Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor

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The androgen receptor (AR) is involved in the development, growth and progression of prostate cancer[1] (CaP). CaP often progresses from an androgen-dependent to an androgen-independent tumor, making androgen ablation therapy ineffective. However, the mechanisms for the development of androgen-independent CaP are unclear. More than 80% of clinically androgen-independent prostate tumors show high levels of AR expression[1]. In some CaPs, AR levels are increased because of gene amplification[2] and/or overexpression, whereas in others, the AR is mutated[3–5]. Nonetheless, the involvement of the AR in the transition of CaP to androgen-independent growth and the subsequent failure of endocrine therapy are not fully understood. Here we show that in CaP cells from a patient who failed androgen ablation therapy, a doubly mutated AR functioned as a high-affinity cortisol/cortisone receptor (AR^{ccr}). Cortisol, the main circulating glucocorticoid, and its metabolite, cortisone, both equally stimulate the growth of these CaP cells and increase the secretion of prostate-specific antigen in the absence of androgens. The physiological concentrations of free cortisol and total cortisone in men[6,7] greatly exceed the binding affinity of the AR^{ccr} and would activate the receptor, promoting CaP cell proliferation. Our data demonstrate a previously unknown mechanism for the androgen-independent growth of advanced CaP. Understanding this mechanism and recognizing the presence of glucocorticoid-responsive AR mutants are important for the development of new forms of therapy for the treatment of this subset of CaP.

Two CaP cell lines with different karyotypes, MDA PCa 2a and 2b, recently established from a bone metastasis from a patient whose CaP showed androgen-independent growth, have been characterized[8,9]. Here, we investigated the mechanism of androgen-independent growth of the MDA PCa 2b cells. Initial experiments using radioligand binding assays with tritiated dihydrotestosterone (DHT), the main prostatic androgen, showed decreased binding by the androgen receptor (AR). Scatchard analyses of the binding of ^{3}H-DHT (Fig. 1*a*) showed that MDA PCa 2b cells expressed ARs at levels similar to those seen in LNCaP cells (a well-characterized, AR-expressing human CaP cell line derived from a lymph node (LN) metastasis[10]), but had a reduced affinity for DHT, to about 2% (dissociation constant (K_d) = 23.3 ± 3.3 nM (n = 3) for MDA PCa 2b; K_d = 0.5 nM for LNCaP). Correspondingly, MDA PCa 2b cells required higher concentrations of DHT for growth stimulation (Fig. 1*b*) than did LNCaP cells, which typically have a bi-phasic growth response to androgens[10]. DHT also induced secretion of prostate-specific antigen (PSA) in MDA PCa

2b cells, with an effective concentration for half-maximum response (EC_{50}) of 100 nM DHT (compared with 0.5 nM in LNCaP cells) (data not shown). These findings demonstrate that MDA PCa 2b cells express a low-affinity AR that is less responsive to DHT than the AR in LNCaP cells in promoting growth and secretion of PSA.

Sequencing of the entire coding region of the AR gene from MDA PCa 2b cells demonstrated two missense mutations in the ligand-binding domain that changed leucine at position 701 to histidine (L701H) and threonine at position 877 to alanine (T877A). As the AR gene is on the X chromosome and as these cells contain only a single X chromosome[9], the two mutations must be on the same allele. Consistently, sequencing analyses of RT–PCR products showed that these two mutations were in the same AR mRNA molecule. Thus, this case differs from a published case in which the two mutations were not found in the same tissue[11].
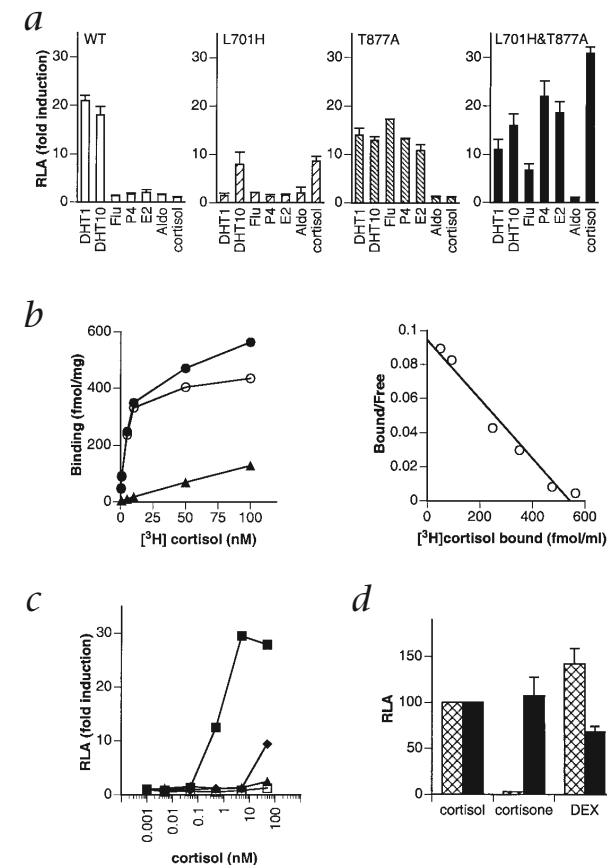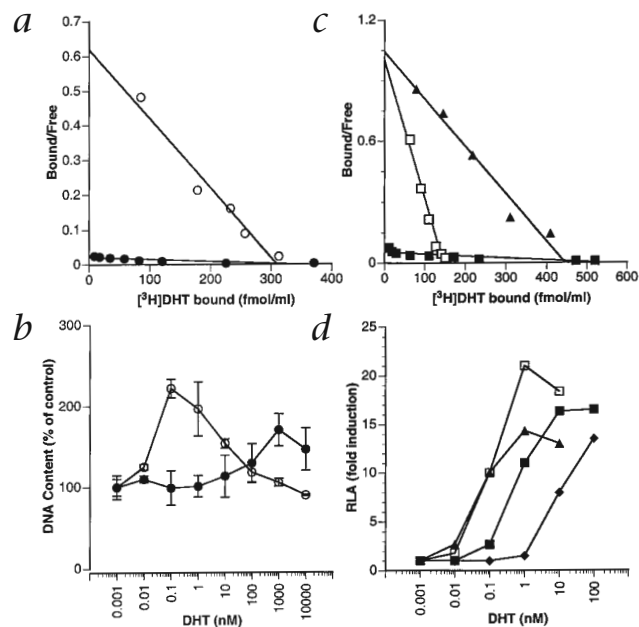
The AR T877A mutation has been identified in LNCaP cells[13] and in some advanced CaP cases[3–5]. The effects of this mutation on AR function have been well documented[13]. Although the AR L701H mutation has also been identified in metastatic CaP specimens[11,12], the biological consequences of this mutation, like those of most AR mutations discovered in metastatic CaP, have not been characterized mainly because of a limited supply of tumor tissue and the difficulty in establishing CaP cell lines.

To determine the individual and combined effects of these mutations on AR function, we recreated the singly mutated AR cDNAs L701H and T877A and AR cDNA with both mutations, L701H&T877A. We expressed the wild-type and mutant AR proteins in COS-7 and CV-1 cells and assessed their ^{3}H-DHT-binding and transactivation properties. The T877A mutant bound DHT with high affinity (K_d = 0.38 ± 0.04 nM), similar to the wild-type AR (K_d = 0.20 ± 0.07 nM) (Fig. 1*c*). In contrast, the L701H mutant failed to show substantial DHT binding. The L701H&T877A double mutant, however, had an affinity for DHT of 2% (K_d =11.80 ± 2.00 nM) that of wild-type AR. In transactivation assays using the androgen-responsive luciferase (Luc) reporter pMMTV–Luc, the T877A mutant had an EC_{50} of 0.04 nM DHT, similar to that of the wild-type AR (Fig. 1*d*), whereas the L701H mutant had an EC_{50} of 10 nM, and the L701H&T877A mutant had an EC_{50} of 0.4 nM. Western blot analyses showed that the expression levels of each mutant AR in transfected cells were similar (data not shown). The luciferase assay (Fig. 1*d*) is more sensitive than the radioligand binding assay (Fig. 1*a* and *c*). Both assays indicate that the L701H mutation decreases the ability of AR to bind and respond to DHT,

Fig. 1 The doubly mutated AR shows decreased androgen binding and responsiveness. ***a***, Scatchard analysis of ³H-DHT binding in LNCaP cells (○; $K_d = 0.5$) and MDA PCa 2b cells (●; $K_d = 23$). ***b***, Growth response to DHT. Control levels (100%) correspond to 8.25 µg and 2.96 µg DNA per well for LNCaP cells (○) and MDA PCa 2b cells (●), respectively. Data represent means ± s.e.m. ($n = 3$). ***c***, Scatchard analysis of ³H-DHT binding to wild-type AR (□), AR T877A (▲) and AR L701H&T877A (■) expressed in COS-7 cells. ***d***, Transactivation assays. CV-1 cells were transiently transfected with pMMTV–Luc and AR expression vectors, as well as pRL–SV40 (Renilla luciferase) to normalize for transfection efficiency. Cells were then treated with DHT and relative luciferase activity (RLA) was determined. □, wild-type; ▲, T877A; ◆, L701H; ■, L701H&T877A. Data represent 'fold induction' over control (no added ligand). The s.e.m. did not exceed 10% for each treatment.

and that ligand binding and androgen responsiveness can be partially restored by the acquisition of the T877A mutation.

As the T877A mutant has a much broader ligand binding profile than the wild-type AR (ref. 13), we examined the transactivation response of each mutant AR to ligands of the class I nuclear receptors, including DHT, progesterone, 17β-estradiol, aldosterone and hydrocortisone (cortisol), as well as an anti-androgen hydroxyflutamide. As predicted from the earlier studies[13], the T877A mutant had substantial transactivation responses to progesterone, 17β-estradiol and hydroxyflutamide (Fig. 2*a*). The L701H mutant responded to DHT and, unexpectedly, to cortisol. The L701H&T877A mutant, however, was activated by all of the ligands except aldosterone. Correspondingly, aldosterone had low binding affinity for the L701H&T877A mutant in competition binding assays. The maximum transactiva-

tion response was with cortisol, and the response of the L701H&T877A mutant to cortisol was much greater (300%) than that of the L701H mutant. Thus, the L701H mutation confers cortisol responsiveness to the AR, and the unique profile of hormone response by the L701H&T877A mutant reflects the combined effects of the two mutations.

As the L701H&T877A mutant showed a greater response to cortisol than to DHT in the transactivation assays (Fig. 2*a*), we re-examined the binding properties of this mutant using ³H-cortisol as the ligand. The L701H&T877A mutant had a specific, saturable and high-affinity binding site for cortisol with a K_d of 4.8 ± 0.1 nM (Fig. 2*b*). This binding affinity was 1,000% greater than the affinity showed by the human glucocorticoid receptor α for cortisol ($K_d = 50$ nM), which we assayed at the same time. Our value is consistent with the K_d reported before[14]. In transactivation assays using the pMMTV–Luc reporter (Fig. 2*c*), the L701H&T877A mutant had an EC$_{50}$ of about 1 nM cortisol, and the L701H mutant had a substantial response to cortisol at a concentration of 10–50 nM. Neither the T877A mutant nor the wild-type AR was responsive to cortisol.

In competition binding assays using ³H-cortisol as the ligand,

Fig. 2 The recreated AR mutants show broadened ligand specificity. ***a***, CV-1 cells were transfected with AR expression vectors (above graphs), the pMMTV–Luc reporter and pRL–SV40 (Renilla luciferase), and were treated with 1 nM or 10 nM DHT (DHT1 and DHT10), or 10 nM hydroxyflutamide (Flu), progesterone (P4), 17β-estradiol (E2), aldosterone (Aldo) or cortisol. Relative luciferase activity (RLA) was assayed. WT, wild-type. ***b***, Specific ³H-cortisol binding (left) and Scatchard analysis (right) of the L701H&T877A mutant expressed in COS-7 cells, showing total binding (●), specific binding (open circles) and nonspecific binding (▲). The calculated K_d is 4.7 nM ($r^2 = 0.969$). ***c***, Transfected CV-1 cells were treated with cortisol, and relative luciferase activity (RLA) was determined. □, wild-type; ▲, T877A; ◆, L701H; ■, L701H&T877A. ***d***, CV-1 cells were co-transfected with pMMTV–Luc and expression vectors for human glucocorticoid receptor α pSG5-GRα (cross-hatched bars) or the L701H&T877A mutant AR (■) along with pRL-SV40. Cells were then treated with 10 nM cortisol, cortisone or dexamethasone (DEX). Data represent relative luciferase activity (RLA; means ± s.e.m.; $n = 3$) due to cortisone or DEX, as a percent of the cortisol-induced activity (set as 100%).
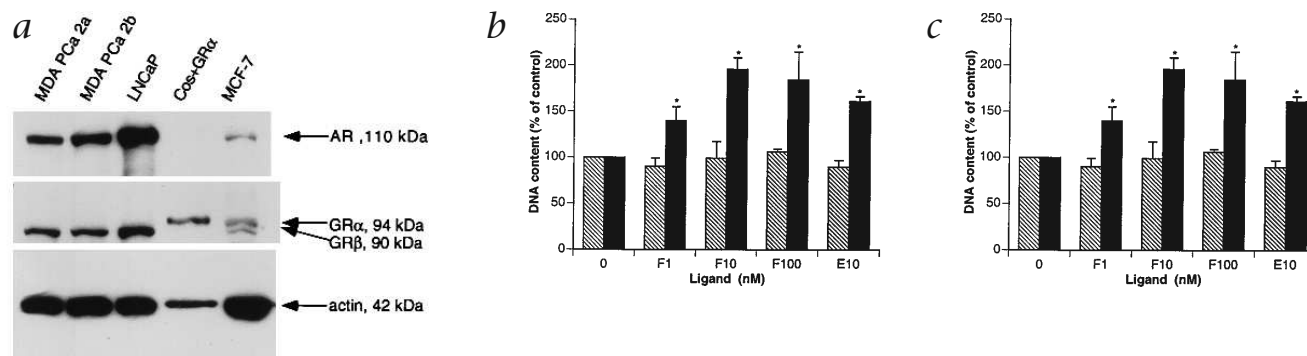
Fig. 3 Glucocorticoids promote growth and secretion of PSA in MDA PCa 2b cells. **a**, Western blot analyses. MCF-7 cells express both glucocorticoid receptors α and β (ref. 20). Above blot, cell lines. Right margin, expected proteins and sizes. GR, glucocorticoid receptor. **b** and **c**, Cells were grown in the presence of 0, 1, 10 or 100 nM cortisol (0, F1, F10 and F100) or 10 nM cortisone (E10) in media 'stripped' of endogenous steroids. ▨, LNCaP; ■, MDA PCa 2b. **b**, Growth, measured by determining the DNA content in each sample. **c**, PSA levels in the conditioned media. Data represent means ± s.e.m. ($n = 3$). *, $P < 0.05$, compared with control.

the L701H&T877A mutant had the highest affinity for cortisol (100%) and cortisone (100%), followed by R1881 (a synthetic androgen; 65%), DHT (41%), dexamethasone (18%), hydroxyflutamide (16%), 17β-estradiol (11%), progesterone (8%), aldosterone (less than1%) and bicalutamide (an anti-androgen; less than1%). Its binding profile for glucocorticoids differs from that of the human glucocorticoid receptor α in that the latter had a higher affinity for dexamethasone, a synthetic glucocorticoid (K_d = 2.2 nM) than cortisol, the natural glucocorticoid. The L701H&T877A mutant also responded to many C_{19} and C_{21} steroids that circulate in the human bloodstream (data not shown), which further distinguishes it from other nuclear hormone receptors. Cortisone, which is a natural metabolite of cortisol and is inactive for human glucocorticoid receptor α, activated the L701H&T877A mutant as efficiently as cortisol (Fig. 2d). This experiment used CV-1 cells, which are deficient in 11β-hydroxysteroid dehydrogenase[15], the enzyme that converts cortisone to cortisol. Thus, the combination of the L701H and T877A mutations effectively transforms the AR into a cortisol/cortisone receptor (AR[ccr]), allowing glucocorticoids to activate androgen-responsive genes in CaP.

To test the effects of cortisol and cortisone on MDA PCa 2b cells, we first determined whether these cells express glucocorticoid receptor, by western blot analysis. Glucocorticoid receptor α was undetectable in MDA PCa 2b cells (Fig. 3a); however, these cells did express glucocorticoid receptor β (Fig. 3a), a truncated form of glucocorticoid receptor that does not bind glucocorticoids or mediate transactivation[16]. In growth studies using androgen-free media (Fig. 3b), both cortisol and cortisone stimulated MDA PCa 2b cell proliferation in a dose-dependent manner. In contrast, neither hormone had any growth-stimulatory effect on LNCaP cells (Fig. 3b), which do not express glucocorticoid receptor α and do not respond to dexamethasone[17]. Furthermore, secretion of PSA, a marker of androgen action, was increased by both cortisol and cortisone in MDA PCa 2b cells but not in LNCaP cells (Fig. 3c). MDA PCa 2a cells also responded to cortisol and cortisone stimulation (data not shown). Our results show that in these CaP cells, cortisol and cortisone, acting through the doubly mutated AR[ccr], promote androgen-independent growth and PSA secretion.

Glucocorticoid activation of a mutated AR may be one of many potential pathways by which CaP cells escape androgen dependence. The mutations described here, L701H by itself or in combination with T877A, convert the AR into a receptor that responds to glucocorticoids by stimulating cell growth and activating androgen-responsive genes, including PSA. PSA is an androgen-dependent marker of CaP progression, and serum PSA levels positively correlate with tumor burden in patients[18]. Our findings indicate the possibility that PSA secretion in some androgen-independent CaPs may reflect glucocorticoid or other hormone stimulation instead of androgen stimulation of the AR. Glucocorticoids circulate at high levels with most cortisol bound to transcortin. In men, the circulating levels of free cortisol (15–45 nM) and total cortisone (39–63 nM) do not decline with age[6,7]. These concentrations exceed the K_d (4.8 nM) of the AR[ccr] receptor and are high enough to sufficiently activate both the L701H mutant and the double-mutant AR[ccr] in transactivation assays. Therefore, these circulating glucocorticoids could substantially activate mutant ARs *in vivo* and promote androgen-independent growth of CaP. We are now testing this using animal models.

To appreciate the frequency with which glucocorticoids promote androgen-independent CaP growth in patients, the prevalence of these AR mutations in metastatic CaP needs to be determined. Although the cells in distant metastases, after androgen ablation fails, often contain AR mutations[1–5], metastatic tissues are not routinely biopsied, and therefore are not readily available.

In conclusion, we have characterized mutations in the AR that may account for a previously unknown mechanism for androgen-independent growth of CaP. Although AR mutants with a variety of 'promiscuities' exist, the recognition of these glucocorticoid-responsive ARs (L701H or L701H&T877A) is an important step in the development of new forms of therapy for the treatment of this subset of androgen-independent CaP.

Methods

Cell culture, cell proliferation and PSA assays. LNCaP and MDA PCa 2b cells were maintained as described[8,9]. Cell proliferation assays were done 6 d after 5×10^4 cells were seeded per 35-mm well and cultured in RPMI-1640 medium supplemented with 5% charcoal-stripped fetal bovine serum and the steroids being studied. Determination of DNA content/well was used as an index of cell proliferation, as described[8]. PSA levels in the conditioned

media were determined by the TOSOH assay, an automated immunoenzymometric assay system (TOSOH Medics, Foster City, California).

Sequencing of genomic DNA. Genomic DNA was isolated from MDA PCa 2b cells, and each exon of the AR gene was amplified by PCR using intronic primers[19]. The PCR products were cloned and DNA was sequenced by the core facility at Stanford University. The data were analyzed using the GCG software (GCG, Madison, Wisconsin). Three independent clones from two preparations of DNA were tested, and the same mutations were found each time.

Sequencing of cDNA after RT–PCR. Total RNA was isolated from MDA PCa 2b cells and cDNA was made using the MuLV reverse transcriptase and an oligo-dT$_{16}$ primer (Roche Molecular Systems, Branchburg, New Jersey). Two gene-specific primers (AR2401, 5'–ACTCTGGGAGCCCGGAAGCTG–3'; and AR3294, 5'–AATGCTTCACTGGGTGTGGAA–3') were used to amplify an intact AR ligand binding domain (exons D–H) by PCR. The RT–PCR product was an 893-base-pair fragment encompassing the AR coding sequence, nucleotides 2401–3294. The RT–PCR products were inserted into a cloning vector, TA-vector (Invitrogen, San Diego, California). A total of 36 clones were screened, and 15 had the 893-base-pair insert. Each positive clone was sequenced in both directions. Every clone contained both L701H and T877A mutations.

Site-directed mutagenesis. Mutations were recreated in the AR cDNA in pSG5-AR (from Z. Culig). pSG5–GR was provided by P. Kushner. The mutants were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). The mutagenic oligonucleotides (Operon Technologies, Alameda, California) used were 5'–GCAGCCTTGCACTC-TAGCCTC–3' for L701H and 5'–GCATCAGTTCGCTTTTGACCT–3' for T877A (mutated bases are underlined). Final constructs were sequenced to confirm the mutations.

Transfection and luciferase assay. Plasmids were transfected into CV-1 (for transactivation) or COS-7 (for ligand binding) monkey kidney cells using lipofectamine (Life Technologies). After this transfection, the CV-1 cells were treated with various ligands for 24 h and luciferase activity was measured using the dual-luciferase assay system (Promega). The experiment was done three times using triplicate wells for each treatment. Triplicate wells contained 1.25 μg pMMTV–Luc (from R. Evans), 0.625 μg expression vectors for AR or glucocorticoid receptor α, and 5 ng pRL-SV40 (as a control for transfection efficiency).

Radioligand binding, Scatchard analysis and competition binding assays. The recreated AR L701H&T877A mutant was expressed in COS-7 cells. Binding assays using ^{3}H-DHT, ^{3}H-cortisol and ^{3}H-dexamethasone, as well as Scatchard analyses using these steroids, were done as described[8]. High-salt extracts (200 μl at a concentration of 0.5–1 mg protein/ml) were incubated with 0–100 nM labeled ligand for 16–20 h at 0 °C. Bound and free hormones were separated by hydroxylapatite. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of a 250-fold excess of unlabeled ligands from the total binding measured in the absence of unlabeled ligands. Competition binding analyses of the double-mutant AR were done in the presence of 20 nM ^{3}H-cortisol with unlabeled ligands at an excess of 1-fold to 100-fold. The relative ability of various compounds to inhibit 50% of ^{3}H-cortisol binding is expressed as the relative binding affinity value, with cortisol set at 100%.

Western blot analysis. Cell extracts containing 50 μg of protein were separated by 4–12% gradient SDS–PAGE and transferred to nitrocellulose membranes. The blots were probed with polyclonal antibodies against AR, glucocorticoid receptors (Santa Cruz Biotechnology, Santa Cruz, California), and actin (Sigma). Peroxidase-conjugated goat antibody against rabbit IgG (Zymed, South San Francisco, California) was used as secondary antibody. The signal was detected using enhanced chemiluminescence (ECL; Amersham).

Statistical analysis. For Fig. 3*b* and *c*, the ANOVA Scheffe's F test was used to assess statistical significance of differences between the treated group and the untreated controls, using the StatView 4.5 program (Abacus Concepts, Berkeley, California). *P* < 0.05 was considered statistically significant.

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