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Epidermal Growth Factor Increases Coactivation of the Androgen Receptor in Recurrent Prostate Cancer*

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Growth of normal and neoplastic prostate is mediated by the androgen receptor (AR), a ligand-dependent transcription factor activated by high affinity androgen binding. The AR is highly expressed in recurrent prostate cancer cells that proliferate despite reduced circulating androgen. In this report, we show that epidermal growth factor (EGF) increases androgen-dependent AR transactivation in the recurrent prostate cancer cell line CWR-R1 through a mechanism that involves a post-transcriptional increase in the p160 coactivator transcriptional intermediary factor 2/glucocorticoid receptor interacting protein 1 (TIF2/GRIP1). Site-specific mutagenesis and selective MAPK inhibitors linked the EGF-induced increase in AR transactivation to phosphorylation of TIF2/GRIP1. EGF signaling increased the coimmunoprecipitation of TIF2 and AR. AR transactivation and its stimulation by EGF were reduced by small interfering RNA inhibition of TIF2/GRIP1 expression. The data indicate that EGF signaling through MAPK increases TIF2/GRIP1 coactivation of AR transactivation in recurrent prostate cancer.

Prostate cancer is a common disease in men that initiates in an environment of abundant circulating androgen. Proliferation of androgen-dependent prostate cancer cells is mediated by the androgen receptor (AR),[1] a ligand-dependent transcription factor that is activated by binding testosterone or dihydrotestosterone (DHT) (1–4). Androgen-induced cell proliferation is associated with the expression of an array of androgen-regulated genes (5, 6). Most prostate cancers regress in response to androgen deprivation therapy or anti-androgen treatment, reflecting their dependence on androgens for growth. Androgen deprivation results initially in reduced AR levels and reduced expression of androgen-regulated genes (7). Nevertheless, prostate cancers recur after a period of remission (8, 9). It has been suggested that prostate cancer recurrence occurs under selective pressure of androgen withdrawal or anti-androgen treatment and results from the clonal expansion of a subpopulation of cells that is independent of androgen for growth (10). The recurrence of prostate cancer after androgen deprivation is a major clinical challenge for improving disease outcome.

An abundance of evidence suggests that the AR is critical to recurrent prostate cancer growth despite reduced circulating androgen levels. AR is expressed in most recurrent prostate cancers (11) and is implicated in cancer growth by a cadre of androgen-regulated genes that continue to be expressed in the absence of testicular androgen (11–15). Recent studies show that selective AR inactivation using a ribozyme inhibits proliferation of LNCaP cells in culture (16). LNCaP prostate tumor growth and PSA secretion were inhibited *in vivo* using antisense oligonucleotides to inhibit AR expression (17).

To account for the functional importance of AR in recurrent prostate cancer, a number of adaptive mechanisms have been proposed (18). Each involves alternative pathways for increased AR activation. One compelling observation is that the AR gene is amplified in ~30% of prostate cancers, and AR levels are increased (9, 19–21). Higher AR expression would be expected to increase the sensitivity of prostate cancer cells to low androgen levels, as reported recently (22).

Mutation of the AR coding sequence is another mechanism for increased AR activation in late stage prostate cancer growth. Unlike mutations that cause androgen insensitivity by inactivating AR (23), most AR mutations identified in prostate cancer retain responsiveness to DHT (24–27). Some of these mutations broaden the ligand binding specificity, allowing additional steroids including adrenal androgens to induce AR transactivation (27–32). For some mutants, such as AR-T877A, in the LNCaP prostate tumor and derived cell lines, antagonists such as hydroxyflutamide gain agonist activity. AR mutations identified in prostate cancer include shorter than average CAG repeat lengths (33) associated with increased AR levels (34). The overall frequency of AR mutations in early stage disease is <5% (35), indicating that AR mutations do not account for prostate cancer initiation in most patients. The frequency of AR mutations increases in late stage recurrent prostate cancer (4, 35, 36) and may reflect adaptive changes to low androgen levels or anti-androgen treatment (37) or is in-

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[1] The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; TIF2, transcriptional intermediary factor 2; GRIP1, glucocorticoid receptor interacting protein; EGF, epidermal growth factor; PSA, prostate-specific antigen; MMTV, mouse mammary tumor virus; Luc, luciferase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; AF2, activation function 2; siRNA, small interfering RNA; TGF, transforming growth factor; ERK, extracellular signal-regulated kinase.

dicative of the inherent genetic instability that characterizes advanced cancer cells.

Another mechanism for recurrent prostate cancer growth is increased expression of nuclear receptor coactivators. The p160 coactivators include steroid receptor coactivator 1 (SRC1) (38), transcriptional intermediary factor 2 (TIF2) (39), also known as SRC2 or the mouse homologue, glucocorticoid receptor interacting protein (GRIP1) (40), and the related SRC3 variants TRAM1, AIB1, RAC3, ACTR, and p/CIP (41). When overexpressed in transient transfection assays, these coactivators increase AR transactivation in the presence of testosterone and DHT (42, 43) or lower affinity adrenal androgens (44). Some of the p160 coactivators have histone acetyltransferase activity that modifies histones to increase chromatin accessibility during gene activation (45). We found increased levels of TIF2 and SRC1 in the majority of a randomly selected set of clinical specimens of recurrent prostate cancer compared with coactivator levels detected in benign prostate (44). Increased coactivator expression is associated with estrogen receptor-mediated gene activation in breast (46, 47) and ovarian cancer (48), suggesting that p160 coactivator overexpression is linked to growth stimulation in cancer cells controlled by sex steroids.

Increased kinase signaling in response to growth factors is another adaptive change implicated in AR transactivation in prostate cancer (49–51). Signaling pathways that trigger increased cyclic AMP-dependent protein kinase A may be a mechanism for increased AR transactivation in recurrent prostate cancer (52, 53). Mitogen activation of AR is reported to occur in the absence and presence of androgen (52) and influences AR phosphorylation (54, 55) and dephosphorylation (56). Insulin-like growth factor-1, keratinocyte growth factor, and epidermal growth factor (EGF) are reported to increase AR transactivation in the absence of androgen (8, 9, 57). Mitogen-activated protein kinase (MAPK) signaling pathways and overexpression of the receptor ErbB2 induced ligand-independent AR transactivation (58). ErbB2 lacks a ligand partner but heterodimerizes with other members of the EGF receptor family to activate MAPK and phosphatidylinositol 3-kinase signaling pathways. Other reports suggest that EGF-induced increases in AR transactivation require the presence of androgen (59, 60). Heregulin-α and ErbB3 were overexpressed in prostate cancer in association with less favorable prognosis in advanced disease (61).

In this report, we investigated the mechanisms involved in an EGF-induced increase in AR transcriptional activity in recurrent prostate cancer cells. We present evidence that an EGF-induced increase in TIF2/GRIP1 phosphorylation is mediated through the MAPK signaling pathway and increases the functional activity of the androgen-activated AR.

EXPERIMENTAL PROCEDURES

CWR22 Tumor Propagation—Androgen-dependent and recurrent CWR22 tumors were originally provided by Thomas G. Pretlow (Case Western Reserve University) and were maintained as xenograft implants in nude mice (13, 62). CWR22 tumors of ~1 cm or less were resected and dispersed by protease digestion. Cell suspensions (10^6 cells/site) were injected subcutaneously with 100 μl of Matrigel™ (BD Biosciences) into nu/nu athymic mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) that were previously implanted with 12.5 mg of sustained release testosterone pellets to normalize circulating levels to ~4 ng/ml testosterone (Innovative Research of America, Sarasota, FL). For analysis of the androgen-dependent CWR22 tumor, tumors were grown to ~0.75 g over ~30 days until the mice were sacrificed by cervical dislocation, and tumors were resected. Testosterone propionate (25 mg/kg/day) in sesame oil (Schein Pharmaceutical, Inc., Port Washington, NY) was injected intraperitoneally, or 150 μg/kg/day EGF in sterile water (BD Biosciences) was injected subcutaneously. For recurrent CWR22 tumors, tumor cell-Matrigel suspensions were injected into castrated athymic mice and grown to ~0.75 g before resection after ~30 days. Tumors were frozen in liquid N_2 and used to prepare protein lysates for immunoblot analysis. Animals were maintained in accordance with National Institutes of Health and University of North Carolina at Chapel Hill animal use guidelines.

Plasmids—Expression vectors were described previously for full-length human AR pCMVhAR (63), AR NH_2-terminal and DNA binding and hinge region fragment pCMVhAR 1–660 (64, 65), the AR N/C interaction mutant pCMVhAR-F*XX*AA/A*XX*AA in which alanine substitutions were introduced into the ^{23}FQNLF^{27} and ^{433}WHTLF^{437} AR NH_2-terminal sequence (66–68), and GAL-AR 624–919 coding for the GAL4 DNA binding domain as a fusion protein with the AR ligand binding domain (69, 70). VPTIF2.1-S736A contained TIF2 residues 624–1287 and a serine to alanine mutation and was prepared by polymerase chain reaction mutagenesis, and the DNA sequence was verified. The following vectors were generously provided: prostate-specific antigen-luciferase reporter PSA-Luc (referred to previously as PSE-Luc) containing the −5220 PSA enhancer and promoter region with an internal deletion of −2876 to −540 (71) from Young E. Whang (University of North Carolina, Chapel Hill, NC) and Lily Wu (University of California Los Angeles), VPTIF2.1 coding for TIF2 residues 624–1287 as a fusion protein with the VP16 activation domain from Heinrich Gronemeyer (Institute of Genetics and Molecular and Cellular Biology) (39, 70), pSG5-GRIP1 and pSG5-GRIP1-S736A from Michael R. Stallcup and Peter J. Kushner (University of Southern California), mouse mammary tumor virus long terminal repeat-luciferase reporter vector (MMTV-Luc) from Stanley M. Hollenberg and Ronald M. Evans (Salk Institute), and the GAL4 luciferase reporter 5×GAL4Luc3 from Donald P. McDonnell (Duke University).

Transient Reporter Gene Assays—Cotransfection assays were performed in the CWR-R1 cell line derived from the CWR22 recurrent human prostate cancer xenograft (22). To study transcriptional activity of endogenous AR, CWR-R1 cells were transfected with 0.5 μg/6-cm dish MMTV-Luc reporter vector in the absence and presence of 0.1 μg of pSG5 empty vector, pSG5-GRIP1, or pSG5-GRIP1-S736A. To study transcriptional activity of transiently expressed wild-type pCMVhAR and pCMVAR 1–660 or pCMVAR-F*XX*AA/A*XX*AA, AR expression vector DNA (10 ng) was transfected with 0.5 μg of MMTV-Luc or 1 μg of PSA-Luc reporter vector as indicated. DNA was transfected into ~75% confluent CWR-R1 cells plated the day before at 10^6 cells/6-cm dish using prostate growth medium without exogenous EGF, containing Richter's improved minimal essential medium (Irvine Scientific, Santa Ana, CA) supplemented with 10 mM nicotinamide, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2% fetal bovine serum. The Effectene transfection reagent (Qiagen, Valencia, CA) was used according to the manufacturer. 1 ml of a DNA reaction mix containing 150 μl of EC buffer (Qiagen), 4 μl of enhancer, 4 μl of Effectene reagent (Qiagen), and 1 ml of 2% serum containing prostate growth medium lacking EGF was added to cell cultures containing 3 ml/dish fresh medium with 2% fetal calf serum without added EGF. The next day, the prostate growth medium was replaced with phenol red-free, serum-free medium (Improved MEM Zinc Option; Invitrogen) with or without the addition of EGF or DHT as indicated, and incubations were continued for 24 h. Cells were harvested in 0.5 ml of lysis buffer containing 25 mM Tris phosphate, pH 7.8, 2 mM EDTA, and 1% Triton X-100 (67). Luciferase activity was measured using an automated LumiStar Galaxy (BMG Labtechnologies, Durham, NC) multiwell plate reader luminometer.

For two-hybrid interaction assays, CWR-R1 cells (1×10^6 cells/6-cm dish) were transfected using Effectene as described above with 10 ng of VPTIF2.1 (VP16 activation residues 411–456 fused to TIF2 residues 624–1287) or the serine to alanine mutant VPTIF2.1-S736A, 100 ng of GAL-AR 624–919, and 0.1 μg of 5×GAL4Luc-3 containing five tandem GAL-4 binding sites and the luciferase coding sequence. Cells were treated with hormone as indicated and analyzed for luciferase activity as described above.

Immunoblot, Immunoprecipitation, and Stability Assays—Monkey kidney COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 20 mM Hepes, pH 7.2, 10% bovine calf serum, penicillin, and streptomycin. COS cells were plated at ~50% confluence (1.2×10^6 COS cells/10-cm dish) and transfected the next day using Effectene reagent. To each plate containing 8 ml of fresh serum-containing medium was added 1 ml of a reaction mix prepared according to the manufacturer to contain, per plate, 2 μg of pCMVhAR, pCMVhAR 1–660, pSG5-GRIP1, or pSG5-GRIP1-S736A as indicated, 300 μl of EC buffer (Qiagen), 16 μl of enhancer, 10 μl of Effectene reagent (Qiagen), and 1 ml of serum-containing medium. After an overnight incubation in 10% serum-containing medium, the medium was exchanged with serum-free, phenol red-free medium. CWR-R1 cells in log phase growth were plated in prostate growth

medium lacking EGF as described. For experiments without transfection, the CWR-R1 cell culture medium was replaced for 24 h with serum-free medium lacking phenol red and containing 0.2% lipid-rich bovine serum albumin (AlbuMax I; Invitrogen). Hormones and growth factors were added as indicated, and cells were incubated for ~20 h with DHT in the absence and presence of EGF. CWR-R1 and COS cells with or without transient DNA transfection were rinsed with phosphate-buffered saline and placed on ice. Cells were scraped into 1 ml of buffered saline on ice, transferred to 1.5-ml microcentrifuge tubes, and centrifuged at 12,000 × *g* for 2 min. The buffer was aspirated, and 50 or 100 μl of RIPA buffer with protease inhibitors was added (RIPA: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and phosphate-buffered saline containing the protease inhibitors 0.02 mg/ml pancreas extract, 0.005 mg/ml Pronase, 0.0005 mg/ml thermolysin, 0.003 mg/ml chymotrypsin, and 0.33 mg/ml papain (Roche Applied Science)). Cell pellets were disrupted by vortexing for 15 s, and lysates were incubated on ice for 15 min. After centrifugation for 15 min at 12,000 × *g*, supernatants were collected, and protein concentrations were determined using the Bio-Rad protein assay. Lysates from CWR22 tumors were prepared as described (72). Proteins (25 or 50 μg/lane as indicated) were separated on 8% acrylamide gradient gels for TIF2/GRIP1 or 10% acrylamide gels for AR and electroblotted after SDS-PAGE to nitrocellulose membranes (NitroBind, 0.22 μm; Osmonics, Inc., Westborough, MA). Rabbit polyclonal AR NH_2-terminal anti-peptide antibody AR32 (73) was used at 0.7 μg/ml, anti-TIF2 mouse monoclonal (BD Biosciences) at 1:1,000 dilution, and anti-phospho-p42/44 antibody (Cell Signaling Technology, Beverly, MA) at 1:1,000 dilution. Incubations with primary antibody were for 1 h at room temperature (AR32 and TIF2) or overnight at 4 °C (pp42/44). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary IgG antibodies (Amersham Biosciences) were used at 1:10,000 dilution for 1 h at room temperature. Specific signals were detected using chemiluminescence (SuperSignal® West Dura Extended Duration Substrate; Pierce).

For TIF2 immunoprecipitation, cells were removed and washed in buffered saline containing 2.5 mM sodium vanadate and 10 mM sodium fluoride. Cell lysates were prepared in 1 ml of RIPA with protease inhibitor (Complete Mini; Roche Applied Science) and phosphatase inhibitor mixtures 1 and 2 (Sigma). 4 mg of protein was immunoprecipitated using anti-TIF2 antibody following a preclear step using normal mouse IgG and Protein G-Plus-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). GRIP-1 antibody (8 μg; NeoMarkers, Inc., Fremont, CA) and 40 μl of Protein G-Plus-agarose was added to precleared lysates, and samples were incubated overnight at 4 °C with rocking. The agarose was pelleted at 1500 rpm for 30 s at 4 °C and washed three times with RIPA containing protease inhibitors and phosphatase inhibitors 2.5 mM sodium vanadate and 10 mM sodium fluoride. The final pellet was resuspended in 40 μl of electrophoresis sample buffer, and protein was fractionated on SDS-containing 8% acrylamide gels (Gradipore Ltd.). Proteins were electroblotted to Immobilon™-P membrane (Millipore Corp., Bedford, MA) overnight. Membranes were incubated with blocking solution (Zymed Laboratories Inc., South San Francisco, CA) containing 2.5 mM sodium vanadate and 10 mM sodium fluoride overnight at 4 °C followed by incubation overnight at 4 °C with a mixture of anti-phosphoserine antibody clone PSR-45 (Sigma) at 1:500 dilution and anti-phosphoserine antibodies 4A3, 4A9, and 16B4 (Calbiochem), each at a dilution of 1:250. Following four 30-min washes with 0.9% NaCl, 0.05% Tween 20, and 10 mM Tris, pH 7.5, membranes were incubated with anti-mouse IgG (Amersham Biosciences) at a 1:10,000 dilution for 30 min at room temperature, and the signal was detected by enhanced chemiluminescence (Supersignal; Pierce).

For AR stability assays, COS cells (1.2×10^6 COS cells/10-cm dish) were transfected with 1 μg of pCMVhAR or pCMVhAR 1–660 using Effectene reagent (Qiagen) as described above. After an overnight incubation in 10% serum-containing medium, cells were rinsed with phosphate-buffered saline and incubated in methionine-free modified Eagle's medium (Sigma) for 30 min. Tran^{35}S-label (100 μCi/dish) (PerkinElmer Life Sciences) was added to the cells with or without DHT or EGF treatment and incubated for 1.5 h. Cells were washed, fresh culture medium with or without hormone was added, and cells were incubated for 0, 8, and 24 h. Lysates were prepared from labeled cells using RIPA buffer containing protease inhibitors. ^{35}S-Labeled AR protein was immunoprecipitated using AR52 IgG (73) and Pansorbin cells (Calbiochem) and analyzed by SDS gel electrophoresis. Autoradiographic signals were quantitated by densitometric scanning using an AlphaImager™ 3400 densitometer and AlphaEaseFC software (AlphaInnotech, San Leandro, CA).

Northern Blot Analysis—CWR-R1 cells (5×10^6/10-cm dish) were plated in prostate growth medium lacking EGF. The next day, cells were washed with phosphate-buffered saline, and the medium was changed to phenol red-free improved MEM zinc option (Invitrogen) containing 0.2% albumin (AlbuMax I; Invitrogen). After an overnight incubation, cells were treated without or with 100 ng/ml EGF for 24 h in the same medium prior to RNA isolation using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (15 μg) aliquots were fractionated on 1% agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled pSG5TIF2 BamHI fragment containing nucleotides 629–869 and a pGEM-18S-4 (Promega) Sp6-generated fragment for control 18 S ribosomal RNA.

RNA Interference—PCR primers to amplify TIF2/GRIP1 target regions were synthesized to incorporate T7 polymerase promoters on the sense and antisense DNA strands. PCR primer sequences were based on the DNA sequence published by Voegel *et al.* (39) as follows with flanking T7 sequence in brackets: TIF2-A nucleotides 197–216, 5′-[TAATACGACTCACTATAGG]CCAGGGCAGAGACAAGAAAG-3′; TIF2-B nucleotides 356–376, 5′-[TAATACGACTCACTATAGG]TTGCACATTTGTCAGGTTTGA-3′. pSG5-TIF2 was used as template for *Taq* DNA polymerase (Invitrogen) to amplify target regions by polymerase chain reaction using the following conditions: 94 °C for 1 min, 55 °C for 30 s, 72 °C for 30 s (30 cycles), and 72 °C for 10 min. PCR products were gel-purified and used as template for *in vitro* transcription using T7 RNA polymerase and the *Silencer*™ siRNA mixture kit (Ambion Inc., Austin, TX). Following DNase and RNase A treatment for 1 h, double-stranded RNA was purified through spin columns and digested with RNase III according to the manufacturer to yield siRNA mixtures. The mixtures of 12–30-bp double-stranded RNAs contain 5′-PO_4, 3′-OH, and 2-nucleotide 3′ overhangs similar to siRNA produced *in vivo*. Recent studies demonstrated that siRNAs generated by RNase III cleavage are efficient for RNA interference in mammalian cells (74, 75). CWR-R1 cells were transiently cotransfected with pCMVhAR, PSA-Luc, and 10 nM siRNA mixtures for TIF2 or with glyceraldehyde-3-phosphate dehydrogenase as a control, using Effectene reagent (Qiagen) as described above. In some studies, cells were cotransfected with pSG5-TIF2 (50 ng/6-cm dish) to overcome inhibition by the siRNAs. Cells were treated with and without DHT and EGF overnight and lysed, and luciferase activity was measured.

RESULTS

Effect of EGF on AR Transactivation in CWR-R1 Cells—The effect of EGF on AR transactivation was determined in the CWR-R1 cell line derived from the CWR22 recurrent human prostate cancer xenograft. Like the CWR22 recurrent prostate tumor, CWR-R1 cells express AR-H874Y that retains wild-type sensitivity to androgens but has increased responsiveness to other steroids (31). In the absence of androgen, EGF at 10, 100, and 500 ng/ml induced a 2.0 ± 0.3-fold increase in transactivation of MMTV-Luc that was independent of the dose of EGF (Fig. 1*A*). Activation in response to EGF alone was negligible compared with the 43 ± 3.5-fold increase in response to 0.1 nM DHT, and the addition of EGF and 0.1 mM DHT increased this further by 3.3 ± 0.6-fold. An androgen-dependent EGF-induced increase in AR transcriptional activity of 1.8 ± 0.2-fold was seen also with transient expression of wild-type AR and a PSA-Luc reporter in CWR-R1 cells, and again the increase in activity was negligible with EGF alone in the absence of DHT (Fig. 1*B*). The PSA-Luc reporter is activated only weakly by endogenous AR in CWR-R1 cells in the presence of androgen (data not shown).

The requirement for an activated AR for a substantial EGF response was supported by transiently expressing the constitutively active AR NH_2-terminal and DNA binding domain fragment AR 1–660 that lacks the ligand binding domain. EGF increased AR 1–660 transactivation of MMTV-Luc and PSA-Luc reporters by 3–4-fold in CWR-R1 cells (Fig. 1*C*). The data suggest that the EGF-induced increase in AR-mediated gene activation depends on an AR that is rendered transcriptionally competent by androgen binding or artificially by deleting the ligand binding domain.

Effects of EGF on AR Levels—To investigate the mechanism whereby EGF increases AR transcriptional activity in the presence of androgen, we determined the effect of EGF on AR

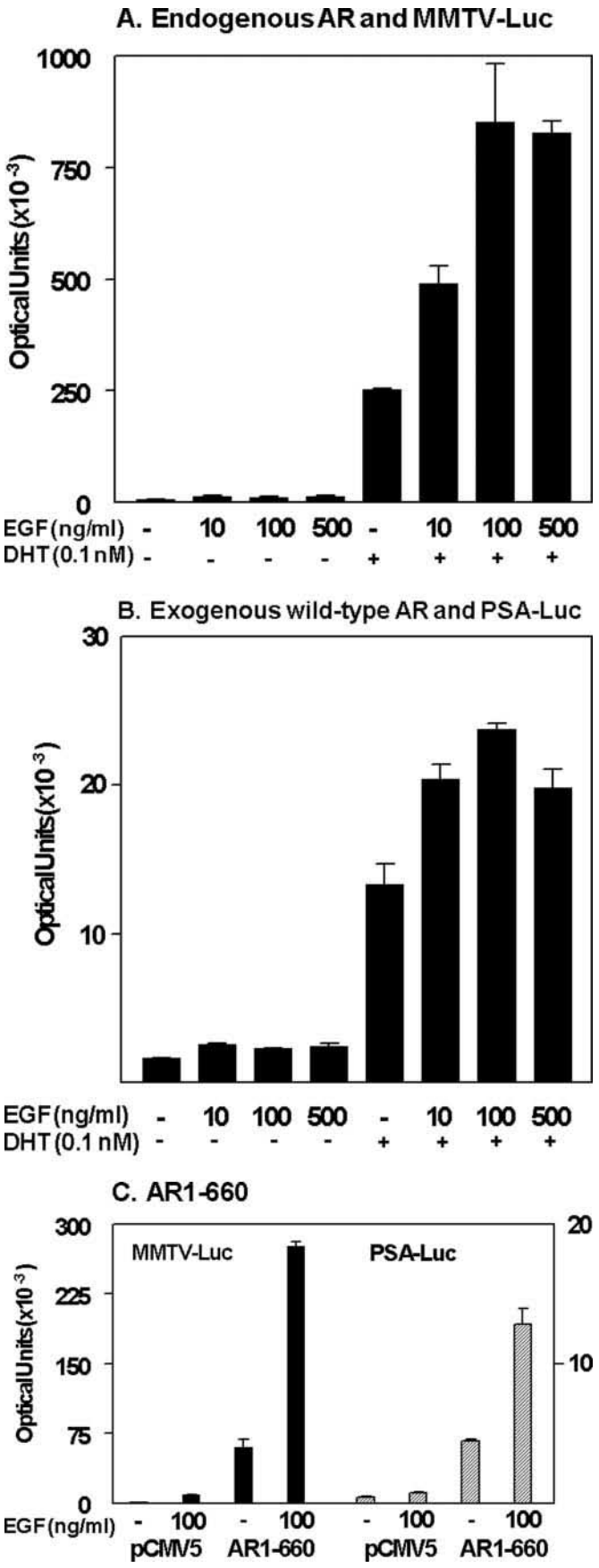

FIG. 1. **EGF stimulation of AR transcriptional activity in CWR-R1 cells.** CWR-R1 cells were assayed as described under "Experimental Procedures" for endogenous AR transactivation using MMTV-Luc (0.5 μg/6-cm dish) (*A*), exogenous wild-type AR activity determined by transfecting pCMVhAR (10 ng/dish) and PSA-Luc (1 μg/dish) (*B*), and constitutively active AR deletion mutant pCMVAR 1–660 (10 ng/dish) that lacks the ligand binding domain with MMTV-Luc and PSA-Luc (0.5 and 1 μg/dish) or 10 ng of empty parent vector pCMV5 (*C*). Cells were incubated for 24 h in the absence and presence of 10, 100, and 500 ng/ml EGF with and without 0.1 nM DHT as indicated. The mean luciferase activity values in optical units plus error of the mean are representative of at least three independent experiments.

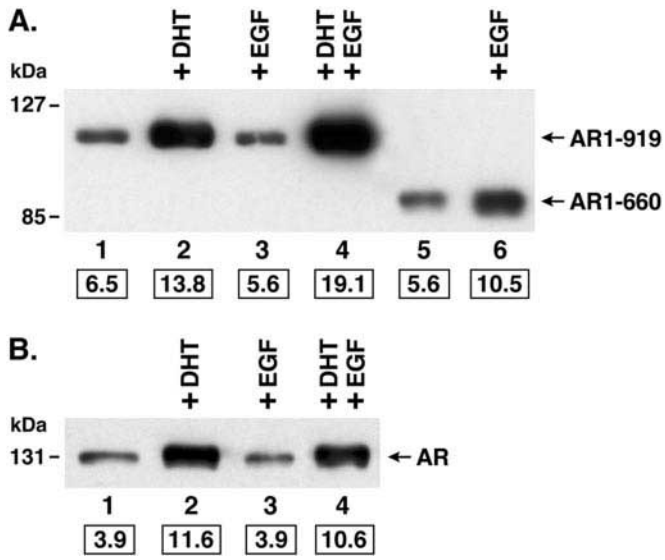

FIG. 2. **Effect of EGF on AR protein levels in COS and CWR-R1 cells.** In *A*, COS cells were transiently transfected as described under "Experimental Procedures" with 2 μg of pCMVhAR coding for full-length AR (*lanes 1–4*) and for the AR NH_2-terminal fragment pCMVhAR 1–660 (*lanes 5* and *6*). Cells were incubated in the absence and presence of 100 ng/ml EGF with or without 10 nM DHT for 24 h as indicated. Immunoblots are shown with 25 μg of protein/lane for COS cell lysates. In *B*, CWR-R1 cells were incubated with and without 100 ng/ml EGF in the absence and presence of 1 nM DHT for 24 h. Extracts were analyzed using 50 μg of protein/lane for CWR-R1 cells. Blots were incubated with AR32, an AR NH_2-terminal specific antibody. Densitometry values for specific bands are included *below* the *lanes* and are representative of more than three independent experiments.

protein levels. COS cells transiently expressing full-length wild-type AR were treated for 24 h in the absence and presence of 10 nM DHT with or without 100 ng/ml EGF. Cell lysates were analyzed on immunoblots using an AR-specific antibody (Fig. 2*A*). Full-length AR protein levels increased 2.1-fold in the presence of 10 nM DHT, reflecting androgen-induced AR stabilization (76), and 2.9-fold in the presence of DHT and EGF (Fig. 2*A*, *lane 4*), but there was no increase in the presence of EGF alone (*lane 3*). The latter result indicated that EGF did not increase transient expression of AR from the pCMVhAR plasmid. EGF also increased by 1.9-fold the level of AR 1–660, a constitutively active AR NH_2-terminal and DNA binding domain fragment (Fig. 2*A*, *lanes 5* and *6*). In CWR-R1 cells, endogenous AR protein levels increased 3.0-fold in the presence of DHT, but a further increase in the presence of EGF was not detectable, and there was no increase in the presence of EGF alone (Fig. 2*B*). Endogenous AR in CWR-R1 cells was shown previously to be relatively stable in the absence of added androgen (22).

A possible effect of EGF on AR stabilization was investigated by determining AR degradation half-times by [^{35}S]methionine pulse-chase labeling at 37 °C in COS cells. The half-time of AR degradation in the absence of DHT (t = 11.3 ± 2.7 h) increased 2-fold in the presence of DHT (t = 22.7 ± 3.8 h), but there was no further increase in the presence of DHT and EGF (t = 24.0 ± 3.0 h). The degradation half-time of AR 1–660, which lacks the ligand binding domain, was 20.0 ± 3.8 h in the absence of EGF and 24.8 ± 2.7 h in the presence of EGF (p = 0.16). The data

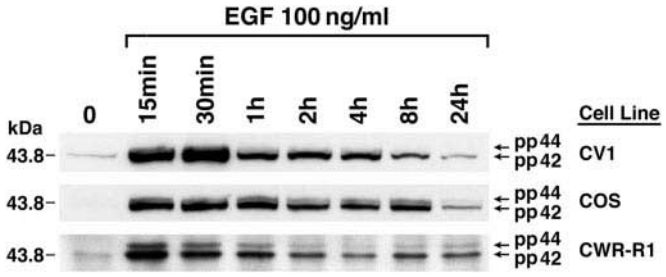

FIG. 3. **EGF-induced MAPK activity.** CV1, COS, and CWR-R1 cells were incubated with 100 ng/ml EGF for the times indicated from 15 min to 24 h. Immunoblots of cell lysates (50 μg of protein/lane) were incubated with phospho-p42/p44 antibody as described under "Experimental Procedures." The data are representative of at least three independent experiments.

suggest that an effect of EGF on AR stabilization could not be detected in COS cells.

Role of MAPK—We determined whether the EGF-induced increase in AR transcriptional activity was associated with an increase in MAPK activity. Growth factor activation of MAPK results in phosphorylation at threonine 202 and tyrosine 204 in ERK1 (pp42) and ERK2 (pp44) that can be detected on immunoblots using phosphorylation-specific antibodies (77). Within 15 min of treating CWR-R1, COS, and CV1 cells with 100 ng/ml EGF, specific phosphorylation of ERK1 (pp42) and ERK2 (pp44) was detected, followed by a decline in signal intensity over 24 h in the three cell lines (Fig. 3). The rapid increase in MAPK activity in CWR-R1 cells in response to EGF was inhibited by pretreatment with 10 μM U0126, a MEK1 and MEK2 inhibitor that prevents phosphorylation of ERK1/2 (data not shown).

The effects of the MEK inhibitor U0126 and the EGF receptor (ErbB1)-specific inhibitor ZD1839 were tested on endogenous AR transactivation in CWR-R1 cells in the absence and presence of EGF using the MMTV-Luc reporter (Fig. 4*A*). Increasing concentrations of U0126 from 0.1 to 2.5 μM inhibited AR transactivation in the absence and presence of added EGF. In contrast, ZD1839 blocked the EGF-induced increase in luciferase activity but had no effect on the transcriptional response in the absence of added EGF. Control experiments indicated that U0126 and ZD1839 did not inhibit general transcription, since the same concentrations of inhibitors in CWR-R1 cells did not reduce the activity of constitutively active reporter vectors pSG5-Luc, pSV2-Luc, or pA3RSV400Luc (78) (data not shown).

The data indicate that the transcriptional capacity of the androgen-activated endogenous AR in CWR-R1 cells is increased by EGF through the MAPK pathway. The kinase inhibitor results suggest that a possible autocrine regulatory loop involving ligands in the EGF family is present in CWR-R1 cells and may not depend solely on the ErbB1 receptor, since the EGF receptor-selective inhibitor ZD1839 did not diminish AR activity in the absence of added EGF. On the other hand, inhibition of transcription by U0126 in the absence of added EGF suggested that other members of the EGF receptor family, such as ErbB2 and ErbB3, mediate signals through the MAPK pathway to increase DHT-dependent AR transcriptional activity in the CWR-R1 recurrent prostate cancer cell line.

The MAPK pathway was also implicated in the EGF-induced increase in AR observed in COS cells in the presence of DHT. ZD1839 (2.5 μM) and U0126 (2.5 μM) blocked the EGF-induced increase in AR levels observed in the presence of DHT (Fig. 4*B*). In contrast, these inhibitors had no effect on DHT-induced AR stabilization (Fig. 4*B*) that was shown previously to require the androgen-induced NH_2-terminal and carboxyl-terminal (N/C) interaction in AR (79). The results support that MAPK signaling acts synergistically with DHT.

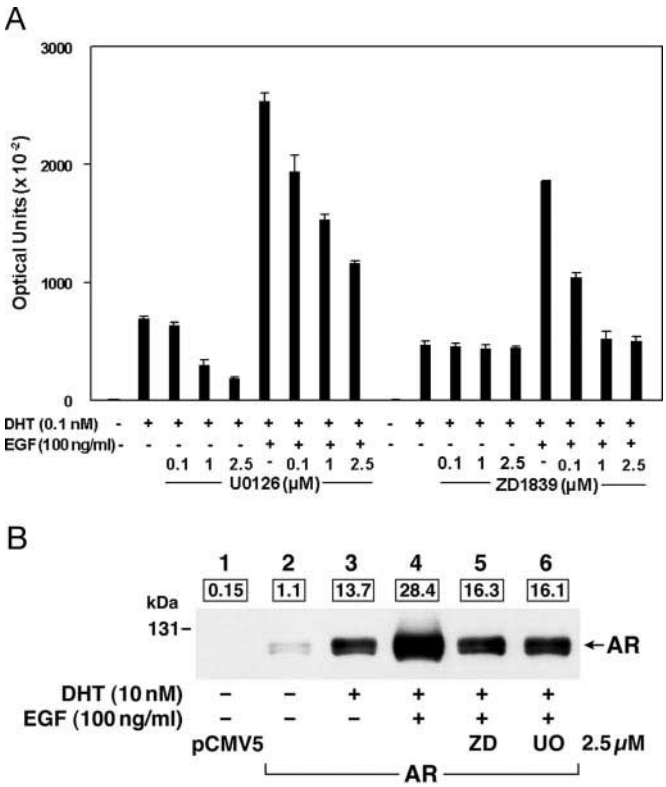

FIG. 4. **Inhibition of AR transactivation and EGF-induced AR stabilization by EGF receptor and MEK inhibitors.** In *A*, transactivation by endogenous AR was determined in CWR-R1 cells transfected with MMTV-Luc (0.5 μg/6-cm dish) as described under "Experimental Procedures." Cells were incubated for 24 h with and without 0.1 nM DHT in the absence and presence of 100 ng/ml EGF and increasing concentrations (0.1–2.5 μM) of the MEK inhibitor U0126 or the EGF receptor (ErbB1) inhibitor ZD1839. Luciferase activity is shown as the mean and error and is representative of at least three experiments. In *B*, pCMV5 empty parent vector (*lane 1*) and pCMVhAR (2 μg of DNA/10-cm dish; *lanes 2–6*) were transiently expressed in COS cells. Cells were treated in the absence and presence of 10 nM DHT with or without 100 ng/ml EGF as indicated. Some of the cells treated with DHT and EGF were also incubated with 2.5 μM ZD1839 (*lane 5*) or 2.5 μM U0126 (*lane 6*). Immunoblots of cell lysates (25 μg of protein/lane) were probed with the AR32 antibody. Densitometry values for specific bands are included *above* the *lanes*.

EGF Regulation of TIF2 Expression—We showed previously that progression to recurrent growth of prostate cancer is associated with increased levels of p160 coactivators (44) that can increase AR activity (68). We therefore investigated expression levels and effects of EGF on endogenous TIF2 levels in several cell lines including the CWR-R1 cell line and in the CWR22 human prostate cancer xenograft. Steady state levels of TIF2 were highest in the CWR-R1 cell line compared with prostate cancer cell lines PC3, LNCaP, and LNCaP-C4-2 and HeLa cells when equal amounts of protein were analyzed by immunoblot (Fig. 5*A*). Lower levels of TIF2 were detected in COS and CV1 cells, and TIF2 was undetectable in a nontransformed human foreskin fibroblast cell line. EGF increased TIF2 levels in CWR-R1 cells after 8 h compared with an earlier increase in COS cells and little change in CV1 cells (Fig. 5*B*). The higher steady state level of TIF2 in CWR-R1 cells and the increase after EGF treatment support the possibility that elevated levels of TIF2 contribute to the EGF-induced increase in androgen-dependent AR transactivation. We ruled out a direct effect of EGF on TIF2 transcription, since Northern blot analysis showed similar levels of TIF2 mRNA in CWR-R1 cells with and without EGF treatment (Fig. 5*C*).

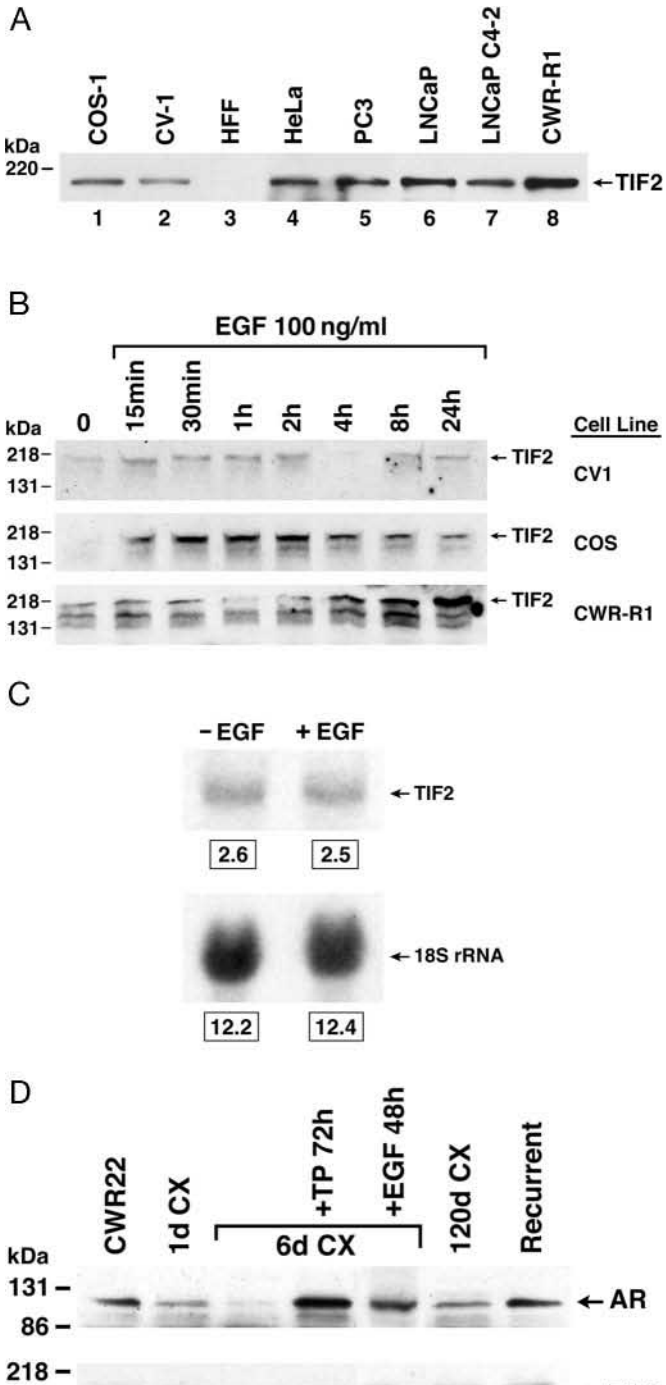

FIG. 5. **Endogenous TIF2 expression and regulation by EGF.** In *A*, endogenous TIF2 levels were assessed in whole cell lysates from the indicated cell lines harvested during logarithmic growth. The immunoblot was performed on cell extracts (50 μg of protein/lane) prepared in RIPA buffer in the presence of protease inhibitors. All cell lines were grown in the absence of supplemental EGF. Blots were probed with anti-TIF2 antibody (BD Biosciences). In *B*, CV1, COS, and CWR-R1 cells were untreated (0) or were incubated with 100 ng/ml EGF for increasing times from 15 min to 24 h as indicated. The blot was probed with the anti-TIF2 antibody. For the Northern blot in *C*, CWR-R1 cells were incubated without and with EGF (100 ng/ml) in the absence of serum for 24 h. RNA was isolated using TRIzol reagent as described under "Experimental Procedures." 15 μg of RNA was fractionated on 1% agarose gels, transferred to a nylon membrane, and hybridized with ^{32}P-labeled TIF2 and 18 S ribosomal RNA cDNAs. Densitometry values of the specific bands are included *below* the *lanes*. In *D*, the levels of AR (*upper panel*) and TIF2 (*lower panel*) were determined by immunoblot of cell lysates from the androgen-dependent CWR22 tumor (*lane 1*), and from CWR22 tumors harvested 1 day (*lane 2*), 6 days (*lanes 3–5*), and 120 days (*lane 6*) after castration and removal of the testosterone implants. The recurrent tumors were propagated in castrated mice and harvested ~45 days later (*lane 7*). Six days after castration, some mice received a single injection of 25 mg/kg testosterone propionate in sesame oil intraperitoneally, and cell lysates were prepared 72 h later (*lane 4*), or mice received a single injection of 150 μg/kg subcutaneous EGF in sterile water, and cell lysates were prepared 48 h later (*lane 5*). Immunoblots of cell lysates (100 μg of protein/lane) were incubated with AR32 or TIF2 antibody. Data in *A* represent two independent experiments, data in *B* represent three experiments, and data in *C* represent 2–6 tumors in each treatment group.

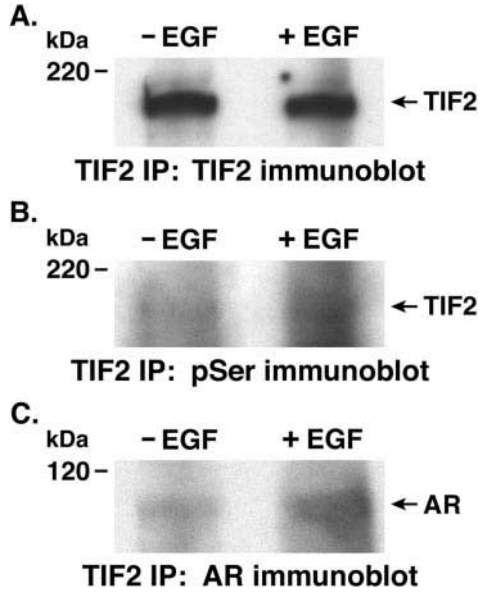

FIG. 6. **Effects of EGF on TIF2 phosphorylation and interaction with AR by TIF2 immunoprecipitation.** CWR-R1 cells were incubated with and without 100 ng/ml EGF in the absence of serum for 24 h. Equal amounts of protein (4 mg) from cell lysates were immunoprecipitated (*IP*) using anti-TIF2 monoclonal antibody as described under "Experimental Procedures." In *A*, immunoprecipitated TIF2 was subjected to immunoblot analysis using anti-TIF2 antibody. In *B*, CWR-R1 cells were incubated with 1 nM DHT in the absence and presence of 100 ng/ml EGF, and equal amounts of cell lysate protein were immunoprecipitated with anti-TIF2 antibody. Shown is an immunoblot using anti-phosphoserine antibodies (*pSer*) to detect phosphorylated TIF2. In *C*, CWR-R1 cells were incubated with 1 nM DHT with and without 100 ng/ml EGF, and equal amounts of cell lysate protein were immunoprecipitated using anti-TIF2 antibodies. The immunoblot was probed with AR polyclonal antibody AR32 to detect AR co-immunoprecipitated with TIF2. The data are representative of at least three independent experiments.

The EGF-induced increase in TIF2 levels was also observed in the androgen-dependent CWR22 tumor that was propagated in nu/nu athymic mice implanted with testosterone pellets (62, 72) (Fig. 5*D*). Six days after castration and removal of the testosterone source, TIF2 was barely detectable in the CWR22 androgen-dependent tumor (Fig. 5*D*, *lanes 1–3*) but increased 4–6-fold when mice were treated 6 days after castration with EGF and analyzed 48 h later (*lane 5*). TIF2 levels also increased 5–10-fold after mice were treated with a single injection of 25 mg/kg testosterone propionate 6 days after castration and analyzed 72 h later (*lane 4*). The increase in TIF2 levels in response to testosterone could be indirect through EGF, since testosterone has been shown to increase circulating EGF levels in castrated mice (80, 81). Testosterone may also increase EGF levels through a direct effect on xenograft tumor cells. In the recurrent CWR22 tumor that develops after prolonged androgen deprivation, TIF2 levels were elevated in the absence of androgen replacement (Fig. 5*D*), suggesting that additional

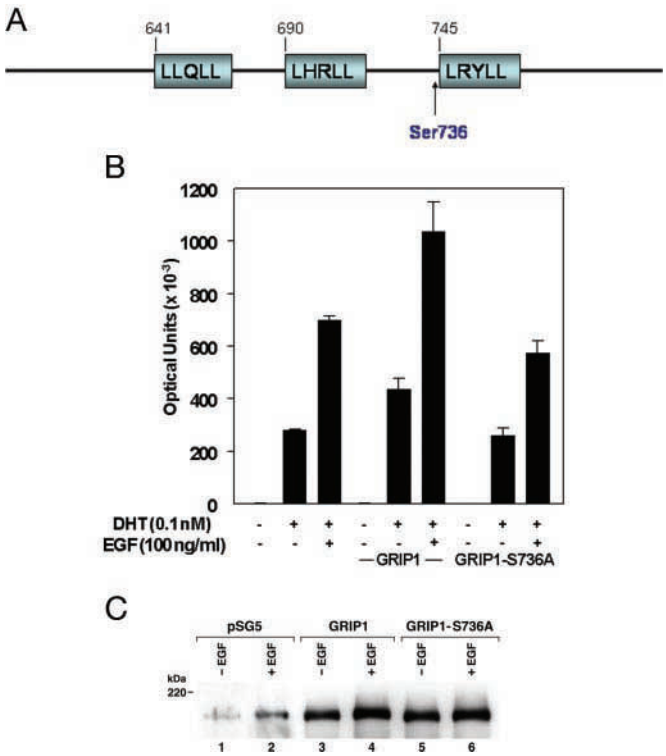

FIG. 7. **Effects of EGF on the AR-TIF2/GRIP1 interaction.** *A*, schematic diagram of the three L*XX*LL motifs of TIF2/GRIP1 and their position relative to the MAPK consensus site at Ser[736] that flanks the third L*XX*LL motif of TIF2/GRIP1, the predominant interaction site for the AR AF2 region (67, 70). Amino acid residues are numbered. *B*, CWR-R1 cells were transfected with MMTV-Luc (0.5 μg/6-cm dish) and 0.1 μg/dish of pSG5 empty vector, pSG5-GRIP1, or pSG5-GRIP1-S736A. Cells were incubated for 24 h in the absence and presence of 0.1 nM DHT with and without 100 ng/ml EGF as indicated. In *C*, immunoblot of endogenous TIF2 (*lanes 1* and *2*), transiently transfected pSG5-GRIP1 (*lanes 3* and *4*), and pSG5-GRIP1-S736A (*lanes 5* and *6*) before and after treatment with 100 ng/ml EGF for 24 h. 50 μg of protein extracts of COS cells were analyzed by immunoblot using the TIF2 antibody as described under "Experimental Procedures." Exposure times of the film were 5 min to detect endogenous TIF2 (*lanes 1* and *2*) and 30 s for transiently expressed TIF2/GRIP1 (*lanes 3–6*).

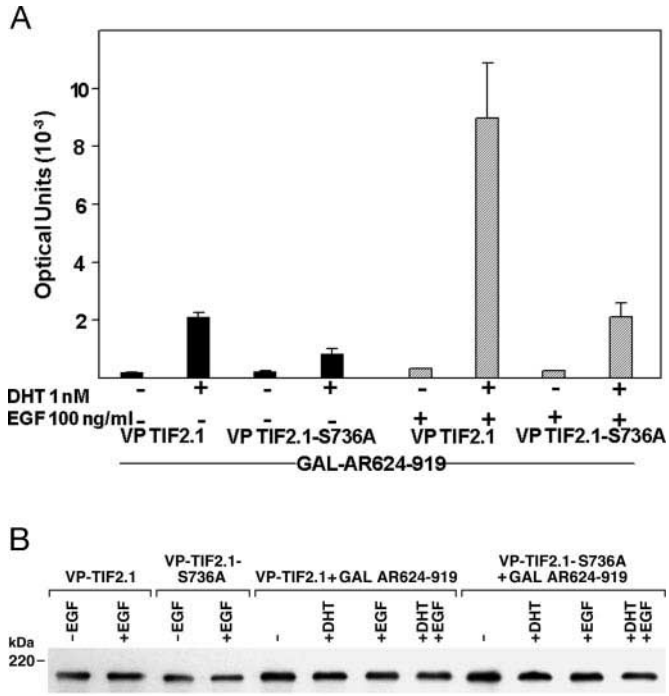

FIG. 8. **Effect of EGF on the interaction between AR and TIF2.** In *A*, two hybrid interaction assays were performed in CWR-R1 cells by cotransfecting 10 ng of VPTIF2.1 (TIF2 residues 624–1287) or VP-TIF2.1-S736A with 100 ng of GAL-AR 624–919 and 0.1 μg of 5×GAL4Luc3. CWR-R1 cells were incubated with or without 1 nM DHT in the absence and presence of 100 ng/ml EGF. The mean and S.E. of luciferase activity are representative of three independent experiments. In *B*, COS cells were transiently transfected as described under "Experimental Procedures" with 1 μg each of VP-TIF2.1 or VPTIF2.1-S736A with and without 1 μg of GAL-AR 624–919 using Effectene reagent. Cells were incubated overnight in the absence and presence of EGF (100 ng/ml) with and without 10 nM DHT. Cell lysates (10 μg of protein/lane) were immunoblotted, and membranes were incubated with anti-TIF2 antibody. The data are representative of three independent experiments.

mechanisms such as autocrine signaling contribute to increased TIF2 levels in prostate cancer tumor progression.

AR levels also increased after testosterone treatment 6 days after castration and to a lesser extent with EGF alone (Fig. 5*D*). In comparison with the tumor analyzed 6 days after castration, the recurring CWR22 tumor growing at 120 days after castration showed higher AR expression that approached the level observed in the recurrent tumor as reported previously (82). The results indicate that androgen and EGF increase TIF2 and AR levels in the androgen-sensitive CWR22 tumor to levels seen in the recurrent tumor.

Mechanism for the EGF-induced Increase in AR Transactivation—The EGF-induced increase in TIF2 levels and AR transactivation led us to investigate TIF2 phosphorylation, since previous studies on the estrogen and progesterone receptors suggested that EGF increases phosphorylation of p160 coactivators (83). Using an anti-TIF2-specific antibody, TIF2 was immunoprecipitated from lysates of CWR-R1 cells incubated in the absence and presence of EGF (Fig. 6*A*). Immunoblotting using phosphoserine antibodies suggested an increase in TIF2 phosphorylation in the presence of EGF (Fig. 6*B*). Moreover, in a separate experiment in the presence of DHT, we found that AR coimmunoprecipitated with TIF2, and the amount of AR in the coimmunoprecipitate increased in the presence of EGF (Fig. 6*C*). The data raised the possibility that EGF-induced phosphorylation of TIF2 increases its interaction with AR.

The proximity of the TIF2 MAPK site Ser[736] and the third L*XX*LL motif of TIF2 (Fig. 7*A*) suggested that EGF-induced phosphorylation of TIF2 might increase AR transactivation by enhancing its interaction with AR. The third L*XX*LL motif of TIF2 is the predominant interaction site among the three L*XX*LL motifs that bind activation function 2 (AF2) in the AR ligand binding domain (68, 78). Coexpression of GRIP1, the mouse homologue of human TIF2 that differs by only 2 amino acids (83), with an MMTV-Luc reporter increased DHT-dependent transcriptional activity of endogenous AR in CWR-R1 cells in the absence and presence of EGF (Fig. 7*B*). When the GRIP1-S736A phosphorylation mutant was expressed, the transcriptional response to DHT alone and to DHT and EGF was similar to that observed in the absence of GRIP1 transient expression. Transient expression levels of GRIP1 and the GRIP1-S736A mutant were similar, as shown in immunoblots of COS cell extracts (Fig. 7*C*, *lanes 3* and *6*), in agreement with a previous report (83), suggesting that expression levels were also similar in CWR-R1 cells. A direct comparison of expression levels of the wild-type and mutant GRIP1 plasmids in CWR-R1 cells was complicated by the relatively high TIF2 levels in CWR-R1 cells (see Fig. 5*A*) combined with low expression of plasmid DNA. As shown above (Fig. 5*B*), EGF increased endogenous TIF2/GRIP1 levels in COS cells (Fig. 7*C*, *lanes 1* and *2*) and the levels of transiently expressed GRIP1 but not the GRIP1-S736A mutant (Fig. 7*C*, *lanes 5* and *6*). The data sug-

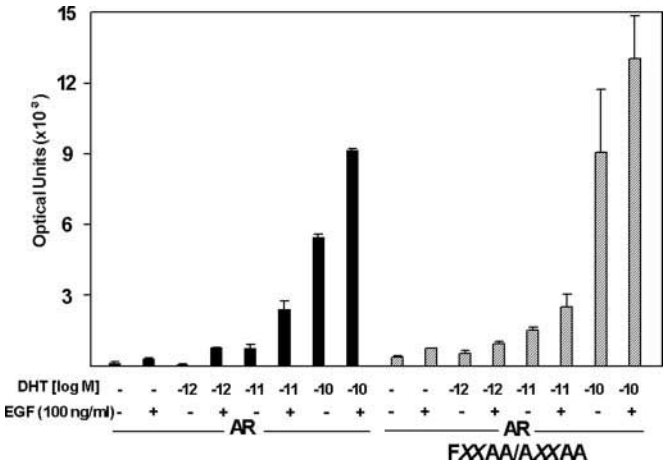

FIG. 9. **Transcriptional activation of PSA-Luc by an AR N/C interaction mutant in CWR-R1 cells.** CWR-R1 cells were transfected with 1 μg of PSA-Luc reporter and 10 ng of pCMVhAR or the AR N/C interaction mutant pCMVhAR-F*XX*AA/A*XX*AA in which ^{23}FQNLF^{27} and ^{433}WHTLF^{437} in the AR NH_2-terminal domain were mutated to FQNAA and AHTAA, respectively (66). Cells were transfected using Effectene as described under "Experimental Procedures" and incubated for 24 h with and without increasing concentrations of DHT as indicated in the absence and presence of 100 ng/ml EGF. The data are representative of three independent experiments.

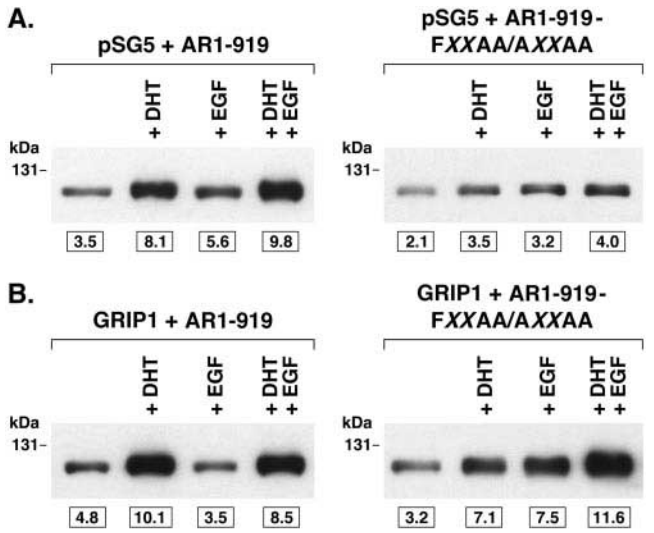

FIG. 10. **Effects of EGF and GRIP1 on expression levels of AR and the AR N/C interaction mutant.** Wild-type and N/C interaction mutant AR levels were determined in COS cells by transient expression of 1 μg of wild-type pCMVhAR or pCMVhAR-F*XX*AA/A*XX*AA in the absence and presence of 1 μg of pSG5 empty vector or pSG5-GRIP1 using Effectene as described under "Experimental Procedures." Cells were treated in the absence and presence of 10 nM DHT with and without 100 ng/ml EGF as indicated. Immunoblots were probed with AR antibody AR32 from cell lysates (25 μg of protein/lane) for wild-type AR and the N/C interaction mutant AR-F*XX*AA/A*XX*AA in the absence (*A*) or presence (*B*) of transiently expressed GRIP1. Densitometry values for specific bands are included *below* the *lanes*.

gest that the EGF-induced increase in AR transcriptional activity is mediated at least in part by phosphorylation of GRIP1 at Ser^{736} and by increased levels of TIF2/GRIP1.

The requirement for GRIP1 phosphorylation in its interaction with AR was evaluated further in the CWR-R1 cell line using a two-hybrid interaction assay. VPTIF2.1 coding for the VP16 activation domain and TIF2 residues 624–1287 (39, 70) was co-expressed with GAL-AR 624–919 coding for the AR ligand binding domain and GAL4 DNA binding domain. The androgen-dependent interaction between GAL-AR 624–919 and VPTIF2.1 was reduced with the phosphorylation site mutant VPTIF2.1-S736A (Fig. 8*A*). The addition of EGF increased the interaction between AR and VPTIF2.1 and between AR and VPTIF2.1-S736A but was greater for wild-type TIF2. The high levels of endogenous TIF2 in CWR-R1 cells probably contributed to some of the activity observed in the presence of the TIF2 mutant. Expression levels of the VPTIF2.1 and VPTIF2.1-S736A plasmid DNA were similar in the absence and presence of GAL-AR 624–919, as demonstrated in COS cells (Fig. 8*B*). The results suggest that EGF increases the interaction between AR and TIF2 through phosphorylation at serine 736 adjacent to the third L*XX*LL motif and probably through additional phosphorylation sites in TIF2.

Role of the AR N/C Interaction—We showed previously that the androgen-dependent AR NH_2- and carboxyl-terminal N/C interaction is mediated by the NH_2-terminal F*XX*LF and W*XX*LF motifs (66) and is required for DHT-induced AR stabilization (67, 84) and slows the dissociation rate of bound androgen (66, 67, 79). The AR-F*XX*AA/A*XX*AA mutant that lacks the N/C interaction activates the MMTV-Luc reporter in CV1 cells to essentially the same extent as wild-type AR, but in CV1 cells this mutant only weakly activates the PSA-Luc reporter compared with wild-type AR (68). We now show that transiently expressed AR-F*XX*AA/A*XX*AA in CWR-R1 cells is equipotent to wild-type AR in activating PSA-Luc (Fig. 9), and EGF increases the androgen-dependent activity.

Previous studies demonstrated that increased expression of TIF2 in CV1 cells could overcome the requirement for the AR N/C interaction in activating the PSA-Luc reporter (68). Since an important functional consequence of the N/C interaction is stabilization of the AR that may be necessary for AR activity *in vivo* (67, 79, 84), we investigated whether increased coactivator levels could replace this function by stabilizing the AR-F*XX*AA/A*XX*AA mutant. Under conditions of low endogenous TIF2/GRIP1 levels in COS cells, DHT had little effect on AR-F*XX*AA/A*XX*AA levels compared with the increase seen with wild-type AR (Fig. 10), as previously reported (67). The addition of EGF only slightly increased AR-F*XX*AA/A*XX*AA levels in the presence or absence of DHT. In contrast, when GRIP1 was overexpressed, there was a striking increase in AR-F*XX*AA/A*XX*AA levels with DHT, which was further increased in the presence of EGF (Fig. 10). It is noteworthy that EGF in the presence of GRIP1 but in the absence of androgen also increased AR-F*XX*AA/AXXAA levels. The results suggest that increased TIF2/GRIP1 expression that can occur in response to EGF and that is frequently observed in recurrent prostate cancer (44) contributes to increased AR-mediated transactivation. Increased levels of TIF2 may compete more effectively for the N/C interaction, resulting in increased coactivator recruitment and AR transactivation.

Effect of TIF2/GRIP1 siRNA on AR Transactivation—To determine whether TIF2/GRIP1 is required for AR transactivation by DHT and EGF in CWR-R1 cells, we used RNA interference to inhibit endogenous TIF2/GRIP1 expression. An siRNA mixture consisting of 12–30 bp of double-stranded RNA coding for TIF2/GRIP1 was transiently transfected into CWR-R1 cells together with pCMVhAR and PSA-Luc. TIF2 siRNA directed at nucleotides 197–376 inhibited AR transactivation of PSA-Luc by 5-fold, whereas a glyceraldehyde-3-phosphate dehydrogenase siRNA mixture had no effect (Fig. 11). Specificity for the TIF2 siRNA inhibition was established by cotransfecting pSG5TIF2 with 10 nM TIF2 siRNA. Partial recovery of AR transactivation of PSA-Luc activity in the presence of overexpressed TIF2 provided evidence that inhibition by TIF2 siRNA was specific.

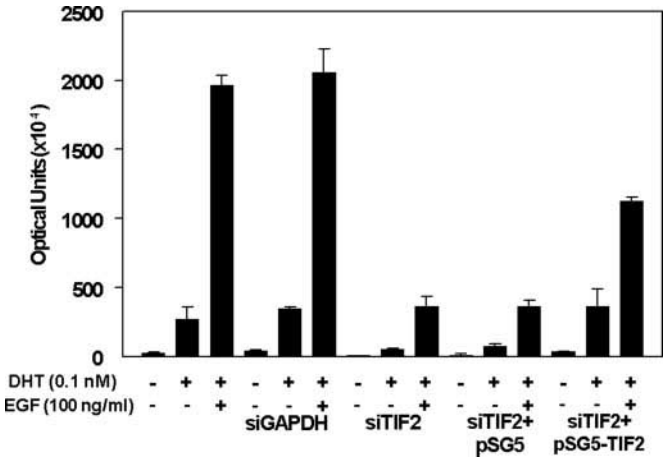

FIG. 11. **Interference of AR transcriptional activity by TIF2 siRNA.** CWR-R1 cells were transfected with the PSA-Luc reporter and pCMVhAR with and without 10 nM glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) siRNA or TIF2 siRNA in the absence and presence of 0.05 μg of pSG5 or pSG5-TIF2 using Effectene as described under "Experimental Procedures." Cells were incubated for 24 h with and without 0.1 nM DHT in the absence and presence of 100 ng/ml EGF. The data are representative of four independent experiments.

DISCUSSION

The recurrent growth of prostate cancer after prolonged androgen deprivation is recapitulated by human xenograft models such as CWR22 (22, 62, 85) and LAPC-4 and LAPC-9 (86) prostate tumors. Like human prostate cancers that are androgen-dependent for growth, these human tumors propagated in nude mice regress following androgen withdrawal by castration but after several months regrow in an environment of low circulating androgen. Most reports implicate a critical role for the AR in recurrent prostate cancer growth and progression despite reduced circulating androgen levels, and some studies suggest that mitogen signaling bypasses the requirement for androgen. In the present report, we provide evidence that EGF signaling through MAPK increases androgen-dependent AR transcriptional activity in the CWR-R1 recurrent human prostate cancer cell line. EGF increased androgen-dependent AR transactivation in association with increased levels of TIF2 in the CWR-R1 cell line. EGF and testosterone each increased AR and TIF2 levels in the androgen-dependent CWR22 xenograft tumor, supporting their complementary relationship. EGF increased TIF2 phosphorylation and the interaction between phosphorylated forms of TIF2 and AR. The effects of EGF were mediated in part through phosphorylation of TIF2/GRIP1 at Ser^{736}. TIF2/GRIP1 in CWR-R1 cells also probably contributed to increased transactivation by the NH_2-terminal activation function 1 of the AR NH_2-terminal DNA binding domain fragment AR 1–660. Reducing TIF2/GRIP1 levels in CWR-R1 cells using inhibitory RNAs resulted in a decreased AR transcriptional response to DHT and EGF. The data provide evidence for a direct link between AR transcriptional activity in recurrent prostate cancer, EGF signaling, and increased p160 coactivator levels. In addition, the studies provide a mechanism to explain the recent finding that a majority of recurrent prostate cancers have elevated levels of TIF2 (44).

Mechanisms described thus far to account for increased AR transactivation in recurrent prostate cancer include ligand-independent activation by mitogen signaling, AR overexpression, and AR mutations. Of these, increased signaling by EGF and TGFα was reported to occur in association with the transition from androgen-dependent to recurrent prostate cancer (87), and prostate cancer cell lines have been shown to synthesize and secrete EGF and related peptides (88). In addition, the EGF family of receptors is expressed in most recurrent prostate cancers (89), with ErbB2 protein expression most frequently reported (90–93). The CWR22 xenograft (62, 94–97) expresses ErbB1, -2, and -3 as shown by reverse transcription PCR (62, 94, 95), and the CWR-R1 cell line expresses ErbB1, -2, -3, and -4.[2] This agrees with reports that the ErbB1 inhibitor ZD1839 reduces the growth of androgen-dependent and recurrent CWR22 cells in culture (98) and that growth in primary cultures of androgen-dependent and recurrent CWR22 cells was inhibited by a monoclonal antibody to ErbB2 that blocked heregulin-induced activation of MAPK and Akt (94).

The link between EGF signaling and AR is supported by a number of previous studies, most of which relied on the overexpression of key signaling molecules. In the LNCaP prostate cancer cell line, increased growth and PSA expression were observed after stable overexpression of ErbB2 through the MAPK pathway (99). Overexpression of ErbB2 was associated with ligand-independent activation of AR in recurrent prostate cancer growth (99). Cell signaling by interleukin-6 in LNCaP cells was mediated by tyrosine phosphorylation of ErbB2 and ErbB3 (100). Stable overexpression of a constitutively active mitogen-activated protein kinase kinase kinase 1-induced apoptosis in LNCaP cells expressing AR, but there was no change in PC3 or DU145 cells that lack AR expression (101). ErbB2 was reported to activate Akt-directed phosphorylation at AR serine residues 213 and 791 in association with growth of recurrent prostate cancer in the absence of androgen (102). MAPK-directed AR phosphorylation was also implicated in hormone-independent AR activation in prostate cancer (99, 101). Overall, the results support the link between the MAPK pathway and AR transcriptional activity.

The data presented in this report indicate that increased AR transcriptional activity occurs in response to EGF in part from an increase in the androgen-dependent association between AR and TIF2/GRIP1. The interaction between p160 coactivator TIF2/GRIP1 and AR is mediated primarily by binding of the third L*XX*LL motif of TIF2 to the AF2 hydrophobic surface in the AR ligand binding domain (103–105). Under normal conditions of low coactivator expression as shown here for a human foreskin fibroblast cell line, AR interaction with p160 coactivators might be limited by the lower binding affinity of the coactivator L*XX*LL motifs to the AR AF2 binding site compared with binding of the AR NH_2-terminal F*XX*LF motif that mediates the androgen-dependent N/C interaction (66, 106). An optimal transcriptional response of AR to TIF2/GRIP1 depends on the interaction between the L*XX*LL motifs and the AR AF2 region (68). We have shown previously that increased levels of p160 coactivators compete for the androgen-induced N/C interaction to gain access to the AF2 region in the ligand binding domain (67). Mutating Ser^{736} adjacent to the third and predominant interacting L*XX*LL motif of TIF2/GRIP1 reduced the interaction between TIF2/GRIP1 and AR, supporting a key role for L*XX*LL motif binding. The importance of phosphorylation at TIF2/GRIP1 serine 736 was shown previously for coactivation of the estrogen and progesterone receptors (83). In the present report, we provide further evidence that this MAPK signaling pathway contributes to increased AR transactivation in recurrent prostate cancer.

We found that transient expression of TIF2/GRIP1 in the presence of DHT and EGF had a stabilizing effect on AR-F*XX*AA/A*XX*AA, an AR with mutations in the NH_2-terminal ^{23}FQNLF^{27} and ^{433}WHTLF^{437} sequences that are required for the androgen-induced AR N/C interaction (66–68). Mutations

[2] C. W. Gregory, W. McCall, X. Fei, Y. E. Whang, F. S. French, E. M. Wilson, and H. S. Earys, unpublished results.

that cause loss of the N/C interaction allow greater accessibility of AF2 in the ligand binding domain to activation by p160 coactivators such as TIF2/GRIP1 (67). Surprisingly, whereas loss of the N/C interaction reduced AR transactivation of the PSA promoter in other cell lines (68), this mutant was as effective as wild-type AR when assayed in the CWR-R1 cell line, supporting the notion that higher levels of TIF2 compensate for loss of the AR N/C interaction.

EGF also increased transactivation by the AR NH_2-terminal and DNA binding domain fragment AR 1–660 that lacks the ligand binding domain. This agrees with previous reports that p160 coactivators interact with multiple regions of steroid receptors, including an interaction between the glutamine-rich region of p160 coactivators and the NH_2-terminal domains of steroid receptors (70, 107, 108). The EGF-induced increased in TIF2/GRIP1 activity in CWR-R1 cells therefore also probably contributes to AR transactivation through interactions with the AR NH_2-terminal region. Previous studies on the effects of EGF on the progesterone and estrogen receptors support increased p160 coactivator activity as a mechanism for growth factor regulation of nuclear receptors (83).

Evidence presented here and previously suggests that recurrent prostate cancer is influenced by autocrine loops involving EGF signaling. Recurrent growth of the androgen-dependent CWR22 xenograft in the absence of testis-derived androgen occurred in the presence of increased expression of TGFα, which could establish an autocrine regulatory loop through the EGF receptor (87). Recurrent CWR22 tumors express high levels of EGF-related ligands compared with the androgen-dependent tumor (94), and increased immunostaining of TGFα was found in recurrent CWR22 xenografts (87). Further support for an autocrine regulatory loop comes from observations that CWR-R1 cells express EGF, heparin-binding EGF, TGFα, and heregulin messenger RNAs.[2] In the present report, we show that ZD1839, an EGF receptor (ErbB1)-specific inhibitor, did not diminish the transcriptional activity of endogenous AR in CWR-R1 cells, whereas the downstream MEK inhibitor U0126 decreased DHT-induced AR transactivation in CWR-R1 cells in the absence of added EGF. These data suggest that an endogenous EGF or TGFα-like ligand induces MAPK signaling independent of the EGF receptor, ErbB1. The ErbB2 and ErbB3 receptors may therefore be key modulators of AR activation in CWR-R1 cells, as reported for heregulin and ErbB3 in advanced prostate cancer (61). EGF-related peptides produced by CWR-R1 cells could interact with other members of the EGF receptor family (50). Our studies did not provide evidence that autocrine signaling through the EGF receptor family can override a requirement for androgen. Rather, autocrine regulation of recurrent prostate cancer cells appears to contribute to androgen-activated AR-mediated gene transcription. Earlier evidence suggested that prostate cancer cells establish an autocrine loop through EGF or TGFα and their receptors (109), and studies cited above indicate that this autocrine loop is present in the recurrent CWR22 xenograft and CWR-R1 cell line.

The apparent requirement for androgen by recurrent prostate cancer cells for AR transactivation raises the question of the source of ligand. In prostate cancer patients that are castrated or treated with luteinizing hormone-releasing hormone agonists to suppress testicular androgen production in response to pituitary luteinizing hormone, adrenal androgens circulate at levels sufficient to serve as precursors for the biosynthesis of testosterone and DHT. Remarkably, testosterone levels in recurrent prostate cancer tissue specimens during androgen withdrawal therapy were found to be similar to levels in benign prostate hyperplasia tissue from untreated patients (110). DHT levels in recurrent prostate cancer tissue were reduced to 10% compared with benign prostate. These results suggest that tissue androgen production may be sufficient in recurrent prostate cancer to activate AR after the withdrawal of circulating androgen. Furthermore, the range of steroids that activate AR in some prostate cancers is increased by certain AR mutations in the ligand binding domain, such as the LNCaP AR mutant T877A and AR-H874Y mutant in the CWR22 tumor and derived cell lines (31). Human prostate cancer xenografts expressing wild-type AR (10, 86) have a pattern of recurrent growth in nu/nu mice like that for recurrent prostate cancer in patients after androgen deprivation by castration, suggesting that tissue androgen or other AR-activating ligands are sufficient in the xenografts to trigger AR transactivation.

Therapeutic strategies for prostate cancer have been aimed recently at inhibiting the EGF signaling pathway. Inhibitors such as GW572016 gained attention in attempts to block tumor progression (111). This approach was effective in targeting ErbB2 for the treatment of breast cancer. But unlike breast cancer, where ErbB2 receptors are frequently overexpressed (112), these receptors are present but not highly expressed in most recurrent prostate cancer specimens (90, 99). Clinical targeting of ErbB2 with antitumor agents such as Herceptin (trastuzumab), a humanized monoclonal antibody to the extracellular domain of ErbB2, was ineffective in advanced prostate cancer patients that were negative for ErbB2 expression (113) but more effective when receptors were overexpressed (114). The anti-ErbB2 antibody Herceptin inhibited growth of androgen-dependent CWR22 and LNCaP xenografts but did not inhibit growth of the recurrent CWR22 tumor (115). A monoclonal antibody that binds a different region from the Herceptin binding site inhibited association of ErbB2 receptor with other EGF receptor family members, blocking heregulin-mediated signaling in androgen-dependent and -independent prostate cancer cell lines (94). Other approaches have been taken to inhibit prostate tumor growth by indirectly targeting the AR. Proliferation of prostate cancer cells and xenografts was reduced by an hsp90 inhibitor and decreased AR, ErbB2, and Akt expression levels, supporting the role of these pathways in androgen-dependent and recurrent tumor growth (116).

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