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A MUTATION IN THE LIGAND BINDING DOMAIN OF THE ANDROGEN RECEPTOR OF HUMAN LNCaP CELLS AFFECTS STEROID BINDING CHARACTERISTICS AND RESPONSE TO ANTI-ANDROGENS

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LNCaP prostate tumor cells contain an abnormal androgen receptor system. Progestagens, estradiol and anti-androgens can compete with androgens for binding to the androgen receptor and can stimulate both cell growth and excretion of prostate specific acid phosphatase. We have discovered in the LNCaP androgen receptor a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand binding domain. Expression vectors containing the normal or mutated androgen receptor sequence were transfected into COS or Hela cells. Androgens, progestagens, estrogens and anti-androgens bind the mutated androgen receptor protein and activate the expression of an androgen-regulated reporter gene construct (GRE-tk-CAT). The mutation therefore influences both binding and the induction of gene expression by different steroids and antisteroids.

Interaction of androgens with their target cells is a process which involves an integrated sequence of molecular events. The hormone binds to a receptor and the receptor is transformed to a DNA-binding form that interacts with the hormone responsive genes. Binding of the transformed receptor to the hormone responsive elements of these genes is an essential step in transcriptional activation. Steroid hormone receptors consist of three domains: an N-terminal part, a DNA binding domain and a steroid-binding domain at the C-terminus. The specificity of hormonal action is accomplished both by the specific recognition of the hormone responsive element by the DNA binding part of the receptor and by the specificity of the hormone-receptor interaction, determined by the ligand binding part of the receptor [1].

LNCaP tumor cells derived from a metastatic lesion of a human prostatic carcinoma contain androgen receptors and respond to androgens with growth in cell culture. In addition, increase in growth rate is observed in the presence of low doses of estrogens and progestagens, but these cells do not contain estrogen or progestagen receptors as has been shown previously with

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specific antibodies against these receptor proteins [2,3]. Contrary to expectation, anti-androgens exert striking stimulatory effects on the proliferation of LNCaP cells [4,5]. The androgen receptors in these cells contain an abnormal binding site with significantly increased binding affinity for progestagenic and estrogenic steroids [3,6].

In this paper we report that the abnormal binding characteristics are due to a point mutation in the ligand binding domain of the androgen receptor and demonstrate that both the abnormal binding characteristics and the induction of gene expression by different steroids and antisteroids is entirely due to this mutation.

MATERIALS AND METHODS

Material: [^{3}H]R1881, s.a., 87 Ci/mmol, unlabeled R1881 and R5020 were purchased from NEN (Boston, US). Triamcinolone acetonide (TAA) was obtained from Sigma (St. Louis, US). Anandron (RU 23908) was a gift from Roussel Uclaf (Paris, France). Cyproterone acetate was a gift from Schering (Berlin, FRG), Tamoxifen (ICI 46,474) was obtained from ICI (Cheshire, U.K.). All other steroids were purchased from Steraloids (Wilton, US). [^{14}C]chloramphenicol was obtained from Amersham (Little Chalfont, UK). Butyryl-CoA was obtained from Sigma (St. Louis, US).

Cell culture: The LNCaP prostate tumor cell line was a gift from Dr. Horoszewicz (Buffalo, NY). These cells were cultured as described previously [6]. COS cells and HeLa cells were cultured in Eagles minimal essential medium (GIBCO) supplemented with 5% (v/v) heat inactivated fetal calf serum (Sera Lab), antibiotics, and non-essential amino acids (GIBCO). Media were changed every 3 or 4 days and cells were passaged once a week by plating out trypsinized cell suspensions. Before transfection (COS cells and HeLa cells) or Western blot analysis (LNCaP cells), cells were cultured in medium with 5% dextran-charcoal treated serum.

RNA Preparation: Total cellular RNA was isolated by the guanidinium isothiocyanate method [7]. cDNA was synthesized using 4 μg of total RNA, 100 ng of oligodeoxynucleotide primer (E8: 5'-AAGGCACTGCAGAGGAGTA-3'), 10 units of avian myeloblastosis virus reverse transcriptase (Promega), and 10 units of RNase inhibitor (RNasin;Promega). Synthesis was done according to the standard protocol (Promega).

DNA Amplification and sequencing: Amplification by the polymerase chain reaction (PCR [8]) took place in 100 μl reaction mixtures containing 1 μg of genomic DNA or 2% of the cDNA-synthesis reaction mixture. PCR mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 μmol of each dNTP, 17 μg of bovine serum albumin, 2 units of Thermus aquaticus (Taq) DNA polymerase (Amersham), and 600 ng of each oligonucleotide. Amplification was performed during 24 cycles; each cycle included denaturation for 1 minute at 92°C, primer annealing for 2 minutes at 60°C and primer extension for 1-5 minutes at 70°C. Amplified fragments were made blunt ended and inserted into the SmaI site of M13mp18 [9] prior to sequencing by the dideoxy chain termination method [10].

Construction of the expression vectors: A human androgen receptor -cDNA expression vector (pAR0) was constructed using the SV40 early promoter and the rabbit β-globin poly-A signal [11]. The pARL expression vector was generated by exchanging the 500 bp EcoRI fragment of pAR0 with the mutant 500 bp EcoRI fragment which was obtained from amplified LNCaP cDNA.

Transfection: Transfection of COS and HeLa cells was done by the calcium phosphate precipitation method [12]. For binding studies 5 dishes with each 1.2×10^6 COS cells were transfected with either 20 μg pAR0 or 20 μg pARL and 20 μg pTZ (Pharmacia) carrier plasmid per dish. For immunoblotting studies

$1.2x10^6$ COS cells were transfected with either 20 μg pARO or 20 μg pARL and 20 μg pTZ carrier plasmid. For transcription regulating studies $5x10^5$ HeLa cells were transfected with either 2.5 μg pARO or 2.5 μg pARL and 2.5 μg p29gtkCAT reporter gene [13].(The p29gtkCAT construct was kindly provided by Dr. Renkawitz). Carrier DNA (pTZ) was added to a total of 10 μg per dish.
Western blot analysis: Androgen receptor was immunoprecipitated from LNCaP and COS cells with a monoclonal antibody against the androgen receptor, subjected to SDS-PAGE electrophoresis, blotted and stained for the presence of receptor as described previously [15,16].
Hormone binding assay: COS cells transfected with either pARO or pARL were collected by scraping in buffer, homogenized and a cytosol fraction was prepared as described previously [6]. The cytosol was incubated overnight at 4°C with 5 nM [^{3}H]R1881 in the presence of unlabeled steroids ranging from 0 to 1000-fold the concentration of the label. Separation of bound and unbound steroid was achieved by protamine sulfate precipitation [6].
CAT assays: One day before harvesting the cells, hormones were added to the cells in concentrations ranging from 10^{-12} to 10^{-7} M. The CAT assay was essentially performed as described [17], using the method of xylene extraction of butyrylated chloramphenicol. The CAT activity per mg of extracted protein was calculated. Background CAT activity (no steroid added) was set at 0%. For each steroid tested, the amount of CAT activity/mg protein after extraction of background activity, was expressed as percentage of the highest level of CAT activity/mg protein that was found for cells incubated with R1881. Background activity was about 5% of the highest levels of CAT-activity (at 10^{-9} to 10^{-7} M R1881).

RESULTS AND DISCUSSION

Exons 2 to 8 coding for the DNA-binding domain and steroid-binding domain of the androgen receptor were amplified from genomic DNA isolated from LNCaP cells, using the polymerase chain reaction (PCR) [8]. Each exon was amplified individually using exon flanking sequences as oligonucleotide primers [14].In case of exon 8 the 3' primer was deduced from the 3' untranslated sequence of the mRNA. Sequences of the fragments were found to be identical to the previously published wild type structure with only one exception: an A to G mutation was found in exon 8. This results in an amino acid change (Thr to Ala) in the steroid binding domain at position 868 (Fig 1). LNCaP cells contain two X chromosomes [2]. Five independent clones derived from genomic DNA all contained the mutated sequence (in 2 separate PCR amplifications). Therefore, it is most likely that LNCaP cells are homozygous for the mutated allele. Sequencing of cDNA obtained from mRNA isolated from LNCaP cells confirmed that the mutant receptor is expressed in these cells. (Recently the same mutation was reported by S.E. Harris [18]). Expression vectors containing either the wild type sequence (pARO) or the mutated sequence (pARL) were transiently expressed in COS cells. Competition experiments performed on the cytosols of these cells, showed that the two receptors had similar affinities for androgenic compounds (dihydro-testosterone, R1881), but showed striking differences in a series of non-androgenic compounds (Fig 2 and Table I). Especially progestagens (progesterone, R5020) and estradiol were bound with high affinity. This

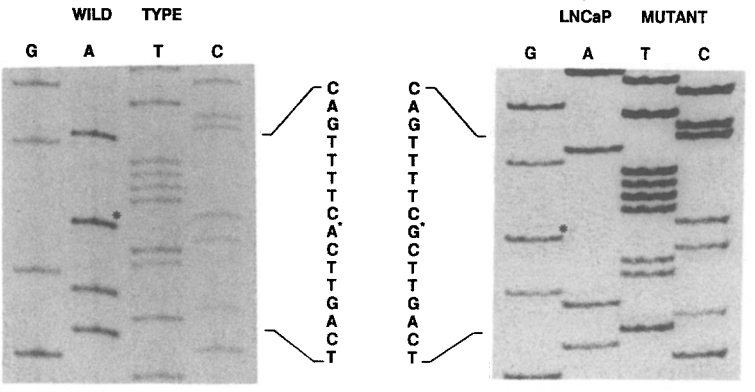

Figure 1.
Sequence comparison of part of exon 8 of the wild type and LNCaP androgen receptor. The asterisks indicate the nucleotide in codon 868 which is an A in the wild type sequence and is substituted by a G in LNCaP sequence.

result indicates that the mutation is responsible for the high affinity of the androgen receptor for these compounds in LNCaP cells. The mutant receptor and wild type receptor, both expressed in COS cells, and the receptor from LNCaP cells were immunoprecipitated with a monoclonal antibody against the androgen receptor. The apparent size of the receptor was 110 kDa on SDS-PAGE (Fig 3), the same as previously found for the androgen receptor in LNCaP cells [19]. This indicates that no major alterations (leading to changed apparent size) of the receptor occur due to the mutation. In addition some bands at lower molecular weight positions were stained, probably due to partial degradation of the receptor in the COS cells.

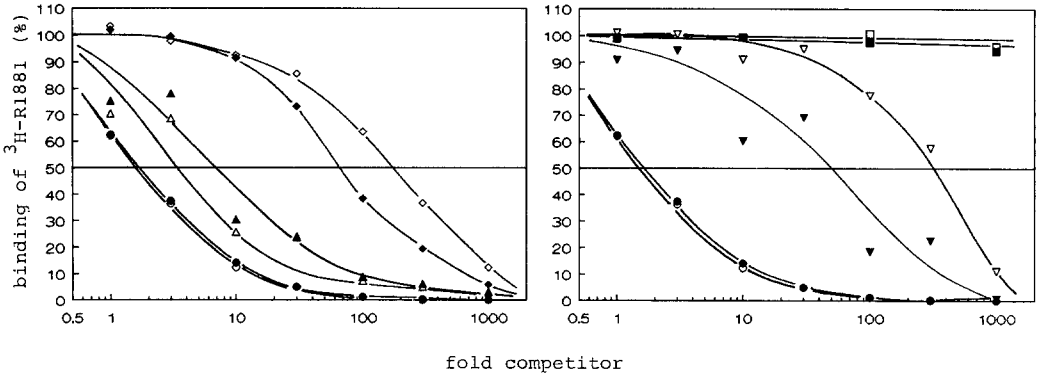

Figure 2.
Competitive binding curves of different steroids for the cytosolic androgen receptor in COS cells transfected with either pAR0 (open symbols) or pARL (closed symbols). The left panel shows: R1881-pAR0 (o); R1881-pARL (•); DHT-pAR0 (Δ); DHT-pARL (▲); estradiol-pAR0 (◇); estradiol-pARL (◆). The right panel shows: R1881-pAR0 (o); R1881-pARL (•);R5020-pAR0 (▽); R5020-pARL (▼); TAA-pAR0 (□); TAA-pARL (■).

Table I
Relative binding affinities of different compounds for the androgen receptor in cytosol fractions of COS cells transfected with either pAR0 or with pARL and, of PC-EW cells (a human prostate tumor cell line), and of LNCaP cells

	RBA value			
	COS cells			
Compound	pAR0	pARL	PC-EW cells	LNCaP cells
R1881	100	100	100	100
Dihydrotestosterone	33.3	29	83	88
R5020	0.5	5	0.3	8.4
Progesterone	0.4	4	0.3	17
Estradiol	1	6	-	2.4
Cyproterone acetate	1.4	2.6	-	4.3
Anandron	0.1	0.4	-	-
Triamcinolone acetonide	<0.1	<0.1	-	<0.1

Competition assays were performed as described in the method section. The relative binding affinity (RBA) is expressed in % as the ratio of the amounts of non-labeled R1881 and competing compound which are needed for 50% inhibition of binding of tritiated R1881. The RBA for R1881 was set at 100% (-, not determined). For comparison, data for PC-EW cells and LNCaP cells are included (from [6]).

Several other mutations of androgen receptors (related to androgen insensitivity syndromes) have been reported, however, these mutations generally lead to decreased or absence of androgen binding affinity for normal sized androgen receptors or absence of binding in the case of mutations leading to receptors of shorter size [20,21].

To investigate whether the mutation described above was not only responsible for the altered binding characteristics of the receptor, but also for the

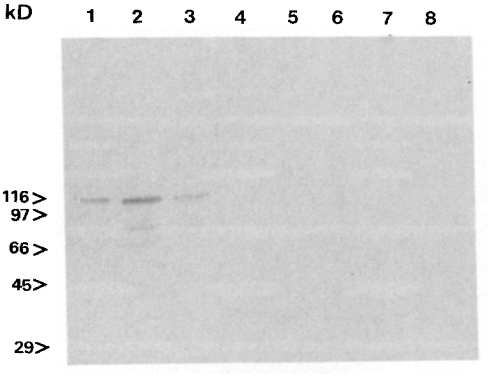

Figure 3.
Immunoblot of androgen receptor immunopurified from LNCaP cells (lanes 1 and 5), from COS cells transfected with either pARL (lanes 2 and 6), or pAR0 (lanes 3 and 7), and COS cells which were not transfected (lanes 4 and 8). Androgen receptors were immunopurified using a specific monoclonal antibody (lanes 1, 2, 3, and 4) or with a non specific antibody (lanes 5, 6, 7, and 8). After SDS-PAGE the proteins were blotted and analyzed with an polyclonal antiserum against the androgen receptor.

stimulatory effects of non-androgenic compounds on the growth rate of LNCaP cells, HeLa cells were co-transfected with pAR0 or pARL and an androgen responsive reporter gene construct. It has been shown that the glucocorticoid responsive element (GRE) can also act as androgen responsive element (see for a review [1]). Therefore, the GRE- driven vector p29gtk-CAT was used for these studies. Androgens (R1881 and DHT) but also progestagens (progesterone and R5020), estradiol, and even anti-androgens (cyproterone acetate and anandron) could induce CAT activity in the cells transfected with pARL, whereas only androgens induced CAT activity in the cells containing the pAR0 construct at low ligand concentrations (Fig 4). The Hela cells we used contain an endogenous glucocorticoid receptor, CAT activity was therefore induced by triamcinolone acetonide both in cells with pAR0 and pARL constructs. Tamoxifen, an anti-estrogen, had no effect on CAT induction.

In conclusion: A single mutation in an essential part of the ligand binding domain of the androgen receptor leads to a decrease in steroid binding specificity and, interestingly, completely reverses the effect of commonly used anti-androgens [22,23]. This mutation provides a tool for further

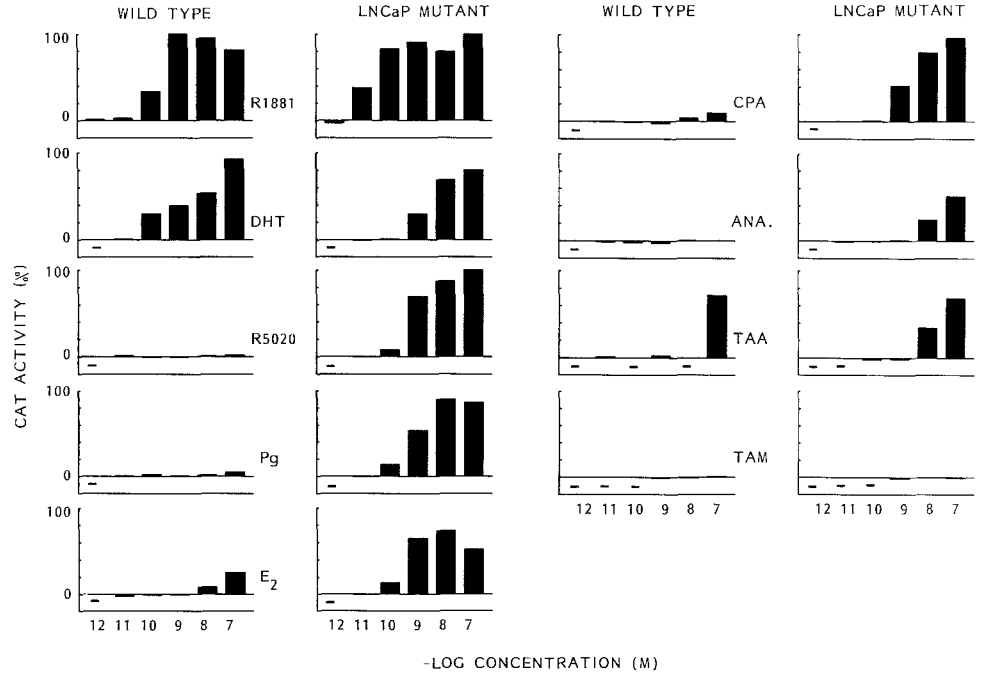

Figure 4.
Induction of CAT activity in HeLa cells after cotransfection with either the wild type androgen receptor or the LNCaP mutant receptor and a GRE-tk-CAT construct. R1881: methyltrienolone; DHT: dihydrotestosterone; R5020: promegestone; Pg: progesterone; E_2: estradiol; CPA: cyproterone acetate; ANA.: anandron; TAA: triamcinolone acetonide; TAM: tamoxifen; -: not determined.

studies on the molecular mechanism of steroid hormone action and anti-androgen blockade of receptor activation and transcription stimulation.

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