Post-Transcriptional Regulation of the Androgen Receptor by Mammalian Target of Rapamycin

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Abstract

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), an ErbB1 ligand and prostate stromal growth factor, is an antagonist of androgen receptor (AR) function. In the LNCaP prostate cancer model, HB-EGF reduced AR protein levels and AR transactivation without affecting AR mRNA level or protein turnover. The signal to attenuate AR was mediated by the mammalian target of rapamycin, as shown by genetic and pharmacologic methods, and was independent of ErbB2/HER-2, extracellular signal-regulated kinase 1/2, and p38 mitogen-activated protein kinase pathways. Additional evidence suggests that AR protein levels are highly sensitive to regulation by cap-dependent mRNA translation. These findings reveal a novel mechanism for regulation of AR by a classic growth factor system and indicate that a rapamycin-sensitive post-transcriptional pathway can attenuate or possibly bypass AR-mediated signaling. (Cancer Res 2005; 65(7): 2547-53)

Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor that regulates gene expression in androgen-responsive tissues. Impairment of normal androgen-AR signaling by genetic defects (e.g., mutation or gene amplification), changes in AR expression, and hypersensitivity of AR to ligand have been linked to the progression to androgen-independent disease in prostate cancer (1).

Evidence indicates that AR intersects with signaling pathways downstream from peptide growth factors and cytokines (1). Insulin-like growth factor I (IGF-I), keratinocyte growth factor, and epidermal growth factor (EGF), were reported to activate AR under androgen-depleted conditions (2), suggesting that AR is capable of acting independently of the physiologic hormone. Recently, the ErbB2/ErbB3 heterodimer was reported to activate AR in a low-androgen environment by a mechanism involving AR stabilization and stimulation of receptor binding to androgen-responsive promoter elements (3).

In contrast, other studies suggest that phosphatidylinositol-3-kinase (PI3K)/Akt pathway activation by soluble growth factors inhibits AR transactivation, resulting in the suppression of AR target genes (4, 5). For example, IGF-I was shown to promote the formation of a complex involving Akt, AR, and Mdm2, resulting in phosphorylation-dependent ubiquitylation and degradation of AR by a proteasome-dependent mechanism (5).

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a prostate stroma-derived growth factor, lowers AR protein levels and inhibits AR transactivation in LNCaP prostate cancer cells *in vitro* and lowers AR levels in LNCaP xenograft tumors that continuously secrete HB-EGF (6). Interestingly, this attenuating effect on AR occurred *in vivo* under conditions of androgen-independent tumor growth, suggesting that EGF receptor (EGFR) activation may inhibit and/or bypass AR-dependent mechanisms of cell proliferation or survival. In the present study, we show that the mammalian target of rapamycin (mTOR), a serine-threonine kinase involved in cap-dependent protein translation, is a mediator of the attenuating effect of the EGF-like growth factor on AR signaling.

Materials and Methods

Cell culture, RNA isolation, and reverse transcriptase-PCR. LNCaP, LNCaP/vector only, and LNCaP/sHB (6) cells were cultured in RPMI supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin at 37°C/5% CO_2. Complete serum-free RPMI or Opti-MEM (Invitrogen, Carlsbad, CA) were used in all experiments. Total RNA isolation and reverse transcription-PCR were done as described (7).

Reagents and antibodies. R1881 was from Perkin Elmer (Boston, MA). Transfection reagents were from Roche (Indianapolis, IN; *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate) or Invitrogen (LipofectAMINE 2000). Human HB-EGF and EGF were from R&D Systems (Minneapolis, MN). Kinase inhibitors, cell cycle inhibitors and cisplatin were from Calbiochem (La Jolla, CA). Rapamycin, MG-132, and lactacystin were from Biomol (Plymouth Meeting, PA). Antibody to AR (PG21) was from Upstate Biotechnology (Charlottesville, VA), and p85 PI3K (B-9) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to p-Tyr-p85 PI3K, Akt/p-Akt (Thr^{308}/Ser^{473}), mTOR/p-mTOR (Ser^{2448}), 4E-BP1/p-4E-BP1 ($Thr^{37/46}$), S6K1/p-S6K1 (Thr^{389}), Erk1/2/p-Erk1/2, eIF4E, and poly(ADP-ribose) polymerase (PARP) were from Cell Signaling (Beverly, MA). Anti-eIF4G antiserum was described (8).

Cell lysate preparations and Western blots. Cell lysates were prepared, and protein concentrations were determined by standard methods (Bio-Rad, Hercules, CA). PBST (0.1% Tween 20) and 5% skim milk was used as a blocking buffer for AR Western blots. PBST and 5% IgG-free bovine serum albumin (Sigma, St. Louis, MO) were used as a blocking and incubating agent for the blots of PI3K, Akt, mTOR, 4E-BP1, S6K1, or Erk1/2, including their phospho-forms. Nitrocellulose membranes were incubated with horseradish peroxidase (HRP)-anti-rabbit or HRP-anti-mouse secondary antibody for 1 hour. Following washes of the membrane, complex formation was detected using Super-Signal (Pierce, Rockford, IL).

Plasmids, DNA transfections, and luciferase assays. The plasmids, pPSA61-Luc, pGRE4-TATA-Luc, and pcDNA3-hAR, were described (9). pIRES-Luc was generated by inserting the PCR product of the firefly luciferase gene from pGL3-*basic* into the *Not*I site of the pIRES vector (BD Biosciences, San Jose, CA). The pIR-AR5′URT-Luc plasmid was obtained by replacing IRES with a PCR fragment containing the AR 5′-untranslated region (UTR) after digesting pIRES-Luc with *Eco*RI/*Xba*I. Ligation was

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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done at 4°C overnight. Orientation and fidelity of the constructs were confirmed by sequencing. pXLJ-cB2-AR5′UTR-NS vector was constructed by inserting the 570-nucleotide (nt) from AR 5′UTR into *Bam*HI site. pXLJ-cB2-ECMV-NS vector was used as a positive control. One-microgram linear pXLJ-cB2-AR5′UTR-NS (test) or pXLJ-cB2-ECMV-NS (control) vector was used *in vitro* transcription/translation reactions. This assay was done using rabbit reticulocite system according to manufacturer protocol (Promega, Madison, WI). ^{35}S-methionine labeled cB2 or NS proteins was visualized by autoradiography. Cells were typically assayed 36 hours after transfection. Firefly and *Renilla* luciferase activities were measured by dual-injection luminometer according to the manufacturer's protocol (Promega). Luciferase activity representing arbitrary numbers was presented as fold induction relative to respective control vector activity.

Immunocytochemistry and microscopy. Cells were seeded in 8-well chamber slides followed by serum depletion for 24 hours. Mock-treated cells were used as controls. Cells were washed with PBS, fixed, and permeabilized with 100% cold methanol on ice for 10 minutes. Primary and secondary antibodies were each incubated for 1 hour. Slides were mounted in medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was detected using a laser scanning confocal microscope (Zeiss, Thornwood, NY).

Akt kinase assay. Cells were serum depleted for 24 hours before lysis in 1% Triton X-100 buffer. For immunoprecipitation of Akt, equal amount of cell extracts were mixed with 20 μL immobilized Akt monoclonal antibody in 200 μL volume followed by overnight incubation at 4°C. Kinase reactions were conducted as described by the manufacturer (Cell Signaling).

Small interfering RNA. For small interfering RNA (siRNA) experiments, 21-nt complementary RNAs were designed and obtained from Dharmacon (Lafayette, CO). mTOR siRNA corresponded to bases 2241 to 2261, and S6K1 siRNA corresponded to nt 217 to 238 relative to the translational initiation codon. siRNAs were transfected using LipofectAMINE 2000 (Invitrogen).

Apoptosis, cell cycle drugs, and 7-methyl GTP-cap assay. For apoptosis assay, serum-depleted cells were incubated with cisplatin for 15 hours as described (10). Apoptosis was assessed by determining the extent of cleavage of PARP using an anti-PARP antibody that only recognizes the 99-kDa cleavage product (Cell Signaling). Cell synchronizations were done according to published methods (11) using 5 μg/mL aphidicolin, 2 mmol/L thymidine, or 1 μmol/L nocodazole. The 7-methyl GTP (m7G)-cap assay was also done as described (11). Briefly, 650 μg total protein were mixed with 30 μL of 50% 7-methyl GTP-Sepharose and incubated for 4 hours at 4°C followed by three washes, and the pellet were mixed with 2× sample buffer. Samples were analyzed by Western blot.

Statistical analysis. Values are expressed as mean ± SD. Where appropriate, an unpaired *t* test was conducted to analyze for differences between treatments. Statistical significance was determined at $P \leq 0.05$.

Results

HB-EGF attenuates androgen receptor expression and function. AR was shown previously down-regulated in LNCaP/sHB cells, which are engineered to continuously express the soluble form of HB-EGF (6). The effect of HB-EGF on AR levels is shown in Fig. 1. Under androgen-depleted conditions, HB-EGF decreased AR protein in a dose- and time-dependent manner, within the range of 0 to 50 ng/mL (Fig. 1*A*). Down-modulation was reproducibly detected at 10 ng/mL. AR protein began to decline 12 hours following treatment with the growth factor and continued to decrease over time. In LNCaP/sHB cells, AR levels were stably reduced in comparison with control (vector only) cells (Fig. 1*B*). A similar observation was made in the aggressive LNCaP subline, C4-2 (data not shown). β-Actin levels were not affected by HB-EGF in any cell background.

HB-EGF had no significant effect on levels of AR mRNA (Fig. 1*C*), indicating that the effect on AR did not originate from an alteration of gene expression. The semiquantitative result shown was confirmed by real-time PCR (data not shown). Promoter-reporter analysis using an AR-responsive promoter showed that reduced AR coincided with a 50% reduction in promoter activity (Fig. 1*D*). The use of a selective EGFR inhibitor, AG1478 (20 μmol/L), completely blocked the decline in AR activity (Fig. 1*D*) and protein (Fig. 1*E*).

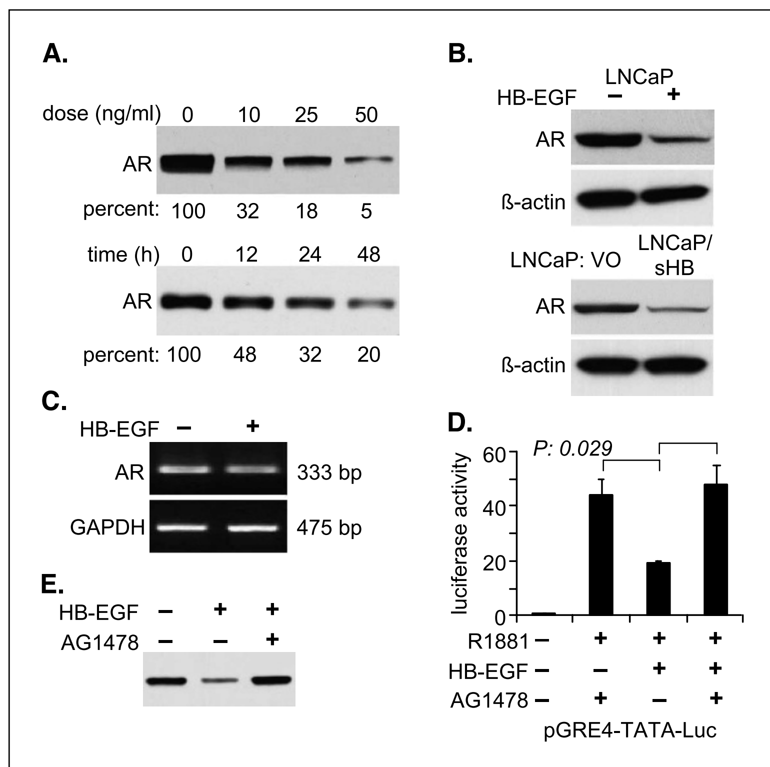

Figure 1. AR expression is inhibited by HB-EGF. *A,* serum-starved LNCaP cells treated with HB-EGF show a dose- and time-dependent decline in AR protein (50 ng/mL HB-EGF used in *bottom* and in *B*, *C*, *D*, and *E*). *B,* comparisons of AR protein levels in LNCaP and engineered LNCaP vector only (*VO*) or HB-EGF-transfected cells (*sHB*). Cells were analyzed after 48 hours of serum starvation. *C,* AR mRNA expression in LNCaP cells. Cells were treated with vehicle or HB-EGF. AR and GAPDH transcripts were analyzed by RT-PCR. *D,* AR-responsive GRE4-TATA-Luc promoter-reporter activity in LNCaP cells: Cells were cotransfected with pCMV-*Renilla*-Luc (internal control), and AG1478-pretreated cells were incubated with 1 nmol/L R1881 +/− HB-EGF in the presence or absence of EGFR inhibitor. At 24 hours, cell lysate was assayed for luciferase activities. Normalized luciferase data are expressed as fold induction with respect to control vector. *GRE,* glucocorticoid response element. *E,* AR in total lysate from cells treated for 48 hours with HB-EGF in the presence or absence of 20 μmol/L AG1478. Representative of at least three experiments.

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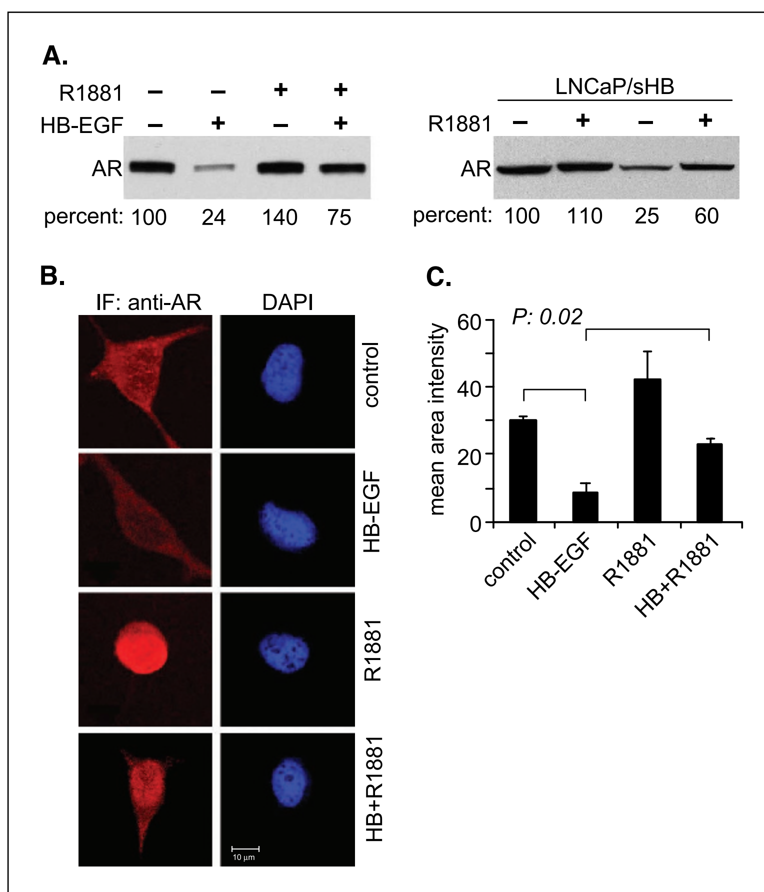

Figure 2. Androgen antagonizes HB-EGF signaling. *A,* effects of 48 hours treatment with 1 nmol/L R1881 on AR expression in parental (*left*, ±50 ng/mL HB-EGF) or HB-EGF-expressing (*right*) LNCaP cells. *B,* confocal microscopic imaging of the AR. Serum-starved LNCaP cells were treated with vehicle, 50 ng/mL HB-EGF, 1 nmol/L R1881, or R1881 + HB-EGF in combination. AR (*red*). Nuclei (*blue*). Bar, 10 μm. *C,* AR signal from confocal images was quantified and expressed as mean area intensity. *Columns,* mean of triplicate determinations; *bars,* ±SD.

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Similarly, AR-regulated prostate-specific antigen (PSA) promoter activation was also reduced by HB-EGF (data not shown). These data indicate that HB-EGF activation of the EGFR results in a reduction in steady-state AR protein and a suppression of AR transactivation activity.

The synthetic androgen, R1881 (1 nmol/L), reversed by 60% to 75% the HB-EGF effect on AR levels, assessed at 48 hours, in both parent LNCaP (Fig. 2*A*, *left*) and LNCaP/sHB cells (Fig. 2*A*, *right*). The suppressive HB-EGF effect on AR protein and the ability of R1881 to antagonize this effect were also observed by immunofluorescence staining of AR (Fig. 2*B*). In addition to differences in AR protein level, the opposing effects of R1881 and HB-EGF were also evident when AR subcellular localization (nucleus versus cytoplasm) was evaluated (Fig. 2*C*). These data indicate that HB-EGF and R1881 are mutually antagonistic with respect to AR expression.

HB-EGF suppresses androgen receptor by activating the phosphatidylinositol-3-kinase/Akt pathway. EGFR/ErbB1 activates the PI3K/Akt and extracellular signal-regulated kinase/mitogen-activated protein (Erk/MAP) signal transduction pathways, which have been linked to AR regulation (12–14) and cell survival in LNCaP cells (14, 15). AG1478 blocked activation of both pathways by the growth factor (Supplementary Fig. 1*A*), confirming that HB-EGF activated PI3K and Erk-MAPK signaling via EGFR. The PI3K inhibitor LY294002 (20 μmol/L) completely ablated the suppressive effect on AR levels by HB-EGF (Fig. 3*A*). In contrast, the Erk1/2 pathway inhibitor, PD98059 (50 μmol/L) did not inhibit the HB-EGF effect on AR (Fig. 3*B*), despite the fact that the drug completely blocked Erk phosphorylation (Fig. 3*B*). Neither drug alone had a detectable effect on AR protein (Fig. 3*A* and *B*).

HER-2/ErbB2 has been shown to regulate AR (12, 13). To determine whether ErbB2 activation results in AR suppression, cells were incubated with or without the selective ErbB2 inhibitor, AG825 (10 μmol/L), before treatment with HB-EGF. AG825 did not prevent suppression of AR by the growth factor (Fig. 3*C*, *inset*, *lane 3*). Similarly, the inhibitor also failed to alter suppression of AR-dependent promoter activity by HB-EGF (Fig. 3*C*). These observations indicate that the primary signal resulting in attenuation of AR function does not require ErbB2.

HB-EGF was shown to activate p38 MAP kinase (MAPK) signaling in LNCaP cells, leading to an enhancement of neuroendocrine features (16). The selective p38 MAPK inhibitor, SB203580 (10 μmol/L) failed to inhibit the attenuating effect of HB-EGF on AR protein (data not shown), suggesting that the suppressive signal does not require the p38 MAPK pathway. Taken together, the above results indicate that an EGFR → PI3K signal is responsible for suppression of AR by HB-EGF. Consistent with this interpretation, Akt activity was up-regulated in HB-EGF-treated LNCaP and in LNCaP/sHB cells (Supplementary Fig. 1*B*).

HB-EGF attenuates androgen receptor without altering protein stability. Activation of the PI3K/Akt pathway was reported to down-regulate AR by the ubiquitin-proteasome pathway (4, 5). To examine whether HB-EGF suppresses AR by stimulating proteasome-mediated degradation, LNCaP cells were treated with the selective 26S proteasome inhibitors, lactacystin, or

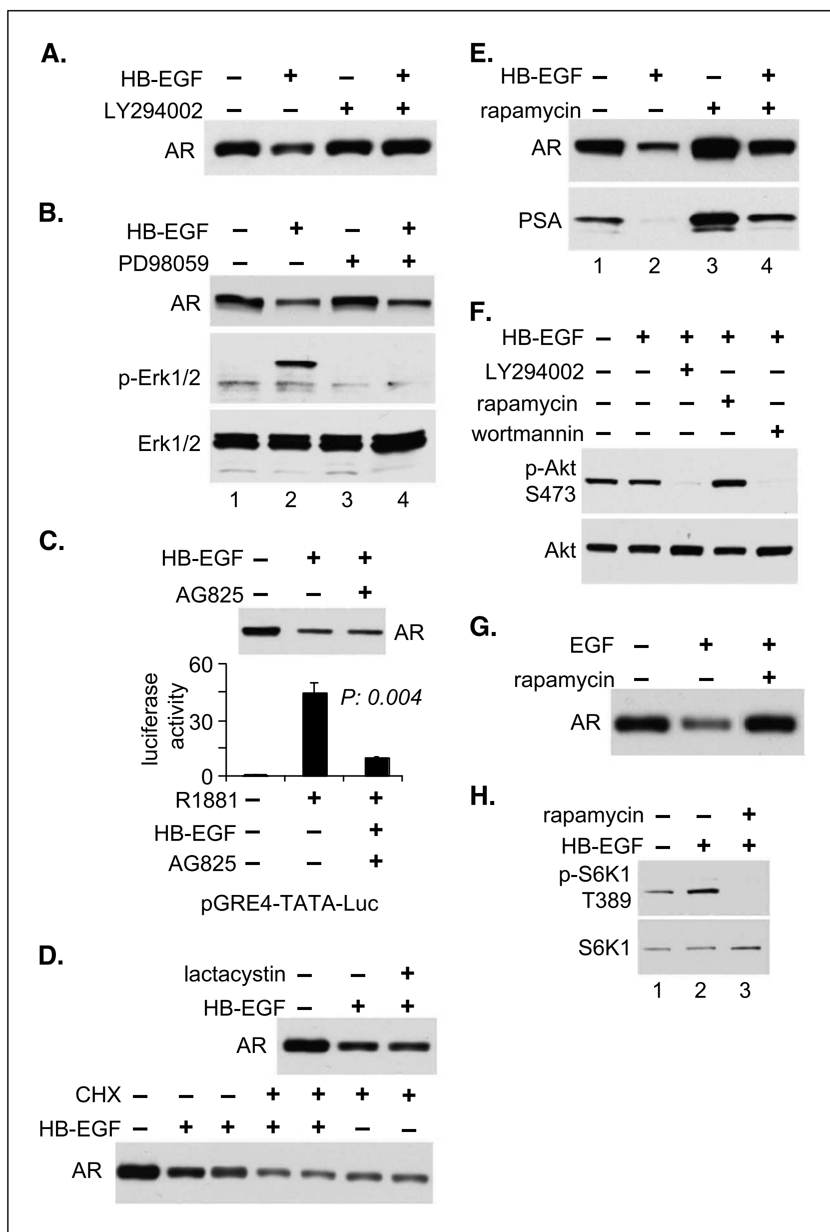

Figure 3. PI3K-mTOR signaling mediates AR downregulation by HB-EGF. *A* and *B,* relative levels of AR following pathway blockade with 20 μmol/L LY294002 or 50 μmol/L PD98059, respectively, as described in Fig. 1 legend 48 hours after treatment. *B,* HB-EGF activates Erk (*bottom*). Serum-starved cells were pretreated with 50 μmol/L PD98059 for 1 hour and Erk1/2 was analyzed 15 minutes after incubation with HB-EGF. *C,* AR-responsive GRE4-TATA-Luc promoter-reporter activity in LNCaP cells: AG825-pretreated cells were incubated with 1 nmol/L R1881 plus or minus 50 ng/mL HB-EGF in the presence or absence of 10 μmol/L AG825. Blot of AR protein levels at 48 hours following HB-EGF alone or HB-EGF plus AG825. *D,* serum-starved cells were pretreated with 10 μmol/L lactacystin or 10 μg/mL cycloheximide (*CHX*) under conditions identical to Fig. 1. *E,* levels of AR at 48 hours following 50 ng/mL1 HB-EGF and 100 nmol/L rapamycin. *F,* phospho-Akt in response to 50 ng/mL HB-EGF, 100 nmol/L wortmannin, 20 μmol/L LY294002, and 100 nmol/L rapamycin. Serum-starved cells were pretreated with inhibitor for 1 hour followed by 50 ng/mL HB-EGF addition for 15 minutes. *G,* serum-starved cells were incubated with 100 nmol/L rapamycin for 1 hour before addition of EGF. Protein lysates were prepared at 48 hours. *H,* effect of 100 nmol/L rapamycin on phosphorylation of S6K1. Representative of at least three individual experiments.

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MG-132 before treatment with HB-EGF. Neither inhibitor altered the suppressive effects of the growth factor on AR (Fig. 3*D* and data not shown), indicating that a mechanism involving stimulation of protein degradation is insufficient to explain the effect of HB-EGF on AR.

To assess whether *de novo* protein synthesis is required for the HB-EGF effect on AR, we determined the effect of treating cells with the protein synthesis inhibitor, cycloheximide, before treatment with HB-EGF. Cycloheximide alone (10 μg/mL) reduced AR levels to an extent comparable to that seen with HB-EGF (Fig. 3*D*), indicating that new protein synthesis is not required for the HB-EGF effect on AR. Furthermore, these data indicate that steady-state AR protein levels can be altered (reduced) by a mechanism involving protein synthesis inhibition.

HB-EGF attenuates androgen receptor by mammalian target of rapamycin signaling. The serine-threonine kinase, mTOR, a regulator of mRNA translation, lies downstream from PI3K/Akt and is specifically inhibited by the bacterial macrolide, rapamycin (17). To examine the potential role of mTOR in AR regulation, LNCaP cells were treated with rapamycin before treatment with HB-EGF. Rapamycin completely abolished the suppressive effect of HB-EGF on AR as well as the inhibitory effect of HB-EGF on PSA expression (Fig. 3*E*) but had no effect on Akt (Ser^{473}) phosphorylation (Fig. 3*F*). Interestingly, rapamycin used alone modestly enhanced AR and PSA expression (Fig. 3*E*). Rapamycin also reversed the suppressive effect of EGF on AR when this growth factor was used in place of HB-EGF (Fig. 3*G*). Rapamycin also inhibited phosphorylation of S6K1, a mediator of translation that lies downstream of mTOR (Fig. 3*H*).

As an alternative to rapamycin, we transfected LNCaP cells with a siRNA duplex targeted to mTOR mRNA. As controls, we used siRNAs encoding (i) a scrambled sequence and (ii) siRNA targeted to S6K1. Transfection of LNCaP cells with mTOR or S6K1 siRNAs

resulted in a dose-dependent decrease in the levels of the respective proteins (Supplementary Fig. 1*C*). In both cases, maximum inhibition was observed with 80 nmol/L siRNA. Reduction in mTOR expression resulted in reduced S6K1 activity, as well as a detectable decrease in apparent molecular weight of 4E-BP1-reactive bands, an indication of reduced 4E-BP1 phosphorylation (18). As expected, S6K1 siRNA affected S6K1 but had no detectable effect on 4E-BP1 or mTOR.

mTOR siRNA significantly reversed (by 70%) the suppressive effect of HB-EGF on AR levels, relative to the scrambled siRNA (Fig. 4*A*). Similarly, inhibition of mTOR by RNA silencing or rapamycin in LNCaP/sHB cells reversed the effect of HB-EGF on AR (Fig. 4*B*). Moreover, although rapamycin inhibited S6K1 phosphorylation (Fig. 3*G*), S6K1 siRNA did not alter the HB-EGF effect on AR (Fig. 4*A*), suggesting that S6K1 is not involved in AR regulation downstream from HB-EGF/EGFR. Taken together, these findings indicate that HB-EGF's suppressive effect on AR is mediated by a mechanism involving mTOR.

Androgen receptor protein levels are regulated by mRNA translation initiation. mTOR regulates protein synthesis by mediating cap-dependent mRNA translation initiation (19). To test independently whether AR protein levels can be altered by this mechanism, we did a series of experiments. Cap-dependent protein translation is inhibited during apoptosis due to caspase-mediated cleavage of the translation scaffolding protein eIF4G (10). To induce apoptosis, LNCaP cells were exposed to increasing concentrations of cisplatin. Apoptosis was assessed by evaluating cleavage of eIF4G and PARP (Fig. 4*C*). AR levels were substantially reduced 15 hours following cisplatin treatment, and this down-regulation correlated with cleavage of the eIF4G protein (*middle*). In contrast, β-actin protein levels were unaffected.

Down-regulation of highly cap-dependent proteins has been reported to occur in a cell cycle–dependent manner, with inhibition stimulated by M-phase arrest, whereas no changes are evident if the cell cycle is interrupted at other points (11). To determine if AR expression is altered specifically with M-phase arrest, LNCaP cells were treated with aphidicolin, thymidine, or nocodazole; these drugs inhibit the cell cycle at the G_1-S boundary, S phase, or M phase, respectively. AR levels were substantially reduced only during M-phase arrest (Fig. 4*D*).

The result in Fig. 3*E* indicates that rapamycin can elevate AR protein levels. To test whether rapamycin treatment of LNCaP cells increases interaction between the translation initiation factor, eIF4E, and the scaffolding protein, eIF4G, with the m7G mRNA cap structure, we used an m7G-Sepharose affinity column to test the ability of these proteins to bind the cap structure. The results showed that rapamycin increased complex formation between m7G and eIF4E (Fig. 5*A* and *B*) and eIF4G (Fig. 5*A*). Furthermore, the degree of this interaction correlated with AR protein level in both engineered and parental LNCaP cells. We also tested the effect of rapamycin on luciferase expression driven by a cytomegalovirus (CMV) promoter coupled to the AR 5′UTR. The AR 5′UTR was capable of stimulating protein expression from a cap-dependent promoter, and rapamycin enhanced this effect (Fig. 5*C*).

Because a small (but biologically significant) number of mRNAs is also translated by an IRES-driven, cap-independent mechanism, we asked whether the AR 5′UTR can promote translation in the context of a bicistronic construct. A 570-nt DNA fragment from the AR

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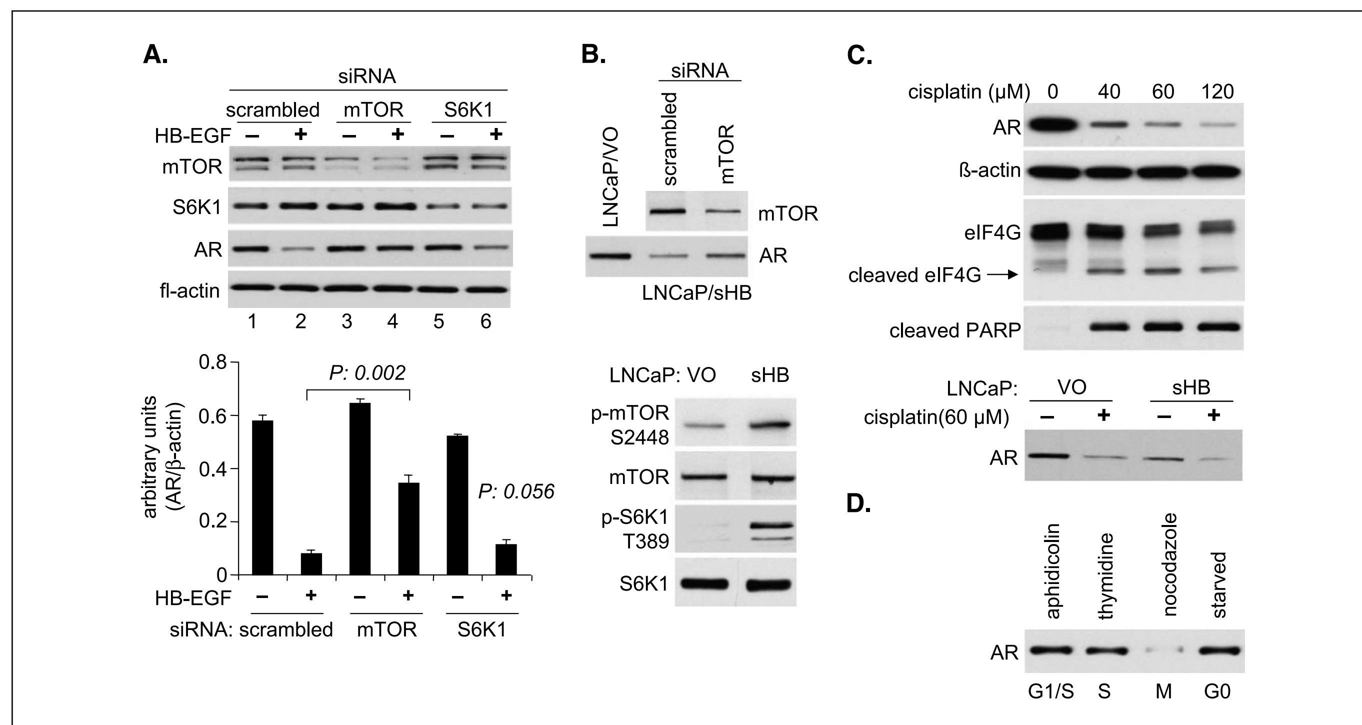

Figure 4. mTOR regulates AR. *A,* analysis of AR levels in the presence of mTOR, S6K1, or scrambled siRNAs. Lysates were prepared 36 hours after transfection. *Bottom, columns,* mean of three determinations; *bars,* ±SD. *B,* effect of mTOR siRNA and 100 nmol/L rapamycin on AR level in HB-EGF-transfected LNCaP cells. Phosphorylation of mTOR and S6K1 are shown (*middle*). Representative of at least three individual experiments. *C* and *D,* levels of AR during cisplatin-induced apoptosis or cell cycle arrest, using aphidicolin (G_1-S), thymidine (S), nocodazole (M), or serum-starvation (G_0), respectively. Lysates were prepared 15 hours after drug treatment. Representative of at least three individual experiments.

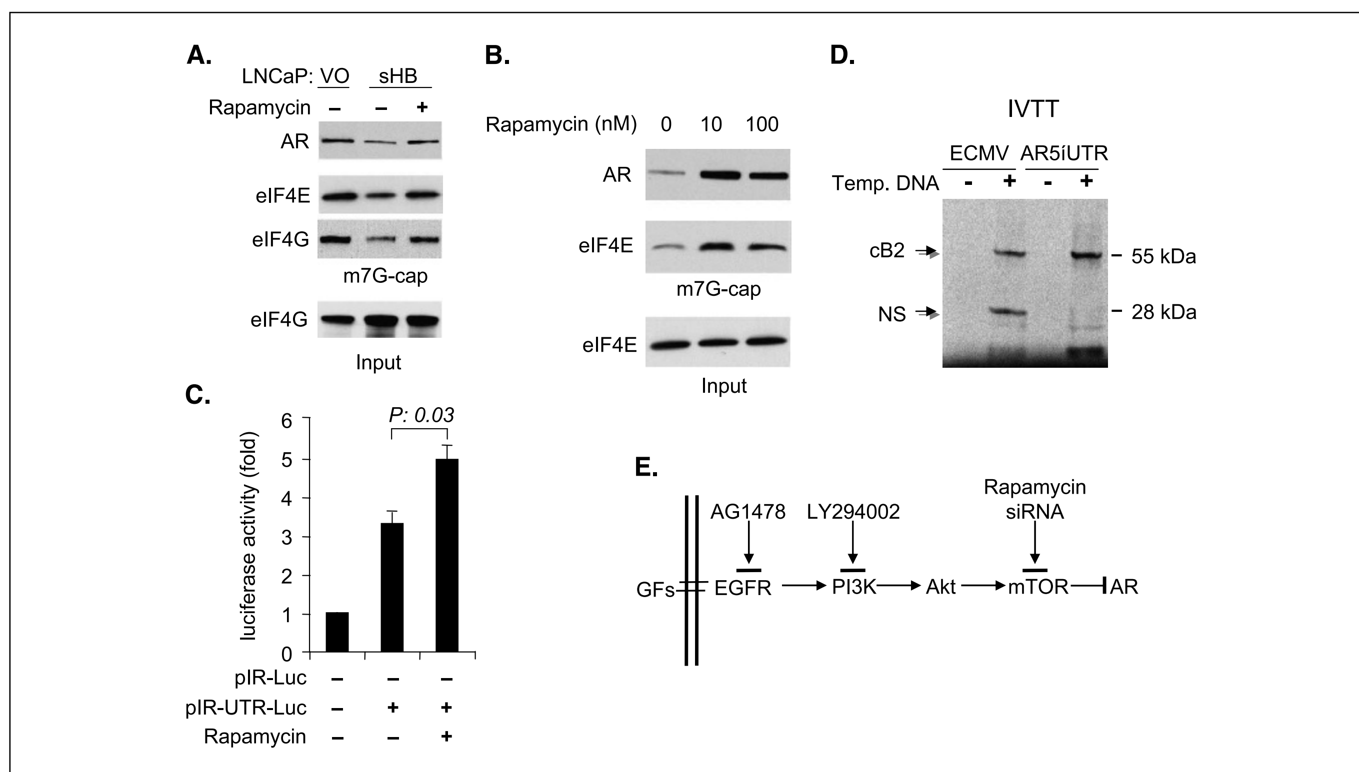

Figure 5. Regulation of interaction between eIF4E and eIF4G with the m7G cap by rapamycin. *A,* effect of 100 nmol/L rapamycin on AR level and on eIF4E and eIF4G interaction with m7G in HB-EGF-transfected LNCaP (*sHB*). *B,* dose-dependent effect of rapamycin on AR level and on eIF4E interaction with m7G in parental LNCaP cells. *C,* effect of rapamycin on luciferase reporter activity in parental LNCaP cells. Cells were transfected with control (pIR-Luc) or plasmid containing the AR 5′UTR (pIR-AR5′UTR-Luc), followed by incubation with 100 nmol/L rapamycin (+) or DMSO (−). *D, in vitro* transcription and translation (*IVTT*) of cB2 and NS proteins by cap-dependent or IRES mechanism, respectively. Experimental details were described in Materials and Methods. *cB2, Xenopus* cyclin B2; *NS,* Influenza virus NS protein. *E,* model representing the signaling cascade that regulates AR expression.

5′ UTR, which was shown in a previous report to play a role in AR translation (20), was placed between the *Xenopus* cyclin B open reading frame (ORF) and the influenza virus NS ORF. Under normal conditions, translation of the second ORF is precluded by the termination of translation of the first ORF. If, however, the sequence contains a functional IRES, as in the case of the EMCV 5′UTR placed between the two ORFs, translation of the NS ORF will take place. In Fig. 5*D*, we show that the EMCV 5′UTR promoted translation of the NS′ protein in an *in vitro* transcription/translation system. In contrast, the AR 5′UTR did not promote translation of the NS ORF, indicating that the AR 5′UTR likely does not possess IRES activity.

Discussion

Our findings show that the EGFR/ErbB1 activating ligand, HB-EGF, inhibits androgenic signaling by a mechanism that is dependent on the kinase mTOR. This mechanism is diagrammed in Fig. 5*E*. This is the first report that the AR can be regulated by mTOR. The attenuating effect of the growth factor on AR was antagonized by androgen, possibly because formation of a ligand-AR complex stabilizes the protein. We also show that this signaling mechanism involves the EGFR and PI3K, which lie upstream from mTOR, but does not require either the Erk or p38 MAPK signaling pathways. AR down-regulation by HB-EGF does not involve changes in AR protein stability, turnover, or alteration of AR mRNA levels but seems to involve regulation of cap-dependent translation of AR mRNA. Consequently, this mechanism is distinct from the ubiquitylation-dependent AR degradation pathway, involving Akt, that has been reported (5). More generally, however, our findings are consistent with the conclusion that down-regulation of AR signaling can result from receptor tyrosine kinase activation by growth factors and that the PI3K/Akt pathway can antagonize AR signaling in some contexts (4).

mTOR regulates mRNA translation initiation by phosphorylating the ribosomal S6 kinase (S6K1) and by regulation of the initiation factor eIF4E (19). mTOR also has a number of other downstream targets (19). Our results indicate that S6K1 is not involved in attenuation of AR by HB-EGF, suggesting that this mechanism involves effects on eIF4E or possibly other proteins involved in formation of the translation initiation complex. Consistent with the possibility that mTOR attenuates androgenic signaling principally by regulating AR mRNA translation rate, the AR mRNA contains a long 5′UTR (1,115 nt), which is characteristic of mRNAs that require elevated levels of eIF4F complex activity. The regulation of AR by translation has not been extensively explored; we are aware of only one report describing translational regulation of AR (20).

In summary, this report identifies a new mechanism of regulation of the AR, a protein important in prostate cancer progression. Our findings show that activation of EGFR/ErbB1 by HB-EGF, one of its soluble ligands, results in attenuation of AR signaling by a post-transcriptional mechanism involving the kinase mTOR. These results are of potential relevance to ongoing clinical trials of EGFR inhibitors and rapamycin analogues, which, our findings imply, could have unanticipated effects on signaling through the AR, particularly under conditions of hormone ablation.

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