Glyceraldehyde-3-phosphate Dehydrogenase Enhances Transcriptional Activity of Androgen Receptor in Prostate Cancer Cells*

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Naoki Harada,¹ Ryoko Yasunaga,¹ Yasuki Higashimura,¹ Ryoichi Yamaji¹‡, Katsumi Fujimoto, Joel Moss, Hiroshi Inui, and Yoshihisa Nakano‡

From the ¹Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 5998531, Japan, the ²Departments of Dental and Medical Biochemistry, Graduate School of Biochemical Sciences, Hiroshima University, Hiroshima 7348553, Japan, and the ³Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Androgen receptor (AR) functions as a transcriptional factor for genes involved in proliferation and differentiation of normal and cancerous prostate cells. Coactivators that bind to AR are required for maximal androgen action. Here we report that increasing the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in a prostate cancer cell line by as little as 1.8-fold enhances transcriptional activity of AR (but not the transcriptional activity of glucocorticoid receptor or estrogen receptor α) in a ligand-dependent manner and results in an increased expression of prostate-specific antigen. Small interference RNA-mediated knockdown of GAPDH significantly attenuated ligand-activated AR transactivation. Immunoprecipitation analysis revealed the presence of an endogenous protein complex containing GAPDH and AR in both the cytoplasm and nucleus. Addition of a nuclear localization signal (NLS) to GAPDH (GAPDH-NLS) completely abolished the ability of GAPDH to transactivate AR. Neither wild-type GAPDH nor GAPDH-NLS enhanced transcriptional activity of mutant AR (ARAC-Nuc) that is a constitutively active form of AR in the nucleus, even though GAPDH-NLS formed a complex with wild-type AR or ARΔC-Nuc. AR transactivation was enhanced by a mutant GAPDH lacking dehydrogenase activity. GAPDH enhanced the transcriptional activity of AR(T875A) activated by an antagonist such as hydroxyflutamide or cyproterone acetate. These results indicate that GAPDH functions as a coactivator with high selectivity for AR and enhances AR transactivation independent of its glycolytic activity. Further, these data suggest that formation of a GAPDH-AR complex in the cytoplasm rather than nucleus is essential for GAPDH to enhance AR transactivation.

Androgens are steroid hormones that are required for the expression of the male phenotype such as male sexual differentiation, the development and maintenance of secondary male characteristics, the initiation and maintenance of spermatogenesis, the maturation of normal prostate, and the development and progression of prostate cancer (1). In mammals, virilization is mediated through two types of steroidal hormones, testosterone and 5α-dihydrotestosterone (DHT). DHT is a more potent androgen whose action is mediated through binding to the androgen receptor (AR). The AR is a member of the nuclear receptor superfamily, which includes estrogen (ER), glucocorticoid (GR), mineralocorticoid, retinoic acid, and vitamin D receptors (2). The nuclear receptors share a common modular structure that is composed of three functional domains: the N-terminal domain (NTD), central DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). Among the nuclear receptors, the primary sequence of the NTD is variable, whereas the structures of the DBD and LBD are more conserved. The ligand-independent activation function-1 and the ligand-dependent activation function-2 are located in the NTD and the LBD, respectively (3, 4). AR, when not bound to a ligand, exists in the cytosol in an inactive state in association with chaperone proteins (e.g. heat shock proteins HSP90 and HSP70). The binding of DHT to the LBD in the AR results in conformational changes of AR, leading to dissociation of the receptor from HSPs (5). The ligand-bound receptor translocates into the nucleus and binds to a specific androgen-response element in androgen-responsive genes, followed by transcriptional activation of these genes. The transcriptional activity of AR is enhanced by recruitment of coactivators (6, 7). Coactivators such as the ARA group (ARA70, ARA55, and ARA267–α), p300/CBP, and p160 family (SRC-1 and GRIP-1) bind to the DBD or LBD of nuclear receptors. Because these domains are conserved among members of the nuclear receptor superfamily, the coactivators enhance the transcriptional activities of multiple nuclear receptors in addition to AR. In contrast, proteins that bind to the variable NTD in AR are expected to superfamily, which includes estrogen (ER), glucocorticoid (GR), mineralocorticoid, retinoic acid, and vitamin D receptors (2). The nuclear receptors share a common modular structure that is composed of three functional domains: the N-terminal domain (NTD), central DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). Among the nuclear receptors, the primary sequence of the NTD is variable, whereas the structures of the DBD and LBD are more conserved. The ligand-independent activation function-1 and the ligand-dependent activation function-2 are located in the NTD and the LBD, respectively (3, 4). AR, when not bound to a ligand, exists in the cytosol in an inactive state in association with chaperone proteins (e.g. heat shock proteins HSP90 and HSP70). The binding of DHT to the LBD in the AR results in conformational changes of AR, leading to dissociation of the receptor from HSPs (5). The ligand-bound receptor translocates into the nucleus and binds to a specific androgen-response element in androgen-responsive genes, followed by transcriptional activation of these genes. The transcriptional activity of AR is enhanced by recruitment of coactivators (6, 7). Coactivators such as the ARA group (ARA70, ARA55, and ARA267–α), p300/CBP, and p160 family (SRC-1 and GRIP-1) bind to the DBD or LBD of nuclear receptors. Because these domains are conserved among members of the nuclear receptor superfamily, the coactivators enhance the transcriptional activities of multiple nuclear receptors in addition to AR. In contrast, proteins that bind to the variable NTD in AR are expected 2 The abbreviations used are: DHT, 5α-dihydrotestosterone; AR, androgen receptor; CPA, cyproterone acetate; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DBD, DNA-binding domain; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; HF, hydroxyflutamide; LBD, ligand-binding domain; NES, nuclear export signal; NLS, nuclear localization signal; NTD, N-terminal domain; polyQ, polyglutamine; PSA, prostate-specific antigen; TPI, triosephosphate isomerase; RLU, relative light unit(s); siRNA, small interference RNA.
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to act more specifically on AR. AR contains a characteristic polyglutamine (polyQ) tract, and the expansion of the polyQ tract results in a reduction in the ability of the AR to activate transcription (8), indicating that conformational changes of the polyQ tract per se have an influence on AR transactivation. Furthermore, ARA24 has been shown to interact with the polyQ tract of AR as a coactivator to enhance AR transactivation (9). ARA24 is identical to Ran, which is involved in nucleocytoplasmic transport (10). These results suggest that intramolecular and/or intermolecular interactions involving the polyQ tract play a critical role in modulation of AR transactivation.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that reversibly catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in the cytosol. Recent studies have demonstrated that GAPDH serves as a multifunctional protein contributing to a number of nonglycolytic cellular processes such as nuclear tRNA transport, apoptosis, DNA repair, and DNA replication in the nucleus, vesicular transport, membrane fusion and microtubule bundling in the particulate fraction (11), and cell spreading in the extracellular space (12). Furthermore, in vitro experiments have shown that GAPDH binds to four gene products (AR, ataxin-1, atrophin, and huntingtin), each produced in a different neurodegenerative disorder (13, 14). A common structural feature of these gene products is a polyQ tract. In fact, GAPDH binds to synthetic polyQ peptides, although binding of GAPDH to AR does not vary with the length of the polyQ tract. Thus, these results indicate that GAPDH interacts with AR through the polyQ tract in the NTD, although the physiological significance of the interaction between GAPDH and AR remains unclear. On the other hand, the expression level of GAPDH mRNA is correlated with the metastatic potential and cell motility of rat prostate cancer cells (15) and increased in late pathological stages of human prostate tumors (16). These results suggest that GAPDH regulates AR transactivation, especially when the expression of GAPDH is up-regulated in cells expressing AR. In the present study, we report that GAPDH enhances the transcriptional activity of AR in a ligand-dependent manner and that the role of GAPDH in AR transactivation is independent of glycolysis. Furthermore, we demonstrate that a protein complex containing GAPDH and AR exists in both the cytoplasm and nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells and human prostate cancer PC-3 and LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in a 5% CO₂/95% air atmosphere at 100% humidity unless otherwise indicated. COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA), and PC-3 and LNCaP cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan).

Plasmids—Human ERα mammalian expression vector, pCAGGS-ERα, was kindly provided by Dr. T. Adachi (Kyoto University, Kyoto, Japan). The coding regions of the human GAPDH, AR, GR, ARA70, and ARA24/Ran cDNAs (GenBank™ accession numbers M33197, M23263, X03225, NM_005437, and NM_006325, respectively) were amplified by two-sequential, nested PCR using human brain (cerebral cortex) Marathon-ready cDNA (Clontech Laboratories, San Jose, CA) as a template and specific primer combinations, followed by cloning into pCR2.1-TOPO vectors. Primers with a typical Kozak sequence and appropriate restriction enzyme sites were designed for the second PCR. The ARA70N cDNA encoding the N-terminal region (amino acids 1–401) of ARA70 was amplified by PCR using ARA70 cDNA as a template and the specific primers. For pcDNA3.1-GAPDH-Myc-His and pcDNA3.1-ARA70N-Myc-His plasmids expressing GAPDH and ARA70N with Myc and His tags at the C terminus, GAPDH and ARA70N cDNAs were subcloned into the EcoRI and BamHI sites and NotI and BamHI sites of pcDNA3.1-Myc-His, respectively. The GAPDH(C151S) cDNA encoding mutant GAPDH substituting Ser for Cys at position of 151 was amplified by PCR using specific primers and the EcoRI-digested fragments of pCR2.1-GAPDH(C151S) (12) as a template and cloned, followed by insertion into the EcoRI and BamHI sites of pcDNA3.1-Myc-His, termed pcDNA3.1-GAPDH(C151S)-Myc-His. The expression vector pcDNA3.1-GAPDH-NLS-Myc-His was constructed by insertion of the DNA fragment encoding three tandem repeat of simian virus 40 large T antigen nuclear localization signal (NLS) in-frame to the BamHI sites of pcDNA3.1-GAPDH-Myc-His, resulting in a fusion protein of GAPDH with NLS, containing Myc and His tags. The GAPDH(258–261A) cDNA encoding GAPDH with a nuclear export signal (NES) containing alanine mutations was synthesized by site-directed mutagenesis of pcDNA3.1-GAPDH-Myc-His using the QuikChange II XL site-directed mutagenesis kit (Stratagene), followed by construction of pcDNA3.1-GAPDH(258–261A)-Myc-His plasmid. For pcDNA3.1-AR and pcDNA3.1-GR plasmids expressing AR and GR with no tags, respectively, AR and GR cDNAs were subcloned into the NotI and BamHI sites of pcDNA3.1-Myc-His. The AR cDNA cloned in the present study contains sequences encoding 25 repetitive polyQ and 18 repetitive polyglycine regions, and the translated protein is composed of 917 amino acids. The ARΔC-Nuc cDNA encoding mutant AR (amino acids 1–658) lacking the LBD was amplified by PCR using specific primers and the AR cDNA as a template, followed by construction of pcDNA3.1-ARΔC-Nuc. AR(T877A) substituting Ala for Thr at position of 877 of AR is found in many prostate tumors, including LNCaP cells (17). Amino acid position 877 of AR(T877A) corresponds to amino acid position 875 of AR cloned in the present study, because AR is polymorphic in its polyQ and polyglycine regions. Therefore, we constructed the expression vector pcDNA3.1-AR(T875A) by site-directed mutagenesis of the pcDNA3.1-AR using the QuikChange II XL site-directed mutagenesis kit. For pCAGGS-ARA24/Ran plasmid expressing ARA24/Ran with no tags, ARA24/Ran cDNA was subcloned into EcoRI sites of pCAGGS (18), which was kindly provided by Dr. J. Miyazaki (Osaka University, Osaka, Japan). Luciferase reporter vectors (pARE2-TATA-Luc and p3xERE-TATA-Luc) in which the pGL3-basic vector has two tandem repeats of an androgen-response ele-
ment and three tandem repeats of an estrogen-response ele-
ment, together with an adenovirus E1b TATA sequence, were
constructed according to the methods of Moilanen et al. (19)
and Legler et al. (20), respectively.

**Reporter Assay**—PC-3 and COS-7 cells were grown to con-
fluence in phenol red-free RPMI 1640 medium supplemented
with 10% dextran-coated charcoal-stripped fetal bovine serum
(steroid-free RPMI 1640 medium). Cells were seeded on
35-mm dishes or 12-well plates and further cultured to 90%
confluence in steroid-free RPMI 1640 medium, followed by
transient transfection using Metafectene (Biontex Laboratories
GmbH, Martinsried/Planegg, Germany). Nuclear receptor
construct and its corresponding luciferase reporter construct
were co-transfected into cells with GAPDH construct
(pcDNA3.1-GAPDH-Myc-His, pcDNA3.1-GAPDH-NLS-Myc-His,
pcDNA3.1-GAPDH(258–261A)-Myc-His, or pcDNA3.1-GAPDH
(C151S)-Myc-His), pcDNA3.1-ARA70N-Myc-His, or pcCAGGS-
ARA24/Ran. Transfection efficiency was normalized using
pRL-TK (Renilla luciferase expression vector, Promega Corp.,
Madison, WI). After 24-h transfection, the medium was
replaced with fresh, steroid-free RPMI 1640 medium supple-
mented with specific ligand or anti-androgen, and cells were
incubated for an additional 24 h. Cells were harvested and lysed,
and firefly and Renilla luciferase activities were determined
with the Dual-Luciferase reporter assay kit and GloMax 20/20
Luminometer (Promega). Data are expressed as relative light
units (RLU, firefly luciferase divided by Renilla luciferase). For
siRNA experiments, PC-3 cells were transfected with GAPDH-
specific siRNA or control siRNA (TAKARA Bio Inc., Shiga,
Japan) using DharmaFECT 1 (Dharmacon, Chicago, IL) for 6 h.
The medium was replaced with fresh, steroid-free RPMI 1640
medium, and cells were incubated for 18 h, followed by tran-
sient transfection with pcDNA3.1-AR, pARE2-TATA-Luc, and
pRL-TK using Metafectene for 24 h.

**Western Blot Analysis**—For detection of PSA, LNCaP cells
were cultured in steroid-free RPMI 1640 medium on 35-mm
dishes. When grown to 70% confluence, cells were transfected
with pcDNA3.1-GAPDH-Myc-His, followed by incubation for
24 h. Cells were further incubated in fresh, steroid-free RPMI
1640 medium supplemented with synthetic androgen, R1881
(methyltrienolone, Dupont, Boston, MA) for 24 h. Cells were
harvested and sonicated in 20 mM Hepes-NaOH, pH 7.5, con-
taining 150 mM NaCl, 0.5% Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM 4-(2-aminoethyl)benz-
esulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin).
COS-7 cells were co-transfected with pcDNA3.1-AR or
pcDNA3.1-ARΔC-Nuc and pcDNA3.1-GAPDH-NLS-Myc-His
for 5 h, followed by incubation for 28 h. Cells were further incub-
ated for 15 h in the presence of 0.2 nM R1881, followed by
harvesting and resuspension in IP buffer. Cell suspension was
incubated for 30 min on ice and centrifuged at 20,000 × g for
20 min at 4 °C. The supernatant is referred to as whole cell frac-
tion. For preparation of nuclear and cytoplasmic fractions,
LNCaP cells were incubated for 15 h in the presence of 0.2 nM
R1881 and resuspended in hypotonic buffer (10 mM Hepes-
NaOH, pH 7.5, containing 10 mM KCl, 1.5 mM MgCl2, 1 mM
dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride,
10 μg/ml leupeptin, and 1 μg/ml aprotinin) and homogenized
by repeated passage through a 23-gauge needle. The homoge-
nate was subjected to differential centrifugation as described
previously (21), except for minor modifications. Nuclei were
sonicated in IP buffer, followed by centrifugation at 20,000 × g
for 20 min. The supernatant is referred to as the nuclear frac-
tion. The post-nuclear fraction was diluted with IP buffer and
incubated for 30 min on ice, followed by centrifugation at
20,000 × g for 20 min. The supernatant is referred to as the
cytoplasmic fraction. The whole cell fraction (LNCaP or PC-3
cells, 1 mg; COS-7 cells, 500 μg), nuclear fraction (333 μg),
or cytoplasmic fraction (1 mg) that had been precleared with pro-
tein G-Sepharose (Amersham Biosciences) for 1 h was incu-
bated with monoclonal mouse anti-AR IgG (2 μg, clone AR441,
Santa Cruz Biotechnology) or control mouse IgG (2 μg) for 1 h
at 4 °C, followed by addition of 60 μl of protein G-Sepharose
(50% slurry) pre-equilibrated with IP buffer. The mixture was
incubated for 4 h at 4 °C, and the resin was washed with IP
buffer. Proteins bound to the resin were analyzed by Western
blotting with monoclonal mouse anti-AR (1/1,000), anti-

**Immunoprecipitation**—For preparation of whole cell frac-
tion, LNCaP or PC-3 cells were incubated for 15 h in the presence
of 0.2 nM R1881, harvested, and resuspended in IP buffer (50 mM
Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM 4-(2-aminoethyl)benz-
esulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin).

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For preparation of whole cell fraction, LNCaP or PC-3 cells were incubated for 15 h in the presence of 0.2 nM R1881 and resuspended in hypotonic buffer (10 mM Hepes-NaOH, pH 7.5, containing 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin). The post-nuclear fraction was diluted with IP buffer and incubated for 30 min on ice, followed by centrifugation at 20,000 × g for 20 min. The supernatant is referred to as the nuclear fraction. The post-nuclear fraction was diluted with IP buffer and incubated for 30 min on ice, followed by centrifugation at 20,000 × g for 20 min. The supernatant is referred to as the cytoplasmic fraction. The whole cell fraction (LNCaP or PC-3 cells, 1 mg; COS-7 cells, 500 μg), nuclear fraction (333 μg), or cytoplasmic fraction (1 mg) that had been precleared with protein G-Sepharose (Amersham Biosciences) for 1 h was incubated with monoclonal mouse anti-AR IgG (2 μg, clone AR441, Santa Cruz Biotechnology) or control mouse IgG (2 μg) for 1 h at 4 °C, followed by addition of 60 μl of protein G-Sepharose (50% slurry) pre-equilibrated with IP buffer. The mixture was incubated for 4 h at 4 °C, and the resin was washed with IP buffer. Proteins bound to the resin were analyzed by Western blotting with monoclonal mouse anti-AR (1/1,000), anti-
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GAPDH (1/5,000, clone 6G5, Biogenesis, Poole, UK) and anti-Myc (1/3,000, clone 9B11, Cell Signaling Technology, Beverly, MA) antibodies and polyclonal rabbit anti-AR antibodies (1/1,000, N-20, Santa Cruz Biotechnology), followed by reaction with the horseradish peroxidase-conjugated goat antimouse IgG (1/3,000) and anti-rabbit IgG (1/3,000), respectively. Immunoreactive bands were detected by Super Signal Chemiluminescent substrate.

Subcellular Fractionation—PC-3 cells in 100-mm dishes were transfected with pcDNA3.1-GAPDH-Myc-His, pcDNA3.1-GAPDH-NLS-Myc-His, or pcDNA3.1-GAPDH(258–261A)-Myc-His using Metafectene, followed by incubation for 24 h. The medium was replaced with fresh RPMI 1640 medium, and cells were incubated for an additional 24 h. Cells were harvested and homogenized by repeated passage in hypotonic buffer through a 23-gauge needle. The cytosolic, nuclear, and particulate fractions were separated by differential centrifugation as described previously (21). Proteins in each fraction were analyzed by Western blotting with anti-GAPDH (1/1,000), anti-triosephosphate isomerase (TPI) (1/1,000) (21), anti-Myc (1/1,000) and anti-lamin B1 antibodies (1/1,000, clone L-5, Zymed Laboratories Inc., San Francisco, CA). Immunoreactive GAPDH or TPI, and Myc or lamin B1 were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG, respectively, followed by detection using Super Signal Chemiluminescent substrate.

Statistics—Data are expressed as mean ± S.D., and Student’s t test for unpaired samples was applied for statistical analysis. Differences were considered significant when p < 0.05.

RESULTS

Enhancement of AR Transactivation by GAPDH—To define the ability of GAPDH to modulate AR-mediated transription, the human prostate cancer PC-3 cell line (an AR-negative cell line) was transiently co-transfected with the pARE2-TATA-Luc reporter plasmid, pcDNA3.1-AR and pcDNA3.1-GAPDH-Myc-His, and the transcriptional activity of AR was measured as luciferase activity. The transcriptional activity of AR was increased with an increasing dosage of pcDNA3.1-GAPDH-Myc-His, which resulted in an increase of recombinant GAPDH-Myc. In contrast, GAPDH had no influence on the AR transactivation in the absence of ligand (Fig. 1A). When the transcriptional activity of AR was determined at various concentrations of R1881 with PC-3 cells overexpressing GAPDH at the same level, the enhancing effect of GAPDH on AR transactivation was observed even in the presence of 0.05 nM R1881 (Fig. 1B). When similar experiments were performed in LNCaP cells (an AR-positive human prostate cancer cell line) or COS-7 cells (an AR-negative African green monkey kidney cell line), AR transactivation was enhanced by GAPDH in both cell lines (Fig. 1C). Prostate-specific antigen (PSA) was used as a biomarker for prostate cancer progression. Because PSA is an AR-mediated endogenous target gene with androgen-response elements, expression of PSA is enhanced by AR transactivation (22). To determine whether GAPDH activates the expression of PSA, GAPDH was overexpressed in LNCaP cells, and then a cell lysate was subjected to Western blot analysis with anti-PSA antibodies. PSA was detected in the presence of R1881, and overexpression of GAPDH resulted in an increased expression level of PSA (Fig. 2). These results indicate that GAPDH enhances the transcriptional activity of AR.

To examine the effect of endogenous GAPDH on AR transactivation, PC-3 cells were treated with GAPDH-specific siRNA, and then the transcriptional activity of AR was determined. siRNA-mediated knockdown of GAPDH resulted in a significant decrease of ligand-induced AR transactivation.

![FIGURE 1. Enhancing effect of GAPDH on AR transactivation.](image-url)
Western blot analyses showed that GAPDH siRNA specifically reduced endogenous GAPDH, but not endogenous /H9251-tubulin. These data demonstrate that endogenous GAPDH contributes to AR transactivation.

Expression Level of Exogenous GAPDH—To assess the amount of exogenous GAPDH required to enhance AR transactivation, the ratio of exogenous GAPDH to endogenous GAPDH in the positively transfected cells was determined. Firstly, to determine the transfection efficiency, PC-3 cells were transfected with pcDNA3.1-GAPDH-Myc-His, fixed, and incubated with anti-Myc IgG. Immunoreactive antibodies were visualized using Alexa Fluor 488-conjugated secondary anti-mouse IgG. The number of GAPDH-Myc-expressing cells was normalized to that of DAPI-stained cells. Values are calculated from data of ten random fields, and results are representative of two independent experiments. In B: Upper panel, whole cell lysates were prepared, and the indicated amounts of proteins were subjected to SDS-PAGE, followed by Western blot analysis with anti-GAPDH antibodies. Lower panel, the intensities of immunoreactive bands for endogenous or exogenous GAPDH were quantified by densitometry, and a standard curve was prepared by plotting the intensity of immunoreactive band for endogenous GAPDH. The intensity of immunoreactive band for exogenous GAPDH in 20 μg of whole cell lysates is indicated by an arrow on the standard curve for endogenous GAPDH. Each graph is representative of two independent experiments.
ratio of the number of recombinant GAPDH-Myc-expressed cells to that of DAPI-stained cells. Approximately 24% of the cells were calculated to express exogenous GAPDH (transfection efficiency = 24%). Subsequently, to determine the ratio of exogenous GAPDH to endogenous GAPDH in whole cells, PC-3 cells were transfected with pcDNA3.1-GAPDH-Myc-His. Whole cell lysates were prepared, and the indicated amounts of proteins in whole cell lysates were subjected to SDS-PAGE, followed by Western blot analysis with anti-GAPDH antibodies (Fig. 4B, upper panel). Densitometry analysis showed that the intensity of immunoreactive endogenous or exogenous GAPDH was quantified, and a standard curve was prepared by plotting the intensity of immunoreactive band for endogenous GAPDH (Fig. 4B, lower panel). Densitometry analysis showed that the intensity of immunoreactive endogenous GAPDH was proportional to the amount of whole cell lysates in the range of 2–10 μg of protein. The intensity of exogenous GAPDH-Myc in 20 μg of whole cell lysates, when quantitated by using a standard curve, corresponded to that of endogenous GAPDH in 3.8 μg of whole cell lysates. Because whole cells are composed of the positively and negatively transfected cells, the amount of lysates from positively transfected cells was calculated to be 4.8 μg in 20 μg of whole cell lysates by taking into account the transfection efficiency. Thus, the ratio of the exogenous GAPDH-Myc molecules to the endogenous GAPDH molecules in the positively transfected cells was 0.81 (3.8:4.8). These results indicate that increasing the expression of intracellular GAPDH by as little as 1.8-fold enhances AR transactivation.

Endogenous GAPDH-AR Complex in Vivo—In vitro experiments have demonstrated that recombinant AR physically interacts with GAPDH (13, 14). To assess whether GAPDH is associated with AR in vivo, LNCaP cells were cultured in the presence or absence of ligand, and whole cell fraction was incubated with anti-AR IgG or control mouse IgG as a negative control. Protein complexes bound to IgG were separated by SDS-PAGE, followed by Western blot analysis. Anti-AR IgG co-immunoprecipitated GAPDH with AR in the presence or absence of ligand, whereas neither GAPDH nor AR was immunoprecipitated with control IgG (Fig. 5A). When whole cell fraction was prepared from PC-3 cells as a negative control, GAPDH was not immunoprecipitated by anti-AR IgG. These results indicate that a protein complex composed of GAPDH and AR is formed in vivo. The ligand-free AR is localized as an inactive form in the cytosol, and the ligand-bound AR is translocated into the nucleus as an active form. GAPDH is localized not only in the cytosolic fraction, but also in the nuclear and particulate fractions. Therefore, to determine the subcellular distribution of the GAPDH-AR complex, the cytoplasmic and nuclear fractions were prepared from LNCaP cells, followed by immunoprecipitation with anti-AR IgG or control mouse IgG. The anti-AR IgG immunoprecipitated endogenous GAPDH-AR complex in both the cytoplasmic and nuclear fractions (Fig. 5B). These results indicate that GAPDH forms a complex with AR in the cytoplasm and nucleus.

Nuclear GAPDH and AR Transactivation—To assess whether nuclear GAPDH plays a critical role in AR transactivation, we constructed a recombinant fusion protein with the simian virus 40 large T antigen nuclear localization signal between GAPDH and Myc tag (GAPDH-NLS-Myc). To evaluate the distribution of GAPDH-NLS-Myc in intact cells, PC-3 cells that had been transfected with GAPDH-NLS-Myc expression vector were immunostained using anti-Myc IgG, followed by Alexa Fluor-labeled secondary anti-mouse IgG. Using immunofluorescent microscopy, we observed that the fluorescent image of GAPDH-NLS-Myc overlaid the DAPI-stained nuclei, indicating a predominant localization of GAPDH-NLS-Myc in the nuclei (Fig. 6A, lower panels). On the other hand, GAPDH-Myc was distributed throughout the cells, although it is partially localized in the nuclei (Fig. 6A, upper panels). To further define the subcellular distribution of GAPDH-NLS-Myc, the PC-3 cells overexpressing GAPDH-NLS-Myc were homogenized, and subcellular fractionation was performed by differential centrifugation. In addition, whole cell fraction was prepared and subjected to Western blot analysis. GAPDH-NLS-Myc was predominantly detected as a nuclear rather than cytosolic protein (Fig. 6B, middle panel). On the contrary, GAPDH-Myc was predominantly detected in the cytosol and partly in the nuclei (Fig. 6B, left panel). The expression patterns of GAPDH-Myc among fractions were consistent with those of endogenous GAPDH. When PC-3 cells were co-transfected with GAPDH-NLS-Myc expression vec-
reported mutant GAPDH construct M3 (23). Compared with GAPDH-Myc, no additional enhancing effect of GAPDH(258–261A)-Myc on AR transactivation was observed, even though more GAPDH(258–261A)-Myc accumulated in the nucleus (Fig. 6, B and C, right panels). Because the cytosolic marker TPI and the nuclear marker lamin B1, respectively, were observed only in each corresponding fraction, there was no contamination between cytosolic and nuclear fractions. These results indicate that addition of NLS to GAPDH, which facilitates the nuclear translocation of GAPDH, completely abolishes the ability of GAPDH to transactivate AR and that disruption of NES in GAPDH, which results in more accumulation of GAPDH in the nucleus, causes no additional enhancing effect on AR transactivation.

**Complexes of GAPDH-NLS and Mutant AR**—Truncated mutant AR (ARΔC-Nuc) corresponding to the previous reported AR1–660 (24) is constitutively localized in the nucleus even in the absence of ligand and exerts androgen action as a constitutively active form of AR. To determine the effect of GAPDH-NLS on the transcriptional activity of ARΔC-Nuc, luciferase assay was determined. Indeed, ARΔC-Nuc exhibited constitutive transcriptional activity similar to ligand-activated wild-type AR. Neither wild-type GAPDH nor GAPDH-NLS enhanced transcriptional activity of ARΔC-Nuc (Fig. 7A). Next, the presence of a nuclear GAPDH-AR complex was studied using GAPDH-NLS and ARΔC-Nuc. COS-7 cells were co-transfected with GAPDH-NLS and wild-type AR or ARΔC-Nuc expression plasmids. Immunoprecipitation with anti-AR IgG revealed that GAPDH-NLS formed a complex with wild-type AR or ARΔC-Nuc (Fig. 7B). These results support the presence of GAPDH-AR complex in the nucleus. Further, the data indicate that, although GAPDH-NLS forms a complex with wild-type AR or ARΔC-Nuc in the nucleus, GAPDH-NLS enhances transcriptional activity of neither wild-type AR nor ARΔC-Nuc.

**Enhancement of AR Transactivation by Mutant GAPDH Lacking Glycolytic Function**—We examined whether the glycolytic function of GAPDH is involved in GAPDH-enhanced AR transactivation. Because the dehydrogenase activity of GAPDH requires Cys-151 of the active center (25), a mutant replacing Ser for Cys at position 151 of GAPDH, GAPDH(C151S), is inactive (12). Therefore, to examine whether GAPDH(C151S) enhances the transcriptional activity of AR, a GAPDH(C151S) expression vector was co-transfected into cells with AR expression vector and reporter vectors. The ability of GAPDH(C151S) to enhance AR transactivation was comparable to that of wild-type GAPDH, indicating that AR transactivation by GAPDH is independent of its dehydrogenase activity (Fig. 8).

**Effects of GAPDH and/or ARA24/Ran on the Transcriptional Activities of AR, GR, and ERO—**ARA24/Ran enhances AR transactivation through binding to the polyQ tract in the NTD of AR (9). To determine whether GAPDH exerts a competitive influence through its polyQ tract on ARA24/Ran-enhanced AR transactivation, reporter assays were performed with PC-3 cells overexpressing both GAPDH and ARA24/Ran. GAPDH and ARA24/Ran enhanced AR transactivation up to 1.6- and 2.2-fold, respectively, whereas co-expression of GAPDH and ARA24/Ran increased AR transactivation up to 4.3-fold, indi-
cating that GAPDH and ARA24/Ran have additive effects on AR transactivation (Fig. 9 A). Some of the coactivators that bind to the DBD or LBD of AR also affect the biological function of other steroid receptors because of the high degree of sequence homology among DBDs or LBDs of the steroid receptor family. To assess the effects of GAPDH on the transcriptional activities of other steroid receptors, the transcriptional activity of GR or ER was determined in a luciferase reporter assay. COS-7 cells were co-transfected with either GR expression vector and pARE2-TATA-Luc or ER/H9251 expression vector and p3xERE-TATA-Luc together with GAPDH-Myc, ARA24/Ran, or ARA70N expression vector. GAPDH did not affect ligand-activated GR or ER/H9251 transactivation, although ARA24/Ran enhanced the transcriptional activity of GR, but not ER/H9251, in a ligand-dependent manner (Fig. 9, B and C). In contrast, ARA70N enhanced the transcriptional activities of GR and ER in the presence of ligand. When the effects of GAPDH, ARA24/Ran, or ARA70N on GR or ER transcriptional activity were tested in PC-3 cells, similar results were observed (data not shown). Although ARA70N is a deletion mutant of ARA70, ARA70N was used as a positive control, because it exhibits stronger transcriptional coactivator activity than full-length ARA70. These results indicate that GAPDH has a preferential selectivity for AR in comparison to ARA24/Ran or ARA70N.

Enhancement of Anti-androgen-activated AR(T875A) Transactivation by GAPDH—Mutation of Thr to Ala at position of 877 in AR, resulting in AR(T877A), enables anti-androgens such as hydroxyflutamide (HF) and cyproterone acetate (CPA) to function as AR agonists (17). In contrast, bicalutamide (Casodex) does not have AR agonist activity of wild-type and T877A mutant ARs (26). We examined the effects of GAPDH on the anti-androgen-activated transcriptional activities with the wild-type AR or AR(T877A). After 24 h of transfection, medium was replenished, and cells were incubated in the presence of 0.2 nM R1881 for 24 h. CPA had marginal agonist activity (2-fold) on wild-type AR, and HF and CPA had substantial agonist activity on AR(T875A). GAPDH substantially enhanced the
transcriptional activity of CPA-activated AR (T875A), and marginally enhanced the transcriptional activity of HF-activated AR (T875A), whereas GAPDH did not enhance the transcriptional activity of the wild-type AR in the presence of the antiandrogens. In contrast, when ARA70N as a positive control was tested, ARA70N enhanced the transcriptional activity of wildtype AR or AR (T875A) in the presence of HF, Casodex, or CPA. These results indicate that GAPDH activates the AR-signaling pathway when cells expressing AR (T877A) such as LNCaP cells (AR (T875A) in the present study) are treated with anti-androgens such as HF and CPA.

**DISCUSSION**

The AR is a ligand-activated transcription factor that mediates the biological responses of androgens, especially DHT. The AR not only mediates prostate development, but also serves as a master regulator of primary prostatic cancer growth. AR-mediated transactivation is enhanced by accessory coactivators (e.g. ARA group, p300, and p160 family) (6, 7). Most of the coactivators bind to DBD or LBD, which have a high homology among members of the nuclear receptor superfamily and serve as a common positive regulator for the transactivation of multiple nuclear receptors. On the other hand, the NTD is variable among nuclear receptors, and AR contains a characteristic region composed of a highly polymorphic polyQ tract. Therefore, the association of AR-binding proteins such as coactivators with a polyQ tract may cause a conformational change that is optimal for the AR transactivation of NTD, thereby specifically enhancing the transactivation of AR. In *in vitro* experiments demonstrate that GAPDH interacts with the
polyQ tract of AR (13, 14), although its physiological significance remains unclear. In the present study, we have demonstrated that GAPDH has a physiological function related to AR transactivation.

GAPDH enhanced AR transactivation in a ligand- and dose-dependent manner and induced expression of endogenous PSA. In PC-3 cells, as little as a 1.8-fold increase in the intracellular level of GAPDH was sufficient to enhance AR transactivation. In contrast, knockdown of endogenous GAPDH using GAPDH siRNA significantly attenuated ligand-activated AR transactivation. GAPDH is ubiquitously expressed in mammalian tissues, whereas AR is expressed in a restricted manner. The present study demonstrates that GAPDH forms a protein complex with AR in cancerous prostate LNCaP cells. These results indicate a novel physiological function of GAPDH on the androgen-signaling pathway in prostate cancer cells. The expression levels of GAPDH mRNA are closely correlated with the metastatic potential and cell motility of prostate adenocarcinoma cells and are at least 15-fold higher in prostatic adenocarcinoma cell lines with high metastatic potential than in normal prostate cells (15). GAPDH activity is increased in an AR-positive prostate cancer cell line, LNCaP cells, after treatment with R1881, but not in an AR-negative prostate cancer cell line, DU145 cells (27). Therefore, the ability of GAPDH to enhance AR transactivation is expected to be more strongly elicited when GAPDH expression is up-regulated in AR-positive cells.

The ligand-free AR predominantly occurs in the cytoplasm as an inactive form, whereas the ligand-bound receptor is translocated into the nucleus and interacts with an androgen-response element in androgen-responsive genes to function as a transcription factor. GAPDH is localized in the nuclear and particulate fractions in addition to the cytosolic fraction (21). The present studies show the presence of a protein complex, including GAPDH and ligand-bound or ligand-free AR. The GAPDH-AR complex was observed in both the cytoplasm and nucleus, suggesting that GAPDH is translocated from the cytoplasm to the nucleus as a protein complex with AR. GAPDH-NLS, although it was constitutively localized in the nucleus, did not enhance AR transactivation. Neither wild-type GAPDH nor GAPDH-NLS enhanced the transcriptional activity of ARΔC-Nuc, which is the constitutively active, nuclear form of AR, even though GAPDH-NLS formed a complex with ARΔC-Nuc. Further, disruption of NES of GAPDH did not affect the ability of GAPDH to enhance AR transactivation. In the present study, we made two NES-mutated GAPDH constructs, GAPDH(258–261A)-Myc-His and GAPDH(266–269A)-Myc-His, corresponding to the previously reported Ala-mutated GAPDH constructs M3 and M5 (23). However, because GAPDH(266–269A)-Myc-His was expressed at a much lower level than GAPDH(258–261A)-Myc-His and wild-type GAPDH-Myc-His and had no influence on AR transactivation (data not shown), GAPDH(258–261A)-Myc-His was used in the present experiments. The nuclear accumulation of GAPDH(258–261A)-Myc-His in prostate PC-3 cells was lower than that of mutant GAPDH M3 in colorectal adenocarcinoma DLD1 cells. The level of nuclear accumulation of GAPDH due to the disruption of NES may be dependent on the cell lines.

Taking into consideration the fact that high grade or metastatic adenocarcinoma expresses a higher level of cytosolic GAPDH, compared with normal tissue or low grade adenocarcinoma, in human prostate tissue (28), formation of GAPDH-AR complex in the cytoplasm rather than nucleus appears to be essential for GAPDH to enhance AR transactivation. The expression of GAPDH is induced by hypoxia in endothelial cells (21), alveolar epithelial cells (29), and prostate cancer cells (30), even though GAPDH is considered as a housekeeping gene. In hypoxic endothelial cells, the expression of GAPDH protein is increased in the nuclear, particulate, and cytosolic fractions. GAPDH activity, however, does not increase sufficiently in proportion to the increase of GAPDH protein in the hypoxic cytosol, suggesting that hypoxia-induced GAPDH protein does not make an equivalent contribution to glycolysis (21). We observed a similar phenomenon in LNCaP cells under hypoxic conditions. Because hypoxia is a common feature of solid tumors, including prostate cancer (31), GAPDH in prostate cancer cells may undergo modification of Cys-151 in the active center during hypoxia and thereby lose its dehydrogenase activity. The present study shows that a GAPDH mutant lacking dehydrogenase activity due to replacement of Cys to Ser at position 151 enhances AR transactivation at the same level as wild-type GAPDH. Therefore, hypoxia-modified GAPDH appears to retain the ability to enhance the AR transactivation even if it loses glycolytic function.

GAPDH had no influence on the transcriptional activities of GR and ERα. ARA70, which is one of the most extensively studied coactivators, interacts with the LBD (32) and NTD (33) of AR, and acts as a coactivator of GR, ER, and the progesterone receptor in addition to AR (32). SRC-1, which binds to the NTD and LBD of AR, functions as a coactivator of general steroid nuclear receptors (e.g., AR, GR, ER, and progesterone receptor) (34). Thus, because ARA70 and SRC-1 bind to not only the NTD of AR but also the LBD, which is highly conserved among members of the nuclear receptor family, it is not surprising that both coactivators appear to enhance the transcriptional activities of general steroid nuclear receptors. On the other hand, in addition to GAPDH, ARA24/Ran binds to the polyQ tract of AR and enhances the transcriptional activity of AR (9). The present study indicates that ARA24/Ran also enhances GR transactivation. Therefore, ARA24/Ran may be associated with other regions of AR (e.g., DBD and LBD), which possess a relatively higher homology to the corresponding region of GR. GAPDH has a higher selectivity for AR than does ARA24/Ran. GAPDH together with ARA24/Ran exerted an additive effect on AR transactivation even though they are predicted to compete for binding to AR. These results suggest that GAPDH and ARA24/Ran interact with AR in different intracellular locations or at different times, or that ARA24/Ran, in the presence of overexpressed GAPDH molecules, binds to additional regions, except for the polyQ tract of AR, to enhance AR transactivation.

In prostate tumor tissues, expression of both ARA24/Ran and PIAS1, which act as coactivators for AR, is increased as is GAPDH, whereas the general coactivators for multiple nuclear

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3 Y. Higashimura, unpublished results.
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receptors, such as SRC-1 and TRAP220, are expressed at similar levels in normal and tumor prostate tissues (35). Contradictory results have been reported recently concerning the expression level of ARα70 in prostate tumor tissues (35, 36). The overexpression of coactivators is predicted to contribute to the progression of diseases related to AR transactivation (e.g. prostate cancer), because coactivators enhance AR transactivation and enable AR to become active at lower concentrations of ligand. Therefore, we suggest that GAPDH as well as ARα24/Ran and PIAS1 play more critical roles in tumorigenesis and progression of androgen-dependent prostate cancer cells than general coactivators for nuclear receptors (e.g. SRC-1 and ARα70).

Androgen deprivation by surgical or chemical castration results in a dramatic regression of prostate tumor growth, because prostate cancer cells are androgen-dependent at early stages. However, most tumors gradually become resistant to androgen deprivation or anti-androgens, and eventually the disease relapses. Although the recurrent cancer is androgen-independent, the mechanisms by which prostate tumors shift from being androgen-dependent to androgen-independent remain unclear. Some of the mechanisms may include mutations in the AR gene that allows the AR to respond to anti-androgens and involve aberrant expression of coactivators that enhance the transcriptional activity of AR even in the presence of low levels of androgens. In recurrent prostate cancer tissue during androgen deprivation therapy, androgens are present at sufficient levels (mean testosterone levels: 2.78 nM; mean DHT levels: 1.45 nM) to activate transcriptional activity of AR (37). Furthermore, an AR mutation, T877A, which is present in LNCaP cells, permits transcriptional responses to anti-androgens (e.g. HF and CPA), whereas wild-type AR is insensitive to anti-androgens. Our study demonstrates that overexpression of GAPDH stimulates AR transactivation in prostate cancer cells even at low concentrations of R1881 (0.05 nM >) and that GAPDH enables AR antagonists such as HF and CPA to behave as strong agonists for AR (T877A). Thus, increased expression of GAPDH in prostate cancer may be one of the mechanisms by which anti-androgens such as HF and CPA fail to suppress tumor growth and instead promote tumor growth.

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