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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 ($2 \times 10^4$ 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 ($2 \times 10^5$ 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 $1024 \times 1024$ 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 ($1 \times 10^5$ 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