Insulin-like Growth Factor-I Inhibits Transcriptional Responses of Transforming Growth Factor-β by Phosphatidylinositol 3-Kinase/Akt-dependent Suppression of the Activation of Smad3 but Not Smad2*

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Insulin-like growth factor-I (IGF-I) and transforming growth factor-β (TGF-β) have been shown to be oncogenic and tumor suppressive, respectively, on prostate epithelial cells. Here we show that IGF-I inhibits the ability of TGF-β to regulate expression of several genes in the non-tumorigenic rat prostatic epithelial line, NRP-152. In these cells, IGF-I also inhibits TGF-β-induced transcriptional responses, as shown by several promoter reporter constructs, suggesting that IGF-I intercepts an early step in TGF-β signaling. We show that IGF-I does not down-regulate TGF-β receptor levels, as determined by both receptor cross-linking and Western blot analyses. However, Western blot analysis reveals that IGF-I selectively inhibits the TGF-β-triggered activation Smad3 but not Smad2, while not altering expression of total Smads 2, 3, or 4. The phosphatidylinositol 3-kinase (PI3K) inhibitor, LY29004 reverses the ability of IGF-I to inhibit TGF-β-induced transcriptional responses and the activation of Smad3, suggesting that the suppression of TGF-β signaling by IGF-I is mediated through activation of PI3K. Moreover, we show that enforced expression of dominant-negative PI3K (DN-p85α) or phosphatidylinositol 3-phosphate-phosphatase, PTEN, also reverse the suppressive effect of IGF-I on TGF-β-induced 3TP-luciferase reporter activity, whereas constitutively active PI3K (p110αCAAX) completely blocks TGF-β-induced 3TP-luciferase reporter activity. Further transfection experiments including expression of constitutively active and dominant-negative Akt and rapamycin treatment suggest that suppression of TGF-β signaling/Smad3 activation by IGF-I occurs downstream of Akt and through mammalian target of rapamycin activation. In summary, our data suggest that IGF-I inhibits TGF-β transcriptional responses through selective suppression of Smad3 activation via a PI3K/Akt-dependent pathway.

TGF-β, for which there are three mammalian isoforms, is a multifunctional autocrine/paracrine growth regulator belonging to a large TGF-β superfamily (1, 2). TGF-β functions as a tumor suppressor of the prostate (3, 4) through a mechanism that is likely dependent on its ability to induce apoptosis and growth arrest (3, 5, 6). A number of studies support that TGF-β plays an important role in the mechanism and regulation of androgen action (7–11). The pattern of cross-talk between androgen and TGF-β, which occurs through multiple steps (8, 9, 11, 12), is likely controlled by androgen receptor co-regulators and other growth modulators such as IGF-I (5, 13). Our laboratory has reported previously that IGF-I is a potent inhibitor of the apoptosis of the NRP-152 rat prostate epithelial cell line induced by TGF-β but not by a variety of other apoptosis inducers (5), suggesting that IGF-I blocks early TGF-β signals that lead to apoptosis.

TGF-β1 signals mainly through a cooperative interaction with cell surface signaling receptors, TβRI, and TβRII (2, 14). TGF-β1 first associates with TβRII, which recruits TβRI to form a ligand/receptor heteromeric complex. The constitutively active kinase domain of TβRII then activates TβRI by transphosphorylation of the GS box in the cytoplasmic domain (15). The activated TβRI then transmits TGF-β signals by binding and activating Smads 2 and 3 through transphosphorylation of their C-terminal SXSX serines (16), a process that requires accessory proteins such as SARA (17), Hrs (18), Dab-2 (19), and ligand-dependent receptor endocytosis (20). Receptor-activated Smads homo- and heterodimerize (21) and then translocate to the nuclear compartment with or without Smad4 (22), where they either directly or indirectly activate transcription through association with Smad response elements (SBEs) (23, 24). TGF-β receptors also activate members of the mitogen-activated protein kinase family, through unknown mechanisms, which together with activated Smads are critical for the mediation of growth arrest and apoptosis induced by TGF-β (25).

IGF-I, which is a potent inhibitor of apoptosis, binds to and activates the receptor tyrosine kinase IGF-IR, a receptor shown to be essential for malignant transformation by a number of servers.

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† The abbreviations used are: TGF-β, transforming growth factor-β; TβRI, TGF-β1 receptor; DMEM/F-12, Dulbecco’s modified Eagle’s medium/Ham’s F-12; IGF-I, insulin-like growth hormone factor-I; LEF-IGF-I, long R3 IGF-I; IGF-IR, IGF-I receptor; DN, dominant negative; PI3K, phosphatidylinositol 3-kinase; CA, constitutively active; SARA, Smad anchor for receptor activation; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SRE, serum response element; SRF, serum response factor; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; SH2, Src homology; SBE, Smad binding element; AP1, activator protein 1; CMV, cytomegalovirus; P38, phosphatidylinositol 3-phosphate; PI, phosphatidylinositol.
IGF-I Inhibits Activation of Smad3

Materials—Materials and their sources were as follows: recombinant human TGF-β1 (R & D Systems, Inc., Minneapolis, MN); anti-phospho-Smad3 antibody (44); anti-p-AKT (Ser-473, number 9271), and anti-AKT (1:1000). DNA was labeled using the T7PdCTP by using Prime® RmT Random Priming Label Kit (Stratagene). Expression level of mRNA was quantified using a PhosphorImager and ImageQuant.

DNA Fragmentation Assay—Internucleosomal DNA fragmentation was detected using the TACS™ apoptotic DNA ladder kit ( Treviglen®). NRP-152 cells were plated at a density of 4 × 10^6 cells/2 ml/well in 6-well plates with GM3 and incubated overnight for attachment. Cells were then treated with or without LR-IGF-1 for 24 h prior to TGF-β1 (10 ng/ml) addition. After 24 h, cells were detached by trypsinization, cell pellets were resuspended in 25 mM phosphate-buffered saline, and DNA was purified as described by the manufacturer. The nicked ends of 1 μg of DNA were labeled for 10 min at room temperature using 2.5 units of Klenow polymerase and 0.5 μM [α-32P]dATP (3 Ci/mmol; PerkinElmer Life Sciences). One-third of the labeled DNA was electrophoresed through a 2% TBS gel stained with 0.5 μg/ml of Euphy II (Santa Cruz Biotechnology, Inc.). Band intensity was determined using GelPro™ images program (Media Cybernetics, Silver Spring, MD).

Northern Blot Analysis—Total RNA purified from NRP-152 cells by the RNeasy total RNA kit (Qiagen) was electrophoresed (5–10 μg/lane) through a 1% agarose gel containing 0.66 M formaldehyde and 0.7 μg/ml ethidium bromide. Equal loading and even transfer were assessed by visualization of the 18 and 28 S rRNAs. To enhance RNA transfer, gels following electrophoresis were soaked in 60 mM NaOH (20 min) and then 10 mM NaCl, 50 mM Tris-HCl, pH 7.4 (20 min). Total RNA was next transferred by capillary action to Hybond N+ membranes (16–20 h with 10× saline/sodium phosphate/ethylenediaminetetraacetic acid). Hybond membranes were cross-linked using UV light and prehybridized, and washed following the Church and Gilbert method (46). The presence of indicated mRNA was detected with cDNA probes labeled with [32P]dCTP using Prime® RmT Random Priming Label Kit (Stratagene). Expression level of mRNA was quantitated using a PhosphorImager and ImageQuant.

IGF-I Inhibits Activation of Smad3

IGF-I is involved in cell growth and apoptosis, including Bad, p70S6k, SH2 domain protein that associates to activated receptor tyrosine kinases. Both PH domains require phosphorylation at Thr-308, whereas a yet unidentified kinase (PDK2) phosphorylates PI3K at Ser-473. Both PI3K and the PI3P phosphatase, PTEN, can inhibit transcriptional responses. Finally, DN-Akt or rapamycin inhibit the IGF-I-promoted suppression of TGF-β-induced 3TP-luciferase, suggesting that suppression by IGF-I is downstream of Akt and mTOR.

EXPERIMENTAL PROCEDURES

Materials—Materials and their sources were as follows: recombinant human TGF-β1 (R & D Systems, Inc., Minneapolis, MN); anti-phospho-Smad3 antibody (44); anti-p-AKT (Ser-473, number 9271), and anti-AKT (1:1000) (Cell Signaling, anti-Smad3 (1:1000) (Transduction Laboratories), anti-Smad4 (1:100,000), anti-Smad3 (1:1000), anti-cyclin D2 (1:1000), anti-TjRI (1:1000), and anti-TjRII (1:1000) (Santa Cruz Biotechnology, Inc.). Anti-p-Smad3 (1:1000) was developed by Dr. Michael Reiss (44). The SuperSignal Chemiluminescence Substrate System (Pierce) was used to visualize protein bands.

Onclones (26–29). Elevated serum IGF-I is proposed to be a good predictor of prostate cancer and may be involved in the etiology of this malignancy (30–33). Other studies show that the IGF-I axis is under androgen control and may be intimately tied into the acquisition resistance to androgens during carcinogenesis (34–38).

Protein kinase B/Akt, which consists of a family of three mammalian isoforms, Akt-α, Akt-β, and Akt-γ, plays a critical role in mediating most, if not all, effects of IGF-I on tumor growth and inhibition of apoptosis (39). All three Akt isoforms have an N-terminal PH domain, a central kinase domain with an activation loop containing a Thr-308 phosphorylation site, and a carboxyl terminal regulatory Ser-473 near the C terminus (39). Once activated either directly by receptor tyrosine kinases or indirectly via insulin-receptor substrate 1, P13K adds a phosphate at the D-3 position of phosphatidylinositol 4-phosphate to generate P1(3,4)P2, which is necessary for the membrane anchor of Akt through the PH domain. PDK1, also a PH domain protein that requires P1(3,4,5)P3 for membrane anchor, phosphorylates Akt at Thr-308, whereas a yet unidentified kinase (PDK2) phosphorylates Akt at Ser-473. Both PH domains require phosphorylation of phosphatidylinositol at the D-3 position via P13K, an SH2 domain protein that associates to activated receptor tyrosine kinases, particularly IGF-IR. How Akt stimulates growth and inhibits apoptosis has been complicated by its many targets involved in cell growth and apoptosis, including Bad, p70S6k, and mTOR, and the androgen receptor (37, 40, 41). PTEN, a recently identified tumor suppressor, is a membrane-associated FYVE finger phosphatase commonly inactivated in many cancers including prostate cancer (42, 43). Inactivation of PTEN causes elevation of P1(3,4)P2 leading to overactivation of Akt, along with increased cell proliferation and decreased apoptosis.

In this report we provide the first evidence that IGF-I suppresses multiple TGF-β signals, using the NRP-152 epithelial cell line. We show that IGF-I receptor signaling selectively suppresses the ability of TGF-β to activate Smad3 but not Smad2. Our data suggest a role for P13K and Akt in the mechanism by which IGF-I blocks TGF-β-induced transcription. P13K inhibitor LY294002, dominant-negative P13K (Δp85α), and the P13P phosphatase, PTEN, can inhibit transcriptional activation of the plasminogen activator inhibitor-1 promoter construct, 3TP-luciferase. LY204002 also reverses the IGF-I-promoted inhibition of Smad3 activation by TGF-β. Moreover, transfection of CA-Akt or CA-PI3K, but not wild-type Akt, mimics IGF-I in blocking TGF-β-induced transcriptional responses. Finally, DN-Akt or rapamycin inhibit the IGF-I-promoted suppression of TGF-β-induced 3TP-luciferase, suggesting that suppression by IGF-I is downstream of Akt and mTOR.

IGF-I and the PI3K/Akt pathway have been shown to regulate mTOR, and the androgen receptor (37, 40, 41). PTEN, a repressor of multiple TGF-β type Akt, mimics IGF-I in blocking TGF-β-induced transcription. Moreover, transfection of CA-Akt or CA-PI3K, but not wild-type Akt, mimics IGF-I in blocking TGF-β-induced transcriptional responses. Finally, DN-Akt or rapamycin inhibit the IGF-I-promoted suppression of TGF-β-induced 3TP-luciferase, suggesting that suppression by IGF-I is downstream of Akt and mTOR.
IGF-I Inhibits Activation of Smad3

RESULTS

We have shown previously (5) that TGF-β1 induces apoptosis of non-tumorigenic NRP-152 cells in vitro. Apoptosis of these cells is strictly dependent on culture conditions, because insulin and IGF-I specifically block whereas dexamethasone enhances the ability of TGF-β1 to induce their apoptosis (5). To study the effects of IGF-I that are independent of IGF-I-binding proteins, which are secreted by these cells, we used an analogue of IGF-I (LR3-IGF-I) that binds to the IGF-I receptor with equivalent affinity as IGF-I but binds poorly to IGF-I-binding proteins. This analogue is about 500-fold more active than IGF-I in blocking TGF-β1-induced apoptosis (Fig. 1A) and can block apoptosis by >95% at all concentrations of TGF-β1 used (Fig. 1B). In contrast to previous reports, in NRP-152 cells LR3-IGF-I is unable to block apoptosis triggered by most inducers of apoptosis tested on these cells (5). These data suggest that IGF-I functions specifically by blocking early signals important to the induction of apoptosis by TGF-β1.

To test our hypothesis that IGF-I is able to inhibit TGF-β signaling, we determined whether IGF-I can also suppress the ability of TGF-β to regulate the expression of several genes in NRP-152 cells. We showed that LR3-IGF-I significantly blocked the down-regulation of the serpin Trespin (see Ref. 13 and Fig. 2A) and the up-regulation of thrombospondin-I (Fig. 2B) and fibronectin (data not shown) expression by TGF-β1 treatment.

To better characterize how IGF-I intercepts TGF-β signaling in NRP-152 cells, we determined whether LR3-IGF-I affects the activity of TGF-β1 on TGF-β-responsive promoters. For this, NRP-152 cells were co-transfected with the plasminogen activator inhibitor-1 promoter reporter construct (3TP-luciferase) and a constitutive promoter construct, cmv-Renilla. TGF-β1 was added after overnight treatment with LR3-IGF-I, and firefly and Renilla luciferase activities were measured 24 h following TGF-β1 addition. When firefly luciferase activity was normalized to Renilla luciferase to cancel differences in transfection efficiency and cell viability by these treatments, our results indicated that LR3-IGF-I significantly inhibits plasminogen activator inhibitor-1 promoter activity induced by TGF-β1 in NRP-152 cells (Fig. 3A). We obtained similar results when this was examined in another IGF-I- and TGF-β-responsive rat prostatic cell line, DP-153 (Fig. 3B). The NRP-154 rat prostatic cell line that undergoes apoptosis by TGF-β3 but that is not blocked by IGF-I (5) was examined similarly. As expected, induction of 3TP-luciferase activity by TGF-β3 in NRP-154 cells was not blocked by LR3-IGF-I (Fig. 3C).

We next investigated the effect of LR3-IGF-I on the activation by TGF-β1 of several minimal promoter response element (SBE, AP1, SRE, SRF, NF-κB) promoter constructors. Similar to 3TP-luciferase activity, LR3-IGF-I significantly blocked the activation by TGF-β of the Smad binding element construct, SBE4Ag-luciferase (Fig. 3D), AP1-luciferase, SRF-luciferase, SRE-luciferase, and NF-κB-luciferase (Fig. 4, A–D). Together, these results suggest that IGF-I targets early TGF-β signaling steps common to the activation of SBE, AP1, SRF, and NF-κB minimal promoter response elements, suggesting that...
TGF-β receptors or Smads are likely targets of suppression by IGF-I.

To determine whether IGF-I suppresses TGF-β receptor function, we first assayed for TGF-β receptor expression on intact NRP-152 cells treated with or without either 2 nM LR3-IGF-I or insulin (1 μM) for 24 h. For this assay, cells were treated with 100 μM 125I-TGF-β1 (100-fold excess cold TGF-β1) for 4 h at 4°C, free ligand was washed out, and the ligand-bound receptors were covalently cross-linked. Following normalization to total cell number, isotopically labeled ligand-receptor covalent complexes were subjected to SDS-PAGE and analyzed by autoradiography. As shown, TGF-β receptors TβRI, TβRII, and TβRIII were not decreased but rather somewhat increased by this treatment (Fig. 5A). The effect of LR3-IGF-I on expression of TβRI and TβRII in NRP-152 cells was also determined by Western blot analysis. This analysis revealed that 24 h of stimulation with 2 nM LR3-IGF-I did not inhibit expression of these signaling receptors but instead slightly increased expression of TβRII (Fig. 5B).

As Smads are the best characterized signaling mediators of TGF-β receptors, we next explored whether the expression of each of these proteins or their activation by TGF-β was inhibited by LR3-IGF-I. For this, NRP-152 cells were pre-treated with or without 2 nM LR3-IGF-I for 24 h and then stimulated with TGF-β1 for 0, 0.5, 1, 2, and 4 h and analyzed for expression of both total and C-terminally phosphorylated Smads 2 and 3 by Western blot analysis (Fig. 6A). In these cells both Smads 2 and 3 were rapidly activated (30 min) by TGF-β1 treatment, with loss of phospho-Smad expression shortly (30 min) following this initial burst. At all these time points, the phosphorylation of Smad3 but not Smad2 by TGF-β1 was significantly inhibited with 2 nM LR3-IGF-I, whereas the levels of total Smads 3 and 2 did not change. In another experiment (Fig. 6B), we showed the suppression of phosphorylated Smad3 by LR3-IGF-I was maintained even after 24 h of TGF-β treatment, whereas phosphorylation of Smad2 by TGF-β1 remained unchanged by LR3-IGF-I during this time. Similarly, such LR3-IGF-I treatment did not alter the expression of total Smads 2, 3, or 4. Interestingly, expression of total Smad3 was reduced by 24 h of treatment with TGF-β1, and this down-regulation of total Smad3 was not reversed by LR3-IGF-I. In contrast, TGF-β1-mediated down-regulation of cyclin D2, detected on the same blot, was completely reversed by LR3-IGF-I (Fig. 6B). These results indicate that IGF-I blocks the ability of TGF-β1 to selectively activate Smad3 but not Smad2.

PI3K/Akt pathways have been reported to mediate most responses of IGF-I (39). We thus explored whether IGF-I inhibits TGF-β responses through a PI3K-dependent pathway, using a highly specific inhibitor of PI3K, LY294002 (49).
transfected these cells with DN-P13K (p85)ase normalized to Renilla luciferase. Each
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TGF-
added 6 h later, and responses were measured following 24 of
imal promoter elements in NRP-152 cells. Effect of LR3-IGF-I (2
LR3-IGF-I, whereas 4
(Ser-473) (Fig. 7
We first showed by Western blot analysis of phospho-Akt
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the suppression of TGF-
/NRP-152 cells. Cells were co-transfected with 25 ng of cmv-Renilla
reporter construct and 2 μg of the above reporter constructs and treated
overnight with LR3-IGF-I or vehicle, followed by TGF-β1 for 24 h before
measuring luciferase. Data shown are relative values of firefly luciferase
ormalized to Renilla luciferase. Each bar represents the average of
triplicate determinations ± S.E.
We first showed by Western blot analysis of phospho-Akt (Ser-473) (Fig. 7A) that pre-treating (2 h) NRP-152 cells with 10 μM LY294002 maximally blocked activation of Akt by 2 nM LR3-IGF-I, whereas 4 μM LY294002 inhibited about 80% of this activation. We next determined whether LY294002 could also reverse IGF-I’s suppression of TGF-β signaling, using 3TP-luciferase and Northern blot expression of thrombospondin-1 and Trespin as endpoints. The duration of pre-treatment of NRP-152 cells with LR3-IGF-I was reduced to 6 h in these experiments because of substantial cell killing with over 48 h of treatment with 10 μM LY294002. Thus, in these experiments NRP-152 cells were treated with 4 or 10 μM LY294002 2 h prior to LR3-IGF-I addition, TGF-β1 was added 6 h later, and responses were measured following 24 of TGF-β1 addition. Here, LY294002 reversed essentially all the suppression of TGF-β-regulated gene expression by LR3-IGF-I (Fig. 7, B-D).

To confirm that the ability of LY294004 to reverse the IGF-I suppression of TGF-β signaling was through PI3K, we co-transfected these cells with DN-P13K (p55αΔSH2-N), CA-Akt (Myc-p110αCAAX), or empty vector control (pSG5), along with the 3TP-luciferase/cmv-Renilla reporters, and after overnight incubation they were treated with 1 doses LR3-IGF-I, 6 h later with 10 ng/ml TGF-β1, and assayed for luciferase 24 later (Fig. 8A). As expected, DN-P13K expression blocked LR3-IGF-I inhibition of TGF-β-induced 3TP-luciferase reporter activity, whereas CA-Akt abolished essentially all this TGF-β-induced reporter activity. Intracellular levels of PI3P are under the regulation of not only PI3K but also PTEN, a phosphatase which specifically removes the 3’ phosphates from PI3Ps (50). Thus, enhanced expression of PTEN is expected to function similar to LY294002 in reversing IGF-I’s inhibition of TGF-β-induced transcription. To test our hypothesis further, we therefore examined the ability of LR3-IGF-I to inhibit TGF-β-induced 3TP-luciferase activity by co-transfecting NRP-152 cells with either pCEP4 empty vector or pCEP4-PTEN (Fig. 8B). Similar to effects of LY294002 or DN-P13K, expression of PTEN completely reversed the suppressive effect of LR3-IGF-I on such TGF-β-induced transcription.

We next explored the role of Akt in mediating the suppressive effect of LR3-IGF-I in TGF-β signaling. This was done similar to the above experiment with PTEN, except cells were transfected with either CA-Akt (Fig. 8C) or DN-Akt (Fig. 8D) expression constructs. Our results clearly show that constitutively active Akt alone inhibits TGF-β-induced 3TP-luciferase to the same extent as treatment with LR3-IGF-I alone, and the active Akt is unable to inhibit 3TP-luciferase much further in the presence of LR3-IGF-I (Fig. 8C). These results suggest that essentially all the inhibitory effect of IGF-I on TGF-β signaling is mediated by the activation of Akt alone. In contrast to activated Akt, DN-Akt partially reversed the suppressive effect of LR3-IGF-I on transcriptional induction by TGF-β1 (Fig. 8D), suggesting that this suppressive effect of IGF-I is at least partially down-stream of activated Akt. As these effects of DN-Akt were not complete, we decided to confirm the role of Akt-mediated signals in this response by testing whether blocking immediate down-stream targets of Akt would also

FIG. 5. LR3-IGF-I does not inhibit expression of TGF-β receptors. Effect of LR3-IGF-I on expression of TGF-β receptors in NRP-152 cells was examined following 24 h of either 2 nM LR3-IGF-I or 1 μM insulin treatment. TGF-β receptors were assayed by cross-linking following binding of 125I-TGF-β1 to cells at 4 °C in the presence or absence of 100-fold molar excess of unlabeled TGF-β1. Cell lysates, normalized to cell number, were subjected to SDS-PAGE and autoradiography (A). Separately, cells were incubated for 24 h in the absence or presence of LR3-IGF-I followed by treatment with TGF-β1 for the indicated times. 20 μg of protein was deglycosylated and subjected to Western blot analysis (B). Data shown are representative of two independent experiments/treatments.
IGF-I Inhibits Activation of Smad3

**Fig. 6.** Effect of LR-IGF-I on TGF-β-induced activation of Smads 2 and 3 and expression of Smads 2, 3, and 4 and cyclin D2. Effect of LR-IGF-I on expression of Smads 2 and 3 or activation of Smad2 and 3 by TGF-β1 in NRP-152 cells was examined in the absence or presence of LR-IGF-I (2 nM) followed by treatment with TGF-β1 (10 ng/ml). Cells were cultured with LR-IGF-I 24 h prior to incubation with TGF-β1 for the indicated time (A). Separately, cells were cultured with LR-IGF-I 6 h prior to incubation with TGF-β1 for an additional 24 h. Expression of Smad2, 3, and 4 and cyclin D2 and activation of Smads 2 and 3 was assayed by Western blot analyses (B). Data in A and B are each representatives of three independent experiments/treatments.

Reverse this IGF-IR-dependent effect. Our results suggest that mTOR, which is activated by Akt, may mediate IGF-IR suppression of TGF-β signals. Rapamycin, which is a highly specific inhibitor of mTOR and inhibits mTOR activity by promoting the association of FKBP12 to mTOR (51, 52), was used to examine this possibility. Pre-treatment of NRP-152 cells with 200 nM rapamycin completely abolished all the suppressive effect of LR-IGF-I on TGF-β-induced 3TP-luciferase activity (Fig. 8E), suggesting that the IGF-I suppression is downstream of Akt and occurs through the activation of mTOR. Moreover, rapamycin does not cooperate with TGF-β (alone) to enhance 3TP-luciferase activity.

We tested our model, that IGF-I blocks the activation of Smad3 through a PI3K/Akt pathway, using LY294002. For this we first performed a time course experiment to determine the minimal time required for 2 nM IGF-I to suppress Smad3 activation following 4 h of treatment with 10 ng/ml TGF-β1. As shown, 1 h of pre-treatment with LR-IGF-I significantly inhibited the activation of Smad3 by TGF-β1 (Fig. 9A). The inclusion of 10 μM LY294002 only 2 h prior to the addition of LR-IGF-I was able to completely reverse IGF-I’s effect on suppressing the activation of Smad3 and to inhibit activation of Akt by LR-IGF-I (Fig. 9B). Finally, we tested whether rapamycin, which reversed the IGF-I suppression of TGF-β-induced 3TP-luciferase, can also reverse the LR-IGF-I suppression of Smad3 activation. As expected, rapamycin blocked the effect of LR-IGF-I on suppression of TGF-β-induced Smad3 activation (Fig. 9C), suggesting that mTOR may mediate this selective suppression of Smad3 activation.

**Discussion**

Here we provide the first evidence that IGF-I inhibits early signals that drive TGF-β transcriptional responses, as demonstrated in NRP-152 and DP-153 rat prostatic epithelial cell lines. This was shown with the use of the IGF-I analogue, LR-IGF-I, which has very weak affinity for IGF-I-binding proteins (53). Thus, our results support that this suppression is through IGF-IR signaling and not through modulation of the activity of IGF-I-binding proteins. Such a distinction is important, because IGF-I may function also by neutralizing the growth inhibitory and apoptotic effects of IGFBP-3 through an IGF-IR-independent mechanism (54). Moreover, our data show that inhibition of PI3K/Akt activity by either the specific PI3K inhibitor, LY294002, or by transfection of either DN-PI3K (p85αΔiSH2-N) or PTEN expression constructs reverses the suppression by LR-IGF-I on TGF-β signaling, whereas the CA-PI3K construct (p110αCAAX) completely abolished this TGF-β response. Further transfection experiments with CA-Akt and DN-Akt constructs or suppression of the activity of an Akt target, mTOR, by rapamycin treatment support that the suppression of TGF-β signaling by IGF-I is mediated through activated Akt (Fig. 8, A and B).

Although our rapamycin data suggest mTOR is involved in the mechanism of TGF-β/Smad3 suppression by Akt, this possibility needs to be investigated further by showing that mTOR is necessary for the IGF-IR suppression of Smad3. An alternative possibility is that rapamycin, which mediates suppression of mTOR activity by forming a complex with FKBP12, can also affect TGF-β responses through the association of FKBP12 to the G5 region of non-activated TβRI (55, 56). However, effective activation of TβRI by rapamycin has been shown to suppress activation of TβRI only by enforced expression of FKBP12 (55, 56) and does not explain effects of rapamycin on TGF-β signals in a number of cell lines examined (57). Thus, if rapamycin reverses IGF-I suppression of TGF-β responses (Fig. 8E) through relieving the interaction of FKBP12 with TβRI in NRP-152 cells, then it is likely that IGF-I/LR3-IGF-I would enhance the expression of FKBP12 or its association to TβRI. This possibility is currently being explored in our laboratory.

Downstream mediators of PI3K signal, including PDK1 (Ser-428), Akt (Ser-473), and mTOR (Ser-2448), result in activation of Akt, p70 S6 kinase, and occur through IGF-IR signaling and not through modulation of the activity of PI3K/Akt-dependent mechanisms. Moreover, IGF-I blocks the induction of apoptosis by TGF-β1, albeit through different mechanisms. In FaO cells, IGF-I was shown to block early TGF-β1 signals but instead to specifically block the activation of caspases by TGF-β1 through a PI3K/Akt-dependent mechanism. On the other spectrum, IGF-I was reported to block...
TGF-β1-induced apoptosis in PC3 cells through neutralizing the ability of IGFBP-3, induced by TGF-β1, to promote apoptosis (54). In addition, LR3-IGF-I, which is unable to bind to IGFBP-3, does not block apoptosis induced by TGF-β1 in PC3 cells, indicating that this effect of IGFBP-3 occurred through an IGF-IR-independent mechanism. The mechanism by which IGFBP-3 triggers the induction of apoptosis is under intense investigation by numerous groups.

Differences between the mechanisms by which IGF-I blocks TGF-β signals are likely to result from differences in cell type. Indeed, one big difference between PC3 cells and NRP-152 cells, other than species differences, is that the former is highly tumorigenic, whereas the latter is non-tumorigenic in athymic mice. Moreover, NRP-152 have a basal epithelial cell pheno-

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**Fig. 7. LR3-IGF-I inhibits activation of TGF-β responses through a PI3K-dependent pathway.** The PI3K inhibitor, LY294002, blocks activation of Akt by LR3-IGF-I between 4 and 10 μM in NRP-152 cells, as measured by Western blot ECL analysis (A). Effect of LY294002 on suppression of TGF-β-induced 3TP-luciferase by LR3-IGF-I (2 nM) in NRP-152 cells was studied using Dual luciferase assay. Cells were co-transfected with 20 ng of cmv-Renilla reporter construct and 1.0 μg of 3TP-luciferase, treated with either vehicle or LY294002 for 2 h, followed by LR3-IGF-I or vehicle for 6 h, and then with TGF-β1 for 24 h before measuring luciferase. Data shown are relative values of firefly luciferase normalized to Renilla luciferase. Each bar represents the average of triplicate determinations ± S.E. (B). Effect of LY294002 on the ability of LR3-IGF-I to reverse inhibition of TGF-β-induced thrombospondin-1 expression (TSP-1; panel C) or TGF-β-repression of Trespin expression (D) was tested in NRP-152 cells (C–E). Cells were then treated with either vehicle or LY294002 (4 μM) for 1 h, followed by LR3-IGF-I or vehicle for 6 h, and then with TGF-β for 24 h before assaying mRNA expression by Northern blot. Expression of thrombospondin-1 and Trespin message was quantified using a PhosphorImager and ImageQuant, and relative mRNA intensities were normalized to that of β-actin.
type (66) and stem cell properties, as evidenced by their ability to transdifferentiate toward a luminal phenotype (67), and can form normal prostatic ducts in vivo in the presence of urogenital sinus mesenchyme (68). Significantly, TGF-β functions as a tumor suppressor in these cells, because blocking TGF-β signaling by stable expression of DN-TPRI triggers their malignant transformation (4, 17).

We show that treatment of NRP-152 cells with LR3-IGF-I for 24 h does not suppress expression of TGF-β receptors or total Smads 2, 3, or 4. Importantly, under these conditions LR3-IGF-I can selectively inhibit the activation of Smad3 but not the activation of Smad2 or loss of Smad3 expression following TGF-β1 treatment. Moreover, LY294002 effectively blocks LR3-IGF-I-mediated suppression of Smad3 activated by TGF-β1. Our results indicate that the PI3K/Akt pathway intercepts activation of Smad3 by TGF-β receptors through a target that remains to be identified. The selectivity by which IGF-I inhibits Smad3 but not Smad2 activation likely resides in differences in the mechanisms by which these proteins are activated by TβRI.

Although SARA, a FYVE finger protein, has been shown to deliver Smad2 to TβRI for activation (17), recent evidence suggests that SARA is not required for the activation of Smad3 (69) and may also not be essential for TGF-β-induced activation of Smad2 in certain cells (70). Another FYVE domain protein, Hrs, cooperates with SARA to deliver Smad2 to TβRI (18). Thus, SARA and Hrs may be focal points by which Smads 2 and 3 are activated differentially.

SARA and Hrs associate to the plasma membrane through the FYVE domain only in the presence of PI3P (18). Thus, treatment of cells with high levels of LY294002 or overexpression of DN-PI3K or PTEN is likely to deplete cellular stores of PI3P and thus block SARA and Hrs from activating Smad2 and possibly Smad3. However, we show that LY294002, DN-PI3K, or PTEN did not inhibit TGF-β signaling or Smad3 activation even after 48 h (see Fig. 7B and Fig. 8A) (data not shown), suggesting that neither SARA nor Hrs is required for the activation of Smad3 in NRP-152 cells. Rather, TGF-β signaling was significantly enhanced by these treatments (see Fig. 7, B–E and Fig. 8A), suggesting that SARA and Hrs may even inhibit Smad3 activation in these cells.

Data presented in this study contrast with that in mammary cancer cells (71, 72), where Akt was activated by TGF-β through a mechanism that was blocked by LY294002 or DN-PI3K. Moreover, LY294002 also blocked the activation of Smad2, 3TP-lux, SBE-luciferase, and epithelial mesenchymal transition (71), presumably through a SARA-dependent pathway. Further work done by that group has demonstrated that Akt mediates the ability of TGF-β to support survival of these cells through inactivation of an Akt substrate, forkhead in rhabdomyosarcoma (72).

Overall, our data show that IGF-IR signaling can not only block TGF-β-induced apoptosis but can also intercept TGF-β signaling at early steps that involve the activation of Smad3. Thus, Smad3 activation is a focal point by which IGF-IR signaling through Akt is able to intercept many effects of TGF-β. Moreover, our data support that Smads 2 and 3 are differentially activated by mutant Akt (K179M) (DN-Akt) (D), 0.8 μg each, were co-transfected with 0.2 μg of 3TP-luciferase and 20 ng of cmv-Renilla reporter into NRP-152 cells. 16 h following transfection, cells were treated with vehicle or 2 nM LR3-IGF-I for 6 h prior to addition of TGF-β1, and luciferase activity was measured after 24 h of incubation with TGF-β1. Each bar represents the mean ± S.E. from triplicate determinations. Results shown are representative of three different experiments.
Fig. 9. LY294002 and rapamycin each reverse the IGF-I suppression of TGF-β1-activated Smad3. NRP-152 cells were cultured with LR3-IGF-I (2 nM) for the indicated time, followed by treatment for 4 h with 10 ng/ml TGF-β1 (A). 10 μM LY294002 (B) or 200 nM rapamycin (C) was added to cells 2 h prior to treatment with LR3-IGF-I and then either 1 h (B) or 24 h later treated with 10 ng/ml TGF-β1 for 4 h. Total cell lysates were subjected to Western blot assay for detection of phospho-Smad3, phospho-Akt, and β-actin as shown. Results are representative of two to three different experiments/treatments. CT, co-treatment.

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...tially activated or inactivated in prostatic epithelial cells, consistent with differential expression of phospho-Smads 2 and 3 shown recently in hepatic stellate cells (44).

Data presented here strongly support our model that IGF-IR signaling is highly oncogenic for prostate, at least in part through suppression of Smad3-dependent gene expression. Further work ongoing in our laboratory in understanding the mechanism by which IGF-I blocks Smad3 signaling is likely to shed light on the mechanism by which IGF-I promotes malignant transformation of prostatic epithelium.

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REFERENCES

1. Roberts, A. B., and Sporn, M. B. (1990) in The Transforming Growth Factor Beta. Handbook of Experimental Pharmacology: Peptide Growth Factors and Their Receptors (Sporn, M. B., and Roberts, A. B., eds) pp. 419–472, Springer-Verlag New York Inc., New York

2. Massagué, J. (1992) Cell 69, 1067–1070

3. Guo, Y., and Kyprianou, N. (1999) Cancer Res. 59, 1366–1371

4. Tang, B., de Castro, K., Barnes, H. E., Parks, W. T., Stewart, L., Bottiger, E. P., Danielpour, D., and Wakefield, L. M. (1999) Cancer Res. 59, 4834–4842

5. Hsing, A. Y., Kodamatsu, K., Benham, M. J., and Danielpour, D. (1996) Cancer Res. 56, 5146–5149

6. Chupak, J. E., Bhat, M., Hsing, A. Y., Ma, J., and Danielpour, D. (2001) J. Biol. Chem. 276, 26614–26621

7. Kyprianou, N., and Isaacs, J. T. (1998) Endocrinology 139, 2124–2131

8. Kim, I. Y., Ahn, H. J., Zelner, D. J., Park, L., Sensibar, J. A., and Lee, C. (1996) Mol. Endocrinol. 10, 107–115

9. Brodin, G., ten Dijke, P., Funa, K., Heldin, C. H., and Landstrom, M. (1999) Cancer Res. 59, 2731–2738

10. Lucia, M. S., Sporn, M. B., Roberts, A. B., Stewart, L. V., and Danielpour, D. (1998) J. Cell. Physiol. 175, 184–192

11. Chupak, J. E., Cornélus, S. C., Pultz, N. J., Jürgensen, J. S., Bonham, M. J., Kim, S. J., and Danielpour, D. (2002) J. Biol. Chem. 277, 1240–1248

12. Kyprianou, N., and Isaacs, J. T. (1989) Mol. Endocrinol. 3, 1515–1522

13. Stewart, L. V., Song, K., Hsing, A. Y., and Danielpour, D. (2003) Exp. Cell Res. 294, 301–312

14. ten Dijke, P., Miyazono, K., and Heldin, C. H. (1996) Curr. Opin. Cell Biol. 8, 139–145

15. Wieser, R., Wrana, J. L., and Massague, J. (1995) EMBO J. 14, 2199–2208

16. Abdollah, S., Mucasia-Silva, M. Tsukazaki, T., Hayashi, H., Attisano, L., and Wrana, J. L. (1997) J. Biol. Chem. 272, 27678–27685

17. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) Cell 95, 779–791

18. Miura, S., Takeshita, T., Asan, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, K. I., Beppu, H., Tsukazaki, T., Hsing, A. Y., and Sugamura, K. (2000) Mol. Cell. Biol. 20, 8546–8555

19. Hoeveer, B. A., Smine, A., Xu, X. X., and Howe, P. H. (2001) EMBO J. 20, 2789–2801

20. Penheiter, S. G., Mitchell, H., Garamszegi, N., Edens, M., Dore, J. J., Jr., and Leof, E. B. (2002) Mol. Cell. Biol. 22, 4759–4769

21. Wu, R. Y., Zhang, Y., Feng, X. H., and Derynck, R. (1997) Mol. Cell. Biol. 17, 2521–2528

22. Xiao, Z., Liu, X., and Lodish, H. F. (2000) J. Biol. Chem. 275, 23425–23428

23. Jonk, L. J., Itoh, S., Heldin, C. H., ten Dijke, P., and Kruijer, W. (1998) J. Biol. Chem. 273, 21145–21152

24. Wrana, J. L. (1998) Miner. Electrolyte Metab. 24, 120–130

25. Yamamura, Y., Hua, X., Bergelson, S., and Lodish, H. F. (2000) J. Biol. Chem. 275, 36295–36302

26. Baserga, R. (1999) Exp. Cell Res. 253, 1–6
