Novel Permissive Role of Epidermal Growth Factor in Transforming Growth Factor β (TGF-β) Signaling and Growth Suppression

**MEDIATION BY STABILIZATION OF TGF-β RECEPTOR TYPE II***

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Transforming growth factor β (TGF-β) signals through TGF-β receptor serine/threonine kinases (TβRI and TβRII) and Smads, regulating cell growth and apoptosis. Although loss of TGF-β receptor levels is strongly selected for during the progression of most cancers, tumor cells frequently escape from complete loss of TGF-β receptors through unknown mechanisms. Here, we provide the first evidence that epidermal growth factor (EGF) signaling, which is generally enhanced in cancer, is permissive for regulation of gene expression and growth suppression by TGF-β in LNCaP prostate adenocarcinoma cells. Our results support that these permissive effects occur through enhanced stability of TβRII mRNA and reversal of TGF-β-mediated TβRII mRNA loss. Changes in stability of TβRII mRNA occur soon after EGF or TGF-β1 addition (optimal within 3 h) and are independent of de novo protein synthesis or transcription. Remarkably, loss of TβRII by TGF-β can be mediated by a kinase-dead TβRII (K277R), as well as by other forms of this receptor harboring mutations at prominent autophosphorylation sites. Moreover, Smad3 small interfering RNA, which blocks TGF-β-induced AP-1 promoter activity, does not block changes in the expression of TβRII by EGF or TGF-β. We have also shown that changes in TβRII levels by EGF are EGF receptor-kinase-dependent and are controlled by signals downstream of MEK1/2. Our findings provide invaluable insights on the role of the EGF receptor-kinase in enhancing TGF-β responses during prostate carcinogenesis.

Transforming growth factor-βs (TGF-β) are multifunctional cytokines that regulate cell cycle arrest, mitosis, differentiation, and apoptosis (1–9). As one of the earliest events in the cellular responses to these multifunctional growth factors, TβRI and TβRII cell surface signaling receptors form a heterotetrameric complex upon binding TGF-βs (10–12). The constitutively active kinase domain of TβRII activates TβRI by transphosphorylation of the GS box in the cytoplasmic domain (13). The activated TβRI then propagates TGF-β signals partly by activating Smads 2 and 3 by phosphorylating their C-terminal serines (14–16). These Smads then translocate to the nucleus where they function either directly as transcription factors or indirectly as transcription co-modulators (16–18).

TGF-β receptors, which function as tumor suppressors in normal and preneoplastic tissues, acquire oncogenic functions during tumor progression. TGF-β receptors are mutated or and expressed at substantially reduced levels in a variety of human cancers including colon, gastric, and prostate cancers, correlating with acquisition of resistance to growth suppression by TGF-β (19–22). Significantly, restoration of TGF-β receptor expression or function can reverse the malignant phenotype in a variety of carcinoma cells (3, 23). Consistent with these observations, suppression of TGF-β signaling by dominant-negative TβRII has been shown to promote malignant transformation of non-tumorigenic cell lines (4, 6).

Despite the apparent selective pressure for carcinomas to lose TGF-β receptors, there appears to be resistance against complete loss of those receptors during the progression of many carcinomas (24), consistent with the conversion of the function of TGF-β to that of an oncogene (25). Thus, the molecular mechanism behind the switch of the function of TGF-β from tumor suppressor to tumor promoter is likely to require a signal for the retention of TGF-β receptors during carcinogenesis.

EGF is a 6-kDa polypeptide that binds to a 170-kDa transmembrane tyrosine kinase receptor (EGFR) expressed on a wide variety of normal and neoplastic cells. Binding of EGF to EGFR causes receptor dimerization and autophosphorylation, leading to activation of a number of downstream signal transduction pathways, such as Ras/Raf/mitogen-activated protein kinase and PI3-kinase/Akt, that mediate cell proliferation, angiogenesis, and apoptosis by EGF (26). Although EGFR appears to be amplified and activated in many cancers, its role in the malignant phenotype is not entirely clear (27). In most cells EGF is growth stimulatory and anti-apoptotic; however, a number of tumor cell lines have been shown to be killed by this peptide through unknown mechanisms (28, 29). Interestingly, EGFR and its homologue HER2 have been reported to control various TGF-β responses both in vitro and in vivo (30–34).

In this study we report the first evidence that the EGF signaling pathway may enhance TGF-β responses through increasing the stability of TβRII, as demonstrated with the most widely used human prostate adenocarcinoma cell model, LNCaP. Although seemingly counterintuitive, the activation of TGF-β responses by EGF is consistent with the general induction of TGF-β ligand by this mitogen (35–37) as well as a requirement of AP-1 and MEK1/2 for many TGF-β responses, including growth suppression (38–41). Elucidation on how EGF can stabilize...
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TβRII mRNA is thus likely to provide mechanistic insight on the conversion of the function of TGF-β from tumor suppressor to tumor promoter.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sources were: LNCaP, DU-145, PC-3 (ATCC); recombinant human TGF-β1 and TGF-β2 (R&D Systems); anti-phospho Smad3 antibody (catalog number 9514), anti-phospho-p44/42 mitogen-activated protein kinase (Thr-202/Tyr-204) [(catalog number 9106), anti-phospho-p70S6K (Thr-389) (catalog number 9205), anti-phospho-c-Jun (Ser-63) (catalog number 9261) (Cell Signaling); anti-TβRII (sc-1700) and anti-Smad3 (sc-8332) (Santa Cruz Biotechnology); peptide-N-glycosidase F (New England Biolabs); DMEM/F12 (1:1, v/v) (Invitrogen); mouse EGF (BioSource International); PD153035, AG1478, SB202190, U0126, PD98059, U0126, PD98059, LY294002, and SP600125 (Calbiochem); rapamycin (BioMol); characterized FBS and DC-stripped FBS (HyClone); pUSE-act-Ras (Upstate Cell Signaling Solutions); and pFC-MEK1, pFC-Ek1, AP-1-luciferase, pFC-MEK1, cis-acting PathDetect™ constructs (Stratagene). For the pCMV5-c-Fos expression vector, the full-length coding region of c-Fos was amplified using High-Fidelity™ (Roche Applied Science) PCR from the pLIB-prostate human cDNA library (Clontech) and inserted into the pCMV5 vector.

**Cell Culture—**LNCaP cells were maintained in DMEM/F12 containing 10% FBS in poly-D-lysine-coated 75-cm² culture flasks (42). In all experiments involving LNCaP cells, unless indicated, cells were attached to polylysine-coated dishes and cultured overnight in DMEM/F12 supplemented with 1% DC-stripped FBS and 15 mM HEPES (42).

In general, all signaling inhibitors were added to cells 1–2 h before the addition of EGF (20 ng/ml), and cells were incubated in the absence or presence of TGF-β1 (10 ng/ml) for up to 48 h. In all time course experiments, the times of EGF or TGF-β addition were varied, with total culture times and all other conditions kept constant for proper control. Vehicles used as controls for TGF-β1 and EGF (4 mM HCl, 1 mg/ml bovine serum albumin, diluted 1000-fold in assay) were shown to have no effect on TβRII level at all treatment times.

**TGF-β Sandwich Enzyme-linked Immunosorbent Assays—**TGF-β proteins were measured by established sandwich enzyme-linked immunosorbent assays (43, 44). LNCaP cells were cultured under serum-free conditions (42) and then treated with various combinations of growth factors and hormones. Serum-free conditions were used for the preparation of conditioned medium to avoid carryover of latent TGF-β1 and EGF (4 mM HCl, 1 mg/ml bovine serum albumin, diluted 1000-fold in assay) were shown to have no effect on TβRII level at all treatment times.

**Adenovirus Gene Delivery—**An adenovirus vector that directs the expression of WT-TβRII (AdMax-WT-TβRII) was constructed using the AdMax system (Microbix Biosystems). The full-length coding sequence of human TβRII (1.7 kb) excised from pCMV5-HA-TβRII (45) was subcloned into the pDC515 adenovirus shuttle vector. Human embryonic kidney 293 cells were co-transfected with 1 µg of pDC515-HA-TβRII and 1 µg of genomic vector pBHGrfDE1,3FLP in 6-well plates, using a standard calcium phosphate precipitation method (42). Following transfection, the cells were maintained in DMEM/F12 containing 2% FBS for ~10 days or until the appearance of viral cytopathic effects. Cells were then lysed by four serial freeze-thaw cycles (on dry ice and 37 °C). Liberated viral particles were further amplified by two-three serial infections through human embryonic kidney 293 cells, according to the manufacturer’s protocol. To titer total viral particles, aliquots of virus stocks were diluted 20-fold in lysis solution (0.1% sodium dodecylsulfate, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA) and incubated for 10 min at 56 °C. The optical density of the samples at 260 nm was used to calculate virus content using the relationship 1.1 × 10¹² virus particles/ml/A₂₆₀ units. All viral preparations were evaluated for expression of recombinant protein by Western blot analysis of transduced NRP-154 and LNCaP cells.

For experiments involving use of adenovirus, LNCaP cells were infected overnight with 1.0–2.5 × 10¹⁰ viral particles/ml (well) in 6-well dishes). Their medium was then replaced to remove residual virus particles before treatment with the indicated factors.

**Northern Blot Analysis—**Northern blot analysis was performed essentially as described (46, 47). In brief, 10 µg of total RNA was electrophoresed and equal loading and even transfer were assessed by visualization of the 18 and 28 S rRNAs. The presence of indicated mRNA was detected with cDNA probes labeled with [³²P]dCTP using PrimeIt® RmT random primer labeling kit (Stratagene).

**RT-PCR—**RT was performed as described (6). The PCR primers applied to detect TβRII expression were 5’-AGCAGCATCCACCG- CACGTTCAAGAG-3’ (forward) and 5’-CTATTTGTATGTCTT TAGGGAGCGCT-3’ (reverse) and yielded a 1.7-kb fragment. Taq polymerase master mix (Promega) was used for PCR amplification of TβRII from 0.1 µg of LNCaP cDNA template, using 32 cycles of the following temperature gradients: 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 2 min. β-Actin, amplified as above for 21 cycles, served as an internal control.

**Western Blot Analysis—**Western blot analysis was performed essentially as described (6, 46). Cells were lysed in cold radioimmune precipitation assay buffer containing a protease inhibitor mixture (Roche Applied Science) supplemented with 1 mM phenylmethylsulfonyl fluoride, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate. The resulting lysates were clarified in 1.5-ml tubes by centrifugation at 16,000 × g for 10 min, and supernatants were quantified by a microtiter BCA protein assay (Pierce) as described before (46). For determination of TβRII levels, aliquots of 10–20 µg (protein) were deglycosylated by incubation with peptide-N-glycosidase F according to the manufacturer’s instructions (New England Biolabs).

**Transient Transfection and Luciferase Assay—**All of these procedures were performed essentially as described (42, 46). In brief, LNCaP cells were plated overnight at a density of 1.25 × 10⁵ cells/ml/well in 12-well plates. Reporter constructs, 1–2 µg, were co-transfected with 12.5–25 ng of CMV- Renilla reporter construct using Lipofectamine-Plus™ followed by treatment with EGF(20 ng/ml) and TGF-β1(10 ng/ml). Luciferase activity was measured using a dual luciferase assay kit (Promega) and a ML3000 microtiter plate luminometer. All luciferase activity was expressed as normalized values of firefly luciferase to Renilla luciferase.

**RESULTS**

**EGF Permits TGF-β Autoinduction and Growth Suppression in LNCaP Cells—**Major interest in the role of TGF-β in the prostate is attributed to the ability of this cytokine to induce cell death, suppress tumor growth, and likely mediate androgen ablation-induced cell death (49, 50). We used the LNCaP cell line, which expresses low to undetectable levels of TGF-β ligands and receptors, to identify factors that may
suppress tumor growth by their ability to up-regulate TGF-β ligands or receptors. We first screened a variety of growth factors and hormones for their ability to enhance expression of TGF-β ligands in these cells under serum-free conditions. Of these, only EGF was able to enhance the expression of TGF-β1 to a level detectable by the most sensitive and specific TGF-β sandwich enzyme-linked immunosorbent assays reported (43). Following a secondary screen of factors that could synergize with EGF to enhance TGF-β1 expression, we found that only TGF-β ligands (TGF-βs 1, 2, or 3) elevated TGF-β1 expression in the presence of EGF (42). We now show that EGF is clearly permissive for the autoinduction of TGF-β1 expression as measured by enzyme-linked immunosorbent assay (Fig. 1A) and Northern blot analysis (Fig. 1, B and C). Use of TGF-β2 treatment rather than TGF-β1 confirmed that changes in TGF-β1 protein levels measured in conditioned medium reflected only that made by LNCaP cells.

The AP-1 complex (commonly composed of dimers of c-Jun and c-Fos, or JunD/Fra-2) and Egr-1 have been shown to mediate TGF-β1 autoinduction by activating its transcription (51, 52). Thus, to understand how EGF permits autoinduction of TGF-β1 in LNCaP cells, we studied the individual and combined effects of TGF-β1 and EGF on c-Fos, c-Jun, Egr-1, and EGFR mRNA expression. Co-treatment of these cells with TGF-β1 and EGF induced c-Fos and Egr-1 mRNA levels, which peaked by 48 h. At this time point, EGF alone induced expression of both these mRNAs. In contrast, TGF-β1 alone (without EGF) was ineffective in modulating these transcription factors. However, TGF-β1 was able to induce the expression of both these mRNAs when this cell line was treated together with EGF (Fig. 1D). Neither TGF-β1 nor EGF added alone or together enhanced the mRNA levels of c-Jun or EGFR. These results show that EGF is permissive not only for TGF-β1 autoinduction but also for TGF-β’s induction of c-Fos and Egr-1.

![Image](https://via.placeholder.com/150)

**FIGURE 1.** EGF is permissive to TGF-β-induced TGF-β1, c-Fos, and Egr-1 expression in LNCaP cells. A, effect of EGF (20 ng/ml), TGF-β2 (2.5 ng/ml), or combination of EGF and TGF-β2 on TGF-β1 production rate (48 h) in serum-free medium (48 h). Data shown are averages ($\pm$ S.D.) of triplicate independent measurements. B and C, EGF permits autoinduction of TGF-β1 at mRNA levels. D, EGF permits TGF-β1-enhanced expression of c-Fos and Egr-1 mRNAs. Total RNA (10 μg) was subjected to Northern blot analysis. Representative blots of three independent experiments are shown (B–D).

![Image](https://via.placeholder.com/150)

**FIGURE 2.** EGF enhances the ability of TGF-β1 to inhibit growth or induce death of LNCaP cells. LNCaP cells (5 × 10^4 cells/well/1.0 ml of DMEM/F12 + 10% DC-FBS) were plated in 12-well dishes, treated with EGF (20 ng/ml) or vehicle, and 24 h later treated with TGF-β1 (10 ng/ml) or vehicle. Cell numbers were assayed 3, 6, 9, and 12 days later using a Coulter Electronics counter. Data shown are averages ($\pm$ S.D.) of triplicate independent measurements.
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FIGURE 3. EGF is permissive to the transactivation of the PAI-1 promoter construct, 3TP-luciferase, by TGF-β1 through a mechanism that involves TβRII expression and the elevation of endogenous TβRII mRNA. A, LNCaP cells were plated in 1% DC-FBS + DMEM/F12 and transiently co-transfected with 3TP-Lux and CMV-RENILIA. β, LNCaP cells were transiently transfected with 3TP-lux, CMV-RENILIA plasmids, and either pMFG-neo or pMFG-neo-DN-TβRII (dominant-negative TβRII). Cells were then treated with EGF (20 ng/ml), TGF-β1 (10 ng/ml), or both EGF and TGF-β1, and luciferase activity was measured 48 h later. Data represent averages of triplicate independent measurements (± S.E.) of firefly luciferase/RENILIA luciferase readings normalized to untreated controls (A, B, C). Endogenous TβRII mRNA in cells not expressing exogenous TβRII, but treated with EGF or vehicle for 24 h, was determined by RT-PCR using primers that yield a 1.7-kb fragment. All data are representative of three independent experiments.

We next performed a cell growth assay to examine whether the permissive effect of EGF also occurred at the level of growth inhibition by TGF-β1. LNCaP cells, plated in DMEM/F12 containing 10% DC-FBS to sustain cell proliferation, were pretreated with or without EGF for 24 h prior to TGF-β1 addition. Cell numbers were determined at days 3, 6, 9, and 12 following the treatment of TGF-β1. Cells cultured with TGF-β1 alone were growth arrested relative to controls that showed a slight increase in proliferation. However, when pretreated with EGF, TGF-β1 promoted death of LNCaP cells, whereas EGF alone actually stimulated growth (Fig. 2). Thus, in LNCaP cells EGF enhances the ability of TGF-β to suppress growth arrest and appears to be permissive to TGF-β-induced cell death/apoptosis.

Endogenous TβRII mRNA Is Enhanced by EGF through a Non-transcriptional Mechanism—To define the mechanism by which EGF can permit TGF-β1 responses we next studied the effects of EGF on TGF-β-regulated transcriptional activity, using a highly TGF-β-responsive plasminogen activator inhibitor-1 promoter-luciferase reporter construct, 3TP-lux. We showed that neither TGF-β1 nor EGF alone activated 3TP-lux, whereas co-treatment with both of these agents induced this promoter activity ~7-fold (Fig. 3A). Consistent with a TGF-β receptor-mediated response, this induction was lost by co-expression of dominant-negative TβRII (Fig. 3B). However, LNCaP cells are reported to be weakly responsive to TGF-β1 due to very low expression of TβRII (53, 54). Not surprisingly, we were unable to detect TβRII expression in these cells even after EGF treatment at the mRNA level by Northern blot or at the protein level by a significantly enhanced Western blot procedure (6). However, the increased sensitivity of detection offered by semi-quantitative RT-PCR revealed that TβRII was elevated ~4-fold following EGF treatment (Fig. 3C), indicating that EGF may enhance TGF-β responses through up-regulating TβRII levels.

FIGURE 4. EGF does not enhance TβRII promoter activity. A–C, LNCaP cells were plated in 1% DC-FBS + DMEM/F12 and transiently co-transfected with CMV-RENILIA and the full-length (~1690 bp) (A) or the deletion (~216 bp) (B, C) of TβRII promoter-luciferase. 20 ng/ml of EGF was added after transfection, and 10 ng/ml of TGF-β1 was added 16 h after. Cells were then incubated for an additional 48 h before dual luciferase assay (A, B). Separately, cells were incubated in the absence or presence of 1 μM MS-275 before being assayed (C). A dual luciferase assay was performed; data shown are averages (± S.E.) of triplicate independent measurements of firefly luciferase/RENILIA luciferase readings normalized to untreated controls (A–C). All data are representative of three independent experiments.
Enhanced expression of TβRII mRNA could occur either through a transcriptional mechanism or through stabilization of the TβRII message. The former possibility was tested by measuring TβRII promoter activity. LNCaP cells were transiently transfected with the full-length (1–1100) or truncated (216–35) TβRII promoter-luciferase constructs, followed by treatment with EGF, TGF-β1, or both EGF and TGF-β1 (Fig. 4, A and B). Although TGF-β1 enhanced (≤2-fold) the activity of only the full-length TβRII promoter construct, EGF had no effect on either construct whether in the presence or absence of TGF-β1 (Fig. 4, A and B). MS-275, a histone deacetylase inhibitor used as a positive control (55), enhanced activity of TβRII (216–35) promoter construct by 8-fold (Fig. 4C). These results suggest that induction of endogenous TβRII mRNA expression by EGF (shown in Fig. 3C) occurs through a non-transcriptional mechanism involving enhanced stability of TβRII mRNA.
**Figure 6.** EGF rapidly enhances the levels of endogenous and of exogenously expressed TβRII protein and mRNA. LNCaP cells plated with 1% DC-FBS + DMEM/F12 were infected with a recombinant adenovirus expressing wild-type TβRII (AdMax-WT-TβRII) (1:200) for 24 h. Medium was replaced (without virus) before various times of treatment with 20 ng/ml of EGF (A and B), and cells were harvested for assaying TβRII by either Western blot (A) or by Northern blot (B) analyses. C, effect of EGF on the endogenous level of TβRII in LNCaP was measured by RT-PCR in a time course experiment similar to that in panel B but without adenoviral infection. In the above time course experiments, the time of EGF addition was varied, with total culture times and all other conditions kept constant for proper control. All data are representative of three independent experiments. Deglycosylated protein (20 µg) and total RNA (10 µg) were subjected to Western blot and Northern blot analyses, respectively (A, B). A representative blot of three independent experiments is shown (A–C).

**Figure 7.** TGF-β suppresses the expression of TβRII protein and mRNA in an EGF-reversible mechanism. LNCaP cells were infected in 1% DC-FBS + DMEM/F12 with a recombinant adenovirus expressing wild-type TβRII (AdMax-WT-TβRII) (1:200) for 24 h. Medium was replaced (without virus) before various times of treatment with 10 ng/ml of TGF-β1 alone (A, B) or by pretreatment with 20 ng/ml of EGF (C, D). Cells were harvested for assaying TβRII by Western blot (A, C) or Northern blot (B, D) analysis. For co-treatment with EGF and TGF-β, EGF was added 0.5 to 24 h prior to TGF-β1. In the above time course experiments, the time of TGF-β1 addition was varied, with total culture times and all other conditions kept constant for proper control. All data are representative of three independent experiments. Deglycosylated protein (20 µg) and total RNA (10 µg) were subjected to Western blot (A, C) or Northern blot (B, D) analyses, respectively. A representative blot of three independent experiments is shown.

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EGF Permits TGF-β1-induced Transcriptional Responses in Cells Overexpressing Exogenous TβRII—Consistent with our hypothesis that enhanced stabilization of TβRII mRNA mediates the permissive effect of EGF on TGF-β responses, EGF can also enhance TGF-β responses in LNCaP cells that overexpress TβRII via transient transfection of a CMV-driven promoter construct, pCMV5-TβRII (Fig. 5A). Although transfection of this TβRII expression vector enabled TGF-β1-induced 3TP-lux activity without EGF, cells overexpressing TβRII retained their exquisite sensitivity to EGF for potentiating this TGF-β response (Fig. 5A). Moreover, EGF also permitted TGF-β1-induced p21CIP1/WAF-1 transcriptional activity 9-fold (Fig. 5B) and enhanced TGF-β1-induced AP-1 basic response element promoter-luciferase construct (AP-1-luc) >40-fold in LNCaP cells overexpressing TβRII, showing maximum activity at 48 h (Fig. 5, C and D). In contrast to TβRII, overexpression of TβRI (ALK5) did not enhance TGF-β responses either with or without EGF (Fig. 5E). Taken together, these results indicate that LNCaP cells retain the permissive effect of EGF even after overexpression of TβRII by a CMV-driven promoter and further support that this EGF effect is mediated by changes in TβRII mRNA stability.

EGF Stabilizes TβRII Expression—We tested our hypothesis that EGF enhances TGF-β responses through elevation of TβRII levels, using LNCaP cells ectopically overexpressing TβRII delivered by either transient transfection or adenoviral infection. Transfected cells were treated with EGF, TGF-β1, EGF + TGF-β1, or vehicle, and 24–48 h later levels of TβRII were measured by Western blot analysis. As expected, TβRII was significantly elevated by EGF in cells transfected with pCMV5-TβRII or infected with AdMax-TβRII (Fig. 6). In time course experiments with these cells, protein levels of this receptor were enhanced by 3 h of EGF treatment, reached a peak at 6 h, and persisted at that level for over 24 h (Fig. 6A). To test whether the changes in TβRII protein levels reflected changes in the expression of TβRII mRNA, total RNA from LNCaP cells treated similarly was subjected to Northern blot analysis. In this experiment expression of TβRII mRNA was increased as early as 1 h of EGF treatment, and robust (>20-fold) increases in this message appeared between 3 and 6 h after EGF treatment. Thereafter, levels of TβRII mRNA dropped for the next 3–6 h and then kept steady up to 24 h (Fig. 6B). The levels of endogenous TβRII mRNA (detected by RT-PCR) in LNCaP cells that were not infected or transfected with TβRII similarly reached a peak induction after 3–6 h of EGF treatment (Fig. 6C). Although with a weaker magnitude, a similar rapid induction in the levels of both endogenous and exogenously expressed TβRII mRNA occurred by EGF in the PC-3, but not the DU-145, androgen receptor-negative human prostate cancer cell lines (supplemental Fig. S1).

TGF-β1 Down-regulates mRNA and Protein Levels of TβRII: an EGF-reversible Mechanism—Interestingly, expression of TβRII at both protein and mRNA levels decreased as early as 3 h following TGF-β1 addition, and a 30-min pretreatment of cells with EGF abrogated the TGF-β1-induced loss of TβRII protein levels (Fig. 7, A–C). Similarly, EGF enhanced TβRII mRNA expression and reversed TGF-β1 down-regulation of TβRII mRNA level following treatment of these cells with EGF for 24 h and then with TGF-β1 for 24–48 h (Fig. 7D). Western blots done in parallel revealed similar changes in TβRII protein levels (supplemental Fig. S2B). To confirm that such expression is not a reflection of changes in CMV promoter activity, we showed that neither EGF nor TGF-β1 altered other CMV-driven constructs (i.e. AdMax-Akt (supplemental Fig. S2B) or CMV–Renilla (data not shown)). The overexpression of Akt also did not change the expression of TβRII in the presence...
or absence of EGF or/and TGF-β1 (data not shown). Taken together, these results suggest that EGF and TGF-β1 enhances and suppresses, respectively, TβRII mRNA levels through changes in mRNA stability rather than in transcription.

**Elevation of TβRII Levels by EGF Are Independent of de Novo Transcription and Protein Synthesis**—The rapidity of the changes in TβRII mRNA levels by EGF or TGF-β1 suggests that such regulation may not require de novo transcription or protein synthesis. To test these possibilities, we first measured changes in TβRII mRNA levels by EGF in the presence of a potent transcriptional inhibitor, actinomycin D. For this, cells overexpressing TβRII via AdMax-TβRII were pretreated with 1 μg/ml of actinomycin D for 1 h and then incubated for the indicated times in the presence or absence of EGF, followed by Northern blot analysis (Fig. 8A). ImageQuant analysis shows that TβRII mRNA was stabilized after 4 h of EGF treatment, with maximum protection by 6 h. We next determined whether de novo protein synthesis was necessary for these changes in the stability of TβRII. For this, cells were pretreated with 1 μg/ml of cycloheximide for 1 h before a 3-h treatment with EGF or TGF-β1. In this experiment, cycloheximide neither blocked nor attenuated the effects of EGF or TGF-β1 on exogenously expressed TβRII mRNA but actually enhanced all levels of TβRII mRNA (Fig. 8B and C). Therefore, changes in TβRII stability by EGF or TGF-β1 do not require de novo protein synthesis.

**Modulation of TβRII Levels by TGF-β1 or EGF Does Not Require TβRII Kinase or Smad3—**To understand the mechanism by which TGF-β1 or EGF is able to modulate stabilization of TβRII, we next investigated whether such stabilization required autophosphorylation of TβRII or the kinase activity of this receptor. LNCaP cells were transiently transfected with expression constructs of TβRII mutated at the kinase domain or prominent sites of autophosphorylation (K277R, S213A, S409A, S416A, and S409A/S416A). Changes in expression of TβRII in these cells after 24 h of treatment with EGF or/and TGF-β1 were then measured by Western blot analysis. Our data clearly show that TGF-β1 treatment suppressed the level of each of the mutant receptors, similar to that of wild-type TβRII. On the other hand, EGF both enhanced the expression of these receptor mutants and reversed their down-regulation by TGF-β1 (Fig. 9A). These data suggest that the kinase activity or autophosphorylation of TβRII is not required for stabilization of TβRII by EGF or destabilization by TGF-β1 despite major differences in the ability of these receptors to mediate induction of AP-1-luc activity by TGF-β1 (supplemental Fig. S3).

To explore the requirement of Smad3 on TGF-β1- and EGF-mediated changes in TβRII levels, we silenced Smad3 expression in LNCaP cells with siRNA oligonucleotides (si-Smad3) (Fig. 9B). Loss of Smad3 by si-Smad3 blocked the ability of TGF-β1 to activate AP-1-luciferase (supplemental Fig. S4) but did not block the ability of EGF to enhance levels of TβRII. However, si-Smad3 significantly elevated TβRII in the non-treated control and, to a smaller extent, in the TGF-β-only-treated group (Fig. 9B). Moreover, si-Smad3 did not enhance the levels of TβRII over that stabilized by EGF alone or by EGF + TGF-β1 (Fig. 9B). These data thus support that the changes in TβRII expression by EGF or TGF-β1 occur through a Smad3-independent mechanism(s). Moreover, these data also suggest that Smad3 may down-regulate the levels of TβRII and reduce the observed effectiveness of EGF to stabilize TβRII levels.

Another interesting observation from Fig. 9B was that expression of Smad3 in the control (scrambled siRNA) group was enhanced by TGF-β1 and further elevated by the inclusion of EGF, but not by EGF without TGF-β1. The latter data suggest that Smad3 is under positive regulatory control by TGF-β1 in these cells. Although elevated expression of Smad3 by TGF-β1 may contribute to ligand-dependent loss of TβRII, the ability of TGF-β1 to reduce levels of TβRII in the absence of protein synthesis (Fig. 8C) further supports the involvement of a Smad3-independent mechanism for enhanced destabilization of TβRII by TGF-β1.

**Stabilization of TβRII by EGF Is EGFR Kinase-dependent and Occurs through a MEK1/2-dependent Pathway—**Autophosphorylation of activated EGFR stimulates a number of intracellular signal transduction cascades, including the Ras/Raf/mitogen-activated protein kinase and PI3-kinase/Akt pathways (56–58). To better understand how EGF stabilizes TβRII expression, we first investigated the kinase dependence of EGFR on regulating TβRII levels, using specific inhibitors of EGFR
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kinase, PD153035 or AG1478. Pretreatment with each of these inhibitors suppressed stabilization of TβRII by EGF either in the presence or absence of TGF-β1 (Fig. 10, A and B), suggesting that EGFR kinase activity is necessary for downstream pathways involved in stabilization of TβRII. We next defined these downstream pathways using selective kinase inhibitors, including U0126 (MEK1/2), PD98059 (MEK1), SB202190 (p38), LY294002 (PI3-K), rapamycin (mTOR), and SP600125 (SAPK/c-Jun N-terminal kinase). Of these, only U0126 abolished EGF-induced TβRII stabilization (Fig. 10, C and D). These inhibitors were functionally tested by their ability to block phosphorylation of downstream substrates such as p44/42 mitogen-activated protein kinase, p70S6K, and c-Jun (Fig. 10, C and D). PD98059 also inhibited the up-regulation of TβRII by EGF, whereas the other agents were ineffective in this respect, thus implicating that MEK1/2 is the mechanism by which EGF enhances TβRII levels.

We further examined whether overexpression of MEK1 could enhance TβRII mRNA, mimicking this EGF effect, and whether U0126 and AG1478 could also suppress TβRII mRNA stabilized by EGF, using Northern blot and RT-PCR analyses (Fig. 10, E and F). Consistent with our Western blot data, TβRII mRNA was significantly elevated by enforced expression of MEK1, and U0126 completely abolished the EGF effect on TβRII mRNA levels (Fig. 10E). Furthermore, U0126 and AG1478 reversed the ability of EGF to enhance the expression of endogenous TβRII mRNA in LNCaP cells not transfected or infected with TβRII (Fig. 10F). As expected, AP-1-luc activity permissively induced by TGF-β1 + EGF was depressed 9- and 16-fold, respectively, by the MEK1/2 inhibitors PD98059 and U0126 (supplemental Fig. S5). Consistently, the p38-kinase inhibitor (SB), which was not able to block TβRII stabilization by EGF, also failed to block AP-1 reporter activity (supplemental Fig. S5).

Consistent with the above results, enforced expression of MEK1 (≥50 ng of the vector) stabilized TβRII levels (supplemental Fig. S6A), and overexpression of active H-Ras and c-Fos, but not Egr-1 or Elk-1, also significantly increased TβRII levels (supplemental Fig. S6B). These results further support that such changes in levels of TβRII occur through a MEK1/2-dependent pathway and may also involve the activation of c-Fos but not Egr-1 or Elk-1. Taken together, these data support that EGF permits the transduction of TGF-β1 signals mainly

![Diagram](https://example.com/diagram.png)

**FIGURE 9.** The ability of EGF and TGF-β to modulate levels of TβRII is independent of the phosphorylation status or kinase activity of TβRII or the expression of Smad3. A, LNCaP cells were transfected with kinase-dead (K277R) and autophosphorylation (S213A, S409A, S416A, or S409A/S416A) mutants of TβRII (48), followed by incubation with or without EGF (20 ng/ml). 16 h later cells were treated with vehicle or TβRII (10 ng/ml) for an additional 24 h and then analyzed for TβRII expression by Western blot. B, separately, cells were co-transfected with TβRII and either 80 nm Smad3 siRNA or scrambled siRNA for 4 h, followed by treatment with or without EGF (20 ng/ml). 16 h later, cells were incubated in the absence or presence of TGF-β1 (10 ng/ml) for an additional 24 h. For Western blot analysis of TβRII, 20-μg aliquots of protein lysates were deglycoylated prior to electrophoresis (A, B). A representative blot of three independent experiments is shown.

![Diagram](https://example.com/diagram.png)

**FIGURE 10.** Modulation of TβRII levels by EGF occurs through an EGFR kinase-dependent and MEK1/2-kinase-dependent pathway. LNCaP cells transiently overexpressing TβRII were pretreated with PD153035 (5 μM) (A); AG1478 (200 μM) (B); rapamycin (R, 200 μM), SB202190 (SB, 10 μM), U0126 (U, 10 μM) (C); or PD98059 (PD, 10 μM), LY294002 (LY, 10 μM), or SP600125 (SP, 10 μM) (D) 1–2 h prior to addition of EGF (A–D) and then incubated with TβRII for 24 h. E, overexpression of MEK1 enhanced endogenous TβRII mRNA levels, and suppression of MEK1/2 activity by U0126 abolished the EGF-mediated stabilization of TβRII mRNA levels. F, effects of U0126 and AG1478 (1 h pretreatment) on induction of endogenous TβRII mRNA by EGF (3 h) in LNCaP cells were assayed by RT-PCR as described in Fig. 3C. A representative blot of three independent experiments is shown (A–F).
through TβRII mRNA stabilization via an EGFR-Ras-MEK1/2-dependent, but Smad3-independent, mechanism.

**DISCUSSION**

In this study we have provided the first evidence supporting a novel function of EGF as a permissive factor for numerous TGF-β responses, including growth suppression. Our data demonstrate that EGF targets TβRII through a previously unreported mechanism, namely via enhanced stabilization of TβRII message. Moreover, this is the first report that TGF-β down-regulates TβRII mRNA by a non-transcriptional mechanism. Such ligand-mediated receptor down-regulation occurs similarly with either wild-type TβRII or kinase-dead TβRII and is reversed by pretreatment with EGF, but not by silencing Smad3 with siRNA. These results suggest that TGF-β destabilizes TβRII mRNA through a non-classical TGF-β signaling pathway that appears to be reversed by EGF. Our results can be distinguished from previous reports of ligand-dependent loss of TβRII protein occurring through receptor-mediated endocytosis (59), which is reported to be required for the transduction of TGF-β signals (60).

Upon activation of EGF by ligand binding and receptor dimerization, EGF initiates the recruitment and phosphorylation of several intracellular substrates, activating multiple signaling cascades such as PI3-kinase, STAT, Ras/MEK, and Rac/PAK/c-Jun N-terminal kinase (26). Using various kinase inhibitors we have shown that EGF controls TβRII levels in LNCaP cells through an EGF kinase-dependent mechanism that involves activation of MEK1 but not PI3-kinase, c-Jun N-terminal kinase, mTOR, or p38-kinase. As expected, Ras or MEK1 cDNA expression constructs enhanced TβRII levels. c-Fos, but not Egr-1, both of which are induced by EGF (Fig. 1D), may be involved in the stabilization of TβRII by EGF, as we showed that enforced expression of c-Fos but not Egr-1 induces TβRII expression (supplemental Fig. S6B). Therefore, our results indicate that an EGF kinase/Ras/MEK1-dependent pathway mediates the permissive action of EGF on TGF-β signaling. Further data suggest that c-Fos, but not Egr-1 or Elk-1, is a potential downstream mediator of this EGF activity, although additional work awaits identification of the actual mediator(s).

The fact that the TβRII expression constructs used here are devoid of the TβRII untranslated regions suggests that regulatory elements for stabilization by EGF are located within the coding region of TβRII rather than within its untranslated regions. This is similar to stabilization of c-Fos, c-Myc, and β-tubulin mRNAs, which are also controlled by sequences within their coding region (61). Specific developmentally controlled proteins have been identified that bind to discreet coding regions of c-Myc and c-Fos mRNAs and regulate their message half-lives (61, 62). One of these coding region instability determinant-binding proteins, whose expression is greatly elevated in ~30% of human breast cancers, has recently been reported to cause mammary tumors in transgenic mice when targeted to the mammary gland with a WAP promoter construct (63). Our data on TβRII constructs harboring mutations at kinase and autophosphorylation sites (K277R, S213A, S409A, S416A, and S409A/S416A) indicate that these sites are not involved in TβRII down-regulation by TGF-β or reversal of such down-regulation by EGF. We are currently exploring the sites of TβRII involved in such mRNA loss, using various deletion constructs of the TβRII coding sequence to help identify potential coding region-binding proteins for TβRII. The results of our cycloheximide experiments suggest that the EGFR or TGF-β controls of such putative coding region-binding protein(s) involve post-translation modification rather than de novo protein synthesis. Identification of such proteins may have therapeutic potential in reversing changes in TβRII levels occurring during carcinogenesis.

Our proposed role of EGF as a stabilizer of TβRII adds a new perspective on the mechanism of cross-talk between EGF and TGF-β. EGF has previously been reported to suppress TGF-β responses in other epithelial cells (30, 64) or be permissive to TGF-β responses in fibroblasts. Particularly striking is the synergism of EGF and TGF-β for growth of NRK-49F rat kidney fibroblast cells on monolayer cultures (35) and in soft agar (65, 66). TGF-β has been suggested to promote EGF responses by inducing levels of EGFR in the NRK-49F cell line (67). We recently found that EGF enhances TβRII expression and TGF-β decreases levels of this receptor in NRK-49F cells (data not shown), similar to LNCaP cells. However, unlike in LNCaP cells, in NRK-49F cells EGF only partially reverses the loss of TβRII expression by TGF-β. NRK-49F cells may behave similarly to human dermal fibroblasts, in which EGF was shown to activate the TβRII promoter through a PI3-kinase-dependent mechanism (68).

EGF has previously been reported to also suppress TGF-β signaling in a variety of cell lines not used in our current study. These may occur through EGF-induced 1) inactivation of Smad2 by phosphorylation of its middle linker region (69), 2) activation of a transcriptional repressor (TGF) through Ras (30), or 3) inhibition of downstream signals of TGF-β-induced apoptosis (e.g. caspase-9 activation) via a PI3-kinase-dependent pathway (31). Taken together, these studies suggest that the manner by which EGF affects TGF-β responses is cell type and context dependent. Our data showing a permissive effect of EGF on TGF-β were obtained using the most widely studied androgen-dependent human adenocarcinoma cell line, LNCaP. Although isolated from a lymph node metastasis, LNCaP cells appear to represent a well differentiated and androgen-responsive prostate carcinoma with neuroendocrine phenotype (70). However, such regulation of TβRII found in LNCaP cells may not be specific to prostate carcinomas with neuroendocrine phenotype, because we showed similar (although reduced in magnitude) responses on the PC-3 human prostate carcinoma cell line, which lacks neuroendocrine behavior.

In conclusion, we propose a new model for control of TGF-β responses by EGF. In our model, EGF may enhance or permit TGF-β responses, such as growth suppression, through TβRII mRNA stabilization controlled by its coding sequence. Our results suggest that the activation of EGFR in certain cancers may function to prevent full loss of TβRII expression in late stage cancers and thereby permit some of the direct oncogenic behavior of TGF-β acquired during tumor progression (25). Moreover, our results suggest that the therapeutic use of EGFR inhibitors for early stage cancer may impede on therapy by relieving the tumor-suppressive effects of TGF-β through decreasing the stability of TβRII. Further work remains to identify targets downstream of EGF, MEK1/2 or c-Fos that control TβRII levels and the TβRII coding region-binding protein(s) involved in such regulation. This effort may provide new insight on the regulation of TβRII expression and disclose novel therapeutic strategies to modulate responses of TGF-β during carcinogenesis.

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