Disruption of Transforming Growth Factor β Signaling by a Novel Ligand-dependent Mechanism

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Abstract

Transforming growth factor (TGF)-β is the prototype in a family of secreted proteins that act in autocrine and paracrine pathways to regulate cell development and function. Normal cells typically coexpress TGF-β receptors and one or more isoforms of TGF-β, thus the synthesis and secretion of TGF-β as an inactive latent complex is considered an essential step in regulating the activity of this pathway. To determine whether intracellular activation of TGF-β results in TGF-β ligand–receptor interactions within the cell, we studied pristane-induced plasma cell tumors (PCTs). We now demonstrate that active TGF-β1 in the PCT binds to intracellular TGF-β type II receptor (TβRII). Disruption of the expression of TGF-β1 by antisense TGF-β1 mRNA restores localization of TβRII at the PCT cell surface, indicating a ligand-induced impediment in receptor trafficking. We also show that retroviral expression of a truncated, dominant-negative TβRII (ΔnTβRII) effectively competes for intracellular binding of active ligand in the PCT and restores cell surface expression of the endogenous TβRII. Analysis of TGF-β receptor-activated Smad2 suggests the intracellular ligand–receptor complex is not capable of signaling. These data are the first to demonstrate the formation of an intracellular TGF-β–receptor complex, and define a novel mechanism for modulating the TGF-β signaling pathway.

Key words: receptor • trafficking • intracellular • signal-transduction • plasmacytoma

Introduction

Among the effects of TGF-β, the regulation of cell growth, cell death, differentiation, and genomic stability appear to be of particular importance (1, 2). The loss of responsiveness to TGF-β represents a significant step in the process of carcinogenesis (3), and several mechanisms underlying the development of TGF-β resistance have been identified (2). These mainly involve a disruption in either the expression or function of components of the TGF-β signaling pathway. TGF-β transduces signals via heteromeric complexes of the type I TGF-β (TβRI)1 and type II TGF-β (TβRII) serine/threonine kinase receptors (4). After ligand binding to TβRII, TβRI is recruited into the complex and activated by TβRII-dependent phosphorylation, thereby enabling it to transduce signals through downstream mediators such as the Smad family of proteins (5). The TGF-β ligand–receptor system has rapidly emerged as an important tumor suppressor pathway that acts to restrain cellular proliferation and to regulate differentiation (1). The first association between resistance to growth inhibition by TGF-β and lack of TβRII receptor expression was reported in retinoblastoma cells (6). Such loss of TβRII expression has since been reported in several types of human cancer, including small cell cancer of the lung (7), hepatoma (8), gastric (9), squamous cell (10), esophageal (11), and breast cancer (12). Known mechanisms underlying receptor down-regulation include mutations associated with the microsatellite instability phenotype (13), mutations of the TβRII gene promoter (14), transcriptional repression by the EWS-FLI1 oncogene (15), and...
DNA methylation of CpG islands in the TβRII promoter (16). Loss of TGF-β receptors at the cell surface has also been described in the absence of gross structural changes, mutations, or transcriptional repression, which suggests that alternative pathways of receptor deregulation must exist.

The role of the TGF-β ligands in disease pathogenesis is more complex. Most tumor cells retain the ability to express TGF-β and often secrete an active form of the ligand. When coupled with a resistance to the inhibitory effects of TGF-β, overexpression of this ligand by the malignant cell could confer a growth advantage through the suppression of immune surveillance (17), promotion of angiogenesis, and stimulation of stroma (18). This potential for TGF-β to exert pro-oncogenic effects in a context in which the tumor cell has an acquired defect in the TGF-β receptor system has been frequently observed in human cancer (19). The production of active TGF-β by plasma cell tumors (PCTs) in mice has been linked to immune dysfunction (20), including the inhibition of cytotoxic T lymphocytes (21). Immune suppression has also been linked to plasma cell production of active TGF-β in the setting of autoimmune disease (22). These studies in the MRL/lpr mouse not only implicate B cells and plasma cells as an important source of circulating active TGF-β, but also provide histoochemical evidence that suggests the activation of TGF-β occurs within the plasma cell (23).

We have previously demonstrated that all PCTs that develop in pristane-primed mice not only secrete active TGF-β, but also uniformly lack the ability to bind exogenous TGF-β at the cell surface (24). In restoring surface TβRII, either by disrupting expression of TGF-β1 with antisense mRNA or by competing ligand binding with a truncated TβRII, we now reveal a novel mechanism whereby the pathologic production of active, intracellular TGF-β impedes receptor localization to the plasma membrane and precludes TGF-β signaling.

Materials and Methods

Cell Culture. Plasmacytoma cell lines (MOPC315, BPC4, TEPC2027, TEPC 1165, and X24) and murine B lymphoma cell lines (CH31, P388, and 8498) were maintained in routine RPMI 1640 media with 10% fetal bovine serum (Biofluids). Recombinant IL-6 (PeproTech) was added at a concentration of 5 ng/ml. Cell lines (CH31, P388, and 8498) were maintained in DMEM containing 10% fetal bovine serum. nant IL-6 (PeproTech) was added at a concentration of 5 ng/ml.

Cell Fractionation. Membrane and cytosolic fractions were prepared according to the methods of Koli et al. (26). In brief, cells were washed with cold PBS, scraped into fractionation buffer (20 mM Tris-HCl [pH 7.4], 2 mM EDTA, 25 mM NaF, 1 mM DTT, 2 mM Na3VO4, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and sheared by repeatedly passing through a 26-gauge needle. After centrifugation at 100,000 × g for 60 min, the soluble fraction (cytosol) was removed and the pellet was resuspended in fractionation buffer containing 0.8% Triton X-100 for 20 min at 4°C. The membrane fraction was cleared from insoluble material by centrifugation at 12,000 × g for 15 min. Triton X-100 was added to the cytosol fraction to yield an 0.8% final concentration. The cytosol and membrane fractions were resolved on 8% SDS-PAGE gels (Novex) and immunoblotted with antibody to TβRII at 1 μg/ml followed by a 1:10,000 dilution of horseradish peroxidase–conjugated goat anti-rabbit secondary antibody. Blots were developed with Super Signal (Pierce Chemical Co.).

Purification and Immunoblotting of γ-Phosphate-linked ATP-Sepharose–purified Lysates. TβRII was extracted from plasmacytoma cell lysates with γ-phosphate–linked ATP-Sepharose (Upstate Biotechnology), which selects for tyrosine and serine/threonine kinases. Eluted kinase-active supernatants were resolved on 8% SDS-PAGE gels (Novex) and immunoblotted with 1 μg/ml of C16 and a 1:10,000 dilution of horseradish peroxidase–conjugated goat anti–rabbit secondary antibody.

In Vitro Kinase Assay. 107 cells were washed in cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer. Lysates were pre-cleared and immunoprecipitated with 2 μg/ml of an anti-TβRII (N-terminal; Upstate Biotechnology). Complexes were captured with 50 μl of protein G–Sepharose for 1.5 h,
and washed five times with RIPA and once with PAN (150 mM NaCl and 50 mM Pipes, pH 7.4). 5 μCi of 32P-γ-ATP (3,000 Ci/mmol; Amersham Pharmacia Biotech) in 50 μl of kinase buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 1 mM NaF, 1 mM NaVO4, and 1 mM DTT) was added to antibody-bound protein G beads and incubated at room temperature for 30 min. Beads were washed three times with RIPA and proteins were eluted in sample buffer, separated on a 10% SDS-PAGE gel (Novex), and immunoblotted with an antibody to full-length receptor (H567; Santa Cruz Biotechnology, Inc.).

Receptor Cross-linking Assay. Analysis of cell surface TGF-β receptor expression by the cross-linking of 125I–TGF-β was performed as previously described (24). Where specified, murine PCTs were acid washed in 150 mM NaCl and 0.1% acetic acid (RPMI 1640, 25 mM Hepes, pH 7.4), resuspended in 1 ml of kinase buffer containing 32P-γ-ATP, and incubated for 30 min at 4°C. The reaction was quenched with 50 mM Tris, pH 8.0, 1 mM EDTA for 60 min, the soluble fraction (cytosol) was removed and the membrane fraction was clarified by centrifugation at 100,000 g for 30 min. The pellet was resuspended in fractionation buffer containing 0.8% Triton X-100 for 20 min at 4°C. Membrane fraction was clarified by centrifugation at 12,000 g for 15 min. Triton X-100 was also added to the cytosol fraction to a final 0.8% concentration. The cytosolic fraction was incubated for 2.5 h with 125I–TGF-β and then cross-linked with diisuccinimidyl suberate (DSS) (Pierce Chemical Co.) added to a final concentration of 3 mM for the final 30 min. The reaction was quenched by the addition of 50 mM Tris-HCl, pH 7.4. Lysates were immunoprecipitated overnight with anti-TβRII (C16). Immunoprecipitates were captured by protein A–Sepharose beads, separated on a 4–20% PAGE gradient gel, and exposed to film.

Analysis of Endogenous, Intracellular TGF-β Ligand–Receptor Complex. 107 cells were washed three times in ice-cold wash buffer (RPMI 1640, 25 mM Heps, pH 7.4), resuspended in 1 ml of the same buffer containing 3 mM DSS, and then incubated for 30 min at 4°C. The reaction was quenched with 50 mM Tris, pH 7.5. Cells were washed three times in cold sucrose buffer (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA) and lysed for 30 min at 4°C in RIPA buffer with protease inhibitors (Boehringer). Lysates were centrifuged at 4°C at 10,000 g in a tabletop Eppendorf centrifuge (model 5415C). Receptor immunoprecipitation was done overnight at 4°C in 1 μg/ml of C16 (Santa Cruz Biotechnology, Inc.). Sample buffer was added with 2-mercaptoethanol in case of immunoblotting with H567 (Santa Cruz Biotechnology, Inc.) and without mercaptoethanol in the case of 1D11 (Gentzeme), and MCA797 (Serotec). Precipitates were run on a 4–20% SDS-PAGE gradient gel and transferred onto polyvinylidene difluoride membranes. Blots were incubated with 1 μg/ml of 1D11 or MCA797 diluted in PBS with 1% BSA or H567 diluted in TBST-milk overnight at 4°C. An additional incubation was done with a 1:10,000 dilution of either a goat anti–mouse or donkey anti–rabbit secondary antibodies. Blots were developed with Super Signal (Pierce Chemical Co.).

Analysis of Smad2. Lysates of cells treated with 2.5 ng TGF-β1/ml in RPMI medium with 0.5% fetal bovine serum were separated on 8% Tris-glycine gels (Novex). Immunoblotting was performed with an anti-phosphoSmad2, rabbit polyclonal antibody (Upstate Biotechnology), followed by a 1:10,000 dilution of goat–anti rabbit secondary (Jackson ImmunoResearch Laboratories) and visualized with Super Signal (Pierce Chemical Co.).

PCTs Express Normal TβRII. Inactivating mutations in TβRII have been described in human malignancies (28, 29). Therefore, to determine if there were any consistent mutations that would impair the processing, expression, or function of this receptor, we sequenced cloned segments of cDNA from TβRII mRNA. The sequencing of cDNAs from five PCTs (BPC4, two variants of MOPC 315, TEP 1165, and X24) and from the spleen of normal BALB/c and C57BL6 mice, showed no consistent bp differences (These sequence data are available from GenBank/EMBL/DDJB under accession no. AF406755). Any differences from the published sequence of TβRII (from NIH3T3 cells) were ascribed to differences in the genetic strain of the mouse (30).

Pristane-induced Plasmacytomas Express a Functional, Kinase-active TβRII. TβRII receptor is a constitutively active serine threonine kinase and autophosphorylates its intracellular domain (31). To characterize the TβRII expressed in the PCT, whole cell lysates were also incubated with γ-phosphate–linked ATP-Sepharose, which selects for tyrosine and serine/threonine kinases. In Western analysis of γ–ATP-Sepharose–selected proteins with TβRII–specific antibodies, an ~70-kd doublet band of TβRII is recognized in the kinase-enriched extracts of the positive controls (Fig. 1 A, lanes 1 and 6) and in each of the four PCTs (Fig. 1 A, lanes 2–5), which suggests that TβRII protein is indeed synthesized by the PCTs. To determine whether the TβRII of the murine PCT has kinase activity, cells from two representative lines (TEPC 1165 and X24) were incubated with 5 μCi of 32P-γ-ATP. Immunoprecipitates of TβRII from lysates labeled with 32P-γ-ATP resolved on an 8% SDS gel demonstrate autophosphorylation of the 70-kd TβRII receptor both in control lymphomas (Fig. 1 B, lanes 1 and 2) and in PCTs (Fig. 1 B, lanes 3 and 4). This suggests that the TβRII of the pristane-induced PCT is capable of autophosphorylation and therefore functional.

TGF-β II Receptor Protein Is Absent in Membrane Fraction but Present in the Cytosol. Because the lack of ligand binding to surface receptors on the PCT is clearly not a consequence of a transcriptional or translational defect (24), we chose to investigate whether this defect represents a problem with localization of TβRII in the plasma membrane. We examined the membrane and cytosolic fractions of the PCTs for the presence of TβRII. Western blotting with an antibody specific for TβRII revealed abundant amounts of receptor protein in the cytosol (Fig. 1 C, lanes 6–8) but not in the membrane fractions (Fig. 1 C, lanes 2–4) of several PCTs. The control cell line CH31 (Fig. 1 C, lanes 1 and 5) shows presence of TβRII both in the membrane and cyto-

Results and Discussion

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Conditioned Media Preparation, Proliferation Assays, and Analysis of TGF-β Production. The methods for the production of serum-free cell supernatants, measurement of DNA synthesis by [3H]thymidine, and TGF-β ELISAs and bioassays have been previously described (24). Quantikine TGF-β1 ELISA kits were purchased from R&D Systems.
described in Koli et al. (26) and as summarized in Materials and Methods. Membrane and cytosolic fractions were prepared from the control CH31 T/PCT cells (lanes 1 and 5) and PCT cells (lanes 2–4 and 6–8) according to the methods described (23) and incubated with antibody to full-length T/PCT cells (lane 3 and 4) are immunoprecipitated with anti-T/PCT cell lysates of control lymphomas (lanes 1 and 2) but not of PCT cells (Fig. 2 C, lanes 3 and 4). As expected, immunoprecipitation with an antibody specific for T/PCT cell lysates were acid washed followed by incubation with 125I–TGF–RII by receptor–ligand affinity labeling in PCTs X24 (lane 2) or 1165 (unpublished data), either before or after acid wash (X24 acid washed, lane 3). Transient exposure of whole cells to low pH does not destroy ligand binding capacity of cell surface receptor as shown with the control cell line (unwashed control, lanes 4 and 5; acid washed control, lane 6). The presence or absence of unlabeled TGF–RII is indicated by − or +, respectively.

**Figure 1.** Murine PCTs contain active intracellular TβRII. (A) Affinity purification of TβRII from whole cell plasmacytoma lysates using γ-phosphate–linked ATP–Sepharose. Products were resolved on an 8% SDS-PAGE gel and immunoblotted with antibody to full-length TβRII. (B) Autophosphorylation of the 70-kD TβRII can be visualized when γ-ATP–labeled cell lysates of control lymphomas (lanes 1 and 2) and PCT cells (lane 3 and 4) are immunoprecipitated with anti-TβRII. (C) TβRII is not detected by Western in membrane fractions of PCTs. Membrane and cytosolic fractions were prepared from the control CH31 (lanes 1 and 5) and PCT cells (lanes 2–4 and 6–8) according to the methods described in Koli et al. (26) and as summarized in Materials and Methods.

**Figure 2.** Intracellular TβRII of the PCT does not bind exogenous ligand. Chemical cross-linking of 125I–TGF–β binding to TβRII in the PCT cell (24) could occur if receptors are occupied by secreted, endogenous ligand. We previously reported the secretion of active TGF–β by multiple PCT cell lines and now provide immunohistochemical evidence of active intracellular TGF–β in the PCT. Using the monoclonal antibody 1D11 that reacts specifically with active and not latent TGF–β, we evaluated sections through in-

**Figure 3.** Active TGF–β is present in the PCT. (A) To demonstrate the presence of active intracellular TGF–β in the murine PCT in vivo, we prepared 4-μm sections through PCT–containing peritoneal granulomas that had been fixed in formalin and embedded in paraffin. Sections were processed by standard methods for histochemical analysis, as previously described (23) and incubated with a biotinylated monoclonal antibody (1D11) that reacts specifically with active but not latent TGF–β. (B) To demonstrate specificity, control sections were incubated with antibody that had been pre-blocked by incubation with recombinant TGF–β1. Arrowheads indicate plasma cells. (C) A ligand–receptor complex cannot be detected at the PCT cell surface. To strip receptor-bound ligand, whole cells were acid washed followed by incubation with 125I–TGF–β for detection of surface TβRII. There is no evidence of TβRII by receptor–ligand affinity labeling in PCTs X24 (lane 2) or 1165 (unpublished data), either before or after acid wash (X24 acid washed, lane 3). Transient exposure of whole cells to low pH does not destroy ligand binding capacity of cell surface receptor as shown with the control cell line (unwashed control, lanes 4 and 5; acid washed control, lane 6). The presence or absence of unlabeled TGF–β is indicated by − or +, respectively.
The formation of active TGF-β1 in the PCT cells expressing a dominant-negative version of the TGF-βRII is competed by excess unlabeled TGF-β1. The cross-linking pattern on antisense X24 is competed by excess unlabeled TGF-β1, indicating that the lack of binding of 125I–TGF-β1 to TGF-βRII cells is due to a direct consequence of blocking ligand production in the PCT. The data presented so far support a hypothesis in which the formation of active TGF-β1 within the PCT cell promotes direct intracellular interaction of ligand with TGF-βRII, effectively trapping the receptor and preventing trafficking to the plasma membrane. If true, the restoration of cell surface localization might occur when the receptor is in relative excess of available ligand. To test this hypothesis, we infected a PCT line with a retroviral vector expressing a dominant-negative version of the TGF-β type II receptor, dnTGF-βRII, which lacks the kinase domain but has an intact extracellular binding domain and a transmembrane domain. Thus, the receptor is capable of binding ligand but is not capable of initiating signaling. We evaluated this line (1165dnRII) for the capacity to bind 125I–TGF-β1 (Fig. 4 D). The parental cell line TEPC 1165 (Fig. 4 D, lanes 1 and 2) consistently lack the ability to bind exogenous 125I–TGF-β1. However, the incubation of the dnTGF-βRII-expressing 1165dnRII cells with 125I–TGF-β1 revealed ligand binding to endogenous receptor at the cell surface (immunoprecipitation of the receptor–ligand complex was performed with an antibody recognizing the C-terminus of wild-type TGF-βRII, thereby distinguishing the endogenous receptor from the dnTGF-βRII; Fig. 4 D, lanes 3 and 4). The ability of the dnTGF-βRII to act as a decoy for endogenous TGF-β is also demonstrated by the ability of 125I–TGF-β1 to bind to TGF-βRII in cytoplasmic extracts of the 1165dnTGF-βRII cells (Fig. 4 D, lane 7). These results indicate that the lack of binding of 125I–TGF-β1 to the intra-

Figure 4. Restoration of cell surface expression of TGF-βRII. (A and B) TGF-β1 antisense expression and disruption of TGF-β production restores the surface expression of TGF-β receptors (A, top). Northern analysis of sense and antisense TGF-β1 transfected cell lines with a 32P-labeled cDNA probe for neomycin (part of the bicistronic message) confirms the expression of sense and antisense TGF-β1 mRNA (A, bottom). Corresponding ethidium bromide–stained gel. (B) Ligand affinity cross-linking studies with 125I–TGF-β show a normal receptor complex in the control CH31 (lane 1), competed by unlabeled TGF-β (lane 2). The restoration of the cell surface expression of TGF-βRII is obtained on transduction of the parental X24 with the antisense TGF-β1 (antisense X24, lanes 3 and 4). The binding and chemical cross-linking of 100 pM 125I–TGF-β to TGF-βRII is competed by a 100-fold excess (1 nM) of unlabeled TGF-β1 and is indicated by +. (C and D) Transfection with a truncated TGF-βRII restores surface localization of endogenous TGF-βRII (C, top). Hybridization with a 32P-labeled cDNA neo probe demonstrates the expression of the dominant-negative TGF-βRII mRNA in the PCT 1165 (C, bottom). Ethidium bromide–stained gel of a 375-bp amplimer specific to the HA-tagged TGF-βRII (D) Chemical cross-linking studies demonstrate the restoration of endogenous TGF-βRII on the cell surface after transduction with the dominant-negative TGF-βRII (1165dnRII). In vitro cross-linking of cytosolic fraction from 1165dnRII demonstrate the presence of free endogenous TGF-βRII that is now available for binding to 125I–TGF-β (lane 7).
cellular TβRII in the parental 1165 (Fig. 2, lanes 3 and 4) is a consequence of receptor binding to active intracellular TGF-β. When endogenous active TGF-β is sequestered by the dnTβRII in the PCT, there is a pool of endogenous TβRII that then becomes available for binding with exogenous ligand (Fig. 4 D, lane 7). Collectively, these data support the conclusion that autocrine, active intracellular TGF-β1 blocks the translocation of TβRII to the plasma membrane in the pristane-induced PCT.

**Intracellular TGF-β Ligand–Receptor Complexes Are Present in the Murine Pristane–induced PCT.** To directly demonstrate the existence of an intracellular ligand–TβRII complex in the PCT, we performed an “intracellular” cross-linking assay by exposing intact PCT cells to the permeable membrane cross-linking reagent, DSS. Unlike traditional TGF-β receptor cross-linking studies that stabilize the interaction of cell surface receptor with 125→TGF-β1, this experiment relies on the presence of active, endogenous TGF-β1 to cross-link with intracellular TβRII. Cross-linked PCT lysates were immunoprecipitated with a TβRII–specific antibody and immunoblotted with either of two distinct monoclonal antibodies specific for TGF-β1, or with a polyclonal antibody raised against the full-length type II receptor. Both anti–TGF-β1 antibodies clearly detected the existence of an identical intracellular ligand–receptor complex (Fig. 5 A, lanes 1 and 4) that was blocked by the pre-incubation of primary antibody with recombinant TGF-β1, and was not detected by incubation with the secondary antibody alone (unpublished data). The same complex was also present when immunoprecipitates were assayed by Western blotting with an antibody raised against the full-length TβRII (Fig. 5 A, lane 5). Because latent TGF-β does not bind TβRII, the demonstration of an intracellular ligand–receptor complex provides clear evidence that TGF-β is being activated intracellularly and is capable of binding TβRII.

**The Intracellular TGF-β Ligand–Receptor Complexes Do Not Signal through Smad2.** The presence of an intracellular ligand–receptor complex raises an important question regarding the potential for signaling to occur in an “intracrine” fashion, outside the context of the plasma membrane. To address this question we looked for the presence of the receptor-activated phosphorylated Smad2 in lysates of both the parental PCTs and in the antisense and dnTβRII–expressing PCT cell lines (Fig. 5, C and D). No phosphorylated Smad2 was detected after the addition of exogenous ligand to the parental cell lines (Fig. 5, C and D), despite the fact that both X24 (unpublished data) and TEPC 1165 contained detectable levels of Smad2 protein. This suggests that the intracellular ligand–receptor complex is incapable of activating Smad2. More importantly, exogenous ligand could induce the phosphorylation of Smad2 not only in the control lymphoma (CH31; Fig. 5 B, top), but also in both the TGF-β1 antisense and dnTβRII–expressing PCT cell lines, in which we have restored receptor expression at the cell surface (Fig. 5, C and D).

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**Figure 5.** An intracellular TGF-β–TβRII complex. (A) Lysates of DSS-treated whole cells were immunoprecipitated with the anti–TβRII antibody (C16) followed by Western analysis with either antibodies to TGF-β1 (1D11, lanes 1–3, or MCA797, lane 4) or full-length TβRII (H567, lane 5). Each primary antibody detected the identical intracellular ligand–receptor complex (Fig. 5 A, lanes 1–3, or MCA797, lane 4) or full-length TβRII (H567, lane 5). Because latent TGF-β does not bind TβRII, the demonstration of an intracellular ligand–receptor complex provides clear evidence that TGF-β is being activated intracellularly and is capable of binding TβRII.

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**Figure 6.** A model for the sequestration of TβRII inside the cell by active endogenous TGF-β1. TGF-β is typically secreted in a biologically latent form and therefore cannot bind to its cognate receptor, TβRII. (A) In a normal cell, the activation of TGF-β1 occurs outside the cell where it can bind cell surface TβRII. (B) In our plasmacytoma model, TGF-β1 is activated within the cell by a currently unknown mechanism and can readily bind TβRII, which consequently contributes to the observed loss of TGF-β receptor expression at the cell surface.
It is worth noting that our use of the truncated dnTβRII has clearly given a result that might not be predicted based on conventional studies in which dominant-negative receptors have routinely been applied. Such dominant-negative constructs have invariably been expressed in cells that have an intact TGF-β signaling pathway. More importantly, the dnTβRII has never been introduced into a cell that spontaneously produces large amounts of active TGF-β or contains an intracellular pool of active TGF-β, such as that present within the PCT cell. However, the fact that one can induce phosphorylated Smad2 in a cell expressing the dnTβRII is not without precedent, as the expression of a similar dnTβRII in the Mv1Lu cell line blocks growth inhibition in response to TGF-β, but not the Smad-dependent induction of fibronectin or the plasminogen activator inhibitor (35). Regardless, it is clear that the endogenous TβRII synthesized by the PCT is capable of transducing a signal when localized in the plasma membrane.

It is also important to note that even upon the restoration of the endogenous receptor to the cell surface we were unable to restore sensitivity to TGF-β-mediated growth inhibition and apoptosis (unpublished data) in either the antisense X24 line or the 1165dn line. It has recently been demonstrated that a similar defect in the membrane localization of TGF-β receptors correlates with insensitivity to the growth inhibitory effects of TGF-β in human mammary epithelial tumors (36). However, this may not be the primary or principal defect underlying TGF-β resistance, especially in the PCT where deregulated expression of c-myc is invariant (37). As the repression of c-myc is critical for TGF-β-induced growth arrest (38), it is possible that our inability to couple Smad2 phosphorylation with either growth inhibition or apoptosis in the antisense X24 and 1165dn lines merely reflects an inability to suppress the expression of c-myc.

In contrast to the majority of growth factors, TGF-β is normally synthesized and secreted in a biologically latent form such that it is unable to bind to its cognate receptor, nor elicit a biological response (39). It is possible that genetic polymorphisms in the TGF-β ligands may result in the altered production and activation of TGF-β. Variants leading to increased circulating TGF-β have been described (40, 41) as well as domain-specific mutations of the TGF-β1 LAP that potentially result in the formation of a constitutively active TGF-β1 (42). We have sequenced the entire coding region for TGF-β1 in two PCTs (MOPC 315 and X24) and found no mutations. Another mechanism that might lead to the aberrant production of active TGF-β involves the increased production of proteases with the capacity to cleave the latent precursor. These include furin-like proteases (43) and matrix metalloproteinases (44, 45). In human myeloma there is significant production of matrix metalloproteinase (MMP)-9, MMP-2, and MMP-1 (46). So far there are no data regarding such protease activity in murine PCTs. This remains an area for future investigation. In this report, we provide the first evidence that the production of active TGF-β within a cell can disrupt autocrine TGF-β signaling via the formation of non-productive intracellular ligand–receptor complexes. In this model, an intracellular sequestration of TβRII by active TGF-β ligand prevents the receptor from trafficking to the cell surface (Fig. 6). The data support the hypothesis that this novel mechanism underlies the consistent absence of TGF-β receptors on the surface of the pristane-induced PCT.

A role for intracellular ligand–receptor interactions in acquired defects in the membrane localization of growth factor receptors has been previously demonstrated in cells transformed by the viral oncoprotein v-sis (47, 48). In these studies, the loss of platelet-derived growth factor receptor at the cell surface was linked to internal activation of the receptor by the v-sis gene product. However, although intracellular autocrine loops have been described for v-sis and for other cytokines (49, 50), our data suggest that intracellular TGF-β ligand–receptor complexes are not capable of initiating signaling, at least through Smad2. This may be due to differences in the trafficking of TβRI and TβRII (33), such that ligand-bound TβRII may not be able to recruit TβRII unless both are present at the cell surface. Alternatively, it may reflect the inability of ligand-bound receptor complexes to recruit cell surface adaptor proteins such as Smad anchor for receptor activation, which are required for the phosphorylation of Smad2 (51). Studies focused on mechanisms of activation of the ligand in the pristane-induced PCT and the endocytic fate of ligand–receptor complexes are currently under investigation.

In conclusion, these data not only support the concept that latency is a key step in regulating the TGF-β ligands, but also demonstrate how the activation of TGF-β within a cell can impact the TGF-β receptor expression, and therefore impact the responsiveness to both autocrine and paracrine sources of the cytokine.

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