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TGF-β Control of Cell Proliferation

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Abstract This article focuses on recent findings that the type V TGF-β receptor (TβR-V), which co-expresses with other TGF-β receptors (TβR-I, TβR-II, and TβR-III) in all normal cell types studied, is involved in growth inhibition by IGFBP-3 and TGF-β and that TGF-β activity is regulated by two distinct endocytic pathways (clathrin- and caveolar/lipid-raft-mediated). TGF-β is a potent growth inhibitor for most cell types, including epithelial and endothelial cells. The signaling by which TGF-β controls cell proliferation is not well understood. Many lines of evidence indicate that other signaling pathways, in addition to the prominent TβR-I/TβR-II/Smad2/3/4 signaling cascade, are required for mediating TGF-β-induced growth inhibition. Recent studies revealed that TβR-V, which is identical to LRP-1, mediates IGF-independent growth inhibition by IGFBP-3 and mediates TGF-β-induced growth inhibition in concert with TβR-I and TβR-II. In addition, IRS proteins and a Ser/Thr-specific protein phosphatase(s) are involved in the TβR-V-mediated growth inhibitory signaling cascade. The TβR-V signaling cascade appears to cross-talk with the TβR-I/TβR-II, insulin receptor (IR), IGF-I receptor (IGF-IR), integrin and c-Met signaling cascades. Attenuation or loss of the TβR-V signaling cascade may enable carcinoma cells to escape from TGF-β growth control and may contribute to the aggressiveness and invasiveness of these cells via promoting epithelial-to-mesenchymal transdifferentiation (EMT). Finally, the ratio of TGF-β binding to TβR-II and TβR-I is a signal controlling TGF-β partitioning between two distinct endocytosis pathways and resultant TGF-β responsiveness. These recent studies have provided new insights into the molecular mechanisms underlying TGF-β-induced cellular growth inhibition, cross-talk between the TβR-V and other signaling cascades, the signal that controls TGF-β responsiveness and the role of TβR-V in tumorigenesis. J. Cell. Biochem. 96: 447–462, 2005.

Key words: TGF-β; growth inhibition; IGFBP-3; TGF-β receptor signaling; IRS; protein phosphatase; tumorigenesis; EMT; ratio of TGF-β binding to TβR-II and TβR-I; endocytosis

Transforming growth factor-β (TGF-β) is a family of structurally homologous dimeric proteins (three mammalian isoforms: TGF-β_1, TGF-β_2, and TGF-β_3) [Roberts and Sporn, 1993; Roberts, 1998]. They regulate multiple biological processes, including cell proliferation, extracellular matrix (ECM) synthesis, angiogenesis, immune response, apoptosis and differentiation [Roberts and Sporn, 1993; Roberts, 1998]. They have been implicated in the pathogenesis of cancer, autoimmune diseases, tissue fibrosis, diabetes, and other disorders [Roberts and Sporn, 1993; Roberts, 1998]. The various biological activities of TGF-β isoforms are mediated by specific cell surface receptors in responsive cells. Multiple cell surface receptors of various sizes have been identified in cultured cells and tissues by cross-linking ^{125}I-labeled-TGF-β (^{125}I-TGF-β) to these molecules in the presence of bifunctional cross-linking reagents. These include type I (TβR-I, MW∼53,000), type II (TβR-II, MW∼70,000), type III (TβR-III, MW∼280,000–370,000), type IV (TβR-IV, MW∼60,000), type V (TβR-V, MW∼400,000), and type VI (TβR-VI, MW∼180,000) receptors as well as several membrane-associated binding proteins (MW∼38,000–190,000) [O'Grady et al., 1991a; Mitchell et al., 1992; Segarini, 1993; Massague, 1998; Moustakas et al., 2002]. TβR-I and TβR-II are Ser/Thr-specific protein kinases and are believed to be primarily responsible for TGF-β-induced cellular responses [Heldin et al., 1997; Massague, 1998; Roberts, 1998]. TβR-III is a proteoglycan-containing membrane glyco-

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protein (also referred to as betaglycan) which presents TGF-β to TβR-II, forms oligomeric complexes with TβR-II and TβR-I and postively and negatively modulates TGF-β-induced cellular responses, in a cell type-specific manner [Eickelberg et al., 2002]. The identity of TβR-IV has not been confirmed by independent studies [Yamashita et al., 1995]. TβR-V coexpresses with TβR-I, TβR-II, and TβR-III in all normal cell types studied thus far [O'Grady et al., 1991a,b] and also serves as the insulin-like growth factor binding protein-3 (IGFBP-3) receptor, mediating IGF-independent growth inhibition by IGFBP-3 in responsive cells [Leal et al., 1997, 1999; Wu et al., 2000]. TβR-VI and other membrane-associated TGF-β binding proteins are expressed only in specific cell types [Mitchell et al., 1992; Segarini, 1993].

One important activity of TGF-β is the transcriptional activation of genes coding for ECM proteins and their regulatory proteins (e.g., collagen, fibronectin, and plasminogen activator-inhibitor-1, PAI-1). This transcriptional activation is mediated by the TβR-I/TβR-II heterocomplex-mediated signaling cascade and has been studied extensively [Heldin et al., 1997; Massague, 1998; Roberts, 1998; Moustakas et al., 2002; Derynck and Zhang, 2003]. In this cascade, following ligand binding directly or via TβR-III presentation, TβR-II (which is constitutively active) recruits TβR-I to form heterocomplexes, resulting in phosphorylation and activation (the cytoplasmic kinase activity) of TβR-I in the heterocomplex. The activated TβR-I then phosphorylates and activates Smad2 and Smad3. The activated Smad2/Smad3 forms oligomers with Smad4 that translocate to the nucleus to regulate expression of target genes.

Another important activity of TGF-β is cellular growth regulation. It inhibits the growth of most cell types, including epithelial cells, endothelial cells, embryonic fibroblasts, and hematopoietic cells but stimulates growth of certain mesenchymal cells (e.g., skin fibroblasts) and some other specific cell types [Roberts and Sporn, 1993; Roberts, 1998]. The growth inhibitory response to TGF-β has been studied in a variety of in vitro cultured cell systems, but the growth inhibitory signaling is not well understood. It is generally thought that, following the TGF-β induction of the TβR-I/TβR-II/Smad2/3/4 signaling cascade, the activated Smad proteins (Smad2/3/4 complexes) target the promoters of the *c-myc* and cyclin-dependent kinase (CDK) genes and repress their transcription in cooperation with nuclear co-repressors. The various Smad protein and transcriptional co-activator (e.g., CREB-binding protein) complexes are also thought to activate the transcription of two major cell cycle inhibitors, CDK inhibitors (CKIs), p15 and p21 [Heldin et al., 1997; Massague, 1998; Roberts, 1998; Moustakas et al., 2002; Derynck and Zhang, 2003]. These inhibit CDK activities associated with the G_1 to S phase progression, prevent phosphorylation of Rb by cyclin-dependent kinases, and arrest cells in G_1.

The transcriptional activation and growth inhibition activities of TGF-β have generally been thought to be mediated by the TβR-I/TβR-II/Smad2/3/4 signaling cascade [Heldin et al., 1997; Roberts, 1998; Moustakas et al., 2002; Derynck and Zhang, 2003]. However, these two activities appear to segregate in several cell types and cells under different culture conditions [Howe et al., 1989; Taipale and Keski-Oja, 1996; Leal et al., 1997; Liu et al., 1997]. This suggests that other signaling pathways are also involved in mediating TGF-β activity. Although Smad2/3/4 signaling can be modulated by other signaling pathways [Mulder, 2000; Derynck and Zhang, 2003; Hayashida et al., 2003], it has been shown to be responsible for mediating the transcriptional activation of ECM synthesis-related genes. Smad2/3/4 responsive elements exist in the promoter regions of all responsive genes. In contrast, the signaling involved in TGF-β-induced growth arrest is more complex than that for transcriptional activation of ECM synthesis-related genes. Many lines of evidence indicate that other signaling pathways, in addition to the prominent TβR-I/TβR-II Smad2/3/4 signaling pathway, are involved in the growth inhibitory response to TGF-β [Howe et al., 1989; Taipale and Keski-Oja, 1996; Wang et al., 1996; Leal et al., 1997; Liu et al., 1997; Hocevar and Howe, 1998; Mulder, 2000; Petritsch et al., 2000]. It has been suggested that the Ras/ERK signaling pathway and PP2A are involved in mediating TGF-β-induced growth inhibition in certain epithelial cell systems [Mulder, 2000; Petritsch et al., 2000]. However, the main signaling pathway which, in concert with the TβR-I/TβR-II heterocomplex-mediated signaling, mediates the growth inhibitory response in epithelial cells remains unknown. TβR-I, -II, -III, and -V

co-express in all normal cell types studied thus far [O'Grady et al., 1991b; Leal et al., 1997; Liu et al., 1997]. Since TβR-III is known not to be involved in TGF-β-induced signaling [Eickelberg et al., 2002], the logical candidate is TβR-V. Many human carcinoma cells express little or no TβR-V [O'Grady et al., 1991b; Leal et al., 1997; Liu et al., 1997]. Growth of these cells is not inhibited by TGF-β. This suggests that TβR-V may be involved in mediating the growth inhibitory response and that its loss may contribute to the malignant phenotype.

TβR-V IS PIVOTAL TO CELLULAR GROWTH INHIBITION BY IGFBP-3 AND TGF-β

The recent identification of TβR-V as the putative IGFBP-3 receptor, which mediates IGF-independent growth inhibition by IGFBP-3 [Leal et al., 1997, 1999], highlights the likely importance of TβR-V in the growth inhibitory response to TGF-β in epithelial cells and other responsive cells. Carcinoma cells are commonly found to express no or low levels of TβR-V. Such cells are insensitive to growth inhibition by either TGF-β or IGFBP-3 [Leal et al., 1997, 1999; Liu et al., 1997]. IGFBP-3 is the most abundant IGF binding protein in the circulation [Firth and Baxter, 2002]. It has been implicated in the actions of retinoic acid, TGF-β and the tumor suppressor gene p53 [Leal et al., 1997, 1999; Firth and Baxter, 2002]. In human plasma, IGFBP-3 forms a ~150-kDa ternary complex with IGF-I and an acid-labile subunit [Firth and Baxter, 2002]. It is produced by many cell types and appears to inhibit cell growth by IGF-dependent and -independent mechanisms. The putative IGFBP-3 receptor that mediates the IGF-independent growth inhibition was first identified as TβR-V in our laboratory [Leal et al., 1997] and subsequently confirmed by others [Wu et al., 2000]. By analogy with TGF-β [Roberts and Sporn, 1993; Roberts, 1998], the dimeric form (non-covalently linked) of IGFBP-3 interacts with TβR-V in mink lung epithelial cells (Mv1Lu cells) and inhibits growth of these cells [Leal et al., 1997, 1999]. The covalently linked dimeric structure is known to be required for TGF-β activity [Roberts and Sporn, 1993; Roberts, 1998]. TGF-β stimulates cellular responses by inducing hetero-oligomerization of TGF-β receptors through its dimeric structure [Roberts and Sporn, 1993; Roberts, 1998]. IGFBP-3 contains a putative TGF-β active-site motif [Leal et al., 1997, 1999]. The binding of IGFBP-3 to TβR-V is blocked by a synthetic TGF-β peptide antagonist (termed TGF-β peptantagonist) containing the TGF-β active-site motif, which prevents TGF-β binding to TGF-β receptors [Huang et al., 1997, 2002]. The IGF-independent growth regulation (including growth inhibition and growth stimulation) of IGFBP-3 shares similar cell-type specificity with TGF-β-induced growth regulation. For example, IGFBP-3 and TGF-β are growth inhibitors for epithelial cells but growth stimulators for fibroblasts [Leal et al., 1997, 1999; Roberts, 1998]. Like TGF-β, IGFBP-3 has been implicated in tumorigenesis and other disorders [Leal et al., 1997; Firth and Baxter, 2002]. These observations indicate that TGF-β and IGFBP-3 share a TβR-V-mediated growth inhibitory signaling cascade and that TβR-V may be a novel signaling receptor. Unexpectedly, structural and functional analyses of TβR-V revealed that TβR-V is identical to the low density lipoprotein receptor-related protein-1 (LRP-1) and that TβR-V/LRP-1 is required for growth inhibition by either IGFBP-3 or TGF-β [Huang et al., 2003]. Genetic evidence and evidence from rescue experiments using H1299 human lung carcinoma cells (which express low levels of TβR-V) and CHO cells deficient in LRP-1 (CHO-LRP-1$^-$ cells) indicate that TβR-V mediates growth inhibition by IGFBP-3 and mediates growth inhibition by TGF-β in cooperation with TβR-I and TβR-II [Huang et al., 2003]. TGF-β inhibits growth of wild-type mouse embroynic fibroblasts (MEF) but not TβR-V-deficient MEF [Huang et al., 2003]. Stable transfection of H1299 and CHO-LRP-1$^-$ cells with TβR-V cDNA confers sensitivity to growth inhibition by either TGF-β or IGFBP-3 [Huang et al., 2003; Tseng et al., 2004]. Both H1299 and CHO-LRP-1$^-$ cells express TβR-I and TβR-II and respond to TGF-β-induced transcriptional activation of ECM synthesis-related genes. This unexpected finding disclosed a previously unrecognized growth inhibitory function of LRP-1, which is best known as an endocytic receptor [Herz and Bock, 2002; Strickland et al., 2002], and may explain the embryonic lethality of LRP-1 null mutation in animals [Willnow and Herz, 1994]. LRP-1 binds many structurally unrelated ligands, of which a few have been reported to regulate cell growth, but the mechanisms underlying this remain unclear [Herz and Bock, 2002; Strickland et al., 2002].

In contrast to other known LRP-1 ligands, both IGFBP-3 and TGF-β can form non-covalently and covalently linked dimers, respectively and both possess the TGF-β active-site motif (WC/SXD). Furthermore, IGFBP-3 and TGF-β bind to TβR-V at specific sites. IGFBP-3 binds to domains II and IV of LRP-1 [Tseng et al., 2004] whereas TGF-β binds to another site of LRP-1 [Huang et al., 2003]. A large excess of several LRP-1 ligands does not affect the binding of IGFBP-3 and TGF-β to TβR-V [Huang et al., 2003]. The structural features shared by TGF-β and IGFBP-3 may explain their unique growth inhibitory activities as compared to other known LRP-1 ligands.

IRS PROTEINS ARE IMPORTANT FOR THE TβR-V-MEDIATED GROWTH INHIBITORY SIGNALING CASCADE

The finding that TβR-V is identical to LRP-1 raises the questions about the mechanism by which TβR-V mediates IGFBP-3-induced growth inhibition and why TβR-V (in addition to TβR-I and TβR-II) is required for TGF-β-induced growth inhibition. LRP-1 is a multifunctional scavenger and signaling receptor [Herz and Bock, 2002; Strickland et al., 2002]. The LRP-1 ligands lactoferrin, urokinase-type plasminogen activator and activated α_2M (α_2M*) have been shown to induce elevations in cyclic AMP and in Ca^{2+} influx in several cell types via different signaling pathways [Herz and Bock, 2002; Strickland et al., 2002]. However, none of these ligands significantly inhibit growth of epithelial cells. Among the growth factors for epithelial cells (aFGF, bFGF, EGF, insulin, and IGF-I), only insulin and IGF-I analogs (with reduced affinity for IGFBP-3) are able to reverse growth inhibition induced by IGFBP-3 in Mv1Lu cells [Huang et al., 2004a]. EGF and FGFs are potent stimulators of the MAP kinase signaling cascade but are incapable of antagonizing IGFBP-3 growth inhibition. Furthermore, PI 3-kinase inhibitors do not affect IGFBP-3-induced growth inhibition in these cells [Huang et al., 2004a]. These observations suggest that the MAP kinase and PI 3-kinase signaling cascades, downstream of IRS proteins, are not involved in IGFBP-3 growth inhibition and in reversal of IGFBP-3 growth inhibition by insulin or IGF-I analogs. Thus, we hypothesized that the insulin receptor substrate (IRS) proteins, the signaling molecules shared by both the insulin receptor and IGF-I receptor signaling cascades, are directly involved in IGFBP-3-induced growth inhibition and its reversal by insulin and IGF-I analogs [Huang et al., 2004a]. To test this hypothesis, we have studied the roles of IRS proteins in IGFBP-3-induced growth inhibition in Mv1Lu cells and 32D murine myeloid cells stably expressing IRS proteins and the insulin receptor. Our studies [Huang et al., 2004a] provided evidence that IRS proteins are critically important for IGFBP-3-induced growth inhibition and, furthermore, that IGFBP-3 inhibits cell growth by stimulating an okadaic acid-sensitive Ser/Thr-specific protein phosphatase (PPase) which dephosphorylates IRS proteins. A key piece of evidence is that stable transfection of 32D cells (which express TβR-V but do not produce endogenous IRS proteins and do not respond to IGFBP-3 growth inhibition) [Huang et al., 2004a] with IRS-1 or IRS-2 cDNA confers sensitivity to growth inhibition by IGFBP-3; this IRS-mediated growth inhibition is completely reversed by insulin in 32D myeloid cells stably expressing IRS-2 and the insulin receptor (IR).

In Mv1Lu cells, a standard model cell system for investigating TGF-β and IGFBP-3 growth inhibition activity, insulin does not affect the TGF-β-induced growth inhibitory response. However, insulin partially reverses TGF-β growth inhibition in the presence of α5β1 integrin antagonists in these cells [Huang et al., 2004b]. Furthermore, stable transfection of 32D cells with IRS-1 or IRS-2 cDNA confers higher sensitivity to growth inhibition by TGF-β; this IRS-mediated sensitivity can be partially reversed by insulin in 32D cells stably expressing IRS-2 and IR [Huang et al., 2004b]. These results suggest that growth inhibition by TGF-β involves IRS proteins. Since TGF-β slightly induces phosphorylation, rather than dephosphorylation, of IRS-2 in Mv1Lu cells, we hypothesize that, like IGFBP-3, TGF-β is capable of stimulating a Ser/Thr-specific PPase associated with TβR-V. We also hypothesize that the TGF-β-stimulated dephosphorylation of IRS-2 by this phosphatase is overshadowed by the phosphorylation induced by TGF-β and presumably mediated by TβR-I/TβR-V [Huang et al., 2004a]. This may be due to a weaker phosphatase activity stimulated by TGF-β as compared to that stimulated by IGFBP-3. This possibility is supported by the observation that

the TGF-β-induced phosphorylation of IRS-2 is overridden by IGFBP-3-induced dephosphorylation of IRS-2 in Mv1Lu cells treated with both TGF-β and IGFBP-3 [Huang et al., 2004a]. This may also explain why TβR-V requires the TβR-I/TβR-II/Smad2/3/4/signaling cascade for mediating TGF-β-induced growth inhibition. The weak phosphatase activity stimulated by TGF-β alone may not lead to growth arrest. In DR26 cells that express TβR-V but lack functional TβR-II, TGF-β only moderately inhibits cell growth at concentrations close to its K_d for binding to TβR-V [Liu et al., 1997].

Upon stimulation of cells with insulin and IGFs, IRS-1 and IRS-2 are tyrosine-phosphorylated and serve as on/off switches to recruit and regulate various downstream signaling proteins in the PI 3-kinase and MAP kinase signaling cascades [Cheatham and Kahn, 1995]. However, the significance of serine phosphorylation of IRS-1 and IRS-2 is not well defined but has been implicated in reducing tyrosine phosphorylation of IRS proteins and the impairment of insulin-induced signaling [Cheatham and Kahn, 1995]. While several phosphorylated serine residues have been identified, the role of serine-specific phosphorylation of IRS proteins in cell growth is unknown [Huang et al., 2004a]. There is no detectable tyrosine- or threonine-specific phosphorylation of IRS proteins in cells stimulated with or without fetal calf serum (1%) [Huang et al., 2004a].

IRS proteins have been characterized as signaling/docking proteins with an exclusively cytoplasmic/membrane localization. However, increasing evidence indicates that IRS proteins can translocate to the nuclei of cells under certain conditions [Sun et al., 2003]. IRS-1 has been localized to the nuclei of hepatocytes in liver [Boylan and Gruppuso, 2002]. To explain the roles of IRS proteins in cellular growth inhibition by IGFBP-3 and TGF-β, a hypothetical model is proposed (Fig. 1). In this model, a cytoplasmic Ser/Thr-specific PPase associates with a fraction of cell-surface TβR-V via its cytoplasmic domain [Huang et al., 2004a]. Following dimeric ligand (IGFBP-3 or TGF-β) binding to TβR-V, the ligand-induced dimerization or oligomerization of TβR-V results in activation of the PPase and the subsequent association of the activated PPase with IRS proteins. The PPase–IRS complexes translocate to the nuclei and then dephosphorylate Rb and other cell cycle regulators, resulting in growth inhibition. The nuclear translocation of the PPase–IRS complexes may be mediated by a nuclear localization sequence (NLS) or a cryptic NLS present in the PPase or by an interacting protein(s) carrying NLS [Sun et al., 2003]. There are no typical NLS motifs found in IRS proteins. The IGFBP-3-stimulated PPase activity alone plays a critical role in IGFBP-3-induced cellular growth inhibition. However, in the case of TGF-β-induced cellular growth inhibition, the TβR-V/PPase/IRS signaling cascade works in concert with the TβR-I/TβR-II/Smad2/3/4 signaling cascade, resulting in potent growth inhibition. Insulin or IGF-I analogs appear to antagonize IGFBP-3-induced growth inhibition by stimulating tyrosine phosphorylation of IRS proteins [Huang et al., 2004a]. This prevents IRS proteins from being dephosphorylated by stimulated PPase and also prevents their nuclear translocation. The insulin or IGF-I-stimulated tyrosine phosphorylation of IRS leads to downstream signaling (PI 3-kinase and MAP kinase). This model has been supported by several lines of evidence: (1) the IGFBP-3- or TGF-β-stimulated PPase activity (as determined using ^{32}P-labeled casein as substrate) is found in the nuclei of Mv1Lu cells treated with IGFBP-3 or TGF-β [Chen et al., manuscript in preparation]. (2) The IGFBP-3- or TGF-β-stimulated PPase activity can be abolished in Mv1Lu cells treated with IGFBP-3 or TGF-β in the presence of TGF-β peptantagonist or insulin [Chen et al., manuscript in preparation]. (3) IRS-2 is resistant to IGFBP-3-stimulated dephosphorylation in Mv1Lu cells co-treated with IGFBP-3 and insulin or ($Q^3A^4Y^{15}L^{16}$) IGF-I (an IGF-I analog with reduced affinity for IGFBP-3) [Huang et al., 2004a], and (4) IRS-2 from cells treated with insulin or ($Q^3A^4Y^{15}L^{16}$) IGF-I is resistant to in vitro dephosphorylation by the IGFBP-3-stimulated PPase activity in cell lysates of Mv1Lu cells treated with IGFBP-3 [Chen et al., manuscript in preparation].

THE TβR-V SIGNALING CASCADE CROSS-TALKS WITH THE TβR-I/TβR-II AND OTHER SIGNALING CASCADES

In Mv1Lu cells, insulin completely reverses growth inhibition by IGFBP-3 whereas it partially reverses growth inhibition by TGF-β in the presence of α5β1 integrin antagonists [Huang et al., 2004a,b]. In Mv1Lu mutant cells

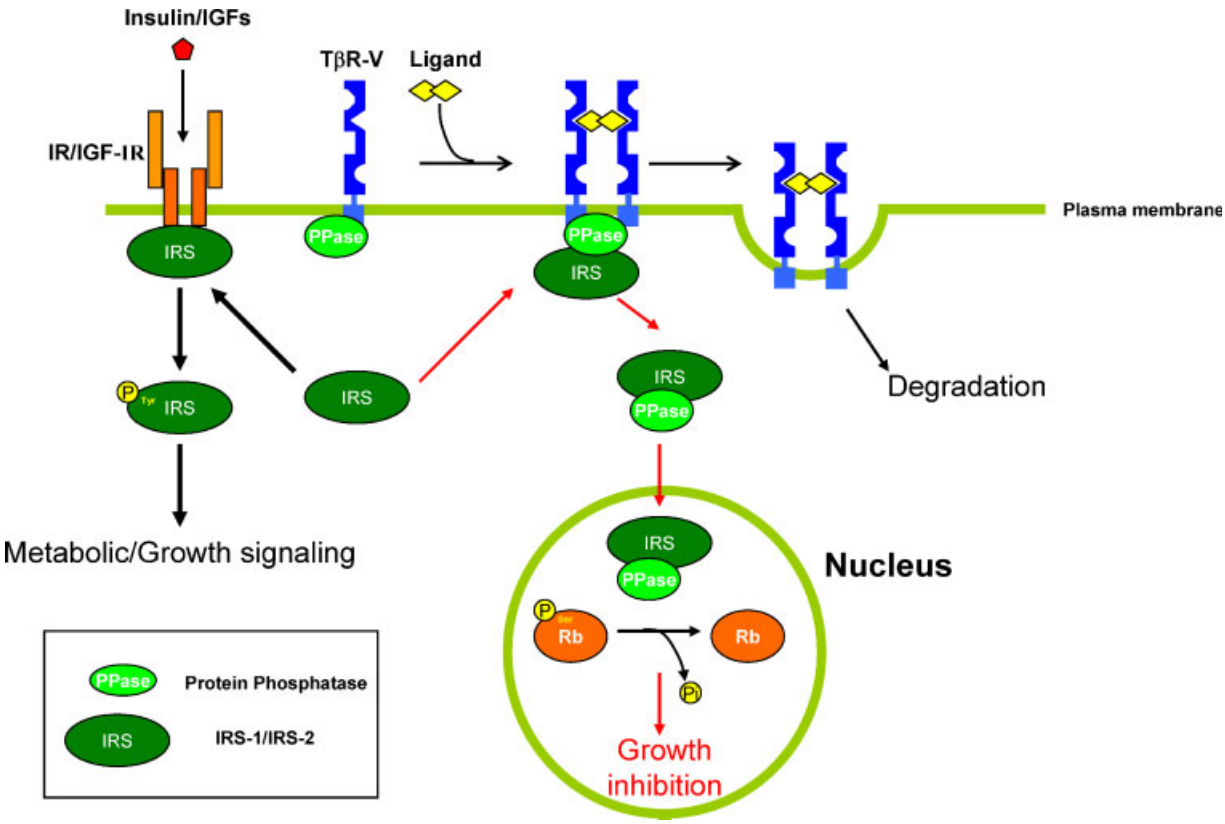

Fig. 1. A model for the mechanism by which IGFBP-3 and TGF-β induce cellular growth inhibition mediated by TβR-V and IRS proteins. Dimeric ligands (IGFBP-3 and TGF-β) stimulate a Ser/Thr-specific protein phosphatase (PP) associated with the cytoplasmic tail of TβR-V by inducing receptor dimerization or oligomerization via their non-covalently and covalently linked dimeric structures. After stimulation by IGFBP-3 or TGF-β, the Ser/Thr-specific protein phosphatase (PPase) forms complexes with IRS proteins (IRS-1 and IRS-2) and translocates with them to the nucleus and dephosphorylates Rb and other cell cycle regulators, resulting in growth arrest. Insulin and IGF-I analogs (with reduced affinity for IGFBP-3) reverse IGFBP-3- or TGF-β-induced cellular growth inhibition by stimulating tyrosine phosphorylation of IRS proteins via interaction with their cognate receptors. The tyrosine phosphorylation of IRS proteins by the insulin receptor (IR) and the IGF-I receptor (IGF-IR) leads to multiple signaling pathways (e.g., PI 3-kinase and MAP kinase) and prevents the formation of the IRS-stimulated PPase complex and subsequent serine-specific dephosphorylation of IRS proteins by stimulated PPase. Under non-stimulating conditions, IRS proteins are mainly phosphorylated at serine residues. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(DR26 cells) that lack functional TβR-II but express TβR-V, the TβR-V-mediated growth inhibitory signaling cascade appears to be functional but the TβR-I/TβR-II signaling cascade is not [Liu et al., 1997]. Insulin also completely reverses growth inhibition by IGFBP-3 in these cells [Huang et al., 2004a]. Based on these results and results published by other investigators [Huang et al., 2004a], we proposed a model for the insulin/IGF-I analog reversal of growth inhibition by IGFBP-3 and TGF-β and for cross-talk of the TβR-V, TβR-I/TβR-II, insulin receptor (IR), IGF-I receptor (IGF-IR), integrin and c-Met signaling cascades. In this model (Fig. 2), IGFBP-3 binds to cell surface TβR-V and induces activation of a cytoplasmic Ser/Thr-specific protein PPase that forms complexes with IRS proteins and dephosphorylates IRS proteins at serine residues. The PPase–IRS complexes then translocate to the nucleus and dephosphorylate cell cycle regulators, leading to growth inhibition. Insulin and IGF-I analogs reverse or antagonize the IGFBP-3 growth inhibition by stimulating tyrosine-specific phosphorylation of IRS proteins and preventing IRS proteins from interaction with the IGFBP-3-stimulated PPase. The IGFBP-3-stimulated TβR-V signaling cascade is negatively modulated by the IR and IGF-IR signaling cascades but independent from the TβR-I/TβR-II signaling cascade. TGF-β also stimulates the activity of the same PPase via interaction with TβR-V. The TGF-β-stimulated PPase activity is weaker than that stimulated by IGFBP-3 possibly

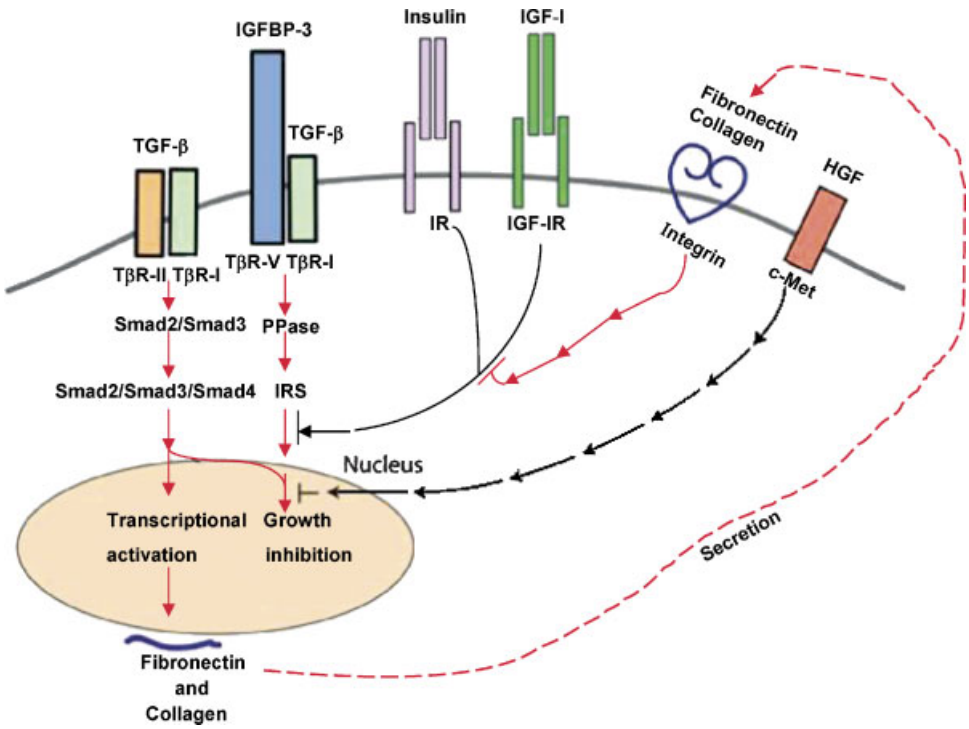

Fig. 2. A model for cross-talk of the TGF-β receptor (TβR-I/TβR-II and TβR-V), insulin receptor (IR), IGF-1 receptor (IGF-1R), integrin and c-Met signaling cascades. The TβR-I/TβR-II/Smad2/3/4 signaling cascade mediates TGF-β-induced cellular responses, including transcriptional activation of ECM synthesis-related genes (e.g., collagen, fibronectin, and PAI-1) and growth inhibition in responsive cells. However, in addition to the TβR-I/TβR-II signaling cascade, the TβR-V/PPase/IRS signaling cascade is required for TGF-β-induced growth inhibition. The TβR-V/PPase/IRS signaling cascade is negatively regulated by the IR and IGF-1R signaling cascades. Insulin or IGFs (IGF-I and IGF-II) attenuate the TβR-V signaling cascade or TGF-β-induced growth inhibition by stimulating tyrosine phosphorylation of IRS proteins. The integrin signaling cascade initiated by ECM proteins induced by TGF-β, impairs the ability of insulin to attenuate the TGF-β-induced TβR-V signaling cascade by down-regulating insulin-stimulated tyrosine phosphorylation of IRS proteins. In diabetes, insulin or insulin signaling defects potentiate TGF-β-induced growth inhibition of responsive cells. High glucose in the plasma and tissues of diabetic patients may enhance both TβR-V and TβR-I/TβR-II signaling via increasing TGF-β production and TβR-II expression. Increased ECM synthesis (which is mediated by TβR-I/TβR-II signaling) further attenuates insulin signaling and enhances TGF-β-induced growth inhibition, resulting in impaired wound healing and accelerated glomerulopathy in diabetic patients. The signaling cascades potentiated in diabetic patients are indicated by red arrows. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

because they bind to different sites in the TβR-V molecule. This weak phosphatase activity stimulated by TGF-β may cause only moderate growth inhibition (~30%–40% growth inhibition at 50 pM TGF-β) in DR26 cells (that are defective in the TβR-I/TβR-II signaling cascade) and requires the TβR-I/TβR-II/Smad2/3/4 signaling cascade for mediating potent growth inhibition (~ 100% growth inhibition at ~10 pM TGF-β) in wild-type Mv1Lu cells [Liu et al., 1997]. In the absence of the TβR-I/TβR-II signaling cascade (e.g., in DR26 cells), TGF-β fails to exhibit transcriptional activation activity and requires higher concentrations (~50 pM) in order to moderately inhibit cell growth [Liu et al., 1997]. By contrast, IGFBP-3 potently inhibits cell growth of DR26 cells [Leal et al., 1997; Huang et al., 2004a]. In carcinoma cells that lack TβR-V but express TβR-I and TβR-II, TGF-β or IGFBP-3 does not inhibit growth. However, TGF-β is capable of inducing TβR-I/TβR-II signaling (which leads to transcriptional activation of ECM synthesis-related genes) in these carcinoma cells. The growth inhibition and transcriptional activation activities of TGF-β appear to segregate in these cells (DR26 and carcinoma cells). The integrin signaling cascade initiated by ECM proteins (e.g., fibronectin and collagen) induced by TGF-β impairs the ability of insulin to block TGF-β-stimulated TβR-V-mediated signaling by down-regulating insulin-stimulated tyrosine phosphorylation of IRS proteins (Fig. 2) [Huang et al., 2004a]. This impairment can be partially reversed by blocking the interaction between fibronectin and α5β1 integrin using α5β1 integrin antagonists

[Huang et al., 2004b]. In cells that do not express α5β1 integrin, insulin partially reverses growth inhibition by TGF-β [Huang et al., 2004b]. The c-Met signaling cascade induced by the c-Met ligand hepatocyte growth factor (HGF) reverses growth inhibition by IGFBP-3 and TGF-β at the sites of cell cycle regulation in the nucleus (Fig. 2) [Taipale and Keski-Oja, 1996; Huang, unpublished results].

In DR26 cells, the TβR-V-mediated signaling cascade appears to be potentiated [Huang et al., 2004a]. In these cells, IGFBP-3 stimulates dephosphorylation of IRS-2 and inhibits cell growth more potently than in wild-type Mv1Lu cells [Huang et al., 2004a]. At ~14 nM (K_d = ~6 nM), IGFBP-3 inhibits growth by ~80%–90% and ~40%–50% in DR26 and Mv1Lu cells, respectively [Huang et al., 2004a]. On the other hand, the deficiency in the TβR-V signaling cascade also potentiates the TβR-I/TβR-II signaling cascade. In cells lacking TβR-V (CHO-LRP-1$^-$ and PEA-13 cells), TGF-β-stimulated PAI-1 expression (which is mediated by the TβR-I/TβR-II signaling cascade) is enhanced as compared to that found in the wild-type cells (CHO-K1 and MEF cells) [Huang et al., 2003; Tseng et al., 2004]. TGF-β (100 pM) stimulates PAI-1 expression by ~ 1.3 and ~ 3.5 fold in CHO-K1 and CHO-LRP-1$^-$ cells, respectively [Tseng et al., 2004]. The potentiation of TGF-β-stimulated PAI-1 expression in CHO-LRP-1$^-$ cells can be reversed by stable transfection of these cells with TβR-V cDNA [Tseng et al., 2004]. These results suggest that the TβR-V signaling cascade cross-talks with the TβR-I/TβR-II signaling cascade, likely in the nuclei of cells. IGFBP-3 does not significantly affect phosphorylation of Smad2/3 in Mv1Lu cells [Leal et al., 1999]. The finding of cross-talk between the TβR-V and other signaling cascades has potentially important clinical implications. For example, in diabetes, insulin or insulin signaling defects may potentiate the TβR-V-mediated growth inhibitory signaling. In addition, high glucose may enhance both TβR-V and TβR-I/TβR-II signaling via increasing TGF-β production and TβR-II expression [Sharma and McGowan, 2000]. Increased ECM synthesis mediated by TβR-I/TβR-II signaling further attenuates insulin signaling (via the integrin signaling cascade) and enhances TGF-β growth inhibitory activity (Fig. 2). The potentiation of TGF-β growth inhibitory activity generated in skin wounds results in prolonged inflammation, attenuated wound re-epithelialization or wound closure and healing [Huang et al., 2004a]. The enhancement of TGF-β growth inhibitory and fibrogenic activities generated in damaged kidney glomeruli causes accelerated glomerulopathy [Huang et al., 2004a]. Impaired wound healing and nephropathy are common clinical problems observed particularly often in diabetic patients. Human carcinomas have frequently been found to associate with fibrosis in the affected tissues. This may be in part due to potentiation of TβR-I/TβR-II signaling by attenuation of the TβR-V signaling cascade in these carcinomas.

ATTENUATION OF TβR-V EXPRESSION IS INVOLVED IN THE PROGRESSION OF TUMORIGENESIS

TGF-β plays a dual role in tumorigenesis—a tumor suppressor in early stages of tumorigenesis and a tumor promoter in advanced stages of tumorigenesis [Derynck et al., 2001; Piek and Roberts, 2001]. The different roles of TGF-β in the process of tumorigenesis are determined by its growth regulatory activity, which is mediated by both TβR-I/TβR-II and TβR-V signaling, rather than its transcriptional activation activity which is mainly mediated by TβR-I/TβR-II signaling. Several pieces of evidence support this proposition: (1) TGF-β is the most potent growth inhibitor known for epithelial cells that express all major TGF-β receptors including TβR-I, TβR-II, TβR-III, and TβR-V [O'Grady et al., 1991a,b; Roberts and Sporn, 1993; Leal et al., 1997; Liu et al., 1997; Roberts, 1998]. (2) All carcinoma cells studied thus far exhibit low-level or no expression of TβR-V [O'Grady et al., 1991b; Leal et al., 1997; Liu et al., 1997; Huang et al., 2003, 2004a,b; Tseng et al., 2004]. Growth of these cells is not inhibited by either TGF-β or IGFBP-3, and (3) the majority of carcinoma cells retain TβR-I/TβR-II signaling and respond to TGF-β-induced transcriptional activation [Derynck et al., 2001; Piek and Roberts, 2001]. While no TβR-V antigen is detected in hepatomas in mice, it is present in the normal parenchymal tissue surrounding the hepatomas [Gonias et al., 1994]. Furthermore, somatic mutations of LRP-1B, a homolog of TβR-V, frequently occur in tumors of various types [Sonoda et al., 2004]. The suppressive activity of TβR-V in tumorigenesis has also been supported by the observation that stable trans-

fection of H1299 human lung carcinoma cells (that express low levels of TβR-V but exhibit typical TβR-I/TβR-II signaling) with TβR-V cDNA confers sensitivity to growth inhibition by TGF-β and attenuates tumorigenicity in nude mice [Huang et al., 2003].

Many carcinoma cells that are no longer sensitive to growth inhibition by TGF-β and IGFBP-3 oversecrete active TGF-β [Derynck et al., 2001; Piek and Roberts, 2001]. Autocrine stimulation of these carcinoma cells by TGF-β results in reversible epithelial-to-mesenchymal transdifferentiation (EMT) and thus enhanced metastatic and invasive potential [Derynck et al., 2001; Piek and Roberts, 2001]. TGF-β is capable of aggravating later stages of tumorigenesis by transforming squamous morphology of carcinoma cells into more aggressive and highly invasive mesenchymal spindle cell types. TGF-β is also capable of promoting tumorigenesis by paracrine stimulation of angiogenesis and immunosuppression. The EMT is characterized by reorganization of the actin cytoskeleton, downregulation of expression of the adhesion and cytoskeleton molecules E-cadherin, ZO-1, vinculin and keratin and expression of vimentin, a mesenchymal marker [Derynck et al., 2001; Piek and Roberts, 2001]. Although TGF-β-induced EMT has been well studied [Derynck et al., 2001; Piek and Roberts, 2001], the molecular mechanism by which TGF-β induces EMT is not completely understood. The TβR-I/TβR-II signaling appears to play an important role in EMT since overexpression of dominant-negative TβR-II prevents EMT in vivo and reverses the mesenchymal phenotype of highly metastatic mouse colon carcinoma cells [Derynck et al., 2001; Piek and Roberts, 2001]. We recently demonstrated that stable transfection of CHO-LRP-1$^-$ cells, which are deficient in TβR-V and have a fibroblastoid spindle morphology, with TβR-V cDNA restores the wild-type squamous morphology and sensitivity to TGF-β growth inhibition [Tseng et al., 2004]. These results indicate that cells lacking TβR-V undergo EMT in the presence of a low concentration of serum without addition of exogenous TGF-β. These results also suggest that TβR-V may negatively regulate EMT, and that loss or reduced expression of TβR-V may endow carcinoma cells with more metastatic and invasive characteristics.

Based on the current concept of tumorigenesis [Derynck et al., 2001; Piek and Roberts, 2001] and the results from our studies [Leal et al., 1997, 1999; Liu et al., 1997; Huang et al., 2003, 2004a,b; Tseng et al., 2004], we propose a model (Fig. 3) for the role of TβR-V in tumorigenesis. In the model, epithelial cells or pre-carcinoma cells that express all major TGF-β receptors are sensitive to TGF-β growth control and TGF-β-induced transcriptional activation. During progression of tumorigenesis, the expression of TβR-V is attenuated. Most of the carcinoma cells become insensitive to TGF-β growth control and some respond to TGF-β mitogenically. These carcinoma cells at this stage retain the TβR-I/TβR-II/Smad2/3/4 signaling cascade, oversecrete TGF-β, exhibit EMT (induced by autocrine TGF-β) and become more metastatic and invasive. A small subset of the carcinoma cells undergo further loss of TβR-I/TβR-II signaling (e.g., via TβR-II, TβR-I or Smad4 mutations). These carcinoma cells fail to respond to TGF-β stimulation and may be less invasive. Such heterogenicity characterizes carcinoma in clinical practice.

A SIGNAL CONTROLS TGF-β PARTITIONING BETWEEN TWO DISTINCT ENDOCYTOSIS PATHWAYS AND RESULTANT TGF-β RESPONSIVENESS

TGF-β is one of the very few potent growth factors/cytokines that is active at subpicomolar concentrations and capable of autoinduction in target cells [Roberts, 1998; Derynck et al., 2001; Piek and Roberts, 2001]. It is regulated at various levels of transcription, post-translation (activation of the latent form of TGF-β) and cell-surface receptor complex formation. Among these regulations, the receptor complex regulation has not been well studied until recently. TGF-β has recently been shown to internalize by two distinct endocytic pathways—clathrin-mediated (K^+ depletion-sensitive) and caveolar/lipid-raft-mediated (nystatin-sensitive) pathways [Di Guglielmo et al., 2003; Mitchell et al., 2004; Le Roy and Wrana, 2005]. Clathrin-mediated endocytosis is the most well-characterized mechanism for cell-surface receptor-mediated endocytosis of ligands. The receptors that undergo clathrin-mediated endocytosis generally contain tyrosine- or di-leucine-based motifs in their cytoplasmic domains. During endocytosis, adaptor protein-2 (AP2), a key component mediating endocytosis, binds to receptors via the internalization motifs and recruits soluble

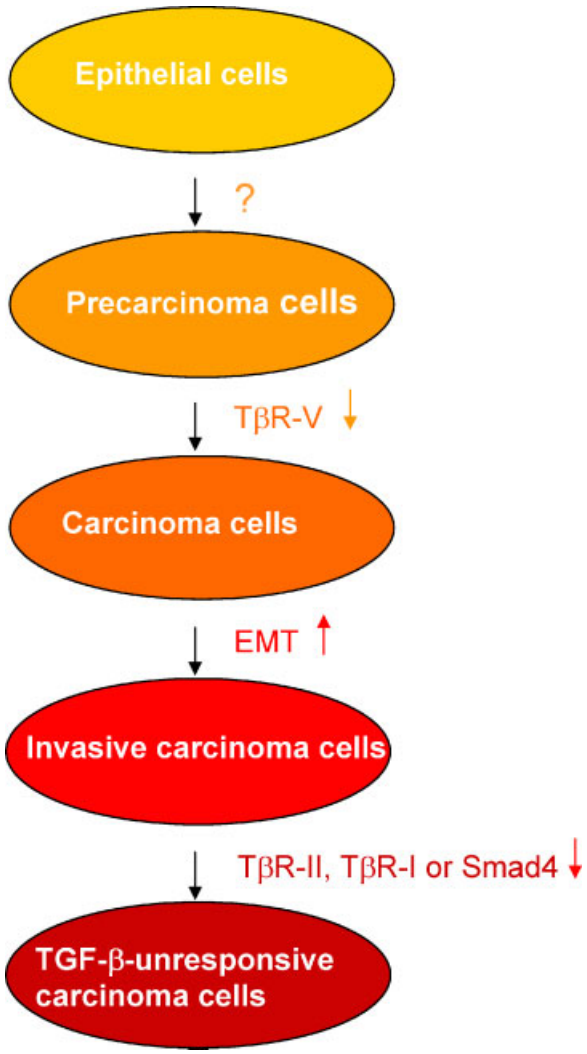

Fig. 3. Requirement of TβR-V expression attenuation in the progression of tumorigenesis. The growth regulatory activity of TGF-β is mediated by both TβR-I/TβR-II and TβR-V signaling in epithelial cells and other cell types. Attenuation or loss of TβR-V expression occurs at the early stages of tumorigenesis and enables carcinoma cells to escape growth control by TGF-β, to gain the ability for reversible epithelial-to-mesenchymal transdifferentiation (induced by autocrine TGF-β) and to become more aggressive and invasive. In a small subset of human carcinoma cells, the gene of TβR-II, Smad4, or TβR-I undergoes mutation and becomes inactivated at the late stages of tumorigenesis. These carcinoma cells become unresponsive to TGF-β. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

clathrin and other endocytotic regulating proteins from the cytoplasm to the plasma membrane. The clathrin triskelia assemble into a polygonal lattice at the plasma membrane to form coated pits that bud and pinch off from the plasma membrane in a dynamin-dependent manner to form clathrin-coated vesicles. Clathrin-coated vesicles are then uncoated and fuse with early endosomes. Receptors recycle back to the plasma membrane through recycling endosomes or are transported to late endosomes and lysosomes for degradation. TβR-II contains a dileucine motif and is present in clathrin coats [Yao et al., 2002]. It has been shown to interact with AP2 and may internalize through clathrin-mediated endocytosis [Yao et al., 2002] Caveolar/lipid-raft-mediated endocytosis is a mechanism of non-clathrin-mediated endocytosis [Le Roy and Wrana, 2005]. Lipid rafts are microdomains of plasma membranes that are enriched in cholesterol and exoplasmically oriented sphingolipids (sphingomyelin and glycosphingolipid). They form liquid-ordered phase domains. Upon integration of caveolin-1, the liquid-ordered phase domains form small flask-shaped invaginations called caveolae. Caveolae contain the same components as those used by other vesicles for budding (for endocytosis) and docking (for exocytosis). They may be associated with caveolin-1. The partitioning of proteins into lipid rafts is dependent on the interactions of the glycosylphosphotidylinositol (GPI) moiety of GPI-anchored proteins with lipid rafts and on protein-protein interactions with lipid-raft protein residents such as caveolin-1. TβR-I has been shown to interact and colocalize with caveolin-1 [Razani et al., 2001]. TβR-I and TβR-II appear to be capable of undergoing caveolar/lipid-raft- and clathrin-mediated endocytosis, respectively. However, following TGF-β binding to TGF-β receptors, the signals which determine partitioning of hetero-oligomeric TGF-β receptor complexes between clathrin-mediated and caveolar/lipid-raft-mediated endocytosis (and thus TGF-β responsiveness) are unknown.

Clathrin-mediated endocytosis of TGF-β and TGF-β receptor complexes has been shown to promote TGF-β-induced TβR-I/TβR-II signaling [Di Guglielmo et al., 2003; Mitchell et al., 2004; Le Roy and Wrana, 2005]. There are two lines of evidence for this: (1) transient transfection of cells with dominant-negative dynamin cDNA prevents localization of TGF-β receptors in EEA1-positive endosomes and TβR-I/TβR-II signaling (as determined by measuring expression of a TGF-β-responsive reporter gene), and (2) depletion of K^+, which inhibits clathrin-mediated endocytosis, attenuates TβR-I/TβR-II signaling. Following TGF-β binding to TβR-II and TβR-I, phosphorylation of Smad2/3 by activated TβR-I in the TβR-I/TβR-II complex

(which is facilitated by SARA) may occur either at the plasma membrane or in endosomes [Di Guglielmo et al., 2003; Mitchell et al., 2004; Le Roy and Wrana, 2005]. However, endosomal localization appears to promote the dissociation of Smad2/3 from SARA and TGF-β receptor complexes and subsequent formation and nuclear translocation of Smad2/3/4 complexes [Runyan et al., 2005]. The functional role of caveolar/lipid-raft-mediated endocytosis is less clear than that of clathrin-mediated endocytosis. Caveolar/lipid-raft-mediated endocytosis has been shown to mediate rapid degradation of TGF-β and TGF-β receptor complexes based on these observations: (1) overexpression of caveolin-1 enhances degradation of TGF-β receptor complexes [Di Guglielmo et al., 2003], and (2) nystatin, a cholesterol sequestering agent, inhibits degradation of TGF-β cross-linked chemically to TGF-β receptors [Di Guglielmo et al., 2003]. On the other hand, caveolae and chimeric GM-CSF receptor (GM-CSFR)/TGF-β receptor (TβR) have been shown not to colocalize in Mv1Lu cells stably expressing chimeric GM-CSFR/TβR receptors (αIβII or αIIβI) that contain the cell-surface domain of the α-chain or β-chain of GM-CSFR and the cytoplasmic kinase domain of TβR-I or TβR-II [Mitchell et al., 2004]. Furthermore, nystatin fails to inhibit the degradation of the chimeric receptors in these cells stably expressing them [Mitchell et al., 2004]. The apparently divergent results of these studies may be explained in two ways. First, the chimeric receptors (αIβII and αIIβI) may form ligand complexes which differ from those of native receptors (TβR-I and TβR-II) [Mitchell et al., 2004]. Therefore, the chimeric receptors may internalize differently than the native receptors. Indeed, the ratio of GM-CSFR binding to βII and αI (or αII and βI), as determined by ^{125}I-labeled GM-CSFR affinity labeling (binding and cross-linking), is >1 and is distinctly different from that (<1) of TGF-β binding to TβR-II and TβR-I in Mv1Lu cells as determined by ^{125}I-labeled TGF-β affinity labeling [Mitchell et al., 2004]. The chimeric receptors may preferentially utilize clathrin-mediated endocytosis and, thus, are less sensitive to inhibitors of caveolar/lipid-raft-mediated endocytosis such as nystatin. Second, the use of TGF-β cross-linked chemically to cell-surface receptors in these studies of TGF-β internalization and degradation mechanisms may be a reason for different results from these studies. It is known that ligand dissociation from the respective receptors in endosomes (because of acidic endosomal pH) is required for targeting of ligands for lysosomal degradation. In addition, the chemical modification of cell-surface proteins by agents used for cross-linking of TGF-β or GM-CSF to native receptors or chimeric receptors could easily affect ligand internalization in cells.

We have recently studied the roles of the two endocytosis pathways in cell-surface receptor-bound TGF-β internalization and degradation in Mv1Lu cells and other cell types by determining the binding of TGF-β to TGF-β receptors (using ^{125}I-TGF-β affinity labeling) and measuring trichloroacetic acid-soluble products of internalized TGF-β in the medium. Our studies indicate that nystatin indeed significantly attenuates TGF-β endocytosis and degradation in Mv1Lu cells and that the ratio of TGF-β binding to TβR-II and TβR-I at the cell surface (as determined by ^{125}I-TGF-β affinity labeling) is an important factor controlling TGF-β partitioning between the clathrin- and caveolar/lipid-raft-mediated endocytosis pathways [Chen et al., manuscript in preparation]. In these cells, the magnitude of TGF-β-induced cellular responses, including the growth inhibitory response and transcriptional activation, is determined by TGF-β partitioning between the clathrin- and caveolar/lipid-raft-mediated endocytosis. The more TGF-β is partitioned into clathrin-mediated endocytosis, the greater the TGF-β-induced responses. Based on these results, we propose a "Dominance" model (Fig. 4) for the signal which controls partitioning of TGF-β and its receptor between clathrin- and caveolar/lipid-raft-mediated endocytosis and resultant TGF-β responsiveness. In this model, following TGF-β binding, TGF-β receptors exist as hetero-oligomeric complexes at the cell surface. When the ratio of TGF-β binding to TβR-II and TβR-I is larger than 1 (i.e., TGF-β binding to TβR-II is dominant), hetero-oligomeric TGF-β receptor complexes preferentially internalize by clathrin-mediated endocytosis, resulting in promotion of signaling and cellular responses and eventually lysosomal degradation. If the hetero-oligomeric TGF-β receptor complexes contain a high percentage of TβR-II (which contains a di-leucine motif and binds to the β2 subunit of AP2) [Yao et al., 2002], clathrin-mediated endocytosis is the preferred (or dominant) internalization mechanism.

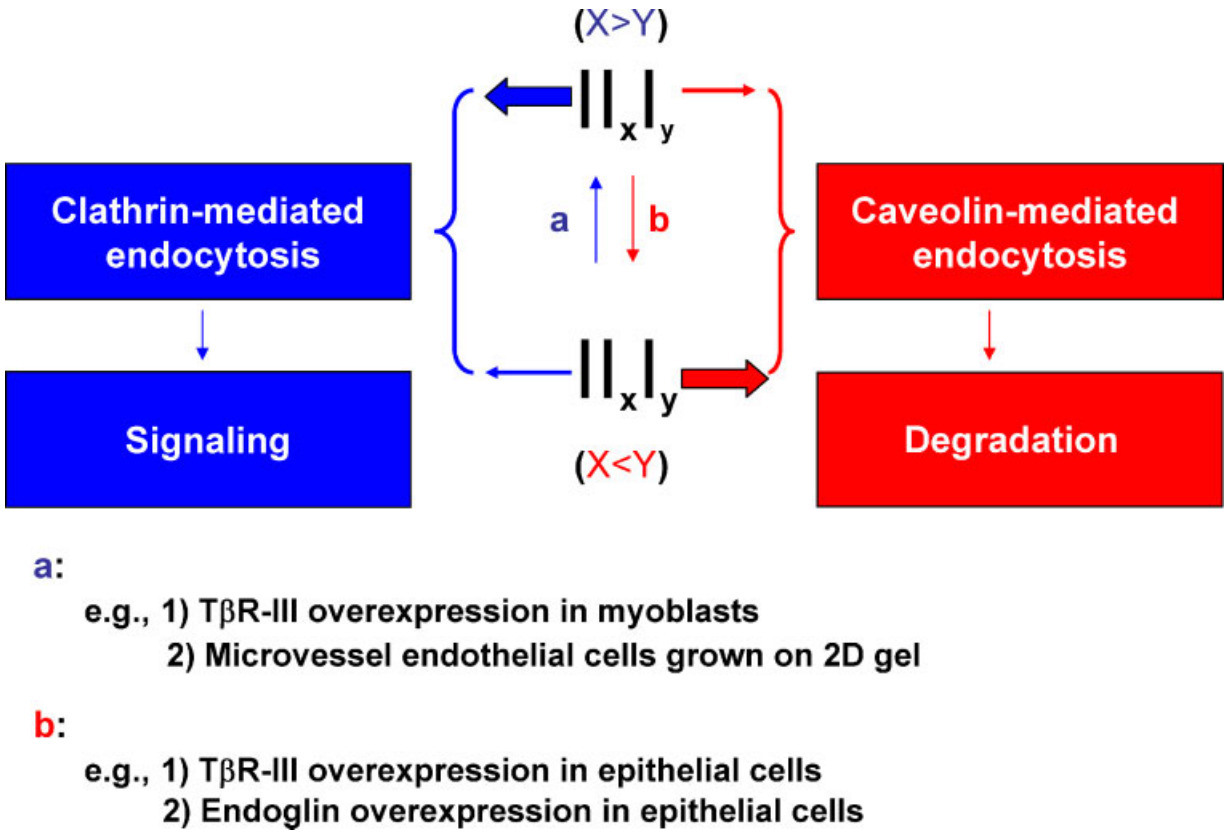

Fig. 4. A ''Dominance'' model for the signal that controls TGF-β partitioning between clathrin- and caveolar/lipid-raft-mediated endocytosis pathways and resultant TGF-β responsiveness. Following TGF-β binding, TGF-β receptors form hetero-oligomeric complexes that contain different percentages of TβR-II (II) and TβR-I (I) (x and y, respectively; as determined by ^{125}I-TGF-β affinity labeling). When $x > y$, receptor-bound TGF-β is preferentially internalized by clathrin-mediated endocytosis, resulting in promoting signaling and cellular responses. When $x < y$, receptor-bound TGF-β is mostly internalized by caveolar/lipid-raft-mediated endocytosis, resulting in rapid degradation of TGF-β and less cellular responses. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

When the ratio of TGF-β binding to TβR-II and TβR-I is smaller than 1 (i.e., TGF-β binding to TβR-I is dominant), hetero-oligomeric TGF-β receptor complexes mainly internalize by caveolar/lipid-raft-mediated endocytosis which is sensitive to nystatin inhibition. This results in rapid degradation and attenuated TGF-β responsiveness. Since the receptor complexes are rich in TβR-I, whose cytoplasmic domain interacts with caveolin-1 [Razani et al., 2001], caveolar/lipid-raft-mediated endocytosis is favored or dominant.

In cells that exhibit growth inhibition when treated with TGF-β, such as epithelial cells and endothelial cells, the TGF-β binding to cell surface receptors (as determined by ^{125}I-labeled TGF-β affinity labeling) generally exhibit a low ratio of TβR-II: TβR-I binding (<1). The magnitude of the cellular responses induced by TGF-β positively correlates with the ratio of TGF-β binding to TβR-II and TβR-I in the same cell types. The TGF-β responsiveness can be potentiated or attenuated by altering the ratio of TβR-II: TβR-I binding through altering expression of TβR-I and TβR-II or changing the cell-surface environment/plasma membrane components in ways known to exist in various pathophysiological conditions. For example, solar UV irradiation reduces TGF-β responsiveness (collagen synthesis) in photoaged skin by decreasing the expression of TβR-II (without affecting TβR-I) and the ratio of TβR-II: TβR-I [Quan et al., 2004]. Active k-ras oncogene can induce TGF-β resistance by decreasing the ratio of TGF-β binding to TβR-II and TβR-I in rat thyroid cells [Coppa et al., 1997]. Smooth muscle cells from atherosclerotic vessels display a low ratio (<1) of TGF-β binding to TβR-II and TβR-I (compared to smooth muscle cells from normal vessels) and exhibit resistance to TGF-β, but

their phenotypes (the low ratio of TβR-II: TβR-I binding and TGF-β resistance) can be reversed by transfection with TβR-II cDNA [McCaffrey et al., 1997]. The TGF-β responsiveness of microvascular endothelial cells grown on 2D gel is higher than that of cells grown on 3D gel due to a greater ratio of TβR-II: TβR-I binding in these cells grown on 2D gel as compared to cells grown on 3D gel [Sankar et al., 1996]. TβR-III overexpression appears to potentiate and attenuate responsiveness to TGF-β in myoblasts and epithelial cells by increasing and decreasing the ratio of TGF-β binding to TβR-II and TβR-I, respectively [Eickelberg et al., 2002]. Furthermore, endoglin overexpression attenuates responsiveness to TGF-β by decreasing the ratio of TGF-β binding to TβR-II and TβR-I [Letamendia et al., 1998].

In mesenchymal cells that generally respond to TGF-β mitogenically, TGF-β is also a potent stimulator of ECM synthesis. The ratio of TGF-β binding to TβR-II and TβR-I is usually >1 in these cells. Clathrin-mediated endocytosis of cell-surface TGF-β may be predominant in these cells. The TGF-β-induced cellular responses in mesenchymal cells may also be determined by the ratio of TGF-β binding to TβR-II: TβR-I as epithelial cells do. Chronic treatment of rat mesangial cells with a high concentration of glucose is known to increase the ratio of TGF-β binding to TβR-II and TβR-I and affect TGF-β responsiveness [Riser et al., 1999]. However, primary dermal fibroblasts from patients with scleroderma exhibit a low ratio (<1) of TβR-II: TβR-I (determined by Western blot analysis) and elevated basal collagen synthesis [Pannu et al., 2004]. A reverse relationship is found between the ratio of TβR-II: TβR-I and the level of basal collagen synthesis in these scleroderma cells as compared to cells from normal patients. Interestingly, transduction of scleroderma fibroblasts with adenovirus carrying dominant-negative TβR-II (cytoplasmic kinase-deletion mutant) fails to attenuate the elevated basal collagen synthesis. No measurement of TGF-β binding to TβR-I and TβR-II (as determined by ^{125}I-TGF-β affinity labeling) and no quantitative measurement of collagen synthesis stimulated by exogenous TGF-β were performed in these studies. Assuming that the ratio of TβR-II: TβR-I (determined by Western blot analysis) reflects the ratio of TGF-β binding to TβR-II and TβR-I in scleroderma fibroblasts, it is possible that increased expression of TβR-I, given a low ratio (<1) of TβR-II: TβR-I binding by TGF-β, in these fibroblasts is caused by feedback from elevated basal collagen synthesis. The low ratio of TGF-β binding to TβR-II and TβR-I may allow cells to decrease the response to exogenous TGF-β stimulation in collagen synthesis. The failure of dominant-negative TβR-II expression to attenuate basal collagen synthesis could be explained by dominant-negative TβR-II formation of nonfunctional heterocomplexes with a fraction of TβR-I while the remaining TβR-I is still capable of forming functional heterocomplexes with endogenous TβR-II that internalize by clathrin-mediated endocytosis [Pannu et al., 2004]. The dominant-negative TβR-II does not contain the functional internalization motif found in native TβR-II. In normal primary dermal fibroblasts, dominant-negative TβR-II overexpression is effective in attenuating basal collagen synthesis because these fibroblasts express a low level of TβR-I. Based on this model (Fig. 4), the dominant-negative TβR-II or soluble TβR-II, should be introduced into the targets (cells and tissues) in a large amount in order to attenuate TGF-β signaling or responsiveness for potential gene or protein therapy of human diseases (e.g., fibrosis). Otherwise, dominant-negative TβR-II or soluble TβR-II might enhance, rather than attenuate, TGF-β responsiveness by increasing the ratio of TGF-β binding to TβR-II and TβR-I (through forming non-functional heterocomplexes with a fraction of TβR-I and thus decreasing TβR-I available to form functional TβR-I/TβR-II hetero-oligomeric complexes) if a large amount of dominant-negative TβR-II or soluble TβR-II is not introduced into the target cells or tissues.

CONCLUSION

The cellular growth inhibitory activity of TGF-β has been implicated in many pathophysiological processes, but the molecular mechanisms are not well understood. Recent characterization of the TβR-V signaling cascade has provided new insights into the mechanism of cellular growth inhibition by TGF-β. The TβR-V signaling cascade appears to cooperate with the TβR-I/TβR-II signaling cascade in mediating TGF-β growth control. The uncoupling of these two signaling pathways may explain the segregation of transcriptional activation and growth inhibition activities of

TGF-β observed in some cell types (e.g., carcinoma cells). The finding of cross-talk between the TβR-V signaling cascade and other signaling cascades has potentially important clinical implications. Insulin or insulin signaling defects and high glucose may up-regulate the TβR-V and TβR-I/TβR-II signaling cascades, resulting in prolonged inflammation, attenuated wound re-epithelialization and neovascularization, as often observed in diabetic foot ulcers. TGF-β is known to play a dual role in tumorigenesis as a tumor suppressor and tumor promoter, depending on the stage of tumorigenesis. The TβR-V signaling is involved in the tumor suppressor activity of TGF-β and its attenuation contributes to the tumor promoter activity of TGF-β in carcinoma cells. Cells deficient in TβR-V exhibit epithelial-to-mesenchymal transdifferentiation (EMT) in the absence of exogenous TGF-β, suggesting that decreased expression of TβR-V may contribute to the aggressiveness and invasiveness of carcinoma cells. Cell-surface TGF-β has recently been shown to be internalized by two distinct endocytosis pathways: clathrin- and caveolar/lipid-raft-mediated endocytosis that promotes signaling and facilitates rapid degradation, respectively. The ratio of TGF-β binding to TβR-II and TβR-I appears to be the signal that controls TGF-β partitioning between these two endocytosis pathways and thus modulates TGF-β responsiveness. Alteration of this ratio provides a mechanism for cells to acquire resistance and sensitivity to TGF-β during physiological and pathological processes.

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