Invitrogen) using each set of primers: Tob1-5E (5′-caggaattcggggagttgaaaccta-3′) and Tob1-3B (5′-cagggatcccggttagccataacagg-3′) for Tob1, Tob2-5E (5′-caggaattccaaggctgtacacgtgc-3′) and Tob2-3B (5′-cagggatcccggttggccagcaccacg-3′) for Tob2, and PC3-5E (5′-caggaattccacccgagacctctca-3′) and PC3-3B (5′-cagggatcccggctggagactgccatc-3′) for PC3. The PCR-amplified fragments were digested with *Eco*RI and *Bam*HI and inserted into the cognate sites of pEYFP-C1 vector (BD Sciences Clontech, Palo Alto, CA). The resulting constructs were designated as pYFP-Tob1, pYFP-Tob2 and pYFP-PC3. To generate Tob1 LXXAA mutant, two leucine residues in the LXXLL motif of the Tob protein were replaced with alanines by using a Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The validity of structure of the constructs was confirmed by DNA sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems Japan Ltd., Tokyo, Japan).

2.3. Immunoblotting

MC3T3-E1 cells were seeded in 60-mm plates and incubated for 24 h with 5% CO_2 at 37 °C. Plasmid DNAs carrying Tob family cDNAs were transfected into the cells. Twenty-four hours after transfection, cells were washed with PBS twice and 400 μl of NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl and 1% NP-40) was added to each dish, followed by rocking for 30 min at 4 °C. Lysates were collected and protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Forty micrograms of each lysate in 1× sodium dodecyl sulfate (SDS)-PAGE sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris–HCl, pH 6.8, 0.01% bromophenol blue) was loaded on a 10% SDS-polyacrylamide gel with wide range Color Marker (Sigma-Aldrich) and electrophoresis was performed at 20 mA for 4 h. Proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Bioscience Corp., Piscataway, NJ) using a Hoefer miniVe unit (Amersham) at 250 V for 1 h at 25 °C. After the membrane was blocked in 1× Block-Ace (Dainippon Pharmaceutical Co., Osaka, Japan), anti-Tob1 mouse monoclonal antibody (IBL-Japan, Fujioka, Japan) or anti-GFP rabbit polyclonal antibody (sc-8334, Santa Cruz Biotechnology Inc., Santa Cruz, CL) was reacted with the membrane in 0.1× Block-Ace for 1 h at 25 °C. Following a brief wash with TBS–Tween 20 (10 mM Tris–HCl, pH 8.0, 0.9% NaCl and 0.05% Tween 20), horseradish peroxidase-linked anti-mouse IgG (Amersham) or anti-rabbit IgG (Amersham) was added in 0.1× Block-Ace as a second antibody and then the membrane was incubated for 45 min at 25 °C. After being washed with TBS–Tween 20, the membrane was reacted with Western blotting detection reagents (Amersham) for 1 min in a dark room. The membrane was then exposed to a sheet of autoradiography film for 1 min and the film was developed and analyzed.

2.4. Functional reporter assay

MC3T3-E1 cells (7×10^4 cells/well), COS-7 cells (1×10^5 cells/well) or PC3 cells (1×10^5 cells/well) were seeded in 12-well plates 20 h before transfection. A reporter plasmid, pGL3-MMTV or pGL3-PSA (0.5 μg/well), and 3 ng/well of pRL-CMV (Promega) as an internal control were cotransfected with 0.1 μg/well of pCMV-hAR and 0.1 or 0.3 μg of the Tob expression plasmid using 2.7 μl/well of Superfect Transfection Reagent (Qiagen K. K., Tokyo, Japan). For ER transcription assay, 0.1 μg/well of pSG5-ERα and 0.1 or 0.3 μg of the Tob expression vector were cotransfected with 0.5 μg of pERE2-tk109-luc as a reporter and 3 ng of pRL-CMV as an internal control. For CBP or TIF2 transfection, 0.1 or 0.3 μg of pcDNA/mCBP or 0.5 μg of pYFP-TIF2 was used for each well. In all transfection experiments, the total amount of transfected DNA was fixed by adding empty vector into the transfection mixture. At 3 h post-transfection, 0.5 ml of medium containing 10% charcoal-treated fetal bovine serum was added with or without steroid hormones. At 24 h post-transfection, cells were rinsed with PBS and lysed in the lysis buffer of a luciferase assay kit (Promega). The luciferase activity was assayed using a Dual-Luciferase Assay System (Promega Corp., Madison, WI) and Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Data were presented as mean ± S.D. One-way analysis of variance followed by Scheffe's test was used for multigroup comparisons. $P < 0.05$ was considered to be statistically significant.

2.5. Coimmunoprecipitation

COS-7 cells (7×10^5 cells/dish) were seeded in 60-mm plates 24 h before transfection. Cells were transfected with 2 μg of plasmids expressing Tob and/or AR and maintained with or without 10^{-8} M of DHT for 24 h with 5% CO_2 at 37 °C. The whole cell extracts were prepared by incubating the cells in 0.3 ml of the NP-40 lysis buffer for 30 min at 4 °C, followed by brief sonication and a centrifugation. Protein concentrations were measured using a BCA protein assay kit (Pierce) and each protein concentration was adjusted to 1 mg/ml. Each lysate (160 μg) was incubated with 3 μg of anti-Tob antibody (IBL-Japan) at 4 °C for 1 h in the NP-40 lysis buffer and further incubated with 25 μl of Protein G Magnetic Beads (New England Biolabs Inc., Beverly, MA) for 1 h at 4 °C. Beads were collected by a magnet and then proteins were eluted in 1× SDS-PAGE sample buffer and subjected to 10% SDS-PAGE. Immunoblotting analysis was performed essentially as previously described (Ohnaka et al., 2001).

2.6. Confocal laser scanning microscopy

MC3T3-E1 cells (2×10^5 cells/dish) were cultured in 35-mm glass-bottom dishes (Asahi Techno Glass Corp., Tokyo, Japan) and transfected with 0.5 μg of pAR-GFP and 1.5–2.5 μg of pYFP-Tob1 using 10 μl/well of Superfect Transfection Reagent. The cells were maintained in α-MEM supplemented with 10% charcoal-treated FBS for 16–20 h and then various steroid hormones were added into the medium.

The cells were observed with an LSM 510 META invert confocal laser scanning microscope (Carl Zeiss Co. Ltd., Jena, Germany) using a 100×, 1.4 numerical aperture oil immersion objective. Images were collected at a 12-bit depth resolution of intensities over 1024 × 1024 pixels. For excitation of GFP and YFP, 488 nm of argon laser was employed and these two emission signals were separated using the emission fingerprinting technique established by Carl Zeiss. Separation of individual emission signals was based on recording of a spectral signature of each emission signal and a digital unmixing procedure using the reference spectra.

3. Results

3.1. Suppression of steroid hormone receptor-mediated transcriptional activation by Tob protein

We investigated whether Tob family proteins modulate the function of steroid hormone receptors in osteoblasts because some of Tob family proteins have one or two copies of an LXXLL motif that is commonly found in interaction domains of coactivators for nuclear receptors (Fig. 1). To examine whether expression of the Tob family proteins influences the transcriptional activation mediated by steroid hormone receptors, cotransfection of AR and Tob expression plasmids was performed with the MMTV-luciferase reporter gene into MC3T3-E1 osteoblastic cells. Expression of Tob family proteins in the transfected cells was confirmed by immunoblotting using anti-Tob1 and anti-GFP antibodies (Fig. 2A). We first examined an effect of Tob1 expression on transactivation mediated by endogenous AR protein, however, ligand-induced increase of luciferase activity was not evident and no significant change of the transactivation was observed by expressing the Tob protein (Fig. 2B). In the wild type AR-transfected cells, remarkable transcriptional activation was observed in the presence of dihydrotestosterone (DHT). Tob1 and Tob2 proteins suppressed this DHT-induced transcriptional activation mediated by AR in MC3T3-E1 cells, whereas expression of PC3 had no effect on the transcriptional activation in spite of carrying two LXXLL motifs (Fig. 2B). As well as AR, transcriptional activation mediated by ERα was also repressed by Tob proteins (Fig. 2C). These Tob1-induced repressions for the steroid hormone receptor-mediated transactivation were also observed in case of different cells (PC3 and COS-7 cells) and a different promoter (PSA promoter) (Fig. 3).

3.2. An LXXLL motif of Tob1 protein is not critical for suppression of AR-mediated transactivation

To investigate whether the suppression of transcriptional activation was elicited through an LXXLL motif in the Tob1 protein, we constructed a Tob1 mutant carrying amino acid substitutions in this motif (LXXAA). As shown in Fig. 4, this Tob1 LXXAA mutant caused the same level of suppression of AR-mediated transactivation as the wild type. This result indicates that the LXXLL motif in the Tob1 protein is not crucial to the suppression of the AR-mediated transactivation. We also found an LXXII motif in the N-terminus of the Tob1 protein, which was known as an interaction domain of some nuclear receptor corepressors (Hu and Lazar, 1999). However, no significant effects were observed by introduction of mutations (LXXAA) at the LXXII motif (data not shown).

3.3. Suppression of the AR-mediated transactivation by Tob was not recovered by overexpression of CBP and TIF2

Several transcriptional coactivators were shown to be commonly involved in various transcriptional factor complexes, and the presence of a competition between these transcription complexes for limited amounts of the coactivators in a cell has been reported (Kamei et al., 1996). CBP/p300 is known to be such a common coactivator. If the Tob protein would extract CBP/p300 from the AR-mediated transactivation function complex, supplementation of the CBP protein might recover from the suppression by the Tob protein. To examine this possibility, CBP was coexpressed with Tob and AR in MC3T3-E1 cells. As shown in Fig. 5A, CBP promoted the AR-mediated transactivation dose-dependently. However, overexpression of the CBP protein had little effect on suppression of AR-mediated transcriptional activation by Tob. We also supplemented another common coactivator protein, TIF2, and no recovery from the suppression was observed (Fig. 5B).

3.4. Immunoprecipitation of Tob protein with AR

To test for a physical interaction between Tob1 and AR, immunoprecipitation was performed using anti-Tob1 antibody. In the cells expressing both AR and Tob1, AR protein

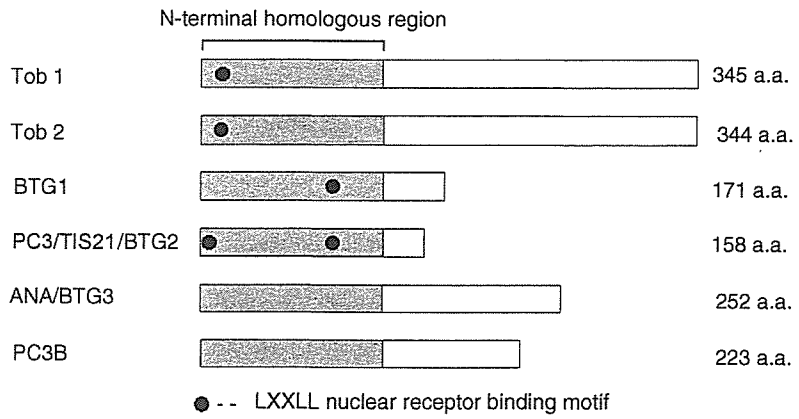
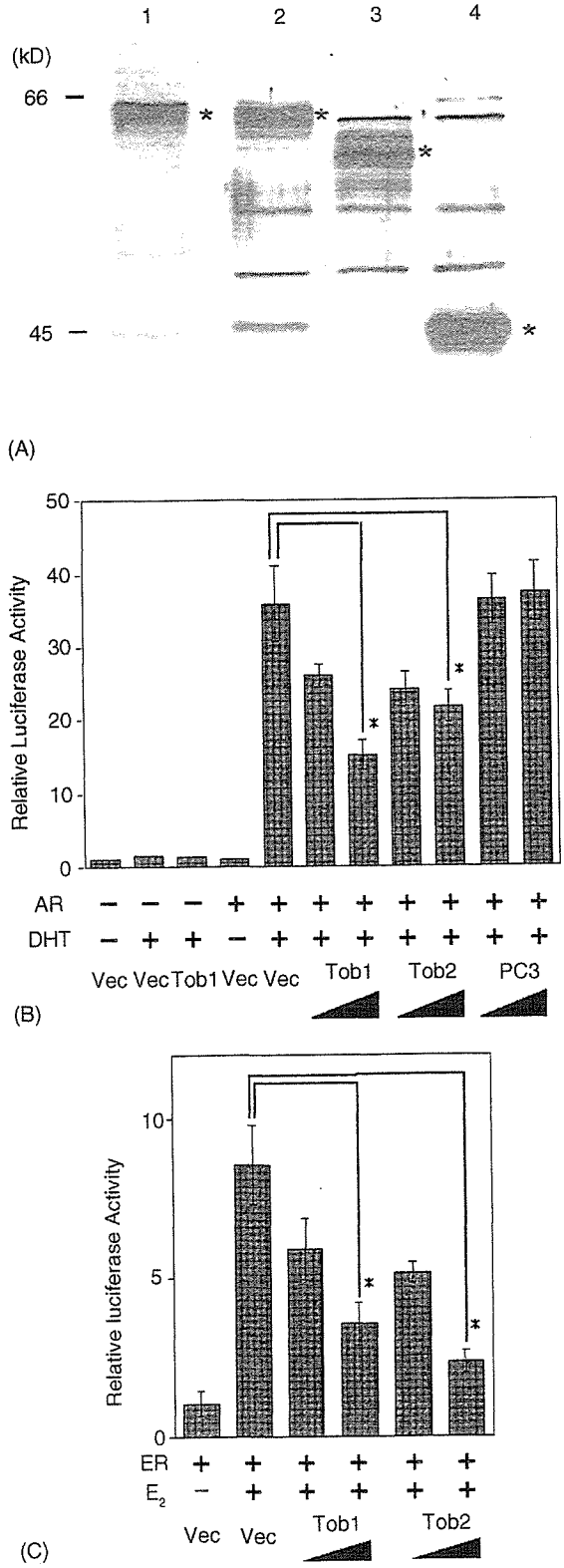


Fig. 1. Schematic representation of Tob family proteins. Six Tob family proteins share homology at the N-terminal region (gray). Filled circles represent LXXLL motifs that are noticed as interaction domains of nuclear receptor coregulators.

was clearly precipitated in the presence and absence of a ligand (Fig. 6, lanes 1–2). However, if Tob protein was not introduced into the cells, only a faint AR band was detected (Fig. 6, lane 3). The small amount of AR might be precipitated with endogenous Tob protein. These results indicate the direct interaction between Tob1 and AR and suggest that a protein-protein interaction between Tob and AR contributes to the repression of AR-mediated transcriptional activation.



3.5. *Effect of Tob1 expression on formation of subnuclear AR foci*

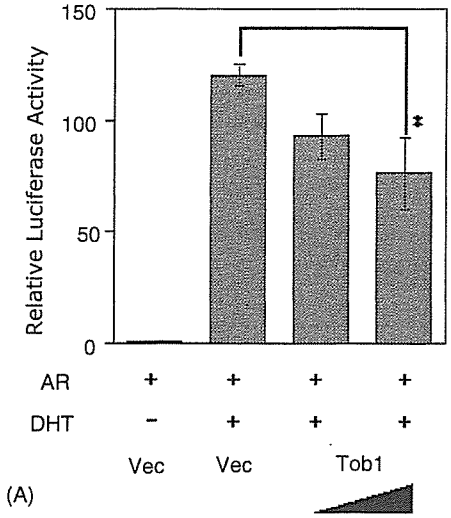
We previously reported that ligand-bound AR was translocated from cytoplasm to nucleus and formed subnuclear foci. This foci formation of AR correlated with AR-mediated transcriptional activation (Tomura et al., 2001; Saitoh et al., 2002). To examine the effect of Tob on the AR foci formation, we observed intracellular localization of the Tob protein. In MC3T3-E1 cells, Tob1 and Tob2 proteins were localized in both nucleus and cytoplasm. Signal strength of Tob1 in the nucleus is higher than that in the cytoplasm, whereas the cytoplasmic signal is dominant in terms of Tob2 (Fig. 7). When the YFP vector, carrying only a YFP cDNA, and pAR-GFP were transfected into MC3T3-E1 cells, AR signal was detected in the cytoplasm before adding DHT (Fig. 8A and C). After the treatment with DHT, AR formed fine foci in the nucleus (Fig. 8D and F). On the other hand, a markedly reduced number of nuclear foci (Fig. 8G and I) or even no foci (Fig. 8J and L) were observed in the DHT-treated MC3T3-E1 cells when both AR-GFP and YFP-Tob1 were cotransfected. No significant change was observed in the intracellular distribution of YFP-Tob1 before and after the addition of DHT. The inhibition of the AR foci formation by Tob1 was well consistent with the suppression of the AR-mediated transactivation function by Tob1 assessed by the luciferase reporter assay.

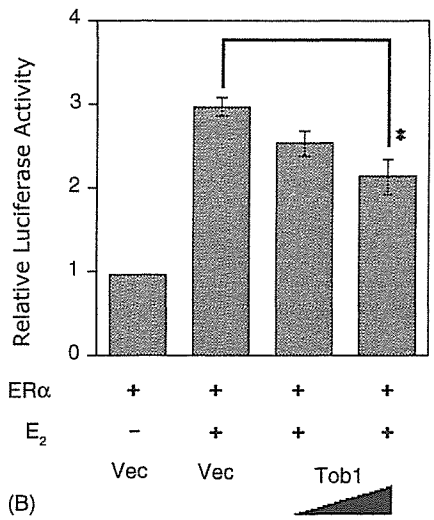
Fig. 2. Tob proteins suppress AR- and ERα-mediated transcriptional activation in osteoblastic cells. (A) Immunoblot analysis of human Tob family-YFP fusion proteins. Cell lysates were collected from MC3T3-E1 cells transfected with the expression vectors for Tob family proteins fused with YFP and were subjected to SDS-polyacrylamide gel electrophoresis (lanes 1 and 2, YFP-Tob1; lane 3, YFP-Tob2; lane 4, YFP-PC3). After proteins were transferred onto the nitrocellulose membrane, anti-Tob1 (lane 1) or anti-GFP (lanes 2–4) antibody was reacted with each membrane for detection of the fusion proteins. Asterisks (*) indicate the YFP fusion protein of each Tob family protein. Positions of protein size markers are shown on the left. (B) Suppression of AR-mediated transcriptional activation by Tob proteins. MC3T3-E1 osteoblastic cells were transfected with 0.1 μg of the AR expression vector, 0.1 or 0.3 μg of the Tob1, Tob2, PC3 expression vector, or the empty vector (Vec), 0.5 μg of pGL3-MMTV and 3 ng of pRL-CMV. The cells were cultured in the absence (−) or presence (+) of 10^{-8} M DHT for 24 h and the luciferase activities were measured. Left three bars show the results of cells without being transfected with the AR expression vector. Bars show the fold change in the luciferase activity relative to the value by the wild type AR without DHT. The average of three independent experiments is shown with the standard deviation. $^{*}P<0.05$. (C) Suppression of ERα-mediated transcriptional activation by the Tob protein. MC3T3-E1 cells were cotransfected with 0.1 μg of pSG5-ERα, 0.1 or 0.3 μg of the pYFP-Tob1, pYFP-Tob2 or the empty vector (Vec), 0.5 μg of pERE2-tk109-luc as a reporter plasmid and 3 ng of pRL-CMV as an internal control. Three hours after transfection, 10^{-6} M estradiol (E_2) was added. Luciferase activity was measured after 24 h incubation. Relative luciferase activity is shown. Bars show the fold change in the luciferase activity relative to the value without the ligand. The mean values and the standard deviation from three independent experiments are shown. $^{*}P<0.05$.

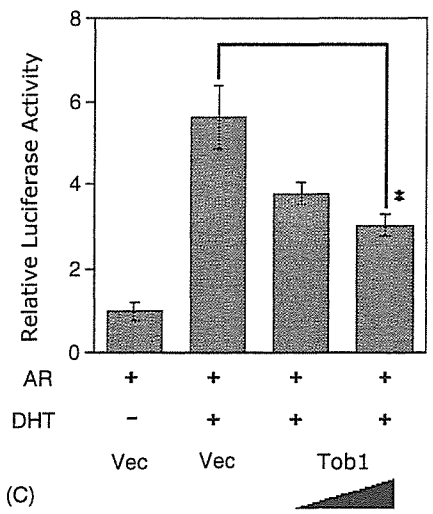
4. Discussion

In the present study, we demonstrated that expression of Tob protein in MC3T3-C1 osteoblastic cells suppressed the ligand-dependent transactivation function of steroid hormone receptors, AR and ERα, and inhibited the ligand-dependent nuclear foci formation of AR.

Some of the Tob family proteins have an LXXLL motif that has been identified in many nuclear receptor coregulators. Through this motif, these coregulators can directly interact with nuclear receptors to modulate the transcriptional activation (Leo and Chen, 2000; Aranda and Pascual, 2001; Cheng et al., 2002; Heinlein and Chang, 2002). It has been reported that two Tob family proteins, BTG1 and BTG2, can either enhance or repress ligand-induced transactivation function of ERα, depending on the promoter context. BTG proteins do not directly interact with ERα, but BTG1 can







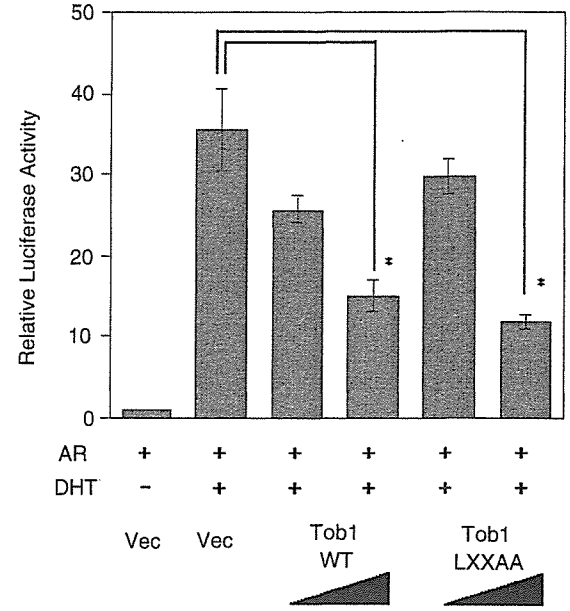
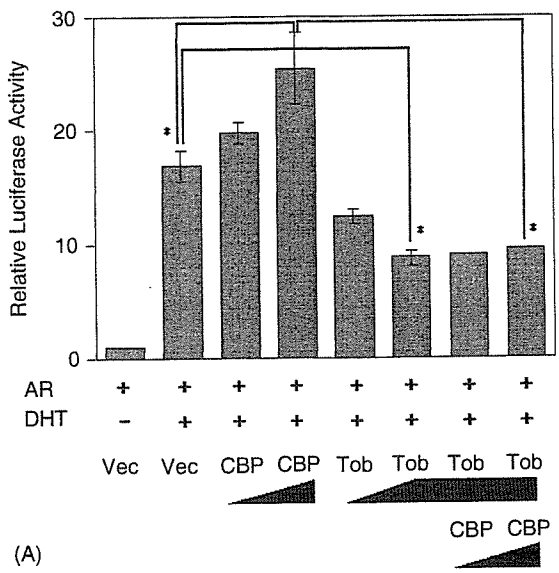


Fig. 4. Suppression of AR-mediated transcriptional activation by a Tob1 LXXAA mutant. The wild type (WT) and mutated (LXXAA) Tob1, carrying amino acid substitutions in its LXXLL motif (LXXLL to LXXAA), were expressed in MC3T3-E1 cells with the AR expression vector and reporter plasmids. The luciferase assay was performed as described in Section 2. The average of three independent experiments is shown with the standard deviation. $^{*}P<0.05$.

Fig. 3. Tob protein-induced suppression of AR- or ERα-mediated transactivation of MMTV (A), ERE2-tk109 (B) and PSA (C) promoter in PC3 (A and B) and COS-7 (C) cells. (A) Prostatic carcinoma cells, PC3, were cotransfected with AR expression vector (0.1 μg/well), 0.5 μg of pGL3-MMTV, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector (Vec). Three hours after transfection, the ligand was added. Luciferase activity was measured after 24 h incubation. (B) PC3 cells were cotransfected with 0.1 μg/well of pSG5-ERα, 0.5 μg/well pERE2-tk109-luc, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector (Vec). After the transfection, cells were treated with or without 10^{-6} M E_2 for 24 h and then luciferase assay was performed. (C) COS-7 cells were cotransfected with pCMV-hAR (0.1 μg/well), 0.3 μg of pGL3-PSA, 3 ng of pRL-CMV and 0.2 or 0.5 μg of the pYFP-Tob1 or the empty vector. After the transfection, cells were incubated in the absence or presence of 10^{-8} M DHT for 24 h and then luciferase assay was performed. Relative luciferase activity is shown and bars represent the fold change in the luciferase activity relative to the value without the ligand. The mean values and the standard deviation from three independent experiments are shown. $^{*}P<0.05$.



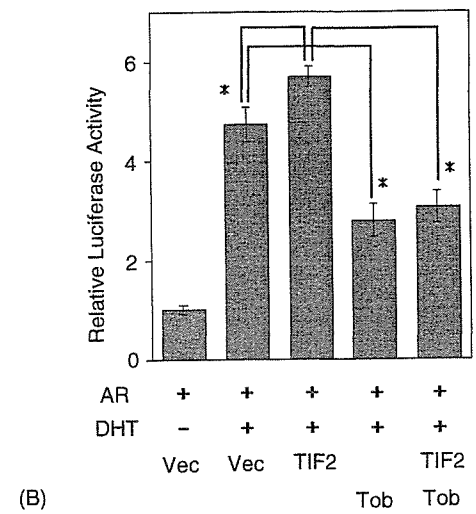


Fig. 5. Effects of coactivator expression on the Tob-mediated repression of the AR-mediated transactivation. (A) pcDNA/mCBP (0.1 or 0.3 μg/well) was cotransfected with pCMV-hAR (0.1 μg/well), pGL3-MMTV and pRL-CMV with or without the Tob expression vector (0.2 or 0.5 μg/well) into MC3T3-E1 cells. The cells were incubated in the absence or presence of DHT for 24 h, and then the luciferase activity was measured. (B) MC3T3-E1 cells were cotransfected with pCMV-hAR (0.1 μg/well), pGL3-MMTV, pRL-CMV, pYFP-Tob1 (0.5 μg/well) and pYFP-TIF2 (0.5 μg/well). After 24 h incubation with DHT, luciferase assay was performed. Bars show the fold change in the luciferase activity relative to the value by the wild type AR without DHT. $^{*}P < 0.05$.

regulate the ERα-mediated transcription through the interaction with CAF1, whose yeast homologue is a component of the CCR4-NOT transcription complex. The mutations of LXXLL motifs in BTG1 abolished both the effect of BTG1 on ERα-mediated transactivation and the interaction of BTG1 with CAF1 (Prevot et al., 2001). Tob1 and Tob2 proteins have also been shown to be associated with the CAF1 protein (Ikematsu et al., 1999), therefore, there is a possibility that the modulation of sex steroid hormone receptor-dependent transcription by Tob proteins would be also mediated by the

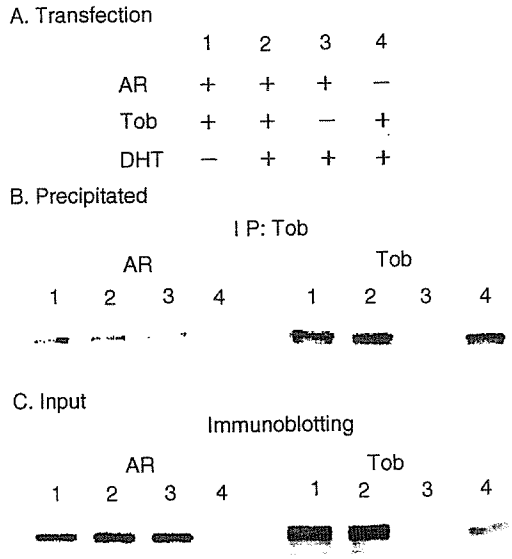


Fig. 6. Coimmunoprecipitation of AR with Tob1 protein. COS-7 cells were transfected with 2 μg of pCMV-hAR and pYFP-Tob1 and then incubated with or without 10^{-8} M DHT for 24 h. The whole cell extracts were subjected to an immunoprecipitation using anti-Tob antibody. Immunoprecipitated fractions were analyzed by immunoblotting as described in Section 2. (A) Transfected (+) and non-transfected (−) expression plasmids, and the presence (+) or absence (−) of ligand in each incubation (lanes 1, 2, 3 or 4) are indicated. (B) AR and Tob in the precipitated fractions were detected by immunoblotting. (C) Input levels of AR and Tob expressed by the transfection (before precipitation) were evaluated by immunoblotting.

CAF1 protein through an LXXLL motif. However, introduction of mutations into an LXXLL motif of the Tob protein failed to abolish the transcriptional repression. Furthermore PC3/BTG2, which carries two LXXLL motifs, failed to repress AR-mediated transcriptional activation in osteoblastic cells. In the amino acid sequence of the Tob1 protein, we also identified an LXXII motif, which was found in nuclear receptor binding domains of corepressors (Hu and Lazar, 1999; Privalsky, 2004). This LXXII motif was also mutated but repression by the mutant Tob was almost equal to that by the wild type protein. These results indicate that, in osteoblastic cells, the LXXLL and LXXII motifs in the Tob protein are not essential for suppression of nuclear receptor-mediated transactivation, nevertheless, the Tob-induced repression of the

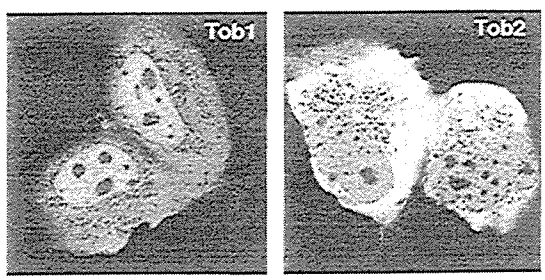


Fig. 7. Localization of wild type Tob1 and Tob2 proteins. YFP-Tob1 or YFP-Tob2 was expressed in MC3T3-C1 osteoblastic cells. Twenty-four hours after the transfection, fluorescent signals in the cells were observed by confocal laser scanning microscopy.

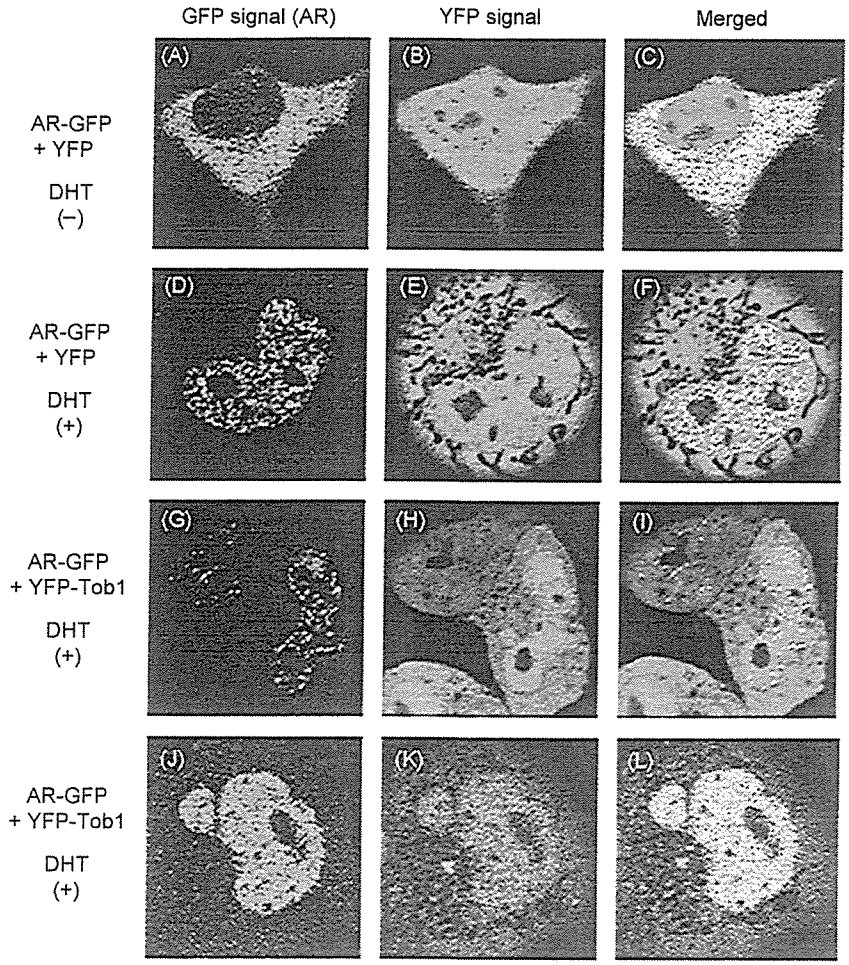


Fig. 8. Subnuclear foci formation of ligand-bound AR and inhibition of the foci formation by expression of the Tob1 protein. The YFP vector (YFP), carrying only a YFP cDNA, and pAR-GFP (AR-GFP) were cotransfected into MC3T3-E1 cells (A–F). Before adding DHT, fluorescent signals from AR-GFP (A) and YFP (B) were collected and the two signals were merged (C). Signals from AR-GFP (D) and YFP (E) in the DHT-treated cells were also collected and the two images were merged (F). MC3T3-E1 cells were cotransfected with pAR-GFP and pYFP-Tob1 (G–L). Fluorescent signals from AR-GFP (G and J) and YFP-Tob1 (H and K) were collected in the presence of DHT, and the two signals were merged (I–L). The experimental conditions were identical between G–I and J–K.

transactivation function of AR was suggested to be mediated by the direct interaction between Tob and AR as demonstrated in the present study by coimmunoprecipitation.

As we previously reported, ligand-dependent intranuclear foci formation of AR closely depends on the receptor being in a transcriptionally active conformation (Tomura et al., 2001; Saitoh et al., 2002). CBP, one of the coactivators for AR, is colocalized with AR at subnuclear foci after treatment with the ligand, and CBP is considered to be essential for the formation of nuclear foci of AR (Saitoh et al., 2002). It was shown that activation of AP-1 or NF-κB repressed AR-mediated transactivation. Extraction of endogenous CBP from the AR-mediated transactivation complex by these transcriptional factors is thought to be a cause of the repression because this repression is relieved by supplementation with exogenous CBP (Frønsdal et al., 1998; Aarnisalo et al., 1998). The dominant negative form of CBP also suppressed AR-mediated transactivation and destroyed AR subnuclear foci, however, cotransfection of a wild type CBP expression vector rescued AR foci formation (Saitoh et al., 2002). In the present paper, we demonstrated that expression of Tob protein inhibited the subnuclear foci formation of AR, being consistent with the results of the luciferase reporter assay. To examine whether CBP is involved in the Tob-induced repression of the transactivation function of AR, CBP was coexpressed with AR and Tob in osteoblastic cells. However, the CBP expression had no effect on the Tob-mediated repression (Fig. 5A). Similarly, the Tob-induced repression of transactivation was not recovered by supplementation of another common coactivator, TIF2 (Fig. 5B). CBP also failed to recover the AR foci formation destroyed by the expression of Tob (data not shown). According to these results, Tob-mediated repression of the AR transactivation function is not due to the sequestration of coactivators from AR transactivation complex. In the present experiments, Tob inhibited the subnuclear foci formation of AR. According to the previous studies including ours, these subnuclear foci are now considered to be the sites where nuclear receptors interact with

coactivators to form (pre)transactivation complexes before binding to DNA (Stenoien et al., 2000; Saitoh et al., 2002). Therefore, Tob seems to inhibit formation of the transcriptionally active complex of AR that should be an earlier step than AR-DNA binding. However, further study will be necessary to elucidate the precise mechanism of the Tob-induced repression of AR-mediated transactivation.

Tob1 knockout mice showed increased bone volume resulting from an increased number of osteoblasts, not due to reduced activity of osteoclasts (Yoshida et al., 2000). Sex steroid hormones are known to promote osteoblast proliferation and differentiation (Hofbauer and Khosla, 1999; Manolagas et al., 2002). Based on our findings that Tob protein represses AR and ERα-dependent transactivation in osteoblastic cells, Tob deficiency would result in upregulation of the transactivation functions of AR and ERα in bone formation. Such elevated function of steroid hormone receptors may also partially contribute to the enhancement of bone volume in Tob-deficient mice.

In conclusion, for the first time, to our knowledge, the present study revealed the repression effects of Tob proteins on sex steroid hormone receptor-mediated transactivation function, thus, Tob proteins would be one of the intracellular modulators for extracellular signals such as BMP-2 and sex steroids.

Acknowledgements

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A zinc finger protein TZF is a novel corepressor of androgen receptor

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Abstract

Steroid hormones control the transcriptional activity of target genes mediated by intracellular nuclear receptors, and these transcriptional activities are modulated by the combination with coactivators and corepressors. We found in this study that testicular zinc finger protein (TZF) that was a nuclear protein with a zinc finger motif of the Cys_2-His_2 type was a novel corepressor of androgen receptor (AR). Fusion protein with green fluorescence protein GFP formed the specific foci in nuclei and TZF-dependent foci were located close to the splicing factor compartment. In addition, TZF was recruited into AR subnuclear foci after the treatment of dihydrotestosterone. Furthermore, we revealed that TZF bound to the activation function-1 (AF-1) domain (N-terminal transactivating domain) of AR protein. Transient over-expression of TZF in COS-7 cells or LNCaP human prostatic cancer cell resulted in decreased AR activity in a ligand-dependent fashion. Moreover, a transcriptional corepressor N-CoR additively decreased the transcriptional activity of AR with TZF. These findings suggest that TZF might be a novel corepressor of AR.

Keywords: Zinc finger protein; Corepressor; Androgen; Androgen receptor; Transcriptional activity

The actions of steroid hormones are mediated by intracellular nuclear receptors whose coordinate activity defines the physiological response [1]. These receptors are all structurally related and constitute a superfamily of nuclear regulatory proteins that modulate gene expression in a ligand-dependent fashion. In the case of androgen receptor (AR), it has been proposed that ligand binds to the cytosolic AR, and then the receptor–ligand complex is relocated to the nucleus with subsequent sequence-specific interaction with hormone responsive elements of target genes under the control of various cofactors to regulate the transcription of target genes [2,3]. A number of transcriptional cofactors (coactivators and corepressors) have been identified and they include the p160 family [4–6], CBP/p300 [7], PCAF/GCN5 [8], and TRAPs/DRIPs [9] as the coactivator, and SMRT/Sin3A [10,11] and N-CoR [12,13] as the corepressor. These transcriptional cofactors are organized in multiprotein complexes and facilitate the access of nuclear receptors and the RNA polymerase II core machinery to their target DNA sequences by chromatin remodeling and histone modification. Transcriptional repression is an intrinsic part of endocrine physiology. However, the mechanism of repression is not fully understood. One reason is the lack of number of corepressors found.

AR, like other steroid hormone receptors, contains the following structural and functional domains: a central DNA-binding domain, a C-terminal ligand-binding domain, and two potential transcriptional activation domains (AF-1 and AF-2). AF-1 is located in the N-terminal region of the receptor and has been shown to act in a

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ligand-independent fashion. By contrast, a ligand-dependent AF-2 domain, which colocalizes with the highly conserved C-terminal ligand-binding domain, is predicted from the sequence similarity among nuclear receptors [14].

Testicular zinc finger protein (TZF) has a nuclear protein with a zinc finger motif [15,16]. Analysis of the open reading frame of cDNA indicates that TZF is a polypeptide of 942 amino acids residues that included three distinct domains, namely, a zinc finger domain of the Cys_2-His_2 type, four basic amino acid-rich domains (putative nuclear-localization sequences), and a myosin II-homology domain. RT-PCR analysis of expression level of mRNAs for mouse TZF shows that transcripts are highly expressed in testis and moderately in adrenal gland, prostate gland, muscle, kidney, and uterus. Fusion proteins with green fluorescence protein (GFP) also demonstrate the nuclear localization of TZF. However, the function of TZF in various organs has not been clear yet. In this paper, we describe that TZF might be a novel corepressor of AR.

Materials and methods

Plasmid constructs. Plasmid vector expressing AR-GFP fusion protein, named pAR-GFP, was constructed as described previously [17]. A plasmid construct carrying GFP-TZF fusion gene, pLP-EGFP-C1-TZF [16], was digested by *Sal*I and *Xho*I to remove the vector sequence and TZF cDNA was separated to two fragments. A 2.3-kb *Sal*I fragment contained an N-terminal part of TZF and a 1-kb *Sal*I–*Xho*I fragment contained a C-terminus of TZF. These two fragments were inserted together into pEYFP-C1 (Clontech) vector or pFLAG-cytomegalovirus (CMV) 2 (Sigma) to produce pYFP-TZF and pFLAG-TZF-CMV. A GFP fragment of pLP-EGFP-C1-TZF was replaced with c-myc tag, to produce pLP-CMV-myc-TZF. The full length of human N-CoR in pEF1-hN-CoR-V5his6 (gifted from Dr. Jun Yanagisawa, University of Tsukuba) was inserted into *Eco*RI and *Not*I sites of pBSSK (Stratagene). *Sal*I enzyme recognition site was introduced into the C-terminus of hN-CoR cDNA in pBSSK by PCR. By digesting with *Eco*RI and *Sal*I restriction enzymes, hN-CoR fragment was subcloned into pFLAG-CMV2 (Sigma), resulting in pFLAG-N-CoR-CMV.

The firefly luciferase reporter plasmid, pGL3-mouse mammary tumor virus (MMTV) [17], and the expression vector for AR (pCMV-hAR) were prepared as previously described [18,19]. A 644-bp of 5′-flanking region of the prostate specific antigen (PSA) gene was amplified by KOD DNA polymerase (Toyobo, Osaka, Japan) using a set of primers: PSAP-N (5′-aggtaccgaattccacattgtttgctgc-3′) and PSAP-C (5′-tccgggtgcaggtggtaagcttgg-3′). The PCR-amplified fragment was cloned into pGL3-Basic vector (Promega, Madison, WI), resulting in pGL3-PSA.

Cell culture. COS-7 cells were purchased from Riken Cell Bank (Tokyo, Japan) and LNCaP human prostatic cancer cell line was obtained from American Type Culture Collection (Manassas, VA). Both cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) with antibiotics and 10% fetal bovine serum (FBS, Cansera International, Canada). HEK293T cells were purchased from Riken Cell Bank (Tokyo, Japan) and were maintained in DMEM supplemented with antibiotics and 10% FBS (Biowest, France).

Immunostaining. COS-7 cells (2×10^4 cells/well) were cultured in the Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL) and transfected with pGFP-TZF using 1 μl/well of the Superfect Transfection Reagent (Qiagen GmbH, Hilden, Germany). Twenty hours after incubation, cells were washed with PBS and fixed with 50% methanol/50% acetone for 2 min at 25 °C. After the cells were blocked in 1× Block-Ace (Dainippon Pharmaceutical, Osaka, Japan), anti-SC-35 mouse monoclonal antibody (Sigma–Aldrich) was reacted with the cells in 0.1× Block-Ace for 1 h at 25 °C. Following a brief wash with TBS–Tween 20 (10 mM Tris–HCl, pH 8.0, 0.9% NaCl, and 0.05% Tween 20), horseradish peroxidase-linked anti-mouse IgG (Amersham Bioscience, Piscataway, NJ) was added in 0.1× Block-Ace as a second antibody and then the cells were incubated for 45 min at 25 °C. After being washed with TBS–Tween 20, cells were mounted in Vectorshield (Vector Laboratories, Burlingame, CA) and examined in the confocal laser scanning microscope (LSM510META, Carl Zeiss, Jena, Germany).

Laser scanning microscopy. COS-7 cells (2×10^5 cells/dish) were seeded in 35-mm glass-bottomed dishes (Asahi Techno Glass, Tokyo, Japan) and transfected with 0.5 μg/dish of a GFP-fusion construct using 5 μl of Superfect Reagent (Qiagen). In case of coexpression studies, cells were transfected with 0.5 μg/dish pAR-GFP and 2.5 μg pYFP-TZF. The cells were incubated for 20 h in DMEM supplemented with 10% of charcoal-treated FBS. Cells were observed before and after the treatment of 10^{-8} M of dihydrotestosterone (DHT) using a LSM510META invert confocal laser scanning microscope (Carl Zeiss) using a 100×, 1.4 numerical aperture oil immersion objective. Images were collected at a 12-bit depth resolution of intensities over 1024×1024 pixels. For excitation of GFP and YFP, 488-nm argon laser was used and each fluorescent signal was separated using the Emission Fingerprinting technique established by Carl Zeiss. A spectral signature of each emission signal was recorded as a reference spectrum and a digital unmixing was performed using the reference spectra.

Immunoprecipitation and immunoblotting. A plasmid expressing myc-tagged or GFP-fused TZF was transfected into HEK293T cells together with expression plasmid for the full-length (1–919 aa) or truncated mutants (AF-1; 1-566 aa, AF-2; 623-919 aa) of AR by using FuGENE6 reagent (Roche), and the cells were maintained with or without 10^{-8} M DHT. Whole cell extracts were prepared from lysed cells in NE buffer (20 mM Hepes–NaOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5% NP-40, and 1:100-diluted protease inhibitor cocktail). The extracts were incubated for 2 h at 4 °C with 20 μl of protein A–Sepharose (Amersham Biosciences) coupled with 10 μg of either N-20 rabbit polyclonal antibody (Santa Cruz Biotechnology) to detect the N-terminal transactivation domain fragment of AR or C-19 rabbit polyclonal antibody (Santa Cruz Biotechnology) to detect the full-length or C-terminal fragment and equilibrated with WB buffer (20 mM Hepes–NaOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5% NP-40, and 0.5% skim milk). After the matrices were washed four times with 180 μl WB buffer and four times with 180 μl WH buffer (20 mM Hepes–NaOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 0.5% NP-40), bound materials were eluted with 60 μl of 2× sodium dodecyl sulfate (SDS) sample buffer, resolved on 10% SDS–polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon-P PVDF membrane (Millipore). Myc-tagged or GFP-TZF was detected with each anti-c-myc mouse monoclonal antibody (9E10; Roche) or anti-GFP mouse monoclonal antibody (GF200; Nacalai Tesque). The full-length or N-terminal transactivation domain fragment of AR was detected with antibody N-20 and the C-terminal fragment was detected with antibody C-19.

Functional promoter assay. COS-7 or LNCaP cells (1×10^5 cells/well) were seeded in 12-well plates at 24 h before transfection. A reporter plasmid, pGL3-MMTV or pGL3-PSA (0.5 μg/well), was cotransfected with 3 ng/ml pRL-CMV (Promega) as an internal control, 0.1 μg/well pCMV-hAR, and 0.5 μg or 1.0 μg of the pFLAG-TZF-CMV2 using 1.7 μl/well of Superfect Transfection Reagent (Qiagen). Three hours after transfection, 0.5 ml of DMEM containing charcoal-treated fetal bovine serum was added with or without steroid

hormones. At 24 h post-transfection, cells were rinsed with PBS and lysed in the lysis buffer of a luciferase assay kit (Promega). The luciferase activity was assayed using a Dual-Luciferase Assay System (Promega) and Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Data were presented as means ± SD. One-way analysis of variance followed by Scheffe's test was used for multigroup comparisons. A value of $P < 0.05$ was considered to be statistically significant.

Results

TZF was located close to the splicing factor compartment

TZF was located in the nucleus and formed larger size but smaller number of dots [16]. Splicing factor complexes also form subnuclear dots whose pattern was similar to that of TZF. We analyzed whether TZF and SC-35, which was shown to be located in the splicing factor complex, were colocalized in the same nuclear dots. To examine an authentic localization pattern of SC-35, immunostaining was performed using anti-SC-35 antibody. SC-35 showed clear dot pattern in the nucleus and TZF was also located close to the SC-35 spots (Fig. 1). TZF was not directly involved the splicing factor complexes but might be a mediator between the splicing factor complex and another protein complex.

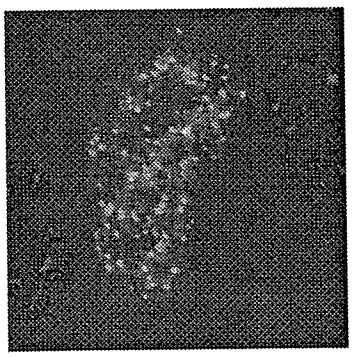

Fig. 1. Subnuclear localization of TZF and SC-35. COS-7 cells were transfected with pGFP-TZF. Twenty-four hours after transfection, cells were fixed with ethanol/acetone and treated with anti-SC-35 antibody for 1 h at 25 °C. After brief wash, second antibody was added for detection. Green signals show the localization of GFP-TZF and red signals show that of SC-35.

TZF was recruited into AR subnuclear foci after the ligand treatment

We previously showed that AR was located in the cytoplasm in the absence of the ligand. After the treatment with the ligand, AR translocated into the nucleus and formed subnuclear fine foci [17,20]. TZF was also shown to localize at the subnuclear foci [16]. To investigate the colocalization of the two proteins, pYFP-TZF and pAR-GFP were cotransfected and cells were observed using confocal laser scanning microscope. As previously reported, AR translocated from the cytoplasm to the nucleus after the treatment of DHT and subnuclear foci formation was observed (Figs. 2A and D). TZF was located in the nucleus and formed larger size but smaller number of dots without DHT compared to the AR foci (Fig. 2B). However, coexpression of AR changed the subnuclear foci pattern of TZF in the presence of the ligand (Fig. 2E). With AR and DHT, TZF formed smaller size but larger number of subnuclear dots that were similar to the AR subnuclear foci (Figs.

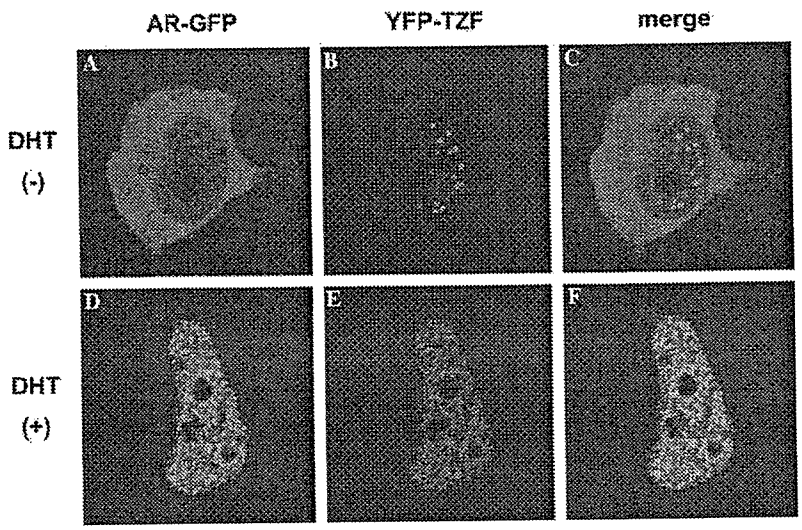

Fig. 2. Ligand-induced colocalization of AR and TZF. COS-7 cells were cotransfected with AR-GFP and YFP-TZF, and observed using the LSM510META laser scanning microscopy before and after the treatment of 10^{-8} M of DHT. Upper panels (A–C) show images before the treatment of DHT and lower panels (D–F) show images after 1 h treatment of DHT. Fluorescent signals for AR-GFP (A,D) and YFP-TZF (B,E) were obtained and two signals were merged (C,F). GFP (A, C, D, and F) and YFP (B, C, E, and F) signals are represented as green and red, respectively.

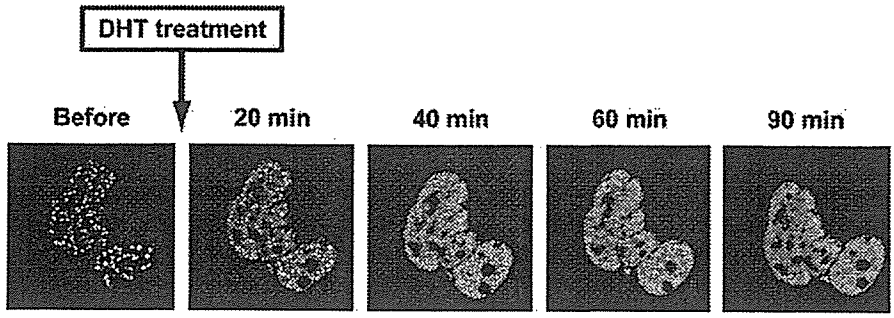

Fig. 3. Time lapse experiment for localization of TZF after the treatment of DHT. COS-7 cells transfected with pYFP-TZF and pCMV-hAR were treated with 10^{-8} M DHT. Images were collected before and 20, 40, 60, and 90 min after the DHT treatment.

2E and F). Time course experiment revealed that since 40 min after the treatment the size of TZF dots started to become smaller with the coexpression of AR (Fig. 3). These data suggested that TZF was recruited into the AR subnuclear foci in the presence of the ligand.

Protein–protein interactions between TZF and AR

Immunocoprecipitation experiments using whole cell extracts were performed to test whether or not TZF binds to AR in living cells. Each plasmid expressing GFP-fused TZF and the full length of AR was transfected into HEK293T cells, and then the cells were maintained with 10^{-8} M DHT. GFP-fused TZF was precipitated with the full-length AR in the presence of DHT (Fig. 4A). Next, a plasmid expressing myc-tagged TZF was transfected into HEK293T cells together with expression plasmid for the AR-(1–532) covering AF-1 or AR-(622–919) covering the ligand binding domain AF-2, and then the cells were maintained with or without 10^{-8} M DHT. Myc-tagged TZF was specifically precipitated with AF-1, which exists in the nucleus without DHT [20], in a ligand-independent fashion (Fig. 4B). By contrast, TZF did not bind to AF-2 in the presence of DHT (Fig. 4C).

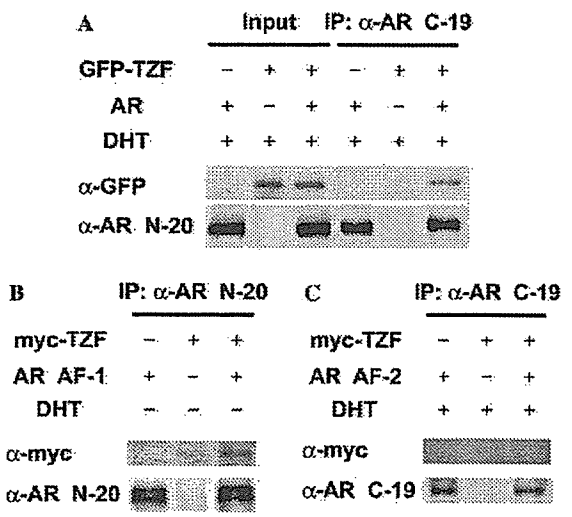

Fig. 4. TZF interacts with the full-length AR or AR-AF-1. (A) Coimmunoprecipitation of GFP-TZF with full-length AR. GFP-TZF and full-length AR were coexpressed in HEK293T cells. The cells were treated with 10^{-8} M DHT, collected at 48 h after transfection, and lysed in NE buffer. Portions of the lysates were immunoblotted with the anti-GFP or the anti-AR N-20 antibody (input), and the rest of the samples were immunoprecipitated with the anti-AR C-19 antibody followed by immunoblotting with the anti-GFP or the anti-AR N-20 antibody (IP). (B) Coimmunoprecipitation of myc-tagged TZF with AR-AF-1 (1–566 aa). (C) Coimmunoprecipitation of myc-tagged TZF with AR-AF-2 (623–919 aa).

TZF repressed AR-mediated transcriptional activation

To examine an effect of TZF on AR-mediated transcriptional activation, we performed luciferase assay using the MMTV-luciferase reporter gene in COS-7 cells. Surprisingly, expression of TZF repressed DHT-induced transcriptional activation by AR in a dose-dependent manner (Fig. 5A). We confirmed the repression by TZF using a different cell line (LNCaP; prostatic carcinoma cells, Fig. 5B) and a different promoter (PSA; prostate specific antigen, Fig. 5C).

N-CoR expression caused no significant effects on TZF-induced repression for AR-mediated transactivation

N-CoR is well known as a corepressor for nuclear receptors. N-CoR is supposed to recruit histone deacetylase into the nuclear receptor complex to repress the AR-mediated transcriptional activation [21]. To examine whether N-CoR was involved in the repression by TZF, luciferase assay was performed using cells coexpressing AR, N-CoR, and TZF. Both N-CoR and TZF repressed the AR-mediated transcriptional activation by itself (Fig. 6). Coexpression of these two proteins exhibited an additive effect in the repression. We have not obtained a clear evidence for direct relationship between TZF and N-CoR in the present study.

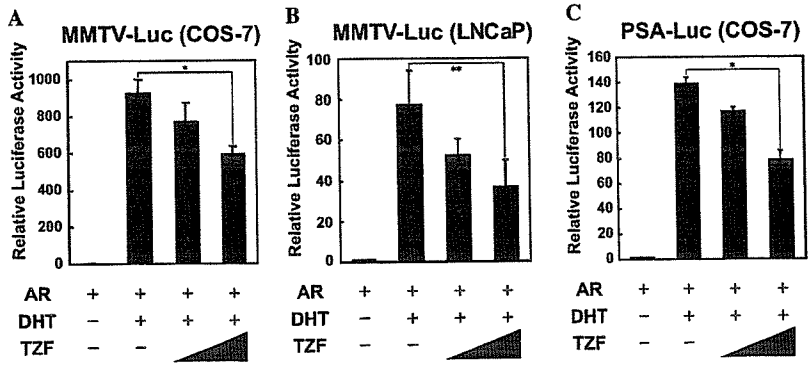

Fig. 5. Repression of AR-mediated transcriptional activation by TZF. (A,B) COS-7 (A) or LNCaP (B) cells were transiently transfected with 0.3 μg pGL3-MMTV, 3 ng pRL-CMV, 0.1 μg pCMV-hAR, and 0.5 or 1 μg pFLAG-TZF-CMV, as indicated. After 24 h incubation with or without 10^{-8} M of DHT, cells were lysed and whole cell extracts were subjected to the luciferase assay. (C) COS-7 cells were cotransfected with 0.3 μg pGL3-prostate specific antigen (PSA), 3 ng pRL-CMV, 0.1 μg pAR-CMV, and 0.5 or 1 μg pFLAG-TZF-CMV and luciferase assay was performed as above. Bars show the fold change in the luciferase activity relative to the value by the wild type AR without DHT. The average of three independent experiments is shown with the standard deviation. $*P < 0.01$; $**P < 0.05$.

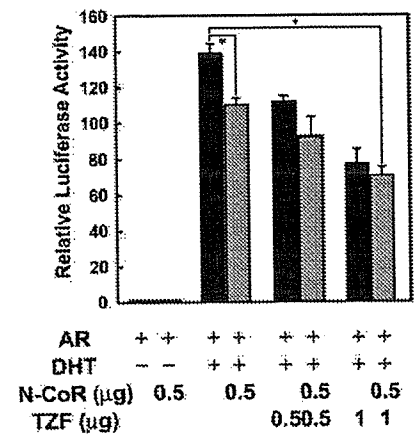

Fig. 6. The effect of N-CoR expression on the repression of AR-mediated transcriptional activation by TZF. COS-7 cells were cotransfected with 0.3 μg pGL3-PSA, 3 ng pRL-CMV, 0.1 μg pCMV-hAR, and 0.5 or 1 μg pFLAG-TZF-CMV with or without 0.5 μg pFLAG-N-CoR-CMV and treated with or without 10^{-8} M DHT. Luciferase assay was performed after 24 h incubation. Solid bars show the values without N-CoR and shadow bars show those with N-CoR. Bars show the fold change in the luciferase activity relative to the value without DHT. The mean values and the standard deviation from three independent experiments are shown. $*P < 0.01$.

Discussion

Transcriptional repression is an intrinsic part of endocrine physiology and contributes to feedback regulation associated with the inhibition of the physiologic response. To date, several investigators have found transcriptional corepressors, such as SMRT [10] and N-CoR [12]. However, there is little information on the effects and action mechanisms of corepressors. In the present study, we demonstrated that a zinc finger protein TZF formed specific foci located close to the splicing factor compartment in nuclei and was recruited into the AR foci in the present of DHT. Functional reporter assays and the immunoprecipitation experiments also showed that the TZF was essentially the AR-AF-1-interacting transcriptional corepressor.

In the nucleus, there exist different sets of functional compartments often called "foci" or "speckles," which include the splicing factor compartment (SFC) that consists of nearly 20–50 large foci [22], and nuclear receptor foci possibly associated with the nuclear matrix structures [17,23,24]. We first visualized TZF-dependent compartment in nuclei and showed that TZF-dependent foci were located close to the splicing factor compartment. Furthermore, TZF interacted to the AR-AF-1 sequence (Fig. 3). It has been known that various molecules, such as SRA [25], BRCA1 [26], and cyclin E [27], bind to the AR-AF-1 and that these molecules are involved in splicing activity of RNA molecules. Together with the finding that TZF-dependent foci were located close to SC-35 compartment, TZF may be involved in the spliceosomal machinery.

Next, we visualized the spatial relationship between TZF-dependent foci and AR foci in the presence and the absence of DHT. Interestingly, by the addition of DHT, TZF was recruited into AR foci from TZF-dependent foci in a time-dependent fashion and, after 90 min, TZF molecules were completely collocated to AR foci (Figs. 2 and 3). The formation of AR foci with the presence of ligand in nuclei plays an important role on its transcriptional activity [17,20]. Therefore, we examined whether TZF was involved in the AR-mediated transcriptional activation. Consequently, we obtained the results that TZF decreased the AR-mediated transcriptional activation. These results suggested that the binding of TZF with AR might play a key role in the transcriptional activity of AR.

AR has been thought to be quite unique among the nuclear receptor superfamily members, because most of its activities are mediated through the ligand-independent constitutive activity of AF-1 function [28]. In addition, the interaction of the N- and C-terminal domain is important for exerting the full AR transactivation capacity [29–31]. Most of the transcriptional cofactors reported are known to interact with AF-2, the number of transcriptional cofactors specifically interacting with the AF-1 sequence of AR is limited. For example, CBP/p300 [7] and SRC-1 [29] interact with both AF-1 and AF-2, and N-CoR [21] and SMRT [32] also bind to AF-2 of AR protein. We, in the present study, showed that TZF interacted with AF-1 domain of AR molecule. Therefore, TZF is a unique corepressor associating with the AR-AF-1 domain.

N-CoR and SMRT contain CoRNR motifs (L/I-X-X-V/I-I) that interact with nuclear hormone receptors. Therefore, we searched the sequence of motifs in TZF protein but were not able to find it. N-CoR is supposed to recruit histone deacetylase into the nuclear receptor complex to repress the AR-mediated transcriptional activation. To examine whether N-CoR was involved in the repression by TZF, we performed luciferase assay using cells coexpressing AR, N-CoR, and TZF. Both N-CoR and TZF repressed the AR-mediated transcriptional activation by itself (Fig. 6). Furthermore, coexpression of these two proteins exhibited an additive effect in the repression. These results show that TZF and N-CoR might act through the different pathways or mechanism on the repression of the AR-mediated transcriptional activation.

To examine an effect of TZF on the AR-mediated transcriptional activation, we performed luciferase assay using the MMTV-luciferase reporter gene in COS-7 cells. Expression of TZF repressed DHT-induced transcriptional activation by AR in a dose-dependent manner (Fig. 5A). We also confirmed the repression by TZF using prostatic carcinoma cells (Fig. 5B) and a different promoter (Fig. 5C). Thus, TZF might be a novel corepressor of AR. Recently, it has been reported that abnormalities of coactivators or corepressors caused several diseases and they were named as cofactor diseases. Rubinstein–Taybi syndrome, that exhibits facial abnormalities, broad thumbs, broad big toes, and mental retardation as the main clinical features, is caused by mutations in the transcriptional coactivator CBP [33]. Some patients with androgen insensitivity syndrome lack a coactivator interacting with the AF-1 region of the androgen receptor [19]. By contrast, repression of transcriptional activation of nuclear receptors plays important roles in acute promyelocytic leukemia and other diseases [34]. Our previous studies revealed that transcripts of TZF were expressed in testis [15]. In particulate, elevated expression of the transcript during testicular development in mice was restricted to spermatocytes at the pachytene stage of meiotic prophase, and to round and elongated spermatids. However, it is unclear now whether the decrease of the transcriptional activation of AR by TZF affects the testis. To fully elucidate the action and mechanism of TZF in the body, we have to wait for the preparation of *tzf*-deficient mouse.

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