The differentiation-related gene 1, *Drg1*, is markedly upregulated by androgens in LNCaP prostatic adenocarcinoma cells

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Abstract A differential display technique was used to identify androgen-regulated genes in LNCaP prostatic adenocarcinoma cells. One of the genes markedly upregulated by androgens proved to be identical to differentiation-related gene 1 (*Drg1*; also described as *RTP*, *Cap43* and *rit42*), a gene whose expression has recently been shown to be diminished in colon, breast and prostate tumors. We show that Drg1 is abundantly expressed in the (androgen-exposed) human prostate and that its expression is stimulated some 14-fold in androgen-treated LNCaP cells. The ligand specificity of the induction reflects the altered specificity of the mutated androgen receptor in LNCaP. In androgen receptor negative tumor lines basal expression is slightly higher than in LNCaP but inducibility is absent. These data suggest that Drg1 is a novel marker of androgen-induced differentiation in the human prostate.

Key words: Differential display; Differentiation; Prostate cancer; Androgen

1. Introduction

Androgens play a pivotal role both in the development of the normal prostate and in the pathogenesis of benign prostatic hyperplasia and prostate cancer, diseases which become increasingly prevalent in our aging Western society [1,2]. Initial studies on the effects and mechanism of action of androgens in the human prostate have focused on secreted proteins such as prostate-specific antigen (PSA) [3]. It is becoming increasingly clear, however, that many other prostatic proteins and functions are under androgenic control [4,5]. We have used a differential display technique to characterize more fully the response of LNCaP prostatic adenocarcinoma cells to androgens [6]. Here we identify one of the PCR fragments upregulated by androgens as Drg1. Initially the only sequence found to be homologous to this fragment was a mouse gene known as *Ndr1* (N-myc downstream regulated gene 1; A. Shimono and H. Kondoh; GenBank acc. no. U60593), a gene isolated by subtraction hybridization between wild-type mouse embryo and mouse embryo carrying an N-myc deletion. The homologous human cDNA has since then be identified in a variety of independent studies also based on the use of differential display. In a first report the corresponding gene was termed RTP (reducing agents and tunicamycin-responsive protein [7]), a homocysteine-respondent gene in vascular endothelial cells. Shortly thereafter it was identified as a differentiation marker of colon epithelial cells, downregulated in colorectal neoplasms (*Drg1*; differentiation-related gene 1 [8]). Very recently an Ni^{2+}-induced gene in lung A549 cells (*Cap43* [9]) and a p53-responsive gene with antiproliferative properties (*RTP/rit42* [10]) proved highly homologous or identical to *RTP* and *Drg1*. In the mean time the mouse gene has also been cloned and characterized as *TDD5*, a gene differentially repressed by testosterone and 5α-dihydrotestosterone (DHT) in a T cell hybridoma [11]. The potential role of the human homologue of *Ndr1*, further conveniently referred to as *Drg1*, as a differentiation marker and its inducibility in a variety of tumor cell lines by apparently unrelated factors stimulated us to explore its response to androgens in LNCaP cells in more detail.

2. Materials and methods

2.1. Cell culture and androgen treatment

The human prostatic adenocarcinoma cell lines LNCaP [12], PC-3 [13] and DU-145 [14] and the breast cancer cell line T47D were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in a humidified atmosphere of 5% CO_2 in air in RPMI-1640 medium (LNCaP, T47D) or in DMEM (PC-3 and DU-145) supplemented with 10% fetal calf serum (FCS), 3 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (all from Life Technologies Inc., Paisley, Scotland) and for T47D: insulin 5 μg/ml. To examine the effects of steroids, cells were preincubated for 2–3 days in RPMI-1640 medium containing 5% FCS pretreated with dextran-coated charcoal (CT-FCS) as previously described [15]. The two recently developed androgen sensitive prostate tumor lines MDA PCa-2a and -2b were kindly provided by Dr. N. Navone and were maintained as described [16]. Androgen responsiveness was evaluated in F-12K medium supplemented with 15% CT-FCS. Natural steroids and dexamethasone were purchased from Sigma Chemical Co (St. Louis, MO, USA). R1881 (methyltrienolone) and mibolerone were obtained from Dupont-New England Nuclear (Boston, MA, USA). Steroids were dissolved in absolute ethanol. Control cultures received similar amounts of ethanol only. Final ethanol concentrations did not exceed 0.1% (v/v).

For the actinomycin D (Sigma Chemical Co., St. Louis, MO, USA) experiment, cells were preincubated for 2 days in RPMI-1640 medium containing 5% CT-FCS, before incubation with fresh medium containing 5 μg/ml actinomycin D for the indicated periods of time.

2.2. mRNA differential display and DNA sequencing analysis

The mRNA differential display polymerase chain reaction (DD-PCR, [17]) was performed using a kit provided by Display Systems (Tandill Ltd, LA, USA) as described earlier [6].

DNA sequencing was performed using Autoread sequencing kits (Pharmacia, Uppsala, Sweden) and an A.L.F. automated sequencer (Pharmacia). The sequences of both strands were determined. Sequences were analyzed using the GCG Wisconsin package provided through the services of the Belgian Embnet Node (BEN). Comparison of DNA homology with the EMBL and the GenBank databases was performed using BLAST [18] and FASTA [19] routines.

2.3. Northern and dot blot analysis

Northern and dot blot hybridizations were carried out as previously described [15]. A radiolabelled Drg1 cDNA probe was prepared as

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follows. The 311 bp DD-PCR fragment (cloned into pGEM-T plasmid; Promega, Madison, WI, USA) was first amplified by PCR using pUC/M13 forward and reverse sequencing primers (Pharmacia, Uppsala, Sweden). Approximately 20 ng of the PCR product was used in a radiolabelling reaction mixture (total volume: 12 μl) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 15 μCi [α^{32}P]-dCTP (3000 Ci/mmol, Amersham International, Buckinghamshire, UK), 50 μM each of dATP, dTTP and dGTP, 0.4 μM 10-mer primer 5′-TGGATTGGTC-3′, 0.4 μM $dT_{11}CG$ primer and 0.025 U/μl Taq DNA polymerase. PCR cycling conditions were as described for differential display analysis [6]. A radiolabelled 18S probe was prepared as described earlier [20]. Hybridization signals were quantified using PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA, USA).

3. Results

3.1. Identification of Drg1 as an androgen-induced gene

One of the DD-PCR bands that was reproducibly induced after treatment of LNCaP cells with 10^{-8} M of the synthetic androgen R1881 was a 311 bp cDNA fragment detected with the 10-mer primer 5′-TGGATTGGTC-3′ and the oligo-dT primer $dT_{11}CG$ (data not shown). This fragment proved 70.4% homologous with the 3′ untranslated region of the mouse Ndr1 cDNA (A. Shimono and H. Kondoh; GenBank acc. no. U60593: bp 2585–2897). During our attempts to sequence the homologous human cDNA, the corresponding sequence was reported by various groups [7–10]. The homology of our 311 bp PCR fragment with these sequences was 97% and a similar homology was observed for the other cDNA sequences available from our laboratory (approximately 1.5 kb) suggesting that the isolated genes were most likely identical.

3.2. Androgen regulation of Drg1 expression in LNCaP cells

Northern blots prepared from LNCaP cells treated or not with androgens were used to study the regulation of Drg1 expression in more detail. The 311 bp cDNA fragment was used as a probe. Dose response curves with R1881 revealed a classical hyperbolic profile (Fig. 1A). Maximal stimulation was observed from a concentration of 10^{-9} M on. In a series of independent measurements at 10^{-8} M R1881 the mean degree of stimulation was 14 ± 3 (mean ± S.D.; $n = 8$). Moreover, the size of the Drg1 mRNA in LNCaP cells (about 3.0 kb) matched that reported for the corresponding mouse and human transcripts (Figs. 2 and 3). Induction became evident after 8–16 h of treatment and culminated after 24 h (Fig. 1B). Thereafter, the level of Drg1 mRNA decreased.

The ligand specificity of the induction reflects the altered specificity of the mutated androgen receptor, characteristic for LNCaP cells [21]. Optimal induction was observed with the synthetic androgens R1881 and mibolerone. The natural and rapidly metabolized androgens 5α-dihydrotestosterone (DHT) and testosterone [22] were somewhat less active (Fig. 1C). Progesterone and estradiol also displayed stimulatory effects. Glucocorticoids were inactive.

Actinomycin D (5 μg/ml) completely prevented androgen induction of Drg1 mRNA after 16 and 24 h, underlining the need for active RNA synthesis (data not shown). To determine whether induction of Drg1 mRNA requires protein synthesis, androgen stimulation was compared in the presence and absence of the protein synthesis inhibitor cycloheximide. At a concentration (1 μg/ml) that clearly prevents stimulation of other androgen-regulated genes such as DBI/ACBP [15], cycloheximide decreased basal expression but did not prevent androgen induction (Fig. 2), suggesting that androgen induction of Drg1 mRNA does not depend on the synthesis of intermediary proteins.

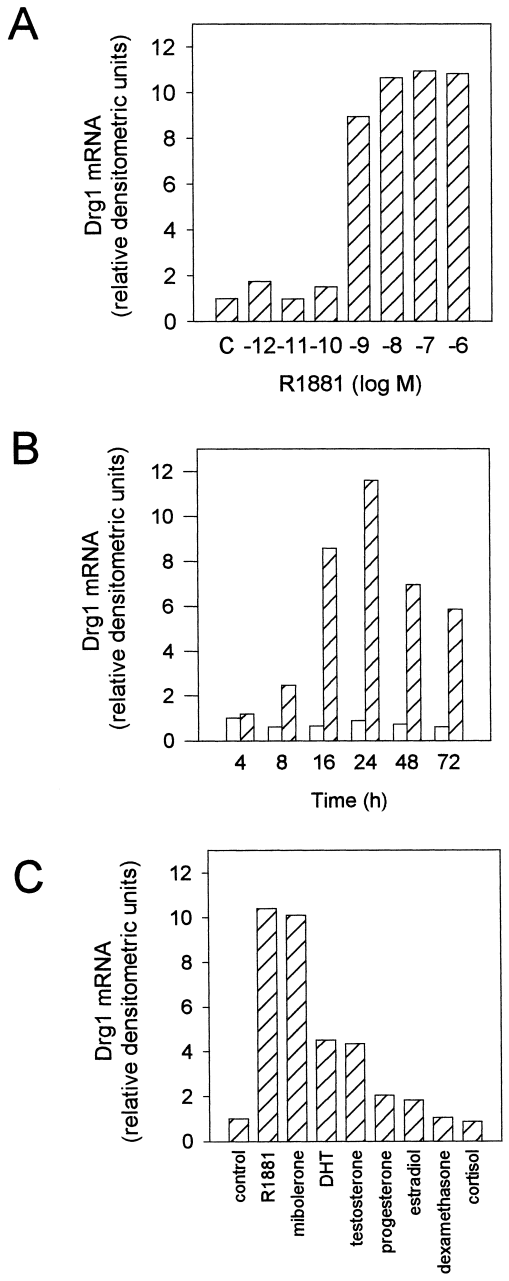

Fig. 1. Androgen dose-dependence (A), time course (B) and steroid specificity (C) of Drg1 mRNA induction in LNCaP. After incubation for 48 h in medium containing 5% CT-FCS, medium was changed and LNCaP cells were incubated with different concentrations of R1881 for 72 h (A), in the absence (open bars) or in the presence (hatched bars) of 10^{-8} M R1881 for the indicated periods of time (B), or for 72 h with 10^{-8} M of the indicated steroids (C). RNA was extracted for dot blot hybridization with a radiolabelled Drg1 probe. Hybridization signals were quantified using PhosphorImaging screens, and mRNA levels were expressed as relative densitometric units, taking the value of vehicle-treated cells as 1.

3.3. Drg1 expression in other cell lines and tissues

To explore the relationship between Drg1 expression, an-

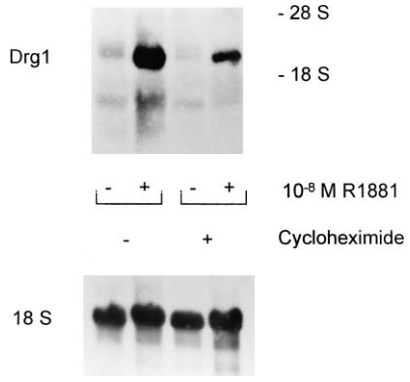

Fig. 2. Effects of cycloheximide on the induction of Drg1 mRNA by R1881 in LNCaP cells. LNCaP cells were cultured for 48 h in medium containing 5% CT-FCS. Medium was changed and cells were incubated for another 24 h without (−) or with (+) 10^{-8} M R1881, either in the absence (−) or in the presence (+) of 1 μg/ml cycloheximide. Twenty μg/lane of total RNA was separated on a denaturing 1% agarose gel, transferred to a nylon membrane and hybridized with a ^{32}P-labelled Drg1 probe (top panel). The positions of Drg1 mRNA and 18S and 28S ribosomal RNAs are indicated. After removal of the probe, the blot was rehybridized with a radiolabelled 18S rRNA probe to demonstrate that similar amounts of RNA were present in all lanes (bottom panel).

drogen responsiveness and degree of differentiation, we also studied basal and androgen-induced expression in two poorly differentiated androgen receptor negative cell lines, PC-3 [13], and DU-145 [14] and in two recently developed androgen receptor positive cell lines (MDA PCa-2a and -2b, [16]). As illustrated in Fig. 3A, PC-3 and DU-145 cells displayed higher basal levels of Drg1 mRNA than LNCaP cells but treatment with 10^{-8} M R1881 did not result in any further stimulation. The MDA cell lines also displayed higher basal expression levels but in these lines a clear response to androgens remained evident (Fig. 3B). The extent of this response, however, was more limited than that observed for LNCaP. Higher basal expression levels and a more limited response to androgens were also observed for other androgen-responsive genes in these cell lines (e.g. PSA; data not shown). Interestingly,

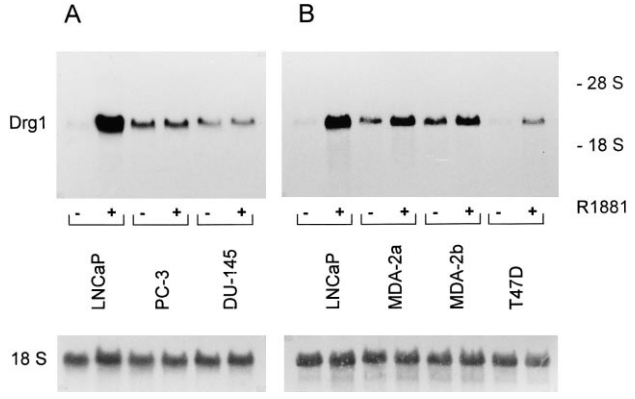

Fig. 3. Expression and androgen regulation of Drg1 in androgen receptor-positive and -negative human cell lines. Drg1 expression in androgen receptor-positive LNCaP cells as compared to (A) androgen receptor-negative cells PC-3 and DU-145, and (B) androgen receptor-positive cells T47D, MDA PCa-2a and -2b. Cells were cultured for 3 days in medium supplemented with 5% (or 15% for MDA PCa-2a and -2b) CT-FCS. Medium was changed and cells were incubated in the absence (−) or in the presence (+) of 10^{-8} M R1881. After 3 days of treatment, Northern blot analysis was performed as described in Fig. 2.

Drg1 expression was also stimulated by R1881 in the breast carcinoma cell line T47D (Fig. 3B).

Northern and dot blot hybridization was used to analyze Drg1 expression in different human tissues. Northern hybridization of a limited number of tissues (spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocytes) yielded data identical to those reported previously [8], including very high expression in the prostate (data not shown). A broader screening using a dot blot containing normalized amounts of poly(A)$^+$ RNA from 50 different human tissues revealed ubiquitous expression with highest levels in prostate followed by spinal cord > substantia nigra > (fetal) kidney > subthalamic nuclei > uterus > medulla oblongata, thalamus, putamen > mammary gland > thyroid gland and placenta (data not shown).

4. Discussion

Using a differential display technique we identified Drg1 as an androgen-induced gene in LNCaP prostatic adenocarcinoma cells. The degree of induction (some 14-fold) is remarkably high. Moreover, comparison of the mRNA expression level in some 50 human tissues revealed the (androgen-exposed) prostate as the organ with the highest level of expression, confirming previous observations on a more restricted number of tissues [8]. Several intriguing points should be noticed. (1) Drg1 (RTP, Cap43, rit42) and its mouse homologue (Ndr1, TDD5) were identified in a variety of experimental settings using differential display [7–11]. The likely explanation is that this gene, despite its ubiquitous expression, is not just a housekeeping gene but that it is subject to marked changes in transcript levels provoked by distinct regulatory factors (reducing agents, tunicamycin, Ni^{2+}, p53, etc.) and that, certainly under stimulated conditions, expression is relatively abundant. The gene appears to be evolutionary well conserved and the limited differences in the reported human sequences can probably be explained by allelic variation. (2) In LNCaP cells Drg1 is upregulated by androgens whereas in a mouse T cell hybridoma and in mouse kidney the homologous gene is downregulated [11]). The response in LNCaP cells is clearly mediated by the androgen receptor as illustrated by the ligand specificity (reflecting the altered specificity of the mutated androgen receptor in LNCaP [21]), the presence of a response in two other androgen receptor positive prostate tumor lines, and the absence of such a response in the receptor negative tumor lines PC-3 [13] and DU-145 [14]. The exact mechanism of gene activation requires further investigation. The fact that actinomycin D blocks induction is compatible with transcriptional control (as also reported in [9]). The finding that cycloheximide reduces basal transcript levels but does not prevent androgen induction resembles earlier observations with PSA and suggests that induction may be direct [15]. Studies on the Drg1 promoter are in progress to analyze its regulation in more detail. It should be noted that the effects of androgens in LNCaP become manifest after a relatively long latent period (8–16 h) as compared to those of all the other inducers described higher (2–4 h). This probably means that several distinct signaling pathways are able to activate Drg1 expression. (3) Earlier data have suggested that Drg1 may be a differentiation marker downregulated in colorectal neoplasms but also in breast and prostate cancers [8,10]. The present data demonstrate that in prostate tumors Drg1 expression

may at least in part reflect androgen responsiveness. Moreover, they show that in poorly differentiated tumor cells Drg1 expression is not completely lost and that basal levels of expression may even be increased. Further studies will be required to define the role of Drg1 in the normal prostate and in prostate cancer. The finding that expression in LNCaP cells is maximal at concentrations of R1881 which promote differentiation and reduce proliferation ($>10^{-9}$ M), rather than at low androgen levels that are optimal for proliferation (10^{-10} M) [23–26], is compatible with a growth inhibitory role observed in other systems [10]. Whether this relationship is casual or causal, however, remains to be investigated.

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