

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- β 1 in the PCT binds to intracellular TGF- β type II receptor (T β RII). Disruption of the expression of TGF- β 1 by antisense TGF- β 1 mRNA restores localization of T β RII at the PCT cell surface, indicating a ligand-induced impediment in receptor trafficking. We also show that retroviral expression of a truncated, dominant-negative T β RII (dnT β RII) effectively competes for intracellular binding of active ligand in the PCT and restores cell surface expression of the endogenous T β RII. 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 (T β RI)* and type II TGF- β (T β RII)

serine/threonine kinase receptors (4). After ligand binding to T β RII, T β RI is recruited into the complex and activated by T β RII-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 T β RII receptor expression was reported in retinoblastoma cells (6). Such loss of T β RII 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 T β RII gene promoter (14), transcriptional repression by the EWS-FLI1 oncogene (15), and

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*Abbreviations used in this paper: dnT β RII, dominant-negative type II TGF- β receptor; DSS, disuccinimidyl suberate; HA, hemagglutinin; MMP, matrix metalloproteinase; PCT, plasma cell tumor; RIPA, radioimmunoprecipitation assay; TBS, Trizma buffer solution; T β RI, type I TGF- β receptor; T β RII, type II TGF- β receptor.

DNA methylation of CpG islands in the T β RII 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 histochemical 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 T β RII, either by disrupting expression of TGF- β 1 with antisense mRNA or by competing ligand binding with a truncated T β RII, 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 for the maintenance of TEPC 1165. Mv1lu cells were maintained in DMEM containing 10% fetal bovine serum.

Constructs and Retroviral Infections. To generate the TGF- β 1 antisense vector, a neomycin-resistant gene containing an internal ribosomal entry site that forms a fusion RNA with antisense TGF- β 1 RNA was subcloned into the BamHI site of the MFG vector (25). To produce retrovirus, the ABOSC packaging cell was transfected with 10 μ g of either the sense or antisense plasmid and cotransfected with 2.5 μ g of pCMV-VSV-G. Retroviral supernatant was collected at 24 h and applied directly to X24 cells. Stable antisense cell lines were selected using G418 and characterized by standard Northern blot analysis and ELISA (TGF- β 1 Quantikine; R&D Systems).

To generate the dominant-negative T β RII vector (dnT β RII), a 567-bp fragment of the human T β RII (nucleotide positions

335–911), with a 5' hemagglutinin (HA) tag inserted at bp 405, was cloned into the BamHI-EcoRI site of pcDNA3 (Invitrogen). The dnT β RII was transfected into TEPC 1165 and expression was determined by reverse transcription PCR with a forward primer specific to the HA tag (TF1: 5' GATGTTCCTGAT-TATGCTAG 3'), and a reverse primer spanning nucleotides 734–759 of the T β RII cDNA (TF2: 5' CATCAGAGCTA-CAGGAACACATGAAG 3') that amplified a 350-bp region.

Immunohistochemistry. Immunohistochemical analysis was performed as previously reported by Caver et al. (23). To demonstrate the presence of active intracellular TGF- β , 4- μ m sections through PCT-containing peritoneal granulomas were fixed in formalin and embedded in paraffin. Slides were submerged in Trizma buffer solution (TBS) containing 0.1% Triton X-100 (Sigma-Aldrich) at room temperature for 15 min followed by TBS for 5 min, methanol for 2 min, and 0.6% (vol/vol) hydrogen peroxide in methanol for 30 min. Slides were subsequently washed at room temperature in methanol for 2 min, TBS for 5 min, and three times in TBS containing 0.1% (wt/vol) BSA for 3 min. After treatment with hyaluronidase (1 mg/ml in 100 mM sodium acetate, 0.85% [wt/vol] NaCl) for 30 min at 37°C, and three washes in TBS/0.1% BSA, slides were treated with an avidin-biotin block (Vectastain) for 15 min at room temperature, rinsed in TBS, and then blocked with 1% goat serum in TBS containing 0.5% BSA for 30 min at room temperature. Slides were incubated with 50 μ g/ml biotinylated anti-TGF β 1 (1D11; R&D Systems) that reacts specifically with active and not latent TGF- β . The primary antibody was biotinylated with avidin-biotin reagent (Zymed Laboratories) according to the manufacturer's instructions. Slides were washed three times in TBS with 0.1% BSA and exposed to ABC complex (Vector Laboratories) followed by 0.05% diaminobenzidine and 0.1% hydrogen peroxide.

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 NaMo₄, 1 μ g/ml aprotinin, and 1 μ g/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 T β RII at 1 μ g/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. T β RII 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 μ g/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 μ g/ml of an anti-T β RII (N-terminal; Upstate Biotechnology). Complexes were captured with 50 μ l 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 μ Ci of 32 P- γ ATP (3,000 Ci/Mmol; Amersham Pharmacia Biotech) in 50 μ l 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 125 I-TGF- β was performed as previously described (24). Where specified, murine PCTs were acid washed in 150 mM NaCl and 0.1% acetic acid according to a standard protocol for stripping ligand from cell surface receptor, as described by Zwaagstra et al. (27). For *in vitro* cross-linking analysis of intracellular receptor, cells were washed with cold PBS and lysed in 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 25 mM NaF, 1 mM DTT, 2 mM NaMo₄, and 2 mM NaVO₄ with protease inhibitors. 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. 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 125 I-TGF- β and then cross-linked with disuccinimidyl 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-T β RII (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 Hepes, 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 with 1 μ g/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 (Genzyme), 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 μ g/ml of 1D11 or MCA979 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- β 1/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.).

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 [3 H]thymidine, and TGF- β ELISAs and bioassays have been previously described (24). Quantikine TGF- β 1 ELISA kits were purchased from R&D Systems.

Results and Discussion

PCTs Express Normal T β RII. Inactivating mutations in T β RII 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 T β RII mRNA. The sequencing of cDNAs from five PCTs (BPC4, two variants of MOPC 315, TEPC 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/DDBJ under accession no. AF406755). Any differences from the published sequence of T β RII (from NIH3T3 cells) were ascribed to differences in the genetic strain of the mouse (30).

Pristane-induced Plasmacytomas Express a Functional, Kinase-active T β RII. T β RII receptor is a constitutively active serine threonine kinase and autophosphorylates its intracellular domain (31). To characterize the T β RII 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 T β RII-specific antibodies, an \sim 70-kD doublet band of T β RII 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 T β RII protein is indeed synthesized by the PCTs. To determine whether the T β RII of the murine PCT has kinase activity, cells from two representative lines (TEPC 1165 and X24) were incubated with 5 μ Ci of 32 P-ATP. Immunoprecipitates of T β RII from lysates labeled with 32 P-ATP resolved on an 8% SDS gel demonstrate autophosphorylation of the 70-kD T β RII 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 T β RII 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 T β RII in the plasma membrane. We examined the membrane and cytosolic fractions of the PCTs for the presence of T β RII. Western blotting with an antibody specific for T β RII 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 T β RII both in the membrane and cyto-

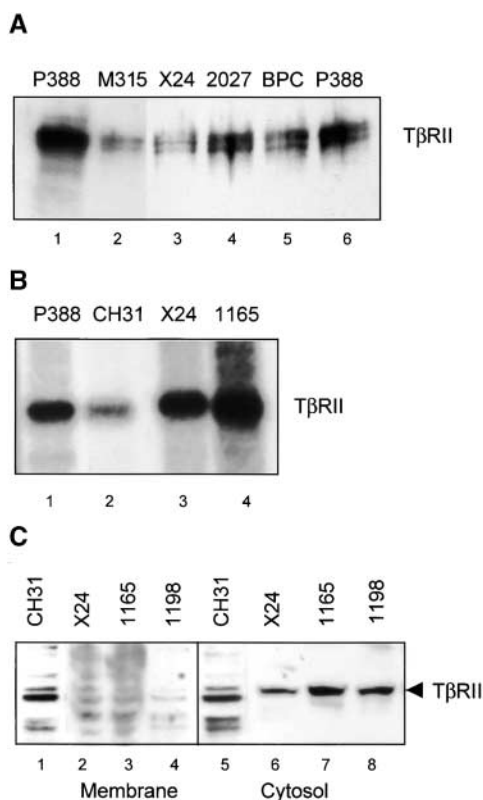


Figure 1. Murine PCTs contain active intracellular TβRII. (A) Affinity purification of TβRII from whole cell plasmacytoma lysates using γ-phosphate-linked ATP-Sepharose. Products were resolved on an 8% SDS-PAGE gel and immunoblotted with antibody to full-length TβRII. (B) Autophosphorylation of the 70-kD 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.

solic fractions. The relative absence of receptor protein in the membrane fractions of PCTs suggests that a defect in receptor trafficking underlies the inability to bind ligand at the surface of the PCT.

¹²⁵I-TGF-β Does Not Bind to TβRII in Lysates of PCT Cells. The absence of membrane TβRII can explain the inability to bind exogenous TGF-β at the cell surface. However, it should be possible to demonstrate the binding of ¹²⁵I-TGF-β to the cytosolic receptor. Receptor cross-linking was performed with ¹²⁵I-TGF-β and cytoplasmic lysates of TEPC 1165 and X24 to investigate whether the intracellular pool of TβRII would bind exogenous TGF-β1. Binding of ¹²⁵I-TGF-β to TβRII was observed in lysates of control lymphoma cells (Fig. 2, lanes 1 and 2) but not of PCT cells (Fig. 2 C, lanes 3 and 4). As expected, immunoprecipitation with an antibody specific for TβRI did not reveal the binding of ¹²⁵I-TGF-β1 to TβRI (unpublished data), nor did anti-TβRII co-precipitate TβRI in this assay, supporting the model in which heteromers of TβRI and TβRII do not form in the presence of ligand until they

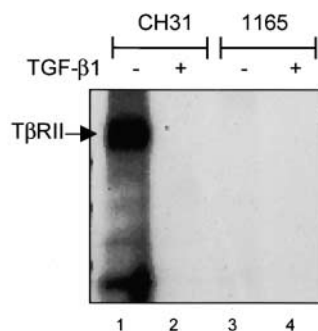


Figure 2. Intracellular TβRII of the PCT does not bind exogenous ligand. Chemical cross-linking of ¹²⁵I-TGF-β to cytoplasmic extracts from the positive control CH31 (lanes 1 and 2), followed by immunoprecipitation with TβRII-specific antibodies reveals ligand binding. In contrast, ligand binding to intracellular receptors could not be detected in cytoplasmic extracts of the parental TEPC 1165 (lanes 3 and 4).

are on the plasma membrane (32, 33). These data suggest that either TβRII expressed by PCTs is not available for ligand binding, or that a unique mechanism is responsible for the sequestration of TβRII within the PCT cell.

Absence of Ligand-TβRII Complex on the PCT Cell Surface. It is possible that the lack 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-

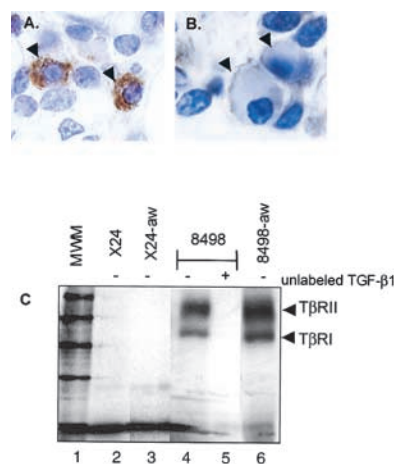


Figure 3. Active TGF-β is present in the PCT. (A) To demonstrate the presence of active intracellular TGF-β in the murine PCT in vivo, we prepared 4-μm sections through PCT-containing peritoneal granulomas that had been fixed in formalin and embedded in paraffin. Sections were processed by standard methods for histochemical analysis, as previously described (23) and incubated with a biotinylated monoclonal antibody (1D11) that reacts specifically with active and 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.

inflammatory granulomas for pristane-primed mice (Fig. 3 A). The specificity of the strong immunohistochemical staining in plasma cells was demonstrated by pre-blocking 1D11 with recombinant TGF- β 1 (Fig. 3 B). Although Western analysis of membrane fractions indicates a true absence of T β RII at the PCT surface, we wanted to clearly exclude the possibility that the lack of 125 I-TGF- β 1 binding on whole PCT cells (Fig. 3 C, lane 2) is secondary to receptor occupancy at the cell surface by the active, endogenous ligand. We performed an acid wash on whole PCT cells, a technique that effectively strips ligand from cell surface receptors and allows for subsequent binding with 125 I-TGF- β 1 (16). The lack of binding of 125 I-TGF- β 1 after the acid wash of both X24 (Fig. 3 C, lane 3) and TEPC 1165 (unpublished data) confirms the true absence of cell surface localization of receptor. Cell membranes are not affected by transient exposure to low pH and 90–95% of TGF- β receptor-bound ligand is removed at low pH without affecting subsequent binding capacity (26). With a control-line cell line (Fig. 3 C, lane 4) we were also able to show that the acid wash of cells does not destroy ligand binding capacity of the receptor.

Production of Autocrine, Active TGF- β 1 Precludes the Translocation of T β RII to the Plasma Membrane in the Murine Pristane-induced PCT. To demonstrate the role of autocrine TGF- β 1 in this PCT phenotype, we examined the effects of blocking endogenous TGF- β 1 production through retroviral expression of antisense TGF- β 1 (Fig. 4, A and B). Antisense expression led to a 10-fold reduction of secreted TGF- β 1 as determined by both ELISA and in a bioassay that evaluates the growth of the TGF- β 1-sensitive Mv1Lu cell line in media conditioned by the PCT (120 pg/ml in sense control vs. 12.4 pg/ml in antisense line). Expression of sense has no effect on the pattern of ligand binding at the cell surface (Fig. 4 B, lane 5). However, the localization of T β RII at the cell surface is restored by the expression of antisense TGF- β 1, as demonstrated by the binding of 125 I-TGF- β 1 to T β RII and T β RI on the antisense X24 PCT cells (Fig. 4 B, lane 3). The cross-linking pattern on antisense X24 is competed by excess unlabeled TGF- β 1 (Fig. 4 B, lane 4) and is similar to that of the control B cell lymphoma CH31 (Fig. 4 B, lanes 1 and 2). The ability to detect cell surface T β RII in antisense X24 and not in cells expressing a control sense vector suggests that the effect is 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- β 1 within the PCT cell promotes direct intracellular interaction of ligand with T β RII, 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 (TEPC 1165) with a retroviral vector expressing a dominant-negative version of the TGF- β type II receptor, dnT β RII, which lacks the kinase domain but has an intact extracellular binding domain and a transmembrane domain (34). Thus, the receptor is capable of binding

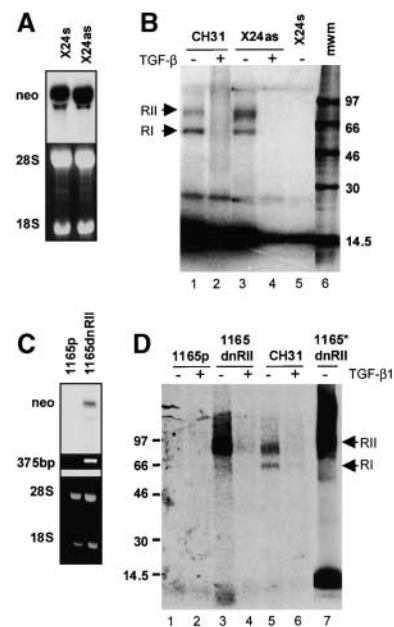


Figure 4. Restoration of cell surface expression of T β RII. (A and B) TGF- β 1 anti-sense expression and disruption of TGF- β production restores the surface expression of TGF- β receptors (A, top). Northern analysis of sense and antisense TGF- β 1 transfected cell lines with a 32 P-labeled cDNA probe for neomycin (part of the bicistronic message) confirms the expression of sense and antisense TGF- β 1 mRNA (A, bottom). Corresponding ethidium bromide-stained gel. (B) Ligand affinity cross-linking studies with 125 I-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 T β RII is obtained on transduction of the parental X24 with the antisense TGF- β 1 (antisense X24, lanes 3 and 4). The binding and chemical cross-linking of 100 pM 125 I-TGF- β to T β RII is competed by a 100-fold excess (1 nM) of unlabeled TGF- β 1 and is indicated by +. (C and D) Transfection with a truncated T β RII restores surface localization of endogenous T β RII (C, top). Hybridization with a 32 P-labeled cDNA neo probe demonstrates the expression of the dominant-negative T β RII mRNA in the PCT 1165 (C, bottom). Ethidium bromide-stained gel of a 375-bp amplicon specific to the HA-tagged T β RII. (D) Chemical cross-linking studies demonstrate the restoration of endogenous T β RII on the cell surface after transduction with the dominant-negative T β RII (1165dnRII). In vitro cross-linking of cytosolic fraction from 1165dnRII demonstrate the presence of free endogenous T β RII that is now available for binding to 125 I-TGF- β (lane 7).

ligand but is not capable of initiating signaling. We evaluated this line (1165dnRII) for the capacity to bind 125 I-TGF- β 1 (Fig. 4 D). The parental cell line TEPC 1165 (Fig. 4 D, lanes 1 and 2) consistently lack the ability to bind exogenous 125 I-TGF- β 1. However, the incubation of the dnT β RII-expressing 1165dnRII cells with 125 I-TGF- β 1 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 T β RII, thereby distinguishing the endogenous receptor from the dnT β RII; Fig. 4 D, lanes 3 and 4). The ability of the dnT β RII to act as a decoy for endogenous TGF- β is also demonstrated by the ability of 125 I-TGF- β 1 to bind to T β RII in cytoplasmic extracts of the 1165dnT β RII cells (Fig. 4 D, lane 7). These results indicate that the lack of binding of 125 I-TGF- β 1 to the intra-

cellular T β RII 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 dnT β RII in the PCT, there is a pool of endogenous T β RII 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- β 1 blocks the translocation of T β RII 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–T β RII 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 I–TGF- β 1, this experiment relies on the presence of active, endogenous TGF- β 1 to cross-link with intracellular T β RII. Cross-linked PCT lysates were immunoprecipitated with a T β RII-specific antibody and immunoblotted with either of two distinct monoclonal antibodies specific for TGF- β 1, or with a polyclonal antibody raised against the full-length type II receptor. Both anti–TGF- β 1 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- β 1, 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 T β RII (Fig. 5 A, lane 5). Because latent TGF- β does not bind T β RII, the demonstration of an intracellular ligand–receptor complex provides clear evidence that TGF- β is being activated intracellularly and is capable of binding T β RII.

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 dnT β RII-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 contain 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- β 1 antisense and dnT β RII-expressing PCT cell lines, in which we have restored receptor expression at the cell surface (Fig. 5, C and D).

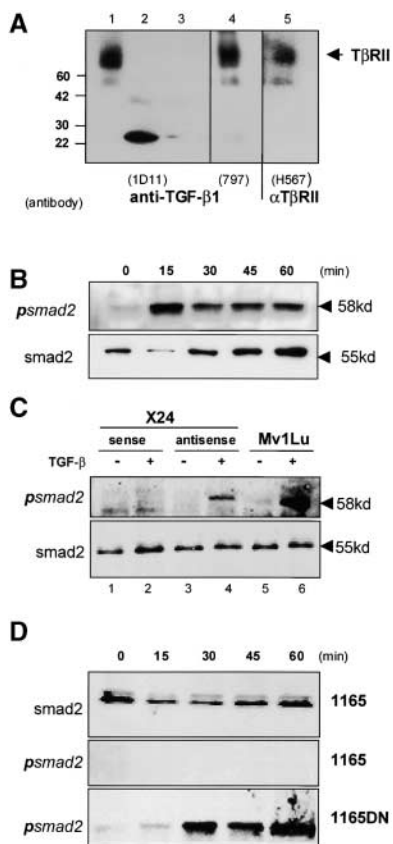


Figure 5. An intracellular TGF- β –T β RII complex. (A) Lysates of DSS-treated whole cells were immunoprecipitated with the anti–T β RII antibody (C16) followed by Western analysis with either antibodies to TGF- β 1 (1D11, lanes 1–3, or MCA797, lane 4) or full-length T β RII (H567, lane 5). Each primary antibody detected the identical intracellular ligand–receptor complexes with recombinant TGF- β 1 loaded as a control (500 ng, lane 2, and 50 ng, lane 3). (B) An \sim 58-kD phosphorylated Smad2 band was induced by TGF- β treatment of the control lymphoma, CH31. (C) TGF- β -induced phosphorylation of Smad2 was detected in the PCT X24 containing the antisense expression vector (lanes 3 and 4), but not in the sense control line (lanes 1 and 2). Phosphorylation of the mink lung epithelial cell line (Mv1Lu) is shown as a positive control (lanes 5 and 6). (D) Similarly, TGF- β -induced phosphorylation of Smad2 was detected in the PCT 1165 containing the dnT β RII expression vector (1165dn, bottom). The result demonstrates the ability of TGF- β to initiate signaling once the endogenous receptor is localized to the plasma membrane.

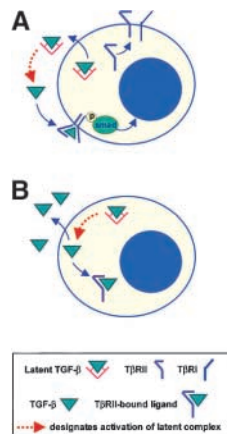


Figure 6. A model for the sequestration of T β RII inside the cell by active endogenous TGF- β . TGF- β is typically secreted in a biologically latent form and therefore cannot bind to its cognate receptor, T β RII. (A) In a normal cell, the activation of TGF- β 1 occurs outside the cell where it can bind cell surface T β RII. (B) In our plasmacytoma model, TGF- β 1 is activated within the cell by a currently unknown mechanism and can readily bind T β RII, 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 dnT β RII 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 dnT β RII 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 dnT β RII is not without precedent, as the expression of a similar dnT β RII 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 T β RII 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- β 1 LAP that potentially result in the formation of a constitutively active TGF- β 1 (42). We have sequenced the entire coding region for TGF- β 1 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 T β RII 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 T β R1 and T β R2 (33), such that ligand-bound T β R2 may not be able to recruit T β R1 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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