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Changes in Androgen Receptor Nongenotropic Signaling Correlate with Transition of LNCaP Cells to Androgen Independence

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ABSTRACT

A cure for prostate cancer (CaP) will be possible only after a complete understanding of the mechanisms causing this disease to progress from androgen dependence to androgen independence. To carry on a careful characterization of the phenotypes of CaP cell lines before and after acquisition of androgen independence, we used two human CaP LNCaP sublines: $LNCaP_{nan}$, which is androgen dependent (AD), and LNCaP-HP, which is androgen independent (AI). In AD $LNCaP_{nan}$ cells, dihydrotestosterone (DHT) stimulated in an androgen receptor (AR)-dependent way a phosphorylation signaling pathway involving steroid receptor coactivator (Src)–mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)-1/2–ERK-1/2–cAMP-response element binding-protein (CREB). Activation of this pathway was associated with increased [^{3}H]thymidine incorporation and resistance to apoptosis. Use of dominant-negative forms of MEK-1/2 and CREB demonstrated in $LNCaP_{nan}$ cells that DHT induced [^{3}H]thymidiine incorporation through a thus far unidentified molecule activated downstream of MEK-1/2, and antiapoptosis through phosphorylation of the transcription factor CREB. In contrast, in AI LNCaP-HP cells, the Src–MEK-1/2–ERK-1/2–CREB pathway was constitutively active. Because it was not further stimulated by addition of DHT, no increase of [^{3}H]thymidine incorporation or apoptosis resistance was demonstrated in LNCaP-HP cells. Additional experiments showed that Src and the scaffold protein MNAR coimmunoprecipitated with AR, indicating a role for Src as an apical molecule in the Src–MEK-1/2–ERK-1/2–CREB pathway. Interestingly, differences between the two cell lines were that in LNCaP-HP cells presence of an AI phenotype and lack of response to DHT were associated with constitutive activation of the protein kinase Src and interaction among Src, AR, and MNAR. In contrast, in $LNCaP_{nan}$ cells, presence of an AD phenotype and ability to respond to DHT were associated with DHT-dependent activation of Src kinase activity and interaction among Src, AR, and MNAR. Intriguingly, in $LNCaP_{nan}$ cells, we found that transcription through the prototypical CREB-responsive promoter c-*fos* could be induced in a DHT-dependent way, and this action was inhibited by the AR antagonist Casodex and MEK-1 inhibitor PD98059. In contrast, transcription through the PSA P/E promoter, a prototypical AR-dependent promoter directly activated by agonist, was obliterated only by Casodex. Additional experiments with genital skin fibroblasts derived from patients with a variety of AR abnormalities indicated that nongenotropic AR signaling does not depend on an intact DNA-binding domain or on the ability of AR to translocate to the nucleus. The results suggest the following: (1) Constitutive activation of the Src–MEK-1/2–ERK-1/2–CREB pathway is associated with the AI phenotype observed in LNCaP-HP cells. (2) Activation of the Src–MEK-1/2–ERK-1/2–CREB pathway is DHT dependent in AD $LNCaP_{nan}$ cells. (3) DHT activation of this pathway is associated with induction of [^{3}H]-thymidine incorporation by a molecule activated downstream of MEK-1/2 and of antiapoptosis through activation of the transcription factor CREB in AD $LNCaP_{nan}$ cells. (4) AR regulates transcription either directly upon ligand binding and nuclear translocation or indirectly through kinase pathways leading to activation of downstream transcription factors. (5) Nuclear translocation and ability of the DNA-binding domain of AR to interact with DNA are not prerequisites for nongenotropic AR activity.

INTRODUCTION

As a member of the superfamily of nuclear receptors, androgen receptor (AR) is a ligand-dependent transcription factor controlling the expression of specific genes (1). At least 60 minutes are necessary for AR to alter gene expression (2). Primarily, positive or negative AR-modulated gene transcription involves an initial phase of ligand binding to the receptor in the cytoplasm, followed by nuclear translocation, contact with the promoter of AR-dependent genes, and the general transcription machinery through a number of coactivators or corepressors. However, increasing evidence indicates that signal transduction pathways known to trigger cell proliferation are activated by steroid receptors by mechanisms other than direct receptor modulation of gene expression (2). This alternative mechanism of action has been dubbed "rapid" or "nongenomic" signaling and in some cases is measurable within seconds after the addition of ligand to cultured cells (3). Rapid signaling by steroid hormone receptors was identified 37 years ago and has now been reported for nearly all nuclear receptor family members (2, 4), including glucocorticoid (5), mineralocorticoid (6), estrogen (ERα and ERβ; refs. 7 and 8), progesterone (9, 10), and ARs (11–15). With the discovery that steroid receptors prevent bone loss in mice (16) and induce *Xenopus* oocyte maturation (14) by using a rapid signaling mechanism, these alternative pathways of steroid hormone action have been firmly established as having important physiologic roles. With the recent identification of a membrane progesterone receptor with the structure of a G protein (17, 18), the potential importance of nongenotropic signaling of steroid receptors has increased even further.

Several breakthroughs have revealed important new insights into the mechanism of AR action, and much of this new knowledge has been applied to the field of prostate cancer (CaP) development and progression. The prostate is a quintessential AR-dependent organ. Clearly, the presence of a functional AR and of normal levels of agonists are *conditio sine qua* for prostate development to the normal adult phenotype (19, 20). Maintenance of the prostate during adulthood also depends on the presence of physiologic concentrations of these ligands, and castration is associated with abrupt prostate involution (apoptosis; ref. 21).

AR signaling plays at least a permissive role in the development of CaP. For instance, some animal models of CaP require normal level of androgens (endogenous or exogenous) for CaP to develop (22), whereas the lack of androgens observed in the eunuch population of China was associated with a lack of CaP development (23). Furthermore, a positive correlation exists between the development of CaP and AR activity. For example, increased AR expression and signaling in the prostate are associated with increased precancerous lesions in a transgenic model (24). *In vitro* experiments have shown that a short-

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ened AR polyglutamine tract is associated with increased AR transcriptional activity (25), and men carrying ARs with shortened polyglutamine repeats have an increased risk of developing CaP (26), metastatic disease (26), and high histologic grade disease (26) at a younger age (27).

In view of this relationship between AR and CaP, one of the most pressing issues in the field is to unravel how this disease becomes androgen independent (AI) in patients who have failed hormonal ablative treatments (28). Many AR-based possibilities have been proposed to explain the development of AI CaP (29). It is becoming clear that AR continues to be active in AI cancers (30), because AR-dependent molecules such as the prostate-specific antigen continue to be produced in the androgen-depleted milieu of patients who underwent androgen ablative treatments (31). AI CaP may involve a mutated AR (32), ligand-independent AR activation (33, 34), altered expression of a variety of coactivators (35), increased AR expression (36), or intraprostatic levels of androgens that retain the ability to activate AR (37). Nevertheless, the possibility that AR may function through mechanisms alternative to the conventional genotropic mode of action (11–14) is intriguing and has not been considered in the context of CaP or in the context of CaP transition to androgen independence. Nongenotropic signaling of AR would open not only a new spectrum of potential mechanisms through which CaP develops and becomes AI, but also a new spectrum of therapeutic targets to treat this deadly disease. A complete understanding of the signaling pathways activated by AR in a nongenotropic fashion could help unravel the intricacies of CaP transition to androgen independence. Based on these assumptions, we initiated a systematic approach to identify pathways of rapid intracellular signaling initiated by AR in CaP cell lines.

MATERIALS AND METHODS

Materials. Chemicals were from Sigma (St. Louis, MO) unless stated otherwise. Hybond ECL nitrocellulose membranes were from Pharmacia Biotech (Piscataway, NJ). The chemiluminescent signals were detected using Pierce West PICO chemiluminescent substrate (Pierce, Rockford, IL). Antibodies included the following: AR (Santa Cruz Biotechnology, Santa Cruz, CA), total extracellular signal-regulated kinase (ERK)-1/2 (Santa Cruz Biotechnology), phospho-ERK-1/2 (Thr^{202}/Tyr^{204}; Cell Signaling, Beverly, MA), mitogen-activated protein/ERK kinase (MEK)-1/2 (Cell Signaling), phospho-MEK1/2 ($Ser^{217/221}$; Cell Signaling), phospho-steroid receptor coactivator (Src; Tyr^{416}; Cell Signaling), total Src (Santa Cruz Biotechnology), cAMP-response element binding-protein (CREB; Cell Signaling), and phospho-CREB (Ser^{133}; Cell Signaling), the monoclonal FLAG M2 peroxidase antibody was from Sigma, and monoclonal and polyclonal β-galactosidase antibodies were from Promega (Madison, WI) and ICL (Newberg, OR). Dihydrotestosterone (DHT) was from Steraloids (Newport, RI). The MEK inhibitor PD98059 was from Sigma and was used at a final concentration of 50 μmol/L. The Src inhibitor PP1 was from AG Scientific (San Diego, CA) and was used at the final concentration of 10 μmol/L. Casodex (bicalutamide) was a gift from Dr. B. Vose (ICI Pharmaceuticals, Macclesfield, United Kingdom) and was used at a variety of concentrations.

Cell Lines and Plasmids. Two subclones of LNCaP cells were used in this study. $LNCaP_{nan}$ were purchased from the American Type Culture Collection (Manassas, VA) and immediately used for the majority of experiments described in this paper. In contrast, LNCaP-high passage (LNCaP-HP) cells were purchased from American Type Culture Collection and passaged more than 60 times before use. All sublines of LNCaP cells were grown in RPMI 1640 in the presence of 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Genital skin fibroblasts strain 1003, 881, and 1017 were derived from patients with a normal male phenotype (1003) or from patients affected by a phenotype of complete androgen insensitivity (881 and 1017). The AR sequences of these cell lines and the functional characterization of the mutations detected have been published previously (38, 39). Genital skin fibroblast strain 1003 has a wild-type AR sequence. Genital skin fibroblast strain 881 contains a mutation inactivating the DNA-binding domain of AR (C574R). Due to a mutation at a splice acceptor site at the 5′ boundary of exon 4, immunoreactive AR was not detected in strain 1017 (38), however immunoreactive AR was present in both strains 1003 and 881 (40). For transcription assays, the following reporter plasmids were used: pSVOAΔ5′ (a gift from Brent Cochram, Tufts University School of Medicine), consisting of a 379-bp murine c-*fos* promoter containing a cyclic AMP-responsive element driving a *luciferase* reporter gene (41); PRL-CMV-TK (Promega, Madison WI), which contains the Renilla luciferase cDNA under the control of the constitutively active CMV promoter; and PSA P/E-luc, in which the luciferase reporter is driven by the AR-dependent 2.4-kb PSA enhancer and 564-bp PSA promoter (31). Hemoagglutinine-tagged MEK-1/2 expression plasmids were a gift of Dr. Natalie Ahn (University of Colorado, Boulder) and consisted of constructs with a wild-type sequence or a dominant-negative mutation (K97M; ref. 42). Wild-type and dominant-negative (containing R287L and R288L mutations, and an insertion of a D in position 290) CREB plasmids, were from BD Biosciences (San Jose, CA; ref. 43). A NH_2-terminally full-length FLAG tagged MNAR plasmid was a gift of Dr. Boris Cheskis (44).

***In vivo* Experiments.** $LNCaP_{nan}$ or LNCaP-HP (5×10^5) were dissolved in 100 μL of a solution containing RPMI 1640/Matrigel 80/20%. Cells were inoculated subcutaneously in the back of six male nu/nu mice, which had previously been castrated. Two inoculations/mouse were performed. Mice were monitored every 3 days for the appearance of subcutaneous xenografts for 30 days. Xenograft size was measured using a caliper, and volumes were calculated by the equation $m_1^2 \times m_2 \times 0.5236$ (where m_1 and m_2 are the smallest and largest diameters; ref. 45).

Immunoblotting. Cells were plated to 80% confluence and allowed to grow for 48 hours. They were serum-starved in the absence of phenol red for 24 hours before being stimulated with steroids or epidermal growth factor (EGF) for the amount of time and at the concentrations indicated for each experiment. In some experiments, inhibitors were administered 60 minutes before the agonists. Cells were harvested in cold PBS, and cell pellets were lysed in a buffer containing 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrphosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L sodium vanadate, and freshly added 1 μg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride. The lysate was centrifuged at $10{,}000 \times g$ for 10 minutes. Approximately 30 μg of protein from the supernatant were loaded per lane. Immunoblot analysis was performed with the antibodies listed above.

Immunofluorescence. Genital skin fibroblast cells strain 1003 and 881 were cultured in minimum essential medium + 10% FBS and 1% penicillin and streptomycin in 6-well plates containing coverslips (1×10^5 cells/well). The day before the experiment, cells were changed to charcoal-stripped FBS. On the day of the experiment, cells were treated for 3 hours with vehicle alone or 10 nmol/L DHT to investigate AR genotropic signaling. After the experiment, cells were washed twice in ice-cold PBS and fixed with 4% formaldehyde in PEM buffer (800 mmol/L PIPES, 5 mmol/L EGTA, and 2 mmol/L $MgCl_2$) for 30 minutes on ice (1:4 dilution of 16% stock). After fixation, cells were washed three times in PEM, incubated with 1 mL of 0.1 mol/L NH_4Cl for 10 minutes to quench autofluorescence and permeabilized with PEM + 0.5% Triton X-100 for 5 minutes. Coverslips were blocked with 5% powdered milk in Tris-buffered saline-tween 20 (TBS-T) buffer plus 0.02% sodium azide for 1 hour at 4°C and then incubated with a polyclonal rabbit AR antibody from Santa Cruz Biotechnology diluted at 1:2500 in 5% BSA overnight at 4°C. After washing off the primary antibody with TBS-T [20 mmol/L Tris-HCl (pH 7.4), 137 mmol/L NaCl, and 0.1% Tween-20), a secondary antibody [Alexa Fluor 546, goat antirabbit IgG (H+L) showing red fluorescence; A-11010; Molecular Probes] diluted in blocking buffer was incubated for 30 minutes at room temperature. After removing the secondary antibody, slides were washed five times with TBS-T and PEM buffers and fixed in 4% formaldehyde. To quench autofluorescence, coverslips were incubated with 1 mL of 0.1 mol/L NH_4Cl for 10 minutes; they were then incubated with PEM + 0.5% Triton X-100 for 5 minutes and counterstained with 1× 4′,6-diamidino-2-phenylindole diluted in TBS-T for 1 minute. A Z-series (0.2-μm steps) of optical sections was digitally imaged on a Delta Vision Deconvolution Microscopy System (Applied Precision, Inc., Issaquah, WA) and deconvolved using a constrained iterative algorithm to generate high-resolution images. All image files were digitally processed for presentation in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Shown in each image is a single focal plane from typical cells. In this experiment, AR shows red fluorescence, and DNA is blue.

c-*fos* and PSA P/E Luciferase Assay. In experiment 1, $LNCaP_{nan}$ cells were plated at a concentration of 0.21×10^6 cells per well in a 12-well plate (Falcon, Franklin Lakes, NJ). The next day, cells were transfected with 1 μg of plasmid pSVOAΔ5′ and 20 ng of the control plasmid PRL-CMV-TK using Tfx-50 (Promega) at 1:2 ratio (plasmid:Tfx) in medium without FBS and antibiotics. One hour later, medium containing serum was added, and the cells were allowed to grow for 24 hours. On day 2, the cells were placed in serum-free, phenol red-free medium for 24 hours. Cells were then stimulated with either 10 nmol/L DHT or 1 μg/mL EGF for 30 minutes. The stimulation time was chosen because the peak rate of CREB-dependent transcription occurs after 20 to 30 minutes (46). In experiment 2, cells were treated in the presence or absence of the antagonists (10 μmol/L Casodex or 50 μmol/L PD 98059) for 60 minutes before the addition of 10 nmol/L DHT. In experiments 3, $LNCaP_{nan}$ cells were transiently transfected with wild-type or dominant-negative CREB expression plasmids in addition to plasmids pSVOAΔ5′ and PRL-CMV-TK and then subjected to the same experimental conditions of experiment 1. Luciferase assays were performed using the Dual-Luciferase Reporter Assay kit (Promega) as per the manufacturer's protocol. The results were expressed as the ratio of luciferase over Renilla activity. Each experiment represents the average from three different wells $\pm$ SD and was repeated a minimum of three times with similar results. $LNCaP_{nan}$ cells from experiment 3 were subjected to Western analysis to confirm that as a result of the transient transfection CREB was overexpressed.

For the PSA P/E luciferase assays, $LNCaP_{nan}$ cells were plated at a concentration of 0.21×10^6 cells per well in a 12-well plate. The next day, cells were transfected with 1 μg of plasmid PSA P/E-luc and 20 ng of the control plasmid PRL-CMV-TK using Tfx-50 (Promega) at 1:2 ratio (plasmid to Tfx) in medium without FBS and antibiotics. One hour later, medium containing serum was added, and the cells were allowed to grow for 24 hours. On day 2, cells were placed in serum-free, phenol red-free medium for 24 hours. Cells were then treated with vehicle, DHT (10 nmol/L), PD98059 (50 μmol/L), or Casodex (10 μmol/L) given 60 minutes before DHT (10 nmol/L) for 8 hours. Cells were then harvested, and luciferase assay was performed as discussed above.

Src Kinase Assay. $LNCaP_{nan}$ or LNCaP-HP cells were serum starved for 48 hours and then incubated with DHT (10 nmol/L) or vehicle for 5 minutes. Cells were washed twice with ice-cold PBS and lysed in 1 mL of lysis buffer for 20 minutes with rocking. Lysis buffer was as follows: 1% NP40, 50 mmol/L HEPES (pH 7.2), 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonylfluoride. Lysates containing 1 mg of proteins were incubated with anti-Src antibody (Santa Cruz Biotechnology sc-18) for 2 hours at 4°C, and immunocomplexes were bound to GammaBind Sepharose beads for 1 additional hour with mixing. Pellets were washed with lysis buffer and assayed for their ability to phosphorylate acid–denaturated enolase as described by Cooper *et al*. (47). Results were visualized by autoradiography and quantitated using densitometry.

Immunoprecipitation. $LNCaP_{nan}$ or LNCaP-HP cells were transiently transfected with 2 μg of FLAG-MNAR plasmid using TransFectin (Bio-Rad, Hercules, CA). Two days post-transfection, cells were starved for 24 hours and then treated with vehicle or 10 nmol/L DHT for 5 minutes. Cells were then lysed in 100 μL of ice-cold lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2 EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L $Na_2H_2P_2O_7$, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, and 1 μg/mL leupeptin on ice. Three hundred μg of lysate protein were suspended in 250 μL of binding buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10% glycerol, 0.05% NP40, 1 $\times$ protease inhibitor (Sigma), and 1 mmol/L phenylmethylsulfonyl fluoride. The FLAG-AR-Src complex was immunoprecipitated overnight at 4°C either with a polyclonal AR antibody (Santa Cruz Biotechnology) plus 5 mL of Ultralink immobilized protein A (Pierce) or with monoclonal anti-FLAG M2 agarose affinity gel (Sigma). Parallel experiments were run as a control, in which the protein lysate was immunoprecipitated with a polyclonal or a monoclonal control antibody (both control antibodies were raised against β-galastosidase). The immunocomplex was washed five times with cold binding buffer, suspended in 1$\times$ loading buffer, and separated on 7.5% acrylamide-SDS gel and transferred to PVDF membrane (Bio-Rad). The portion above M_r 90,000 was probed either with the anti-AR antibody or with anti-FLAG M2-peroxidase conjugate (Sigma). The portion below M_r 90,000 was probed with anti-Src antibodies. To minimize detection of IgG heavy chain in immunoblot-containing material immunoprecipitated with the polyclonal anti-AR antibody, a monoclonal anti-Src was used (Santa Cruz Biotechnology). Conversely, when immunoprecipitation was done with the monoclonal anti-FLAG antibody, a polyclonal anti Src was used (Santa Cruz Biotechnology).

Prevention of Staurosporine-Induced Apoptosis. In experiment 1, 1×10^4 LNCaP-HP and $LNCaP_{nan}$ cells were plated in a 96-well plate. After 1 d, cells were placed in medium without serum and phenol red. After 1 d, cells were treated with staurosporine (STS; 100–400 nmol/L) for 3 hours. In parallel experiments, cells were treated with DHT (10 nmol/L) or EGF (5 ng/mL) alone or together with STS (which was added to the plate 5 minutes after DHT or EGF). In additional experiments, $LNCaP_{nan}$ cells were transiently transfected with wild-type or dominant-negative CREB expression plasmids (experiment 2) and subjected to the same experimental conditions of experiment 1. Cells were then subjected to the Cell Death Detection ELISA^{PLUS} (Roche Diagnostic Corporation, Indianapolis, IN) using a GENios Multi-Detection Reader (Phenix, Hayward, CA). Results are expressed as A_{405} and corrected according to the manufacturer's specification. They represent the average $\pm$ SD of three plates and were repeated a minimum of three times. A similar experiment was performed with genital skin fibroblast cell lines 881, 1003, and 1017, with the only difference being that these cell lines were incubated with STS for 4 instead of 3 hours.

Thymidine Incorporation Assay. In experiment 1, 5×10^4 $LNCaP_{nan}$ or LNCaP-HP cells/well were seeded in a 12-well plate on day 0 and grown in RPMI 1640 + 10% FBS + 1% penicillin and streplomyan. After 24 hours, cells were placed in serum-free and phenol red-free medium. After 24 hours, cells received treatment with DHT (10 nmol/L) or vehicle, and after 5 minutes, they were switched back to serum and phenol red-free medium. In experiment 2, additional plates were treated with Casodex (10 μmol/L) or PD98059 (50 μmol/L) for 60 minutes + DHT (10 nmol/L) for the last 5 minutes and subsequently switched back to serum and phenol red-free medium. In additional experiments $LNCaP_{nan}$ cells were transiently transfected with wild-type or dominant-negative CREB expression plasmids (experiment 3), or with wild-type or dominant-negative MEK-1/2 expression plasmids (experiment 4) and subjected to the same experimental conditions of experiment 1. Fifteen hours later, 1 μCi of [^{3}H]thymidine was added for 1 hour. Cells were then rinsed with PBS, and harvested in a glass tube in which they were rinsed twice with PBS and three times with 10% TCA and pelleted in 100 μL of 0.5 N NaOH/0.5% SDS, of which 10 μL were counted in a scintillation counter. The disintegration per minute numbers were multiplied by 10 and represent DNA synthesis in 50,000 cells. Each experiment consisted of at least three plates and was repeated at least two times. [^{3}H]Thymidine incorporation assays were also performed with genital skin fibroblast cell lines 881, 1003, and 1017. The experiments were done essentially as described in experiments 1 for LNCaP cells (+ or −10 nmol/L DHT), with the only difference being that [^{3}H]thymidine was added 10 hours after DHT.

Additional Evidence Suggesting a Role for CREB Signaling in LNCaP-HP Cells. Using previously published technology (48), we tried to create a LNCaP-HP cell line stably transfected with the dominant-negative form of CREB to verify whether this would antagonize the AI phenotype of this cell line. Despite several attempts, we were unable to select such a cell line, indicating that CREB signaling is vital for the survival of LNCaP-HP cells.

RESULTS

Dihydrotestosterone Does Not Induce Phosphorylation of Extracellular Signal-Regulated Kinase-1/2 in LNCaP-HP Cells. Because rapid signaling of many steroid receptors involves phosphorylation of ERK-1/2, we used several CaP cell lines to determine whether this pathway was activated by DHT in an AR-dependent way. The effects of DHT were evaluated in cells that had grown in medium devoid of serum for at least 24 hours. We initially examined whether AR activated the ERK-1/2 pathway using a HP LNCaP cell line (LNCaP-HP) that had been split more than 60 times in our laboratory. After serum starvation, LNCaP-HP cells were treated with DHT or EGF at a variety of concentrations and for a variable period of time.

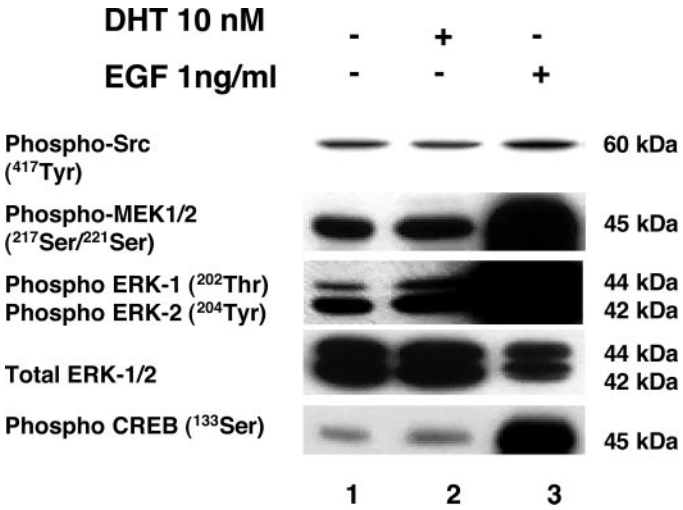

Fig. 1. Lack of DHT-mediated induction and presence of EGF-mediated induction of Tyr^{417}-Src, Ser^{217}/Ser^{221}–MEK-1/2, Tyr^{202}–Tyr^{204}–ERK-1/2, and Ser^{133}-CREB phosphorylation in LNCaP-HP cells. LNCaP-HP cells were seeded and grown for 24 hours in RPMI + 10% FBS + 1% penicillin and streplomyan. They were then cultured for an additional 24 hours in the same medium without serum and stimulated for 5 minutes with vehicle (*Lane 1*), DHT 10 nmol/L (*Lane 2*), or EGF 1 ng/mL (*Lane 3*). Total ERK was inserted to control for equal loading. The experiment shows that less protein was loaded in the lane stimulated with EGF (see blot probed with total ERK-1/2 antibody), and yet a much larger amount of phospho-Tyr^{417}-Src, Ser^{217}/Ser^{221}–MEK-1/2, Tyr^{202}–Tyr^{204}–ERK-1/2, Ser^{133}-CREB was detected after EGF stimulation. No DHT-dependent induction of these phospho-proteins was detected.

Fig. 1 shows basal levels of ERK phosphorylation at baseline. Five minutes of stimulation with 1 ng/mL EGF, but not with 10 nmol/L DHT, resulted in additional ERK-1/2 phosphorylation. Although DHT was still unable to induce any change in ERK-1/2 phosphorylation throughout a 24-hour experiment, EGF continued to increase ERK-1/2 phosphorylation for an additional 6 hours (not shown). Furthermore, no induction of ERK-1/2 phosphorylation was observed using a range of DHT concentrations (0.1–100 nmol/L, not shown). Phosphorylation of Src and MEK-1/2, two molecules located upstream in the pathway leading to ERK-1/2 activation, and of CREB, which is a target of active ERK-1/2, showed a similar behavior to that of ERK-1/2 (Fig. 1; data not shown). Use of Casodex (10 μmol/L) did not change the phosphorylation pattern of the molecules shown in Fig. 1, whereas the MEK-1/2 inhibitor PD98059 (50 μmol/L) completely inhibited phosphorylation of MEK-1/2, ERK-1/2, and CREB (not shown). This experiment demonstrated that ERK-1/2 signaling was constitutively active in LNCaP-HP cells and that EGF, but not DHT, was capable of inducing additional stimulation. Of the two inhibitors used, only PD98059 interrupted this signaling pathway.

Dihydrotestosterone Induces Phosphorylation of Extracellular Signal-Regulated Kinase-1/2 in $LNCaP_{nan}$ Cells. LNCaP cells typically do not grow as xenografts in a castrated host (49), but studies performed with LNCaP-HP cells indicated that they had lost some of their initial characteristics, as they grew efficiently as xenografts in castrated mice. Therefore, based on the acquisition of androgen independence by LNCaP-HP cells, a new LNCaP cell line [designated $LNCaP_{nan}$ (low passage)] was purchased from the American Type Culture Collection. As expected, $LNCaP_{nan}$ had reduced ability to form tumors in castrated hosts, and they formed tumor in only one of 12 inoculations (8.3%) compared with 12 of 12 (100%) with LNCaP-HP. Fig. 2 shows that addition of DHT to the culture medium of $LNCaP_{nan}$ cells for 5 minutes was associated with induction of ERK-1/2 phosphorylation in a biphasic way. The effect was seen between 0.01 and 10 nmol/L DHT (Fig. 2*A*, *Lanes 3-6*), whereas it decreased when supra-saturating concentrations of hormone (100 nmol/L DHT) were added (Fig. 2, *Lane 7*). Because maximal ERK-1/2 stimulation occurred at 10 nmol/L, this concentration was used in subsequent experiments. The pattern of ERK-1/2 activation was also studied in response to 10 nmol/L DHT during a 24-hour experiment (Fig. 2*B*).

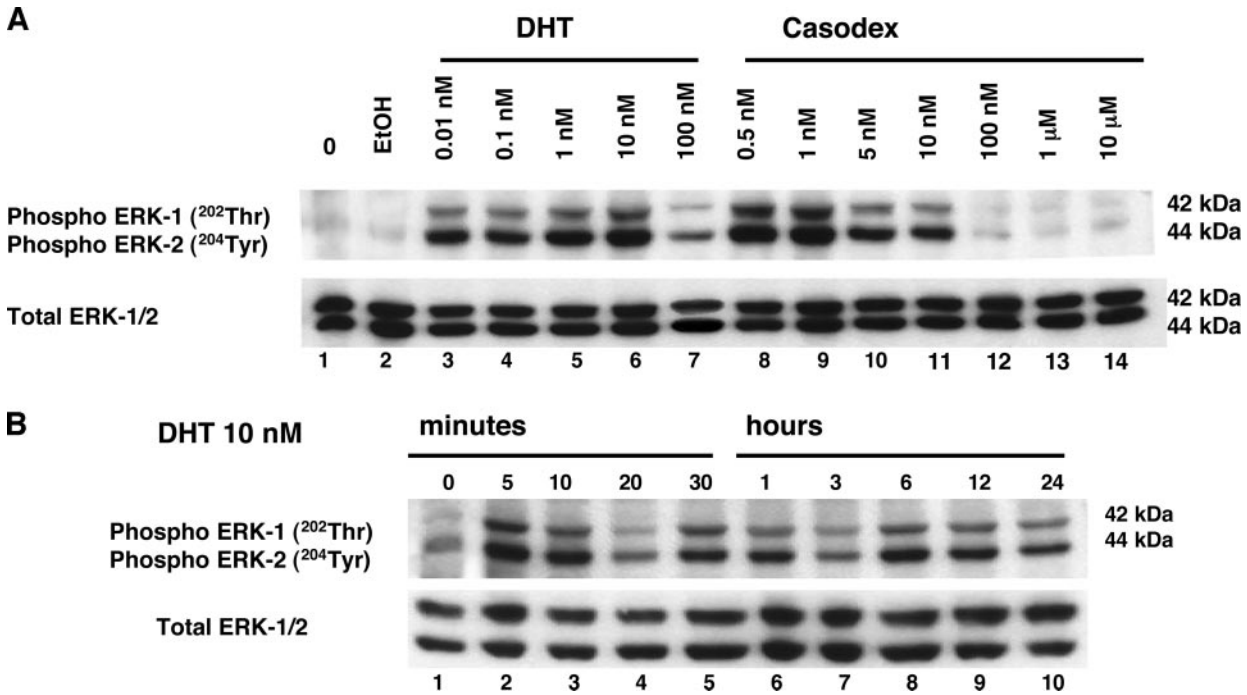

Fig. 2. *A*, activation of the ERK-1/2 pathway in $LNCaP_{nan}$ cells. $LNCaP_{nan}$ were seeded in RPMI + 10% FBS + 1% penicillin and streplomyan for 24 hours. Subsequently, they were cultured in RPMI without phenol red and FBS for additional 24 hours and treated with increasing concentrations of DHT or Casodex for 5 minutes. Immunoblot analysis for phospho-Tyr^{202}/Tyr^{204} ERK-1/2 was done by using a specific antibody. The same blot was analyzed with an antibody for total ERK-1/2 to control for equal loading. *B*, induction of Tyr^{202}/Tyr^{204} ERK-1/2 phosphorylation by DHT. $LNCaP_{nan}$ were seeded in RPMI + 10% FBS + 1% penicillin and streplomyan for 24 hours. Subsequently, they were grown for 24 hours in RPMI 1640 without phenol red in conditions of serum starvation. Cells were then incubated with 10 nmol/L DHT for the amount of time indicated. *Top panel*, a Western analysis of Tyr^{202}/Tyr^{204} phospho-ERK. *Bottom panel*, the same blot after incubation with an antibody recognizing total ERK to control for equal loading.

Addition of DHT to the medium of $LNCaP_{nan}$ cells starved for 24 hours in RPMI 1640 without phenol red and serum was associated with a peak of ERK activation after 5 minutes. A lower degree of ERK activation was observed at subsequent time points during the 24-hour experiment, with temporary declines after 20 minutes and 3 hours (Fig. 3, *Lanes 4* and *7*).

Effect of Casodex on Extracellular Signal-Regulated Kinase-1/2 Signaling in $LNCaP_{nan}$ Cells. To determine whether AR is required to obtain activation of ERK-1/2, we used Casodex, a well-characterized AR antagonist. As shown in Fig. 2*A*, *Lanes 8–14*, also Casodex had a biphasic effect on ERK activation. At concentrations between 0.5 and 10 nmol/L, Casodex was a powerful inducer of ERK-1/2 phosphorylation, whereas at concentrations above 100 nmol/L, no effect was detected. To answer the question of whether Casodex might be an agonist between 0.5 and 10 nmol/L and an antagonist between 100 nmol/L and 10 μmol/L, DHT (10 nmol/L) was given in combination with Casodex at low (1 nmol/L) or high (10 μmol/L) concentrations (Fig. 3*A*). Under these experimental conditions, 10 μmol/L Casodex worked as an antagonist of DHT-induced ERK-1/2 activation (Fig. 3*A*, compare *Lanes 3* and *4*). When DHT (10 nmol/L) plus Casodex (1 nmol/L) were compared with 10 nmol/L DHT alone (Fig. 3*A*, compare *Lanes 2* and *3*), a stronger degree of ERK-1/2 phosphorylation was present, suggesting that Casodex at lower concentrations worked as an agonist and contributed to ERK-1/2 phosphorylation in association with DHT.

Molecules Activated Upstream of Extracellular Signal-Regulated Kinase-1/2 in $LNCaP_{nan}$ Cells. MEK-1/2 is known to be upstream in the signaling pathway leading to ERK-1/2 activation. In our experiments, phosphorylation of MEK-1/2 was demonstrated in a time frame compatible with the observed DHT-dependent activation of ERK-1/2 [Fig. 3*A*, compare *Lanes 1* (control) and *3* (5 minutes after 10 nmol/L DHT); not shown]. Supporting the fact that AR mediates activation of this signaling pathway, Casodex at inhibitory (10 μmol/L) concentrations in combination with 10 nmol/L DHT prevented phosphorylation not only of ERK-1/2, but also of MEK-1/2 (Fig. 3*A*, compare *Lanes 3* and *4*). In contrast, agonistic concentrations of Casodex (1 nmol/L) given with 10 nmol/L DHT did not change the status of MEK phosphorylation compared with cells treated with DHT alone (Fig. 3*A*, compare *Lanes 2* and *3*). The observation that a molecule located upstream of ERK1/2 was activated in response to DHT was complemented by experiments with the MEK-1 antagonist PD98059 (Fig. 3*B*). Addition of PD98059 pre-

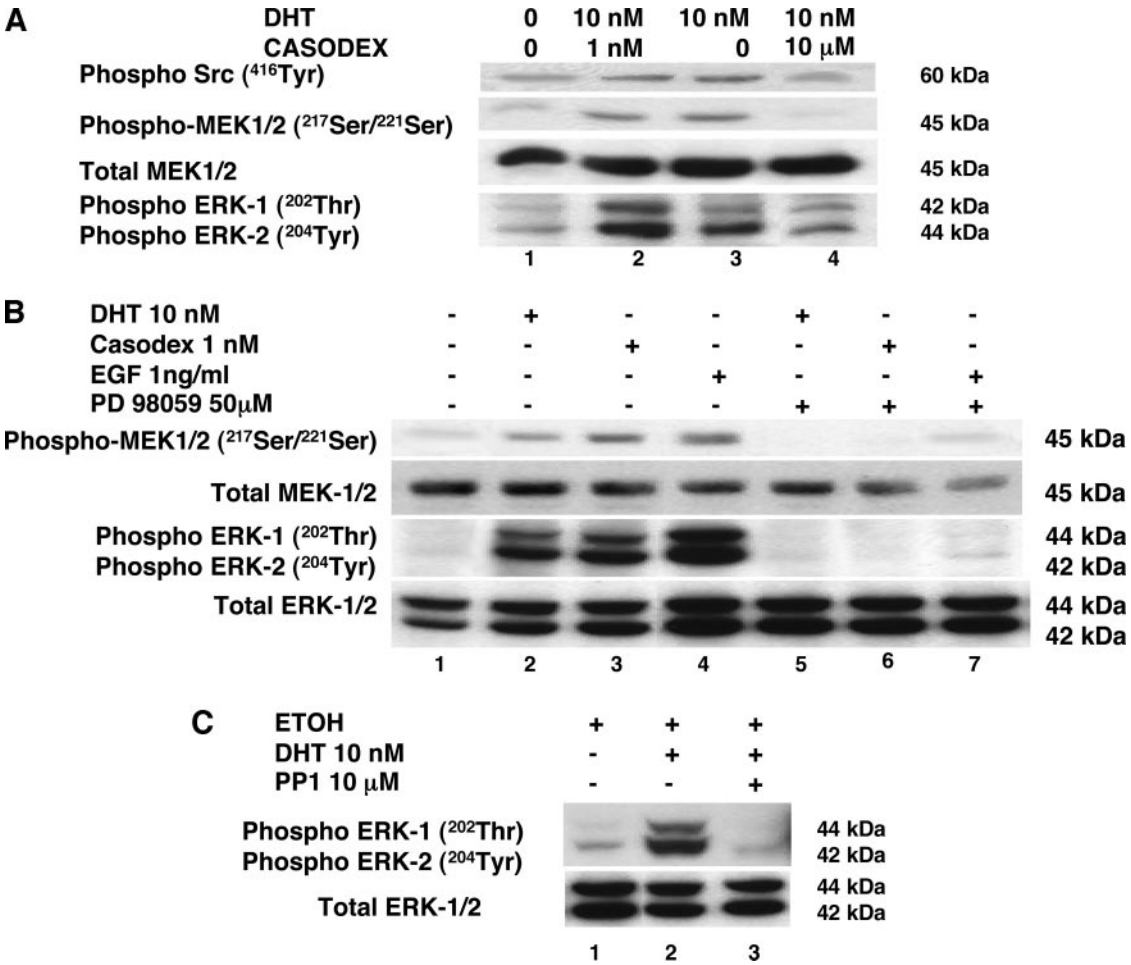

Fig. 3. *A*, inhibition of Src, MEK, and ERK phosphorylation by antagonistic concentrations of Casodex. $LNCaP_{nan}$ cells were starved for 24 hours in RPMI 1640 in the absence of serum and phenol red. Cells were then treated with vehicle (*Lane 1*), DHT 10 nmol/L + Casodex 1 nmol/L (*Lane 2*), DHT 10 nmol/L (*Lane 3*), or DHT (10 nmol/L) + Casodex (10 μmol/L; *Lane 4*). In *Lanes 2* and *4*, cells were preincubated with Casodex (at 1 nmol/L or 10 μmol/L) for 1 hour, and then DHT was added for 5 minutes. Lysates were used for immunoblot analysis of Tyr^{416} phospho-Src, Ser^{217}/Ser^{221} phospho-MEK-1/2, Thr^{202}/Tyr^{202} phospho-ERK-1/2 using specific phosphoantibodies. Total MEK-1/2 was measured to control for equal loading of each lane. *B*, effect of the MEK inhibitor PD98059 on ERK-1/2 and MEK-1/2 phosphorylation induced by DHT (10 nmol/L), Casodex (1 nmol/L), and EGF (1 ng/mL). $LNCaP_{nan}$ cells were grown for 24 hours in RPMI 1640 without phenol red in conditions of serum starvation. Cells were then incubated with 10 nmol/L DHT, 1 nmol/L (agonistic concentrations) Casodex, and EGF 1 ng/mL (the positive control) for 5 minutes alone or preceded by PD98059 50 μmol/L for 60 minutes. The same blot was analyzed with an antibody for total ERK-1/2 to control for equal loading. *C*, same experiment as in *B* performed with 10 μmol/L of the Src inhibitor PP1, which was given 60 minutes before 10 nmol/L DHT.

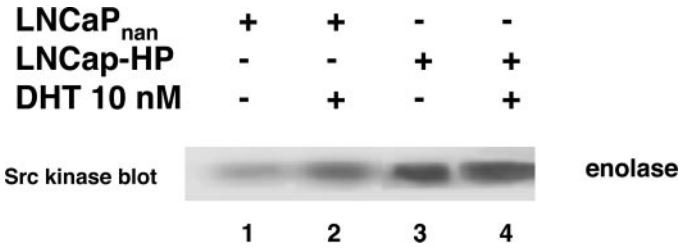

Fig. 4. Src kinase assay in $LNCaP_{nan}$ and -HP cells. Cells were grown for 24 hours in RPMI 1640 without phenol red in conditions of serum starvation. Cells were then incubated with 10 nmol/L DHT or vehicle for 5 minutes and subjected to Src kinase assay using enolase as a substrate. The intensity of the signal was quantitated by densitometry. Addition of DHT in $LNCaP_{nan}$ resulted in 7-fold increase in the phospho-enolase signal. Addition of DHT did not change the intensity of the enolase signal in LNCaP-HP cells. Under control conditions, the signal was 12-fold stronger in LNCaP-HP compared with $LNCaP_{nan}$ cells.

vented DHT-induced phosphorylation of MEK-1/2 and ERK-1/2 (Fig. 3*B*, compare *Lanes 2* and *5*). Similarly PD98059 inhibited MEK-1/2 and ERK-1/2 activation mediated by agonistic concentrations of Casodex (Fig. 3*B*, compare *Lanes 3* and *6*), and 1 ng/mL EGF (the positive control; Fig. 3*B*, compare *Lanes 4* and *7*). In support of the possibility that additional molecules located upstream of MEK-1/2 may be involved in this pathway, DHT-induced phosphorylation of ERK-1/2 was inhibited when $LNCaP_{nan}$ cells were preincubated with the Src inhibitor PP1 (Fig. 3*C*, compare *Lanes 2* and *3*). Based on this and on the fact that Src activation was previously reported to mediate nongenotropic signaling of a variety of steroid receptors including AR (10, 12, 13), we tested whether Src was the initial mediator of the cellular response leading to AR-mediated ERK-1/2 phosphorylation. This possibility was supported by the observation that Tyr^{416} phospho-Src, a functionally active form of Src, increased in response to DHT or DHT plus Casodex (at agonistic concentrations) compared with control conditions [Fig. 3*A*, compare *Lane 1* (control) with *Lane 2* (DHT 10 nmol/L) or *Lane 3* (DHT 10 nmol/L + Casodex 1 nmol/L)] and that this activation step was inhibited by Casodex at antagonistic concentrations [Fig. 3*A*, compare *Lane 2* (DHT 10 nmol/L) with *Lane 4* (DHT 10 nmol/L + Casodex 10 μmol/L)]. In addition, using immunoprecipitated Src incubated with enolase and 5 μCi of [γ-^{32}P]ATP, we found that enolase phosphorylation increased in a DHT-dependent manner in $LNCaP_{nan}$ cells (Fig. 4, compare *Lanes 1* and *2*) by a factor of seven. In contrast, a 12-fold higher baseline Src kinase activity that was not further inducible after addition of DHT was detected in LNCaP-HP cells (Fig. 4). These results indicated that Src (and a downstream pathway consisting of MEK-1/2–ERK-1/2) was inducible by DHT in androgen-dependent (AD) $LNCaP_{nan}$ cells, whereas it was constitutively active in AI LNCaP-HP cells, where it could be further stimulated by EGF, but not DHT (Fig. 1). The level of activation of this pathway induced by DHT in $LNCaP_{nan}$ cells was similar to that observed in LNCaP-HP cells under control conditions.

Pathway Activated Downstream of Extracellular Signal-Regulated Kinase-1/2. We next focused our analyses on proteins known to be downstream targets of active ERK-1/2 and that are also responsible for intracellular activities compatible with the general assumption that AR mediates cellular proliferation and survival. These molecules included the transcription factor CREB (50) and the downstream target of CREB, c-*fos* (51). Fig. 5*A* shows that the active form of CREB, Ser^{133} phospho-CREB, was detected in $LNCaP_{nan}$ cells after 5 and 30 minutes of DHT stimulation and returned to baseline levels after 60 minutes. The same blot was analyzed with an additional antibody, which recognizes phospho-ERK-1/2, and a pattern of phosphorylation similar to that of Fig. 2 was demonstrated (not shown). DHT-dependent CREB phosphorylation was prevented at inhibitory concentrations of Casodex [Fig. 5*B*, compare *Lanes 2*

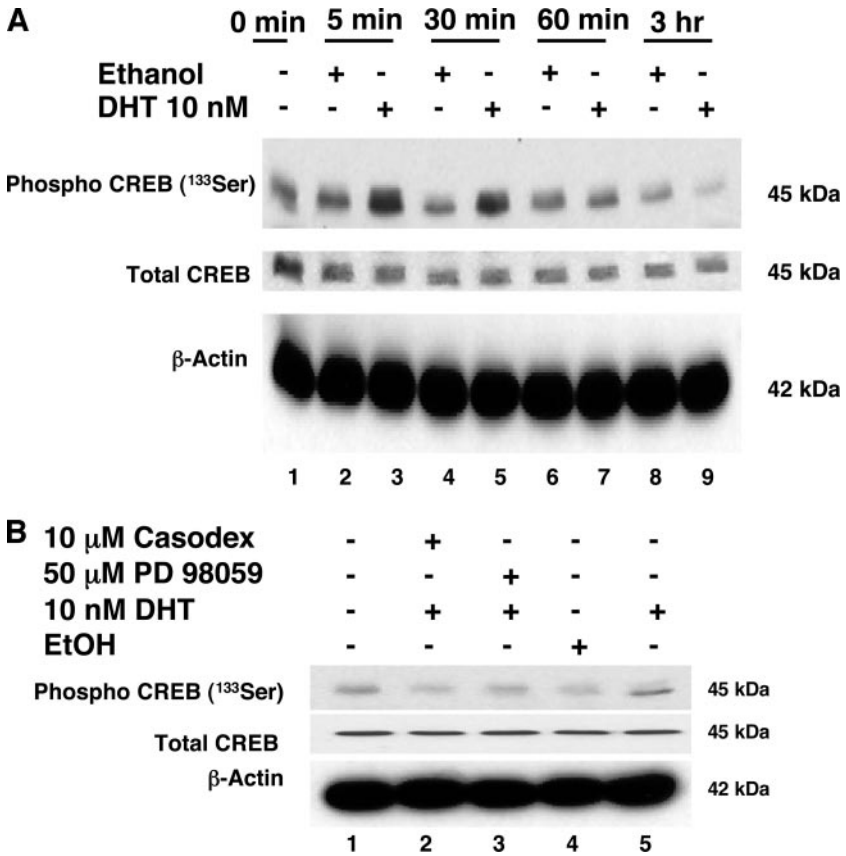

Fig. 5. Phosphorylation of the transcription factor CREB after treatment with DHT. *A*. $LNCaP_{nan}$ were seeded in RPMI + 10% FBS + 1% penicillin and streplomyan for 24 hours. They were then grown for 24 hours in RPMI in the absence of serum; they were then treated with DHT (10 nmol/L) or vehicle alone for 5 minutes to 3 hours. Cell extracts were sized in a gel and analyzed with antibodies for Ser^{133} CREB and total CREB or β-actin to control for equal loading. *B*. $LNCaP_{nan}$ were seeded in RPMI + 10% FBS + 1% penicillin and streplomyan for 24 hours. Cells were then grown in the absence of serum for 24 hours. They were then treated with Casodex (*Lane 2*) or PD98059 (*Lane 3*) for 60 minutes and then with DHT (10 nmol/L; added to *Lanes 2*, *3*, and *5* for 5 minutes). Cell extracts were sized in a gel and analyzed with antibodies for Ser^{133} CREB and β-actin to control for equal loading. The additional faint band detected in the phospho-Ser^{133} CREB blot in *Lane 6* has not been conclusively identified.

(DHT + Casodex) and 5 (DHT alone)] and PD 98059 [Fig. 5*B*, compare *Lanes 3* (PD98059 + DHT) and 5 (DHT alone)]. Because Src–Mek-1/2–ERK-1/2 signaling was constitutively active in LNCaP-HP cells, we tested whether CREB was expressed as a constitutively active molecule. Fig. 1 shows that similar to Src, MEK-1/2, and ERK-1/2, CREB was also constitutively phosphorylated and unresponsive to DHT. Because upon Ser^{133} phosphorylation CREB becomes an active transcription factor and is known to up-regulate the expression of c-*fos* mRNA (51), $LNCaP_{nan}$ cells were transiently transfected with a plasmid containing the 379 bp of the murine c-*fos* promoter linked to a *luciferase* reporter gene. Luciferase activity was up-regulated by DHT and EGF (the positive control; Fig. 6*A*), and this effect was inhibited by pretreatment with 10 μmol/L Casodex or 50 μmol/L PD98059 (Fig. 6*B*) or by transfection with a dominant-negative CREB plasmid (Fig. 6*C*).

Differences between Genotropic and Nongenotropic Gene Activation by Androgen Receptor. Because DHT-mediated transcription of the *c-fos* promoter was inhibited by Casodex and PD98059, we hypothesized that an AR-initiated, nongenotropic pathway was involved in which not AR but CREB was responsible for transcription. We next repeated this experiment using a reporter plasmid driven by the PSA P/E promoter, which is directly activated by AR upon nuclear translocation through a genotropic mechanism. Fig. 6*D* shows that PSA P/E activity was induced by DHT and inhibited by Casodex; however, in contrast to the *c-fos* promoter, PD98059 was a poor inhibitor of luciferase activity. Fig. 6*D* represents the synthesis of six experiments, and we found that PD98059 inhibited DHT-induced luciferase activity only by 30% compared with 85% inhibition in the experiment shown in Fig. 6*C* in which the *c-fos* promoter was used.

Evidence in Support of a Ternary Complex Containing Androgen Receptor, MNAR, and Src. If Src was indeed the apical molecule mediating the cross-talk in AR and ERK-1/2 signaling, it would be reasonable to expect that AR and Src interact. A molecule mediating the cross-talk between genotropic and nongenotropic signaling of the estrogen receptor (ER) has recently been identified in the scaffold protein MNAR, which interacts with both ER and Src in an estradiol-dependent way (44). Based on this, we hypothesized the presence of trimeric complex in which MNAR interacts with both AR and Src, and we performed experiments in which antibodies for AR or FLAG were used to immunoprecipitate lysates obtained from FLAG-MNAR–transfected $LNCaP_{nan}$ or LNCaP-HP cells harvested under baseline conditions or 5 minutes after stimulation with DHT. The experiment shown in Fig. 7*A* demonstrated the formation of a trimeric complex consisting of MNAR, Src, and AR after addition of DHT to the culture medium [Fig. 7*A*, compare *Lanes 1* and *3* (control conditions) with *2* and *4* (after addition of DHT for 5 minutes)]. In contrast, in LNCaP-HP cells, this complex was present under control conditions, and its formation was not further stimulated by DHT (Fig. 7*B*). These experiments demonstrated that Src kinase activity and its as-

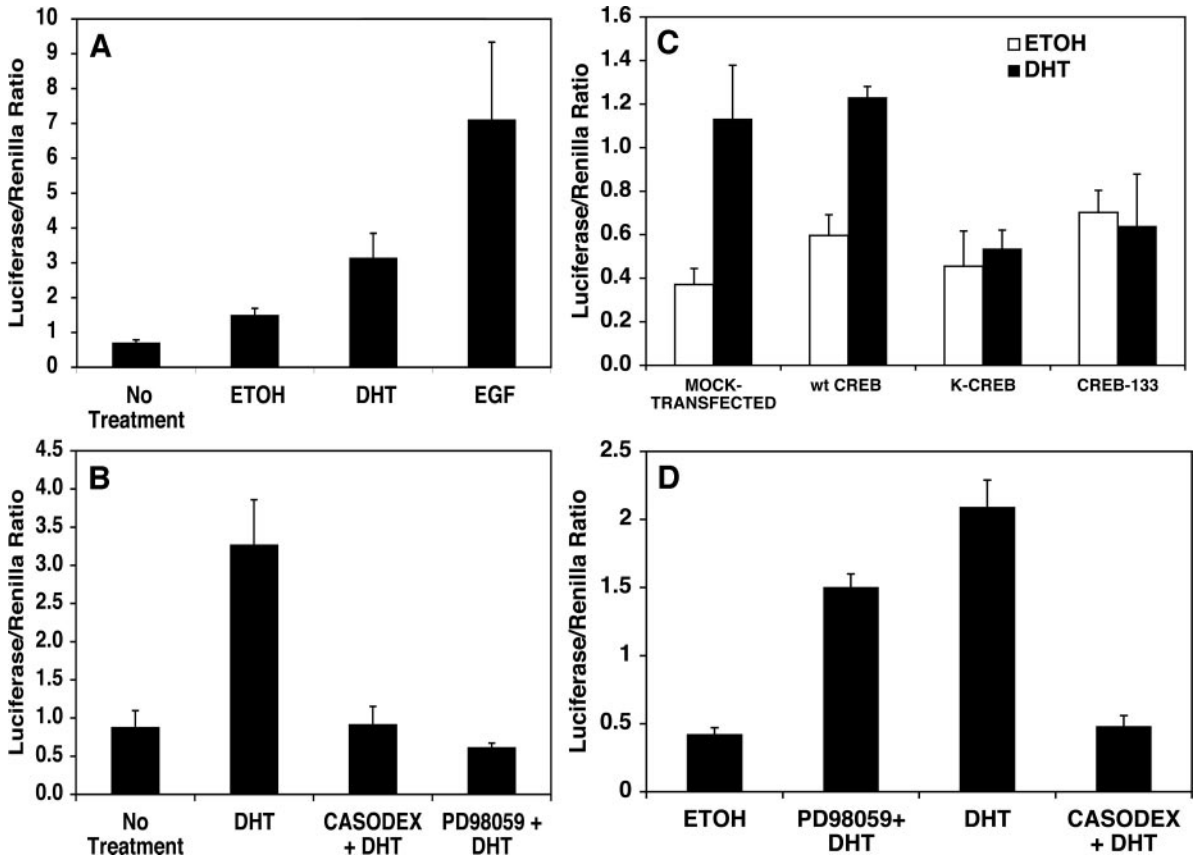

Fig. 6. *A*, induction of luciferase activity by DHT and prevention by Casodex (10 μmol/L) or PD98059 (50 μmol/L) in $LNCaP_{nan}$ cells transfected with plasmid pSVOAD5, containing the initial 379 bp of the c-*fos* promoter and with a constitutively active Renilla luciferase plasmid. After transfection, cells were treated with DHT(10 nmol/L) or EGF (1 ng/mL) alone for 30 minutes or with DHT (10 nmol/L) alone or + the inhibitors Casodex (10 μmol/L) or PD 98059 (50 μmol/L), which had been added 60 minutes earlier (*B*). *C*. In addition, cells were transfected with an empty vector, or vectors containing wild-type (*wt*) or dominant-negative CREB cDNAs and treated with or without DHT 10 nmol/L for 30 minutes. *D*. Luciferase activity induced in LNCaP cells transfected with plasmid PSA P/E luciferase. $LNCaP_{nan}$ were seeded in RPMI + 10% FBS + 1% penicillin and streplomyan for 24 hours. They were then grown for 24 hours in RPMI in the absence of serum. Cells were then transfected with plasmid PSA P/E luc and with a constitutively active Renilla luciferase plasmid. After transfection, cells were treated with DHT (10 nmol/L) alone for 8 hours or + the inhibitors Casodex (10 μmol/L) or PD 98059 (50 μmol/L), which had been added 60 minutes earlier. The results show that DHT induces luciferase activity and that this activity is completely inhibited by pretreatment with Casodex and only modestly by pretreatment with PD98059. Results are expressed as a ratio of luciferase over Renilla activity.

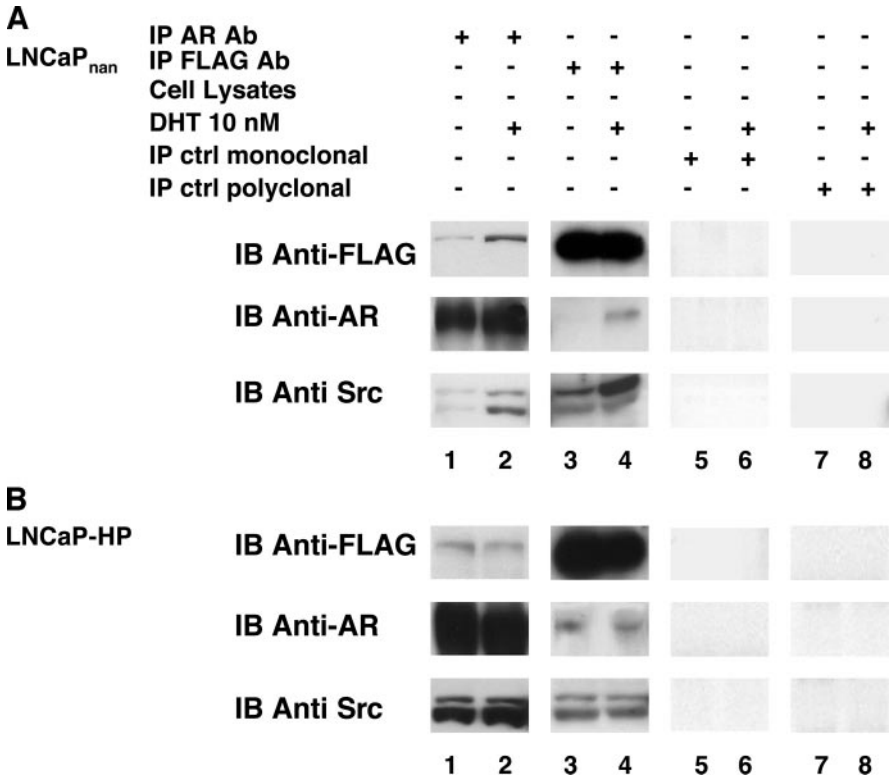

Fig. 7. Coimmunoprecipitation of AR, Src, and MNAR in $LNCaP_{nan}$ (*A*) and LNCaP-HP (*B*) transfected with a full-length FLAG-MNAR plasmid. $LNCaP_{nan}$ and LNCaP-HP were grown for 24 hours in RPMI 1640 without phenol red in conditions of serum starvation. Cells were then incubated with 10 nmol/L DHT or vehicle for 5 minutes. Lysates containing 300 μg of proteins were immunoprecipitated with antibodies for AR or for FLAG (*Lanes 1-4*). The resulting immunoprecipitated material was then subjected to immunoblot analysis with antibodies for AR, FLAG, or Src. As a control, the same lysates were immunoprecipitated with control monoclonal or polyclonal antibodies (*Lanes 5-8*) and subjected to immunoblot analysis with antibodies for AR, FLAG, or Src.

sociation with AR and MNAR are DHT dependent in AD $LNCaP_{nan}$ cells and constitutively present in AI LNCaP-HP cells.

Activation of the Src–MEK-1/2–ERK-1/2–CREB Pathway Signals Antiapoptosis in $LNCaP_{nan}$ Cells. We performed experiments to determine whether activation of the DHT–AR–Src–MEK-1/2–ERK-1/2–CREB pathway regulates survival (antiapoptosis) and cell proliferation in $LNCaP_{nan}$ cells. The antiapoptosis experiment was done using STS, a protein kinase inhibitor and powerful inducer of apoptosis in LNCaP cells (52), at increasing concentrations (100, 200, and 400 nmol/L) for 3 hours. Cells receiving STS alone showed a dose-dependent increase in apoptosis (Fig. 8*A*). Treatment with DHT and EGF prevented $LNCaP_{nan}$ cell apoptosis induced by STS regardless (at all doses) to near baseline levels (Fig. 8*A*). Fig. 8*B* shows that unlike $LNCaP_{nan}$, LNCaP-HP cells were resistant to STS. If activation of the DHT–AR–Src–MEK-1/2–ERK-1/2–CREB pathway is indeed responsible for the observed rescuing effect of DHT on STS-induced apoptosis in $LNCaP_{nan}$ cells, one would anticipate that a dominant-negative form of CREB would be disruptive. In agreement with this possibility, $LNCaP_{nan}$ cells transiently transfected with a dominant-negative CREB mutant were not rescued by DHT from the apoptotic effect of STS (Fig. 8*C*).

Activation of the Src–MEK-1/2–ERK-1/2 Pathway Signals Proliferation in $LNCaP_{nan}$ Cells, but This Effect Is Not Mediated by Activation of CREB. Cell proliferation was studied by using a [^{3}H]thymidine incorporation assay. Cells growing in starving medium were treated with DHT for 5 minutes, and the assay was performed 16 hours later. A significant increase in thymidine incorporation was noticed in $LNCaP_{nan}$ cells treated with DHT (Fig. 9*A*). This effect was completely abrogated if cells were pretreated with Casodex or PD98059 (Fig. 9*A*). Paradoxically, decreased thymidine incorporation was noticed in LNCaP-HP cells in response to DHT, and no significant changes were evident if DHT was preceded by PD98059 or Casodex (Fig. 9*B*). These data support the hypothesis that DHT-induced $LNCaP_{nan}$ proliferation depends on activation of the ERK-1/2 pathway. In agreement with this idea, DHT-induced $LNCaP_{nan}$ cell proliferation was prevented when the experiment was performed using cells that had been transfected with a dominant-negative MEK-1/2 cDNA (Fig. 9*C*). However, DHT-mediated proliferation of $LNCaP_{nan}$ cells was not inhibited by transfection with a dominant-negative form of CREB, suggesting that factors downstream of MEK-1/2 other than CREB mediate the observed DHT-dependent proliferation of this cell line (Fig. 9*D*).

Activation of Nongenotropic Androgen Receptor Signaling Is Inducible in Other Cell Lines and Does Not Require Androgen Receptor Translocation to the Nucleus. To demonstrate that activation of nongenotropic AR signaling is not a cell line–dependent phenomenon, we used three genital skin fibroblast cell lines. In these cell lines, induction of ERK-1/2 phosphorylation by DHT was a function of AR expression. In strain 1017, in which we know that AR is not expressed due to a mutation at the splice acceptor site at the 5′ boundary of exon 4 (38), no induction of ERK-1/2 phosphorylation was present (Fig. 10*A*). In contrast, in strain 1003 and 881 in which AR is expressed, there was a clear induction of ERK-1/2 phosphorylation in response to DHT, and interestingly, the magnitude of induction was higher in 881 cells (Fig. 10*A*). We next asked the question of where AR is localized when it induces activation of this pathway. Fig. 10*B* and *C* shows that under control conditions, AR was predominantly localized in the cytoplasm in both strains 881 and 1003, whereas after stimulation with DHT or the synthetic androgen mibolerone, it translocated to the nucleus in strain 1003 but not 881. The fact that more phospho-ERK was induced in strain 881 compared with 1003 suggests that lack of nuclear translocation may contribute to the apparent increase in nongenotropic AR signaling after induction with agonist ligands. Finally, we asked the question of whether AR-induced proliferation and protection from apoptosis described in $LNCaP_{nan}$ cells occur via a strictly ERK-1/2–dependent mechanism that does not also require AR genotropic signaling, and we repeated the experiments shown in Figs. 8 and 9 with the three genital skin

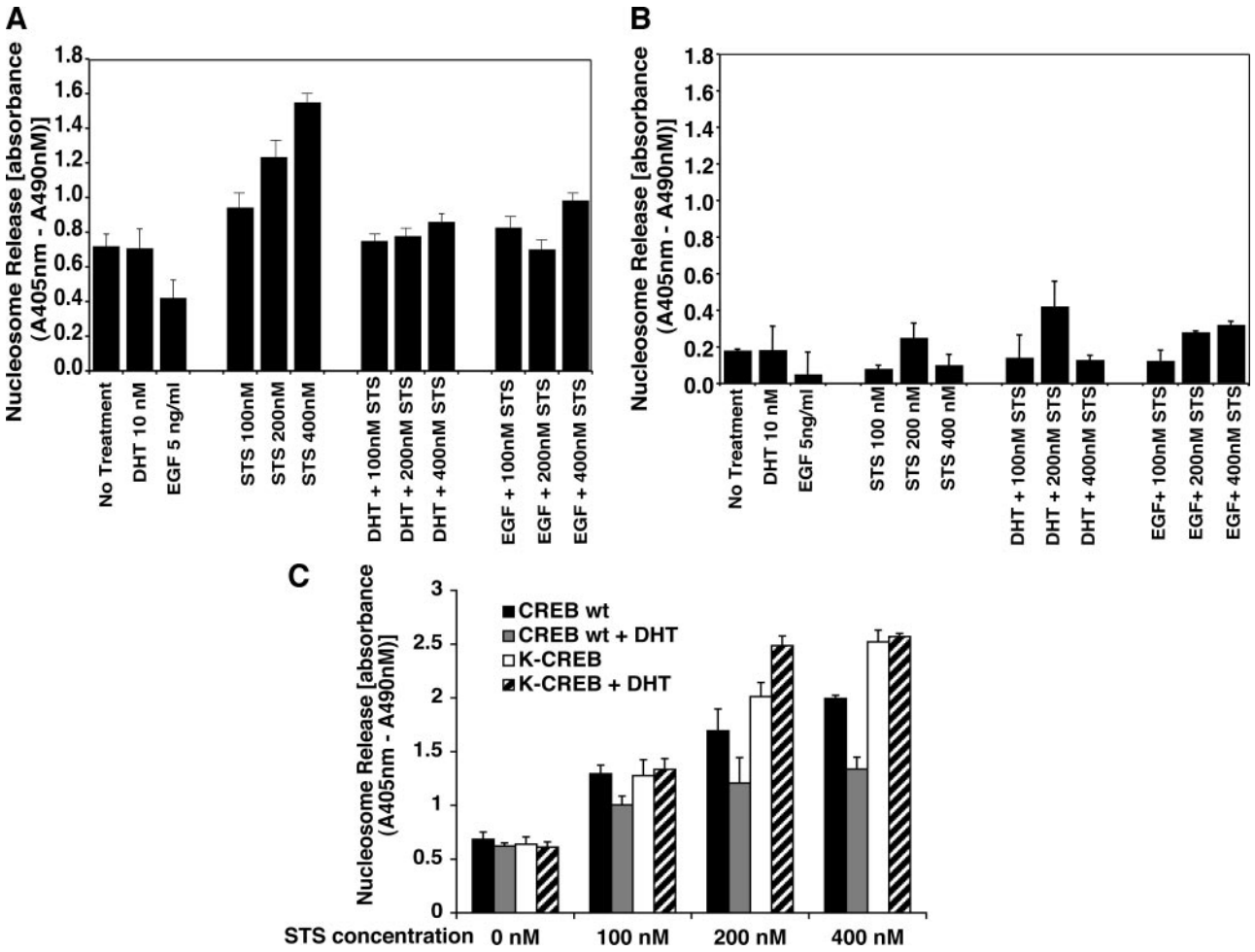

Fig. 8. DHT and EGF prevent STS-induced apoptosis in $LNCaP_{nan}$ but not in LNCaP-HP cells. On day 0, 1×10^4 $LNCaP_{nan}$ (*A*) and LNCaP-HP (*B*) cells were plated in a 96-well plate. After 1 d, cells were placed in medium without serum and phenol red. After 1 d, cells were treated with STS (100–400 nmol/L) for 3 hours. In parallel experiments, cells were treated with DHT (10 nmol/L) or EGF (5 ng/mL) alone or together with STS (which was added to the plate 5 minutes after DHT or EGF) for 3 hours. Cells were then subjected to the Cell Death Detection $ELISA^{PLUS}$ (Roche Diagnostic Corporation). Results are expressed as A_{405} and corrected according to the manufacturer specification. They represent the average ± SD of three plates and were repeated a minimum of three times. In *C*, $LNCaP_{nan}$ cells were transiently transfected with a wild-type (*wt*) or a dominant-negative (*K-CREB*) CREB plasmid and then subjected to the same experimental conditions of *A* and *B*.

fibroblast cell lines. Fig. 10*D* and *E* shows that these activities were inducible in strains 1003 and 881 but not 1017. Because strain 881 has an AR mutation causing the functional inactivation of the DNA-binding domain known to completely impair AR genotropic signaling (39), these data suggest that a purely nongenotropic mechanism mediates these mitogenic and antiapoptotic effects of AR.

DISCUSSION

$LNCaP_{nan}$ cells were found to have an AD phenotype characterized by responsiveness to DHT stimulation in [^{3}H]thymidine incorporation and antiapoptosis assays and by the inability to grow in castrated nude mice as xenografts. Addition of DHT to serum-deprived culture media resulted in induction of a pathway that involved Src–MEK-1/2–ERK-1/2–CREB in $LNCaP_{nan}$ cells. ERK-1/2 phosphorylation was obtained using concentrations of DHT of 0.01 to 10 nmol/L, although it decreased when supra-saturating concentrations of hormone (100 nmol/L) were added. Considering that the K_d of the wild-type AR for DHT is reportedly between 0.2 and 2 nmol/L (53, 54) and that the receptor is saturated at 6 nmol/L DHT (53), one can conclude that the observed activation of ERK-1/2 occurred within low to physiologic concentrations of DHT, whereas it was inhibited almost to baseline when supra-physiologic concentrations were used. Similarly to DHT, Casodex showed a biphasic pattern in its ability to activate ERK-1/2 signaling in $LNCaP_{nan}$ cells. At higher concentrations Casodex acted as an antagonist and confirmed that initiation of DHT-mediated ERK-1/2 activation occurred through the interaction between AR and DHT. In contrast, at low concentrations, Casodex acted as an agonist. The common feature is that at low concentrations, both DHT and Casodex were agonistic, whereas at higher concentrations, they were inhibitory. These observations are intriguing, and they essentially agree with those of Castoria *et al.* (55), who recently demonstrated in NIH3T3 cells that association of AR with Src, or with phosphatidylinositol 3 phosphate, could be induced only at very low concentrations of the AR agonist R1881. Although we do not have a clear understanding of this phenomenon, one could argue that low occupancy of the receptor may be associated with decreased ability to translocate to the nucleus and that under these circumstances, the receptor may logistically be better positioned to activate the Src pathway, which generally originates from the plasma membrane. The fact that subcellular localization of AR may affect its ability to activate nongenotropic signaling was confirmed by the observation that in genital skin fibroblast strain 881, more ERK-1/2 phosphorylation was induced by DHT than in wild-type genital skin fibroblast strain 1003 and that this corresponded to inability of the mutated

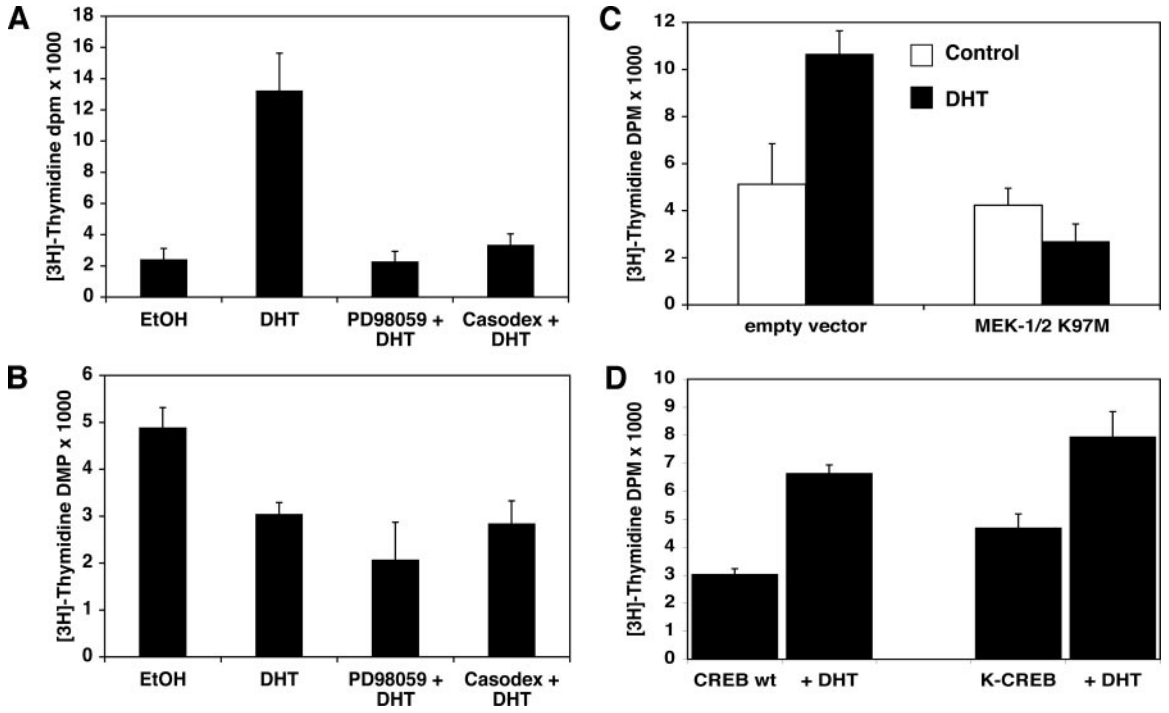

Fig. 9. Thymidine incorporation assay in $LNCaP_{nan}$ (*A*) and LNCaP-HP (*B*). In *C*, $LNCaP_{nan}$ cells were pretransfected with an empty plasmid or a plasmid containing a dominant-negative MEK-1/2 cDNA. In *D*, $LNCaP_{nan}$ cells were pretransfected with a plasmids containing wild-type (*wt*) or dominant-negative CREB (*K-CREB*) cDNAs. In a 12-well plate on day 0, 50,000 $LNCaP_{nan}$ or LNCaP-HP cells per well were seeded and grown in RPMI 1640 + 10% FBS + 1% penicillin and streplomyan. After 24 hours, cells were placed in serum-free and phenol red-free medium. After 24 hours, cells received treatment with DHT (10 nmol/L) or vehicle, and after 5 minutes, they were switched back to serum-free and phenol red-free medium. In parallel experiments, additional plates were treated with Casodex (10 μmol/L) or PD98059 (50 μmol/L) for 60 minutes + DHT (10 nmol/L) for the last 5 minutes and subsequently switched back to serum-free and phenol red-free medium. Fifteen hours later, 1 μCi of [^{3}H]thymidine was added for 1 hour. Cells were then rinsed with PBS, harvested in a glass tube in which they were rinsed twice with PBS and three times with 10% TCA, and pelleted in 100 μL of 0.5 N NaOH/0.5% SDS, of which 10 μL were counted in a scintillation counter. The numbers were multiplied by 10 and represent DNA synthesis in 50,000 cells. Each experiment consisted of at least three plates and was repeated at least two times.

receptor of this strain to translocate to the nucleus. Other interesting conclusions derived from our studies with strain 881, which contains a mutation inactivating the DNA-binding domain of the receptor (C574R; ref. 39), are that AR does not need a functioning DNA-binding domain to activate nongenotropic signaling and that the observed effects on proliferation and survival are purely mediated by activation of nongenotropic AR signaling, because they were observed also in this cell line, which was derived from a patient with complete androgen insensitivity. A final observation in $LNCaP_{nan}$ cells was that progression of DHT-induced ERK-1/2 signaling was inhibited not only by Casodex but also by the Src inhibitor PP1 and by the MEK-1 inhibitor PD98059.

In contrast, the Src–MEK-1/2–ERK-1/2–CREB pathway was not stimulated in LNCaP-HP cells, which had an AI phenotype characterized by lack of response to DHT stimulation on the [^{3}H]thymidine incorporation assay and on the antiapoptosis assay and by the ability to grow in castrated nude mice as xenografts. In LNCaP-HP cells, this pathway was constitutively active; treatment with DHT was unable to induce additional stimulation, whereas PD98059 but not Casodex maintained the ability to interrupt its progression.

The most important findings of this paper, which further our understanding of the mechanisms regulating transition of LNCaP cells to AI, were as follows: (1) the association between AI and the constitutive activation of the Src–MEK-1/2– ERK-1/2–CREB signaling pathway in LNCaP-HP cells; (2) the association between AD and the inactivity of this pathway in $LNCaP_{nan}$ cells; and (3) the ability of DHT to elicit activation of this pathway in $LNCaP_{nan}$ cells. From a biological point of view, these differences resulted in the presence of DHT-dependent [^{3}H]thymidine incorporation, and resistance to the apoptotic effect of STS in $LNCaP_{nan}$ but not in LNCaP-HP cells. This difference can be explained based on the fact that LNCaP-HP cells were already maximally stimulated to proliferate and resist apoptosis by the constitutive activation of this pathway, and, unlike $LNCaP_{nan}$, they could not be further stimulated to survive, or to proliferate upon addition of DHT. The experiments performed with genital skin fibroblast strains 1003, 881, and 1017 confirmed that the observed effects on proliferation and survival were purely mediated by activation of nongenotropic AR signaling.

Because the kinase Src is one of the upstream molecules of the ERK-1/2 pathway, it represents a candidate molecule that could interact with AR and mediate AR-dependent stimulation of the ERK-1/2 pathway. An additional participant of this pathway could be the protein MNAR, which was previously shown by the Cheskis laboratory to mediate the cross-talk between ERK-1/2 and ER signaling (44). By performing immunoprecipitation, we provided two important observations: (1) AR and Src coimmunoprecipitate with each other and with MNAR, indicating that the point of contact between AR and ERK-1/2 signaling occurs at the level of AR and Src and that MNAR is the scaffold molecule facilitating their interaction; and (2) formation of the AR-Src-MNAR complex was constitutively active in LNCaP-HP cells and DHT dependent in $LNCaP_{nan}$.

To further elucidate aspects related to the mechanism of ERK-1/2 activation in LNCaP-HP and $LNCaP_{nan}$ cells, a quantitative Src kinase assay was performed before and after stimulation with DHT. A stronger level of Src kinase activity was found in the LNCaP-HP than in $LNCaP_{nan}$ cells in the absence of DHT. After addition of DHT, Src kinase activity remained unchanged in LNCaP-HP, but increased significantly in $LNCaP_{nan}$ (although not to the same level as LNCaP-HP). This observation suggested that the disregulated step responsible for the constitutive activation of ERK-1/2 signaling in LNCaP-HP lies

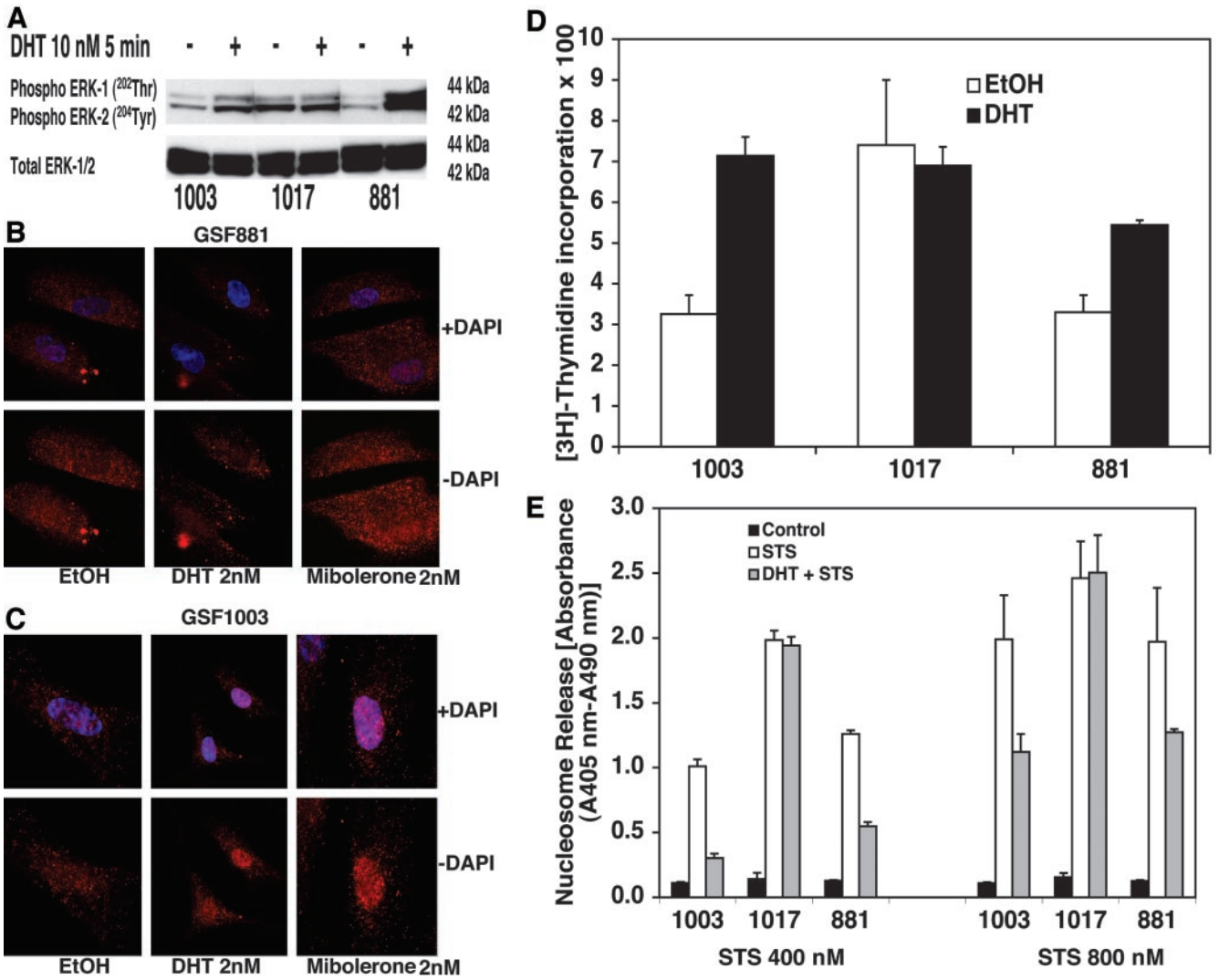

Fig. 10. *A*, presence of DHT-induced ERK-1/2 activation in genital skin fibroblast (*GSF*) cell lines 1003 and 881, but not 1017. Although in genital skin fibroblast 1003 containing a wild-type (*wt*)AR there is DHT-dependent nuclear translocation of AR (*red fluorescence*; *C*), this is lacking in genital skin fibroblast 881 containing an inactivating mutation of the DNA-binding domain causing transcriptional inactivation of the receptor (*B*). *D* and *E*, induction of [^{3}H]thymidine incorporation and prevention of STS-induced apoptosis by DHT in genital skin fibroblast cells strains 1003 and 881, but not 1017. *EtOH*, ethanol.

at the level of one of the mechanisms regulating activation of the protein kinase Src. Although this mechanism has not yet been unraveled, it is tempting to speculate that before conditions of androgen independence arise, as in LNCaP$_{nan}$ cells, AR has the ability to stimulate Src in a DHT-dependent way, and this stimulation sets in motion ERK-1/2 signaling, which is inhibited by Casodex or PD98059. In contrast, when conditions of androgen independence have arisen, as in the case of LNCaP-HP cells, Src kinase activity becomes constitutively active. Under these conditions, Src activity does not require DHT, and the ensuing constitutive ERK-1/2 signaling can be inhibited by PD95059 but not Casodex. Increased Src activity in cancer is not unique to LNCaP-HP cells; it has already been described in colon and breast cancer; and it has been attributed to elevated expression levels, increased activity, or activating mutations of Src (56–62). Current studies in our laboratory are directed at understanding the mechanisms causing constitutive Src activation in LNCaP-HP cells and its role in the transition of CaP to androgen independence.

That constitutive activation of ERK-1/2 signaling is important *in vivo* in CaP comes from studies performed by Gioeli *et al*. (63), who found increased expression of phospho-ERK-1/2 by immunohistochemistry in patients with AI CaP, and by Price *et al*. (64), who demonstrated increased expression and activity of ERK-1/2 in any type of CaP examined. The recognition that changes in the activity of ERK-1/2 signaling correlate to the status of androgen dependence of *in vitro* models of CaP comes from several studies. For instance, Bakin *et al*. (65, 66) have shown that manipulation of the ERK-1/2 signaling pathway is associated with acquisition of an AI phenotype when constitutive activation of Ras is induced in an AD LNCaP subline (65) and with restoration of androgen sensitivity when Ras signaling is attenuated in AI C4-2 cells (66). Similarly, Voeller *et al*. (67) demonstrated transition to an AI phenotype in LNCaP cells stably expressing v-rasH. The essence of our studies and this previous work is similar, as each associates AI phenotypes with acquisition of constitutively active ERK-1/2 signaling. The differences were that their systems were artificial, because they were established by stable transfection of constitutively active (65, 67) or dominant-negative (66) Ras cDNAs in LNCaP or C4-2 cells, respectively. Altogether, the data described here and in the papers of Bakin *et al*. (65, 66) and Voeller *et al*. (67) are complementary, because Src is located upstream of Ras in the signaling pathway leading to ERK-1/2 activation.

Important events occurring downstream of ERK-1/2 were detected

and include an increase in CREB phosphorylation at Ser^{133} in $LNCaP_{nan}$ and were dependent on a functioning DHT–AR–MEK-1/2–ERK-1/2 pathway. CREB is a nuclear effector of multiple signaling pathways activated through phosphorylation by the catalytic subunit of protein kinase A (68) and in response to Ca^{2+}/calmodulin-dependent and stress activated signaling pathways (69). It is also well established that CREB is a downstream target of active ERK-1/2 through the mediation of the Rsk kinase Rsk-2 (51). Studies have shown that after becoming phosphorylated at Ser^{133}, phospho-CREB becomes an active transcription factor and stimulates transcription of a wide variety of genes (46), including c-*fos* (51). In our hands, transcription through the c-*fos* promoter was stimulated in a DHT-dependent way in $LNCaP_{nan}$ cells, and similarly to CREB phosphorylation, it was inhibited by both the AR antagonist Casodex and the MEK inhibitor PD98059. Using dominant-negative MEK-1/2 and CREB constructs, we performed experiments to understand whether DHT-dependent CREB activation is indeed responsible for the activation of proliferation and survival in $LNCaP_{nan}$ cells. These experiments have shown that CREB is the mediator of the observed antiapoptotic effect of DHT but not of its mitogenic effects. The identity of the molecule downstream of MEK-1/2 causing increased thymidine incorporation in $LNCaP_{nan}$ cells is currently unknown. In contrast to $LNCaP_{nan}$, CREB phosphorylation was constitutive in LNCaP-HP cells, and its signaling was essential for cell survival, as we were unable to select a LNCaP-HP line after stable transfection with a plasmid containing a dominant-negative form of CREB.

Transcription through the c-*fos* promoter was inhibited by Casodex, PD98059, and dominant-negative CREB, although through the PSA P/E promoter, it was inhibited predominantly by Casodex. The minor inhibitory effect of PD98059 on transcription through the PSA P/E promoter was probably due to inhibition of a ERK-1/2–dependent phosphorylation step necessary for the activation of the SRC1 (70), which is necessary in every cell for the achievement by this molecule of its full transcriptional potential as a coactivator of several steroid receptors (including AR) acting through the genotropic pathway. The observed different sensitivity to Casodex or PD98059 suggested that AR induces transcription through two mechanisms: (1) an indirect mechanism in which CREB is the downstream target, and (2) a direct mechanism(s) in which AR, upon nuclear translocation, directly induces transcription of a network of genes such as the *PSA* gene.

In conclusion, we have identified that a constitutively active kinase pathway in a CaP cell line is associated with its transition to androgen independence. The presence of this constitutively active kinase pathway was related to unresponsiveness to manipulation with AR agonists or antagonists. Although the apical molecule of this pathway was identified as the protein kinase Src, additional work is required to understand the mechanism causing its constitutive activation. It is anticipated that answers to this question, and determination of the full array of downstream events associated with activation of CREB or other molecules of this pathway, will eventually help define the intricacies of CaP transition to androgen independence. Importantly, continued focus on the rapid signaling component of AR action may suggest new effective therapeutic targets.

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Changes in Androgen Receptor Nongenotropic Signaling Correlate with Transition of LNCaP Cells to Androgen Independence

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