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

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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), 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 (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.

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† The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; TIF2, transcriptional intermediary factor 2; GRIP1, 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.

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 expression. In fact, more than 5% of patients exhibit AR mutations in prostate cancer (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 in-
EGF-induced increase in TIF2/GRIP1 phosphorylation is measured in prostate cancer cells. We present evidence that an EGF-induced increase in AR transcriptional activity in association with less favorable prognosis in advanced disease (25 mg/kg/day) in sesame oil (Schein Pharmaceutical, Inc., Port cervical dislocation, and tumors were resected. Testosterone propionate for analysis of the androgen-dependent CWR22 tumor, tumors were obtained release testosterone pellets to normalize circulating levels to Indian Indianapolis, IN) that were previously implanted with 12.5 mg of sus- biosciences) into nu/nu athymic mice (Harlan Sprague-Dawley, Inc., growth stimulation in cancer cells controlled by sex steroids. suggesting that p160 coactivator overexpression is linked to transcriptional intermediary factor 2 (TIF2) (39), also known as SRC1 in the majority of a randomly selected set of clinical specimens of recurrent prostate cancer compared with coacti- vator 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 transactiva- tion 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 transacti- vation (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 transactiva- tion require the presence of androgen (59, 60). Heregul- in-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 im- plants in nude mice (13, 62). CWR22 tumors of ~1 cm or less were resected and dispersed by protease digestion. Cell suspensions were introduced into the 23FQNLF27 and 433WHTLF437 AR XX AA/A 660 (64, 65), the AR N/C binding domain (69, 70). VPTIF2.1-S736A contained TIF2 residues GAL-4 DNA binding domain as a fusion protein with the AR ligand binding domain (69, 70). VPTIF2.1-S736A contained 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. Kashner (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×GAL4Luc 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/cm2 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-FXXAAXXXA, AR expression vec- tor 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 106 cells/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 mg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2% fetal bovine serum. The Ecotropic transfection reagent (Qiagen, Valencia, CA) was used according to the manufac- turer. 1 ml of a DNA reaction mix containing 150 µl of EC buffer (Qiagen), 4 µl of enhancer, 4 µl of Ecotropic reagent (Qiagen), and 1 ml of 2% serum containing prostate growth medium lacking EFG 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 3 ml red-free, serum-im- proved 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 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 luminoimeter.

For two-hybrid interaction assays, CWR-R1 cells (1 x 106 cells/6-cm dish) were transfected using Ecotropic 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 DNA binding sites and the coding sequence. Cells were treated with hormone as indicated and analyzed for luciferase activity as described above.

Immunoblot, Immunoprecipitation, and Stability Assays—Mouse 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% fetal bovine serum, penicillin, and streptomycin. Cells were seeded at ~50% confluence (1.2 x 106 COS cells/10-cm dish) and transfection the next day using Ecotropic 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. After 300 µl of EC buffer (Qiagen), 5 µl of Ecotropic 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
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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; 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 supernatant 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 pancreatin, 0.025 mg/ml Trypsin/Pronase, 0.0001% β-mercaptoethanol, 2.5 mM sodium vanadate, 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/GRIPI1 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 NH2-terminal anti-peptide antibody AR32 (73) was used at 0.7 μg/ml, anti-TIF2 mouse monoclonal (BD Biosciences) at 0.7 μg/ml, 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 inhibitors (Sigma). 4 μg 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 pre-cleared 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 (Gрадiopore Ltd.). Proteins were electroblotted to Immobilon membranes (Millipore Corp., Bedford, MA) and were blocked overnight. Membranes were blocked with incubation 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 (73) 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 using enhanced chemiluminescence (Supersignal; Pierce).

For AR stability assays, COS cells (1.2 × 106/10-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. TransfT3-label (100 μCi/dish) (PerkinElmer Life Sciences) was added to the cells with or without DHT or with or without EGF and incubated for 30 min. Cells were washed twice with 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. 32S-Labeled AR protein was immunoprecipitated using AR52 IgG (73) and Pansorbin cells (Calbiochem) and analyzed by SDS gel electrophoresis. Autoradiograms were quantitated by densitometric scanning with AlphaImager™ 3400 densitometer and AlphaEaseFC software (Alphalnnotec, San Leandro, CA).

Northern Blot Analysis—CWR-R1 cells (5 × 106/10-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 with or without 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 32P-labeled pSG5/TIF2 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/GRIPI1 target regions were synthesized to incorporate T7 polymerase promoters on the sense and antisense DNA strands. PCR primer sequences were based on the published TIF2 sequence published by Voegel et al. (39) as follows with flanking T7 sequence in brackets: TIF2-A nucleotides 197–216, 5’-TAATACGACTCACTATAGGGCCAGGGCAGACAGAAGG-3’, TIF2-B nucleotides 356–376, 5’-TAATACGACTCACTATAGGGTGCCACATTGGTCAGGTGGTTGA-3’. pSG5-TIF2 was used as template for T7 RNA 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 for 30 (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, and anti-manufacturer to yield siRNA mixtures. The mixtures of 12–20 bp double-stranded RNAs contain 5’-PO4, 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. 1A). 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 transactivation 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. 1B). 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 NH2-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. 1C). 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

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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. 2A). 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. 2A, 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. 2A, 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. 2B). 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 ($t = 11.3 \pm 2.7$ h) increased 2-fold in the presence of DHT ($t = 22.7 \pm 3.8$ h), but there was no further increase in the presence of DHT and EGF ($t = 24.0 \pm 3.0$ h). The degradation half-time of AR 1–660, which lacks the ligand binding domain, was 20.0 $\pm$ 3.8 h in the absence of EGF and 24.8 $\pm$ 2.7 h in the presence of EGF ($p = 0.16$). The data 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 ng/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.
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. 4A). 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. 4B). In contrast, these inhibitors had no effect on DHT-induced AR stabilization (Fig. 4B) that was shown previously to require the androgen-induced NH2-terminal and carboxyl-terminal (N/C) interaction in AR (79). The results support that MAPK signaling acts synergistically with DHT.

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. 5A). 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. 5B). 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. 5C).
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. 5D). Six days after castration and removal of the testosterone source, TIF2 was barely detectable in the CWR22 androgen-dependent tumor (Fig. 5D, 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. 5D), suggesting that additional 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.

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. 5D). Six days after castration and removal of the testosterone source, TIF2 was barely detectable in the CWR22 androgen-dependent tumor (Fig. 5D, 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. 5D), suggesting that additional
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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. 5D). 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). 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).

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. 6A). Immunoblotting using phosphoserine antibodies suggested an increase in TIF2 phosphorylation in the presence of EGF (Fig. 6B). Moreover, in a separate experiment in the presence of DHT, we found that AR communoprecipitated with TIF2, and the amount of AR in the communoprecipitate increased in the presence of EGF (Fig. 6C). The data raised the possibility that

EGF-induced phosphorylation of TIF2 increases its interaction with AR.

The proximity of the TIF2 MAPK site Ser736 and the third LXXLL motif of TIF2 (Fig. 7A) suggested that EGF-induced phosphorylation of TIF2 might increase AR transactivation by enhancing its interaction with AR. The third LXXLL motif of TIF2 is the predominant interaction site among the three LXXLL 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. 7B). 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. 7C, 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. 5A) combined with low expression of 5×GAL4Luc3. CWR-R1 cells were incubated with or without 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).

In A, two hybrid interaction assays were performed in CWR-R1 cells by cotransfecting 10 ng of VP-TIF2.1 (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 VP-TIF2.1-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.

EGF-induced phosphorylation of TIF2 increases its interaction with AR.

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fected with $1/H9262$ CWR-R1 cells were trans-
interaction mutant in CWR-R1 cells. androgen-dependent interaction between GAL-AR 624 was co-expressed with GAL-AR 624 – VP16 activation domain and TIF2 residues 624 using a two-hybrid interaction assay. VPTIF2.1 coding for the tional phosphorylation sites in TIF2.

Role of the AR N/C Interaction—We showed previously that the androgen-dependent AR NH2- and carboxyl-terminal N/C interaction is mediated by the NH2-terminal FXXLF and WXXLF 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-FXXAA/AXXXA 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-FXXAA/AXXXA 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-FXXAA/ AXXXA mutant. Under conditions of low endogenous TIF2/ GRIP1 levels in COS cells, DHT had little effect on AR-FXXAA/ AXXXA levels compared with the increase seen with wild-type AR (Fig. 10), as previously reported (67). The addition of EGF only slightly increased AR-FXXAA/AXXXA levels in the presence or absence of DHT. In contrast, when GRIP1 was overexpressed, there was a striking increase in AR-FXXAA/AXXXA 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-FXXAA/AXXXA 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.
In the present report, we provide evidence that EGF signaling bypasses the requirement for androgen. Reduced circulating androgen levels, and some studies suggest that mitogen signaling might be limited by the lower binding affinity of the coactivator LL motif binding. The importance of phosphorylation at Ser736 adjacent to the third and predominant interacting LXXLL motifs of TIF2/GRIP1 reduced the interaction between the AR NH2-terminal FXXLF motif that mediates the androgen-sensitive N/C interaction (66, 106). An optimal transcriptional response of AR to TIF2/GRIP1 depends on the interaction between the LXXLL 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\textsuperscript{736} adjacent to the third and predominant interacting LXXLL motif of TIF2/GRIP1 reduced the interaction between TIF2/GRIP1 and AR, supporting a key role for LXXLL 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-FXXA/AXXAA, an AR with mutations in the NH2-terminal 23\textsuperscript{FQNLF}\textsuperscript{27} and 433\textsuperscript{WHTLF}\textsuperscript{437} sequences that are required for the androgen-induced AR N/C interaction (66–68). Mutations

\textsuperscript{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 NH2-terminal and DNA binding domain fragment AR 1 region of p160 coactivators and the NH2-terminal domain of the ligand binding domain. This agrees with previous reports that recurrent prostate cancer is influenced by autocrine loops involving EGF on the progesterone and estrogen receptors support independent and -independent prostate cancer cell lines (94). Other approaches have been taken to inhibit prostate tumor growth by 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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