

# Modulation of Androgen Receptor Transactivation by Gelsolin: A Newly Identified Androgen Receptor Coregulator<sup>1</sup>

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## ABSTRACT

The partial agonist effect of antiandrogens has been well documented, and such effect is amplified by derived mutant androgen receptors (ARs) in prostate cancer cells. Here we report the identification of gelsolin (GSN) as an AR-associated protein. Hydroxyflutamide (HF), as well as androgens, can promote the interaction between AR and GSN in a dose-dependent manner. GSN interacts with AR DNA-binding domain and ligand-binding domain via its COOH-terminal domain. Immunolocalization studies show that GSN interacts with AR during nuclear translocation. Functional analyses additionally demonstrate that GSN enhances AR activity in the presence of either androgen or HF. Two peptides representing partial regions of the AR DNA-binding domain and the ligand-binding domain can block the GSN-enhanced AR activity. The expression of GSN is enhanced in LNCaP cells, LNCaP xenografts, and human prostate tumors after androgen depletion. Increasing expression of GSN enhances the AR activity in the presence of HF. Together, these data suggest that the weak androgenic effect of HF may be amplified by increasing the amount of GSN after androgen ablation treatment. Therefore, blockage of the interaction between AR and GSN could become a potential therapeutic target for the treatment of prostate cancer.

## INTRODUCTION

The AR<sup>4</sup> is an intracellular mediator of androgen action and belongs to the steroid receptor superfamily (1). Androgen-bound AR can coordinate with various coregulators to bind to AREs for the regulation of its target gene expression (2, 3). Several AR coregulators, such as ARA24, ARA54, ARA55, ARA70, ARA160, ARA267, Rb, SRC-1/NcoA-1, TIF-2/GRIP1, and SRC-3/ACTR, were isolated and characterized (4–13). However, the *in vivo* function of these coregulators, especially their linkage to AR-related diseases, remains largely unclear.

Prostate cancer is the second leading cause of death in American men (14). Androgens and AR have been well documented to correlate with prostate cancer progression (15). Androgen ablation therapy with medical/surgical castration in combination with antiandrogens (flut-

amide or bicalutamide) remains the mainstream therapy to treat metastatic prostate cancer (16, 17). However, the majority of prostate cancers undergoing such androgen ablation treatment develop “antiandrogen withdrawal syndrome,” in which patients with disease progression and increasing PSA expression improve after antiandrogen withdrawal (18, 19). Furthermore, a majority of tumors progress from an androgen-dependent to an androgen-independent state (20). Because a majority of prostate tumors at this late stage still express AR (21, 22), it is possible that factors other than androgen may activate AR and contribute to prostate cancer progression. Several mechanisms have been proposed including the following. First, the cross-talk between AR and HER-2/*neu* pathway suggests that signals stimulated by growth factors can activate AR (23). Second, mutant ARs (mtAR)s, which have broad ligand specificity, and respond to other steroids and antiandrogens, have been detected in prostate tumors (24, 25). Third, AR coregulators may enhance AR transactivation induced by residual amounts of androgen or antiandrogen (6, 7, 26). Among several AR coregulators, ARA70 and ARA55 can enhance the androgenic effect of HF, the active metabolite of flutamide (26). ARA55 has higher expression in prostate cancer compared with normal prostate (6). To date, there is no report demonstrating ARA55 or ARA70 expression being regulated after androgen ablation treatment. The increasing expression of TIF-2 and SRC-1 after androgen deprivation has been proposed to play a role in tumor progression, but they weakly promote the androgenic effect of HF (27).

GSN is an actin severing protein well characterized in its function for cytoskeleton reorganization, cell morphology, and motility (28, 29). Because GSN is identified as a substrate for caspase-3, its dual roles in promoting apoptosis and protecting cells from apoptosis are proposed (30, 31). Several reports have indicated that GSN expresses differentially in various cancers, including prostate cancer (32, 33). However, its roles in carcinogenesis remain unclear. Here, we characterized the novel function of GSN, which associates with AR and promotes AR transactivation in the presence of HF as well as androgens. Furthermore, the expression of GSN increases in prostate cancer after androgen ablation.

## MATERIALS AND METHODS

**Yeast Two-Hybrid Screening.** pAS-mtARt877s (aa 595–918) and human prostate cDNA in pACT2 plasmid (6) were sequentially transformed into the yeast Y190. Positive clones were selected under -3 synthetic dropout medium (tryptophan, leucine, and histidine were eliminated) with 50 mM 3-aminotriazole and 10  $\mu$ M HF, then confirmed by  $\beta$ -gal assays. Once positive clones were isolated, they were incubated under -1 synthetic dropout medium (leucine was eliminated) with 10  $\mu$ g/ml cycloheximide to drop out the bait plasmid. Y190 colonies expressing only the candidate library plasmid were mated with Y189 (Clontech) expressing the bait plasmid or pAS2 empty vector to verify the specific interaction in yeast.

**Yeast Liquid  $\beta$ -Gal Assays.** Y190 yeast cells were transformed with pAS2-mtARt877s (aa 595–918) and pACT2-GSN (aa 281–731). Transformants were selected by their growth in the presence of 100 nM DHT, 10  $\mu$ M

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<sup>4</sup> The abbreviations used are: AR, androgen receptor; PSA, prostate-specific antigen; ARE, androgen response element; DHT, 5 $\alpha$ -dihydrotestosterone; HF, hydroxyflutamide; P, progesterone; E2, 17 $\beta$ -estradiol; EtOH, ethanol; GST, glutathione S-transferase; GSN, gelsolin; GSNc, gelsolin COOH terminus; DBD, DNA-binding domain; LBD, ligand-binding domain; ARDL, androgen receptor DNA-binding domain to ligand-binding domain; ARL, androgen receptor ligand-binding domain; ARD, androgen receptor DNA-binding domain; ARN, androgen receptor NH<sub>2</sub> terminus; LUC, luciferase; mt, mutant; T, testosterone; GR, glucocorticoid receptor; MMTV, mouse mammary tumor virus; aa, amino acid;  $\beta$ -gal,  $\beta$ -galactosidase; DAPI, 4,6-diamidino-2-phenylindole; ER, estrogen receptor<sup>3b</sup> PPAR, peroxisome proliferating activator receptor  $\gamma$ ; CD, charcoal dextran-treated.

HF, 1  $\mu$ M P, 1  $\mu$ M E2, or EtOH vehicle, and assayed for liquid  $\beta$ -gal assays as described previously (8).

**GST Pull-Down Assay.** The plasmids expressing GST-GSN and GST-GSNc fusion proteins were constructed by inserting PCR amplified GSN and GSNc cDNA into pGEX-KG plasmid (34). GST-GSN, GST-GSNc fusion proteins, and GST control protein were purified as instructed by the manufacturer (Amersham Bioscience, Piscataway, NJ). The purified GST fusion proteins and beads were suspended in 100  $\mu$ l NETN buffer (20 mM Tris/pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.5 mM Nonidet P-40, 1 mM DTT, 8% glycerol, 1 mM PMSF) with EtOH or 1  $\mu$ M DHT. Resuspended GST protein-bound beads were incubated with 5  $\mu$ l *in vitro*-translated [<sup>35</sup>S]methionine-labeled AR, AR ARDL, ARL, ARD, or ARN by TNT coupled reticulocyte lysate system (Promega, Madison, WI). After incubating for 2 h at 4°C, glutathione beads were washed with NETN buffer four times, protein complexes were loaded in 9% SDS-PAGE, and visualized by PhosphorImager (Molecular Dynamics Amersham Bioscience).

**Transfection Studies.** The cDNA encoding GSN COOH-terminal (aa 281–731) in the pACT2 plasmid isolated from yeast was inserted into pSG5-Gal4 (DBD; constructed by Dr. Ryusuke Nakao, Kyushu University, Fukuoka, Japan). AR fragment (aa 36–918) was inserted into pCMX-VP16 (a gift from Dr. Don Chen, Boston University, Boston, MA). For GSN expression vector, a full-length cDNA fragment of GSN from LKCG, a gift from Dr. David J. Kwiatkowski (Harvard Medical School, Boston, MA), was inserted into pSG5. Dr. Michael L. Lu (Harvard Medical School) provided the p(ARE) 4-LUC plasmid. Dr. Atsushi Mizokami (University of Kanazawa, Kanazawa, Japan) provided the pGL3-PSA6.0LUC plasmid. The expression plasmids of AR peptides were constructed by inserting the PCR amplified cDNA fragment of ARD into pFlag-CMV (Sigma Chemical Co., St. Louis, MO) and the fragments of ARL into pCDNA-flag plasmid. Cell culture conditions, transfection protocol, and LUC assay are as described previously (35).

**Immunocytofluorescence and Confocal Microscopy.** COS-1 cells were seeded, transfected, and processed for immunocytofluorescence as described previously (36), then incubated with 1:200 dilution of anti-AR polyclonal antibody (NH27) and 1:100 dilution of mouse monoclonal anti-GSN antibody (Sigma) for 45 min, followed by Texas-red-conjugated goat antirabbit antibody (ICN, Costa Mesa, CA) and fluorescein-conjugated goat anti-mouse antibody for 45 min at room temperature. Slides were then washed, mounted with VECTASHIELD mounting medium with DAPI (Vector Lab, Burlingame, CA), and photographed under 40-fold magnification with a Leica TCS SP Spectral Confocal Microscope (Leica, Bannockburn, IL).

**Preparation of Cellular Protein and Western Blots.** CWR22R, LNCaP, DU145, PC-3, PC-3 (AR2; the PC-3 cells stably transfected with AR expression plasmid; Ref. 36), C2C12, COS-1, and HTB-14 cells were collected, suspended in lysis buffer, and centrifuged. Aliquots corresponding to 50  $\mu$ g protein of each sample were loaded onto a 10% SDS-PAGE for Western blotting. After blotting, the membrane was blocked with 5% nonfat milk and probed with GSN antibody for 1 h at a dilution of 1:1000 in PBS containing 0.1% Tween 20. After washing with PBS containing Tween 20, the membrane was incubated for 60 min with the secondary antibody, washed again, and the immunoreactive bands were visualized for alkaline phosphatase activity with the 5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium phosphatase substrate (Bio-Rad Laboratories, Hercules, CA).

**Animal Study.** LNCaP ( $3 \times 10^7$ ) cells in Matrigel (Becton Dickinson, Franklin Lakes, NJ) were inoculated into the dorsal region of nude mice. At 11 weeks after cell inoculation, one group of mice ( $n = 3$ ) was castrated, whereas another group ( $n = 3$ ) underwent sham operation. A representative LNCaP xenograft of each group was harvested 6 weeks after castration or sham operation. The xenograft tissue was fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Immunohistochemical analyses were then performed as described below.

**Immunohistochemical Analysis.** Sixteen patients were treated with androgen ablation therapy before prostatectomy, whereas 11 patients underwent prostatectomy alone. Human prostate tumors were fixed in 10% neutral-buffered formalin, processed routinely, and embedded in paraffin. The slides of tissue sections stained with H&E were reviewed by a pathologist (K. A.) and mapped for cancer lesions. Localization of GSN protein expression was investigated on 5  $\mu$ m serial sections of tumor specimen. Slides were deparaffinized, rehydrated, and incubated with 3% (v/v) hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. The sections were then blocked with

BSA for 15 min and incubated for 3 h at 37°C with rabbit polyclonal anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA) or GSN antibody at a dilution of 1:100. Mouse immunoglobulin was used as the negative control in place of the primary antibody. The bound primary antibody was visualized by avidin-biotin-peroxidase detection with the DAKO kit (DAKO Corp., Carpinteria, CA) according to the manufacturer's instructions, and nuclei were stained with hematoxylin. Serial sections were then scanned for GSN expression under a light microscope at magnifications of  $\times 40$ ,  $\times 100$ ,  $\times 200$ , and  $\times 400$ . Only cancer cells with intense staining were counted as positive. The area containing the greatest number of positive cancer cells was selected for each sample. The number of positive cells per 500 cancer cells was counted and expressed as a percentage. GSN or AR expression level was graded as no expression (0%), low expression (<50%), and high expression ( $\geq 50\%$ ). The Mann-Whitney *U* test was used for analyses of GSN expression in human prostate cancer samples.

## RESULTS

**Cloning of Gelsolin as an AR-associated Protein.** To determine whether any AR-associated proteins are potentiated in the presence of antiandrogen, we applied yeast two-hybrid system to screen AR interacting proteins in human prostate cDNA library using mtARt877s as bait in the presence of 10  $\mu$ M HF. The mtARt877s was identified from a patient with androgen-independent prostate cancer, and its altered hormone specificity was demonstrated previously (25). Because HF can activate this mtAR (24), which was also confirmed in our laboratory (data not shown), we chose the LBD of mtARt877s as bait.

One of the positive cDNA clones was isolated accordingly, and its cDNA sequence was identical with the COOH terminus (aa 281–731) of human GSN. The clone also interacted with wild-type ARL in the presence of 100 nM DHT or 10  $\mu$ M HF in yeast two-hybrid assays (data not shown). In this study, we isolated only GSN, but not other AR interacting proteins identified previously using either wild-type AR-androgen or mtARt877 androgen. The other positive clones (two cDNAs) contain small cDNA fragments (<0.3 kb), and have not been deposited in public data banks.

**Ligand-enhanced Interaction between AR and Gelsolin.** To determine whether AR interacts with GSN in a ligand-enhanced manner, we first applied the yeast liquid  $\beta$ -gal assay. Y190 yeast cells were transformed with pAS-mtARt877s (aa 595–918) and pACT2-GSN (aa 281–731). HF, DHT, E2, and P promoted significant interaction between mtARt877s and GSN compared with EtOH (Fig. 1A). These results indicate a broad specific ligand-enhanced interaction between mtARt877s and GSN. We next tested the interactions between GSN and AR by mammalian two-hybrid assays, which are sensitive enough to detect relatively weak interactions. A VP16 fusion protein containing AR (aa 36–918) and a Gal4-GSN (aa 281–731) were coexpressed in COS-7 cells in the presence of T or HF (Fig. 1B). T promoted significant interaction between AR and GSN in a dose-dependent manner starting at the concentration of 10 nM. Likewise, HF induced significant interaction of these proteins starting at 1  $\mu$ M, a serum concentration achieved in the treatment of prostate cancer. The ligand-enhanced interaction of Gal4-GSN (aa 281–731) and VP16-AR (aa 36–918) were also confirmed in PC-3 cells (data not shown).

**Interaction Domains Are Located in Gelsolin COOH Terminus and AR DBD-LBD.** According to yeast and mammalian two-hybrid assays, GSN COOH-terminal interacts with AR. We additionally identified the interaction domains between GSN and AR by *in vitro* GST pull-down assay. We constructed plasmids for expressing GST-GSN (full-length GSN), and GST-GSNc (COOH-terminal product generated after caspase-3 digestion). AR was truncated to several fragments according to the functional domain (Fig. 2A) and expressed *in vitro*. The results from the GST pull-down assays indicate ARD and

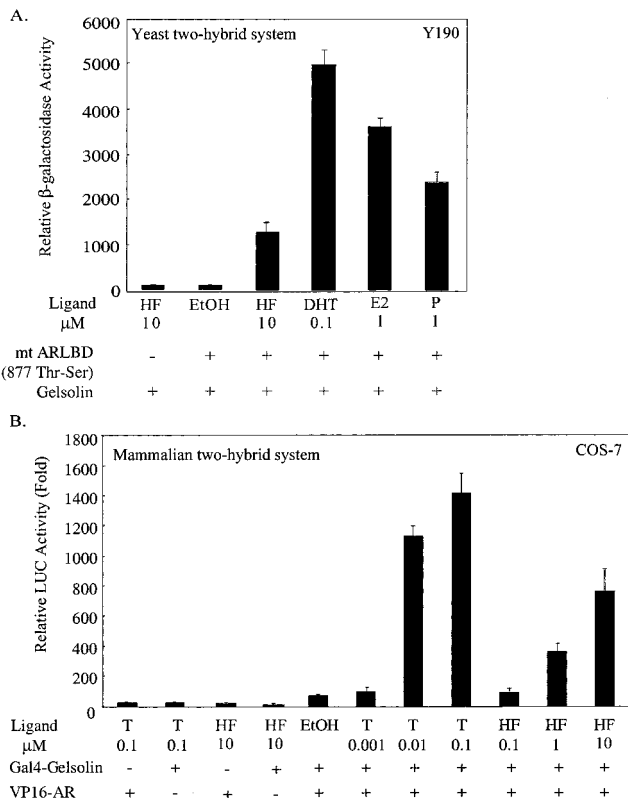


Fig. 1. AR interacts with GSN in two-hybrid assays. *A*, Y190 yeast cells were transformed with Gal4(DBD) fused with the COOH terminus (aa 595–918) of mtAr877s and Gal4AD fused with GSN (aa 281–731). Transformants were selected by their growth in the presence of DHT, HF, P, E2, or EtOH vehicle, and assayed for liquid  $\beta$ -gal activity as described previously (8). *B*, COS-7 cells were transfected with expression vectors for COOH terminus (aa 281–731) of GSN fused with Gal4, AR (aa 36–918) fused with VP16, pG5-LUC reporter, and internal control pRL-CMV reporter. Relative LUC activity was determined as Gal4-LUC activity relative to control LUC activity; bars,  $\pm$ SD.

ARL, but not ARN, interact with both GSN and GSNc (Fig. 2, *B* and *C*). The weak binding and little ligand effect shown in GST pull-down assays are possibly because of the lack of essential components for stabilizing the complex of AR and GSN in this assay system.

**Gelsolin Promotes AR and GR Activity in a Ligand-enhanced Manner.** To address the functional significance of the interaction between AR and GSN, we performed reporter gene assays by transient transfection of GSN and AR expression plasmids into human prostate cancer DU145 cells. To reduce the potential artificial effect from any single reporter gene assay, we applied three different AR target gene promoters, PSA, MMTV, and four repeats of ARE, in separate reporter gene assays. Transfection of full-length GSN enhanced AR transactivation by 2–3-fold in the presence of 10 nM DHT, but had no significant effect in the absence of DHT in these three different reporter gene assays (Fig. 3*A*). To test whether GSN also affects other steroid receptors, we performed reporter gene assays of GR, ER- $\alpha$ , and PPAR- $\gamma$  with overexpression of GSN. In this assay system, GSN promoted GR in the presence of ligand and promoted PPAR- $\gamma$  in the absence of ligand, but had no effect on ER- $\alpha$  transactivation (Fig. 3*B*).

**AR Peptides Block Gelsolin from Enhancing AR Activity.** Because GSN may depend on its association with AR to modulate AR activity, we designed AR peptides to disrupt the interaction between AR and GSN. AR peptides covering either the whole or partial DBD domain are D, D1, and D2. The others designed by dissecting 12 helices of ARL are H1–2, H3, H4–5, H6–7, H8–9, H10–11, and H12 (Fig. 4*A*). GSN-enhanced AR activity was demonstrated by reporter gene assays. Cotransfection of D, D1, or H1–2 peptides suppressed GSN-enhanced AR activity (Fig. 4*B*, Lanes 3, 4, and 6). Several

peptides in other regions of ARL also reduced AR activity but blocked GSN effect to a much lesser degree. Together, these data suggest that D1 (aa 551–600) and H1–2 (aa 665–695) may represent the major sites to suppress GSN-enhanced AR transactivation via interruption of the interaction between AR and GSN.

**Temporary Colocalization of AR and Gelsolin after Androgen Treatment.** GSN is known to be an actin-binding protein and mainly localized in the cytoplasm. AR is an androgen-dependent transcriptional factor and moves from the cytosol into the nucleus in an androgen-dependent manner. We investigated the subcellular localization of these two proteins by indirect immunofluorescence study in COS-1 cells cotransfected with full-length cDNAs of both AR and GSN followed by treatment with DHT and HF (Fig. 5). Both proteins localized mainly in the cytosol in the absence of androgen. After a 15-min treatment with 10 nM DHT, both proteins translocated into and around the nucleus (DAPI staining indicates the location of nucleus). Then, 30 min after the treatment, GSN surrounded the nucleus, whereas AR accumulated inside the nucleus. Similarly, colocalization of GSN and AR was observed under 30-min 1  $\mu$ M HF treatment when most of AR translocated into the nucleus (where AR, GSN, and DAPI signal colocalized around the nucleus), which may be because of weak androgenic activity of HF. As a control, COS-1 cells transfected with cDNA of GSN alone were treated with 1  $\mu$ M DHT, showing no obvious changes in the subcellular localization of GSN (data not shown). Therefore, we did not believe that DHT causes translocation

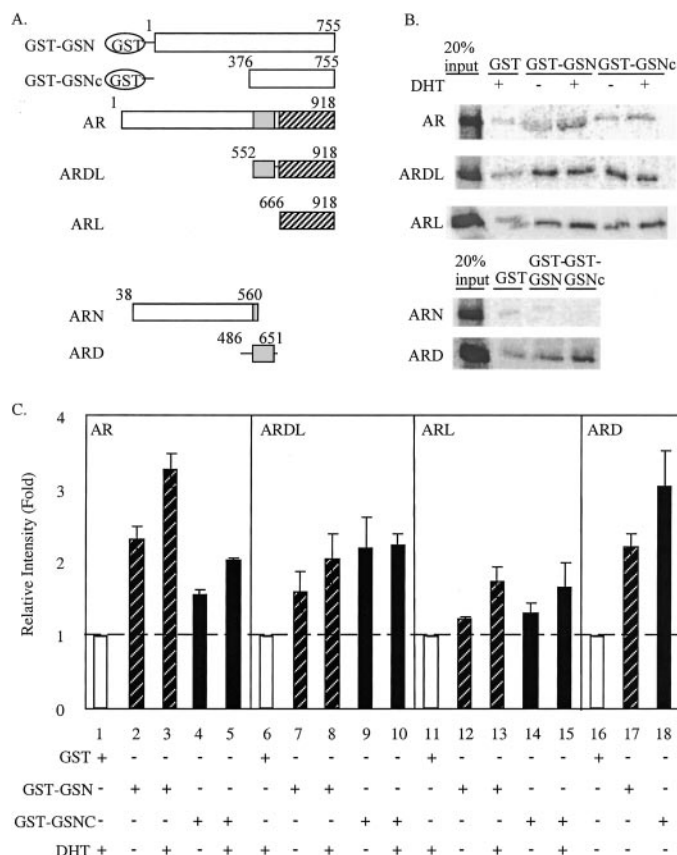


Fig. 2. The interaction domain between GSN and AR. *A*, the diagram of GST-GSN fusion proteins and AR functional domain, ARDL, ARD, ARL, and ARN, used in GST pull-down assay. *B*, GST fusion proteins were expressed and purified by glutathione-conjugated beads. AR fragments *in vitro* translated and labeled by [<sup>35</sup>S]methionine were incubated with GST proteins. Protein complexes pulled down by GST proteins were separated on SDS-PAGE and visualized by PhosphorImager. *C*, the pulled down proteins were quantified by using IQMac v1.2 software, and the relative amount of GST pulled down protein was calculated. Result shown here is from two independent experiments; bars,  $\pm$ SD.

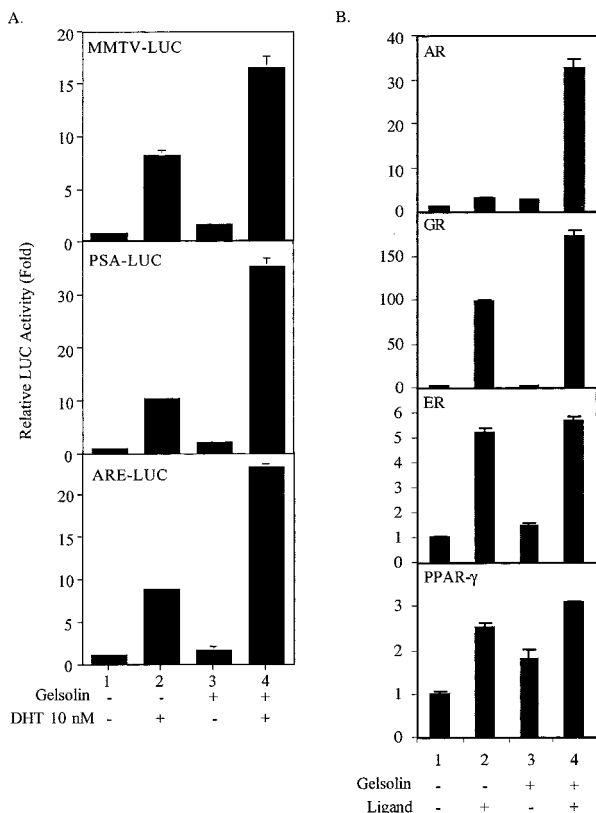


Fig. 3. GSN overexpression enhances transcriptional activity of steroid receptors. A, DU145 cells were cotransfected with pSG5-AR, pSG5-GSN, pRL-SV40, and reporter genes as indicated using SuperFect. Cells were treated with EtOH or DHT and then lysed for LUC activity assay. B, DU145 cells were cotransfected with pSG5-AR, GR, ER- $\alpha$ , or PPAR- $\gamma$ , and their respective reporters, ARE-LUC, MMTV-LUC, ER response element-LUC, and PPAR- $\gamma$  response element-LUC. Cells were treated with EtOH or ligand (10 nM DHT, 10 nM dexamethasone, 10 nM E2, or 1  $\mu$ M 15-deoxy- $\Delta$ 12,14-prostaglandin J2) and then lysed for LUC activity assay. The reporter LUC activity from AR reporter gene was normalized by control LUC activity. After measuring the LUC activity, values relative to Lane 1 were calculated. Results are the mean of three independent experiments; bars,  $\pm$ SD.

of GSN in the absence of AR. These results indicated that GSN interacts with AR temporarily at the time of nuclear translocation of AR under either DHT or HF treatment.

**AR and Gelsolin Coexist in Prostate Cancer Cells and Tissues.** Western blotting assays additionally confirmed that AR and GSN coexist in the same cell. GSN expression can be detected in CWR22R and LNCaP cell lines (Fig. 6A). As CWR22R and LNCaP cell lines are well documented as expressing mutated ARs (37), our data showing GSN expression in these two cell lines demonstrated that AR and GSN coexist in the same cell. In addition to CWR22R and LNCaP cells, GSN also expresses in two other prostate cancer cell lines, PC-3 and DU145, which are AR-negative. Human prostate cancer specimens from patients treated with or without androgen ablation were then used to demonstrate the codistribution of AR and GSN. Both GSN and AR were expressed in the nucleus of some but not all of the cancer cells in the same lesion (Fig. 6E, panels b and d).

**Androgen Ablation Enhances Gelsolin Expression in Prostate Cancer Cells.** To determine whether androgen ablation has an impact on GSN expression, we first used LNCaP cell culture as an *in vitro* assay model. Because HF does not act as an antagonist in LNCaP cells because of endogenous mtAR, we treated LNCaP cells with bicalutamide instead of HF to block androgen action. Then, we showed increased expression of GSN in LNCaP cells treated with bicalutamide (Fig. 6B Lane 2 versus Lane 1). LNCaP cells cultured in androgen-deprived CD-FCS supplemented medium also show in-

creased GSN expression compared with those cultured in androgen containing FCS supplemented medium (Fig. 6B, Lane 3 versus Lane 1). This result suggests that androgen ablation increases GSN expression. We additionally confirmed this observation by using LNCaP xenograft nude mice as an *in vivo* assay model. LNCaP xenografts in castrated nude mice show growth arrest after castration and no apparent regrowth of tumor for up to 6 weeks before harvest (Fig. 6C). In contrast, xenografts in the control group continue to grow after sham operation. Those viable cancer cells that represent LNCaP xenografts were confirmed by H&E staining (Fig. 6D, panels a and b). Immunostaining of GSN in these LNCaP xenograft cells show that GSN expression is much more intense in the xenografts of castrated nude mice (Fig. 6D, panel d) as compared with the control group (Fig. 6D, panel c) suggesting that androgen ablation by castration may

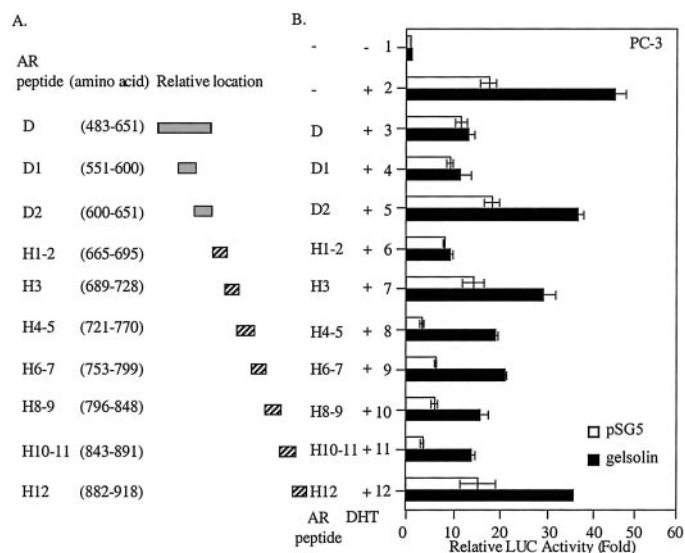


Fig. 4. Overexpression of AR peptides interrupts GSN enhancing AR activity. A, the AR peptide name, the amino acids, and the relative location in the AR protein are shown in the scheme. B, PC-3 cells were cotransfected with AR, pSG5 (□), or pSG5-GSN (■), MMTV-LUC, pRL-SV40, and flag-AR peptides expression plasmids using SuperFect. Cells were treated with EtOH or DHT and then lysed for LUC activity assay as described in Fig. ; bars,  $\pm$ SD.

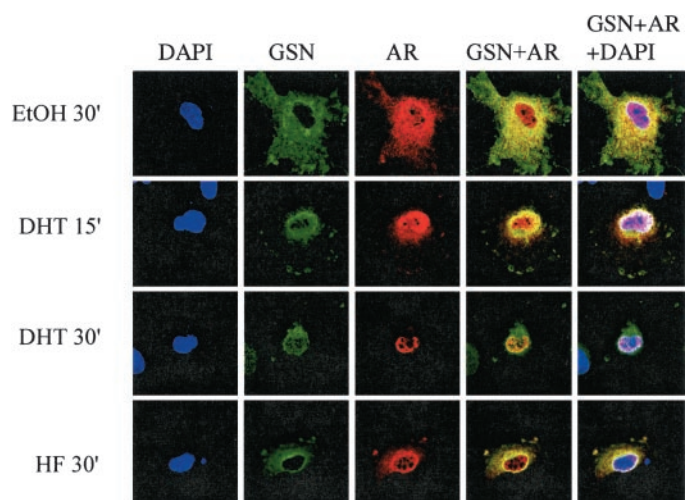


Fig. 5. Time course of subcellular colocalization of AR and GSN after treatment of DHT or HF. COS-1 cells were transfected with expression vectors of full-length AR and full-length GSN for 16 h then cultured in 10% CD-FCS supplemented medium. After 24 h, cells were treated with EtOH, 10 nM DHT, or 1  $\mu$ M HF for the indicated time, and fixed for immunofluorescence study as described in "Materials and Methods." Green fluorescence represents GSN, red fluorescence represents AR, and DAPI represents the nucleus.

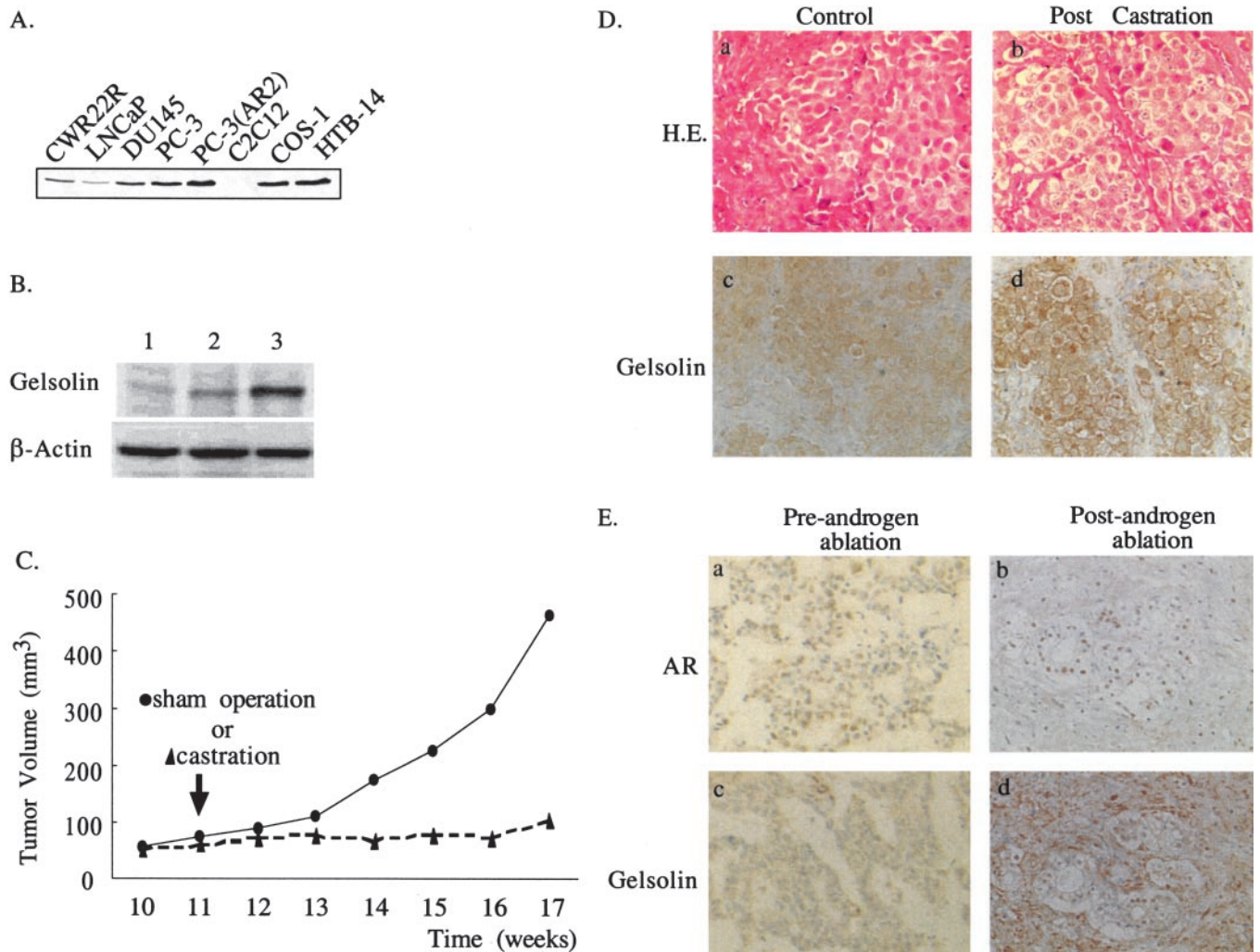


Fig. 6. GSN expression is increased in prostate cancer after androgen ablation. *A*, Western blot analysis for GSN in human prostate cancer cell lines, CWR22R, LNCaP, PC-3, DU145, and other cell lines, C2C12, COS-1, and HTB-14. *B*, LNCaP cells were maintained under 10% normal FCS for 7 days (Lane 1), 10% normal FCS plus 1  $\mu$ M bicalutamide for 7 days (Lane 2), and RPMI 1640 containing 10% CD-FCS for 6 months (Lane 3). Ten  $\mu$ g of protein was loaded on each lane.  $\beta$ -Actin levels were used to assess the integrity of protein lysate and to ensure that equivalent amounts of total protein were loaded. The result was a representative of three independent experiments. *C*, growth curves of LNCaP xenografts in nude mice after castration ( $\blacktriangle$ ,  $n = 3$ ) versus sham operation ( $\bullet$ ,  $n = 3$ ). Data indicate median size of each group. Tumor size was measured weekly before harvest. *D*, LNCaP xenografts in nude mice after castration (*b* and *d*) versus sham operation (*a* and *c*). H&E staining (*a* and *b*). Immunohistochemical staining of GSN (*c* and *d*). *E*, human prostate cancer specimens treated with (*b* and *d*) or without (*a* and *c*) androgen ablation therapy. Immunohistochemical staining of AR (*a* and *b*) and GSN (*c* and *d*).

increase GSN expression. This conclusion was additionally supported using human prostate cancer specimens from patients treated with and without androgen ablation therapy. GSN expression is higher in cancer cells after androgen ablation therapy than in those cells without androgen ablation therapy (Fig. 6E, panels *c* and *d*). Among 16 patients treated with androgen ablation therapy, 9 (56%) patients showed high expression, and 7 (44%) patients showed low expression. Among 11 patients treated with surgery alone, 1 (9%) patient showed high expression, and 7 (64%) patients showed low expression, whereas 3 (27%) patients showed no expression. These results indicated that androgen ablation therapy was significantly associated with high GSN expression in prostate cancer ( $P = 0.0043$ ). For AR, 14 patients (88%) treated with androgen ablation therapy and 8 patients (72%) treated with surgery alone showed high expression. There was no significant association of AR expression with androgen ablation therapy. Nine patients (56%) treated with androgen ablation therapy show high expression of both GSN and AR.

**Gelsolin Reduces the Capacity of HF in Suppressing AR Activity.** We additionally tested the effect of GSN on AR activity in the presence of HF in an agonist mode. HF has a partial agonist effect at

a concentration  $>1 \mu$ M. Cotransfection of GSN and AR into DU145 cells enhanced HF-AR activity in both AR reporter genes, ARE-LUC and PSA-LUC (Fig. 7A). As the results show, HF-AR activity enhanced by overexpressing GSN (Fig. 7A, Lanes 3 and 6, black bars) is similar to DHT-AR activity in the absence of GSN (Fig. 7A, Lanes 2 and 5, white bars). This suggests that increasing expression of GSN in prostate cancer cells after androgen ablation therapy can maintain AR activity, in the presence of HF, to a level similar to that before therapy.

We additionally examined whether GSN could enhance AR in a condition mimicking the combined medical/surgical castration and flutamide treatment in prostate cancer patients. For this experiment, medium containing normal 10% FCS, which contains low levels of androgen, instead of CD-FCS, was used. The degree of AR transactivation in the presence of low-level androgen is shown in Lane 1 of Fig. 7B. Addition of 100 nM HF can then inhibit 80% of AR transactivation (Fig. 7B, Lane 2 versus Lane 1). Further addition of GSN can then enhance the activity of AR and reduce the inhibition capacity of HF on AR activities to 40% (Fig. 7B, Lanes 3–4 versus Lane 1). Because the concentration of HF used here was considerably low, the

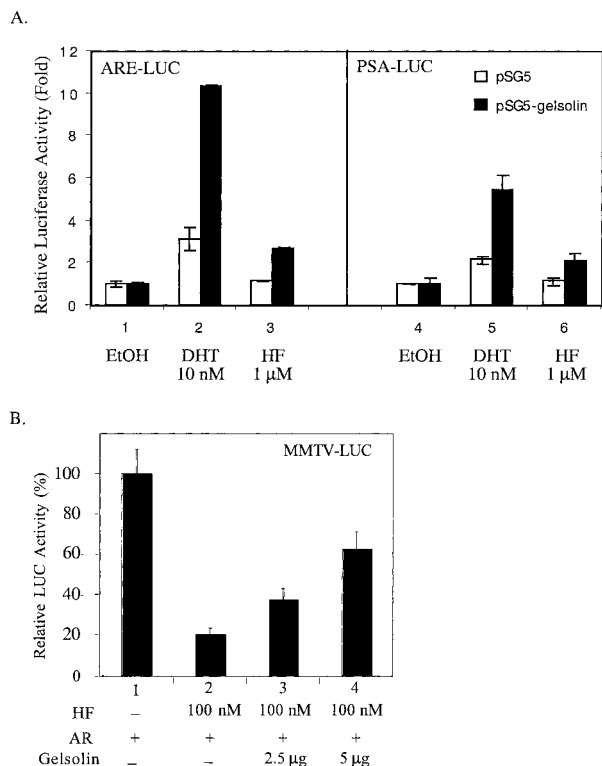


Fig. 7. GSN promotes the AR activity in the presence of HF. *A*, DU145 cells were cotransfected with pSG5-AR, either ARE-LUC or PSA-LUC, and empty vector pSG5 or pSG5-GSN as indicated. EtOH, DHT, or HF was added in the 10% CD-FCS. *B*, DU145 cells were transfected with pSG5-AR, either empty vector pSG5, or pSG5 plus increasing amounts of pSG5-GSN as indicated. EtOH or HF was added in the normal FCS-supplemented medium. Relative LUC activities were calculated using the activity of AR in the absence of pSG5-GSN and HF as 100%; bars,  $\pm$ SD.

enhanced activity of AR by GSN is still smaller than that in the absence of HF. However, this suggests that increasing expression of GSN may help AR gain transcriptional activity during androgen ablation therapy.

## DISCUSSION

Gelsolin is a multifunctional actin-binding protein that has been implicated in cell motility, signaling, apoptosis, and carcinogenesis (29, 30). In this study, we demonstrate a novel function of GSN as an AR-associated protein. Other actin-binding proteins, such as filamin (38) and supervillin (35), have also been characterized to function as AR coregulators and modulate AR activity. Early reports have linked actin-associated proteins to the signal transduction pathway in the nucleus (39, 40). Whereas some reports showed the nuclear localization of GSN in differential endothelial cells (41), immunostaining data suggested that GSN was located mainly in the cytosol. As GSN lacks the nuclear localization signal, it is possible that it could be cotranslocated into the nucleus with binding to other proteins. This is in agreement with our finding that when GSN and AR are cotransfected into COS-1 cells, the GSN was present in and around the nucleus temporarily after DHT treatment (Fig. 5). Therefore, like the other actin binding protein, filamin, GSN may be able to interact with AR at the time of its nuclear localization to facilitate the nuclear translocation of AR (38).

The role of GSN in carcinogenesis is unclear. Several studies reported significantly lower levels of GSN expression in breast, lung, and bladder cancer (42–44). However, higher expression of GSN was reported to be associated with a higher risk of recurrence in lung cancer (45), and was shown in renal cystadenomas and carcinoma in

a murine model of tuberous sclerosis (46). Although a previous study (44) indicated decreased expression of GSN in prostate cancer, all of the clinical samples examined were obtained from patients without androgen ablation therapy. Therefore, their study is not in conflict with our results showing increased expression of GSN in prostate cancer after androgen ablation therapy.

The increased expression of GSN we observed after androgen ablation suggests that signals up-regulating GSN expression may be involved in prostate cancer progression. With findings that GSN functions as an AR-associated protein, we hypothesize that GSN may participate in prostate cancer progression. The mainstream therapy of advanced prostate cancer, androgen ablation therapy with surgical/medical castration plus antiandrogen, reduces androgen concentration and antagonizes androgen AR-induced tumor growth. However, in <3 years, most of advanced prostate cancers become resistant to this type of therapy. One possible explanation could be that surgical/medical castration reduces the androgen concentration but increases the expression level of AR coregulators, such as GSN, in prostate cancer cells (Fig. 6, *D* and *E*). This increased expression of GSN could then enhance the AR activity under HF with low levels of androgen treatment (Fig. 7) to induce the expression of PSA, which is an androgen-regulated clinical marker for prostate cancer. Our hypothesis is that GSN promotes the activity of AR liganded with either HF or small amounts of androgen. If this hypothesis is correct, then blockage of the interaction between AR and GSN could become a valuable therapy for the advanced prostate cancer. In this regard, our data showing that two peptides, D1 (aa 551–600) and H1–2 (aa 665–695) located within AR DBD and LBD, respectively, can block GSN-enhanced AR activity, suggests that these peptides may potentially serve in therapeutic applications for the treatment of prostate cancer progression. These two small peptides may also interfere with functions of other AR coregulators, in particular, those associated with the same site of AR. Although the mechanism of how androgen depletion up-regulates GSN expression remains unclear, manipulation of either association with AR or expression of GSN in prostate cancer may elucidate how GSN participates in prostate cancer progression.

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