Transforming growth factor-β type-II receptor signalling: intrinsic/associated casein kinase activity, receptor interactions and functional effects of blocking antibodies

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The transforming growth factor β (TGF-β) family of growth factors control proliferation, extracellular matrix synthesis and/or differentiation in a wide variety of cells. However, the molecular mechanisms governing ligand binding, receptor oligomerization and signal transduction remain incompletely understood. In this study, we utilized a set of antibodies selective for the extracellular and intracellular domains of the TGF-β type-II receptor as probes to investigate the intrinsic kinase activity of this receptor and its physical association in multimeric complexes with type-I and type-III receptors. The type-II receptor immunoprecipitated from human osteosarcoma cells exhibited autophosphorylation and casein kinase activity that was markedly stimulated by polylysine yet was insensitive to heparin. Affinity cross-linking of ^{125}I-TGF-β1 ligand to cellular receptors followed by specific immunoprecipitation demonstrated that type-II receptors form stable complexes with both type-I and type-III receptors expressed on the surfaces of both human osteosarcoma cells and rabbit chondrocytes. Pretreatment of the cultured cells with an antibody directed against a distinct extracellular segment of the type-II receptor (anti-TGF-β-IIR-NT) effectively blocked the ^{125}I-TGF-β labelling of type-I receptors without preventing the affinity labelling of type-II or type-III receptors, indicating a selective disruption of the type-I/type-II hetero-oligomers. The anti-TGF-β-IIR-NT antibodies also blocked the TGF-β-dependent induction of the plasminogen activator inhibitor (PAI-1) promoter observed in mink lung epithelial cells. However, the same anti-TGF-β-IIR-NT antibodies did not prevent the characteristic inhibition of cellular proliferation by TGF-β1, as determined by [^{3}H]thymidine incorporation into DNA. The selective perturbation of PAI-1 promoter induction versus cell-cycle-negative regulation suggests that strategic disruption of TGF-β type-I and -II receptor interactions can effectively alter specific cellular responses to TGF-β signalling.

INTRODUCTION

Secretory polypeptides of the transforming growth factor type β (TGF-β) superfamily have profound effects on the physiology of tissue development and repair [1], exerting potent control of cellular proliferation, differentiation, cell adhesion and/or extracellular matrix gene and protein expression in a variety of different cell types [2]. Numerous studies have demonstrated a role for TGF-β in wound healing [1] leading to the first clinical applications of exogenous TGF-β [3] and the promise of future utility in the repair of bone, surgical wound healing [4] and the treatment of diabetic ulcers and burns [5]. Blocking or neutralizing endogenous TGF-β may also reduce dermal scarring during the course of normal wound healing [6] and may ameliorate lung fibrosis occurring in response to bleomycin [7].

Indeed, TGF-β produces a number of seemingly contrasting effects, exerting either growth-promoting or -inhibitory influences, which depend largely on the type and/or the developmental status of the responding cells [2]. TGF-β is a potent mitogen of early-stage fibroblasts and osteoblasts [2]; however, the molecular mechanisms by which TGF-β stimulates mesenchymal cell growth have not yet been fully defined. TGF-β is also a potent growth inhibitor and immunosuppressive agent, with potential clinical applications in the treatment of both proliferative and autoimmune disorders [1]. In lung, TGF-β is a potent inhibitor of cell-cycle progression in epithelial cells [8], inhibits embryonic lung-branching morphogenesis and n-*myc* expression [9] and is strongly implicated in the aetiology of pulmonary fibrosis [10]. However, as with mesenchymal cell mitogenesis, the specific mechanisms by which TGF-β exerts these pleiotropic effects remain to be completely elucidated.

All three of the major TGF-β receptor types have been cloned [11–13]. The type-III receptor [13–15], TGF-β-IIIR or betaglycan, is a transmembrane proteoglycan with a molecular mass greater than 250 kDa which contains a short C-terminal cytoplasmic tail that is not expected to participate directly in TGF-β signalling. Binding TGF-β through its extracellular protein core rather than through its glycosaminglycan chains, the type-III receptor appears to function by presenting TGF-β to the type-II receptor (TGF-β-IIR) via physical interaction which induces a higher affinity for TGF-β in TGF-β-IIR [14,15]. Antibodies directed against the C-terminus of TGF-β-IIR immunoprecipitate ^{125}I-TGF-β-affinity-labelled TGF-β-IIIR and TGF-β-IIR, without co-precipitation of the type-I receptor (TGF-β-IR), demonstrating the presence of heterotypic TGF-β-I and IIR receptor complexes [14,15].

TGF-β-IR and TGF-β-IIR share a common overall molecular structure with sequence similarity in the extracellular cysteine-

Abbreviations used: TGF-β, transforming growth factor β; TGF-β-IR, TGF-β type-I receptor; PAI-1, plasminogen activator inhibitor; TGF-β-IIR-CT, C-terminus of TGF-β-IIR; TGF-β-IIR-NT, N-terminus of TGF-β-IIR; RA, retinoic acid; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; DTT, dithiothreitol.

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rich domains and the intracellular serine/threonine-specific protein kinase domains [11]. TGF-β-IR exhibits a molecular mass of approx. 50–60 kDa, whereas TGF-β-IIR exhibits one of approx. 75–80 kDa, the size difference being attributed to extensions at the N- and C-termini of mature TGF-β-IIR [12]. Both receptors are involved in signalling through their intrinsic serine/threonine kinase activity, as has been shown directly for TGF-β-IIR using single-residue mutagenesis in the kinase domain [16]. Moreover, TGF-β-IR requires TGF-β-IIR in order to bind ligand, and TGF-β-IR and TGF-β-IIR interact physically in hetero-oligomeric complexes [16,17]. However, hetero-oligomeric receptor complexes have been observed in the absence of ligand, and type-II receptors can also form stable homodimers in a variety of mammalian cells. Thus, although the functional specificities of the TGF-β-IR–TGF-β-IIR and TGF-β-IIR–TGF-β-IIR complexes remain to be further characterized, it appears that TGF-β-IIR plays a pivotal role in mediating TGF-β binding and in orchestrating TGF-β responses.

Both TGF-β-IR and TGF-β-IIR mediate biological responses by mechanisms involving their intrinsic kinase activities. Indeed, the type-I receptor is transphosphorylated and activated by the type-II receptor. Moreover, overexpression of dominant-negative TGF-β-IIR mutants lacking the cytoplasmic signalling kinase domain results in nearly complete loss of TGF-β-dependent growth inhibition but does not affect TGF-β-dependent stimulation of the synthesis of fibronectin, plasminogen activator inhibitor (PAI-1) and oncogene mRNA [18]. Thus under some circumstances receptor-specific signalling can occur, growth inhibition being transduced by TGF-β-IIR and matrix gene regulation by TGF-β-IR.

For the present study we prepared anti-peptide antibodies directed against the C-terminus (anti-TGF-β-IIR-CT) and N-terminus (anti-TGF-β-IIR-NT) of TGF-β-IIR, peptide motifs that are essentially missing from TGF-β-IR. These antibodies were utilized to characterize further the enzymology of TGF-β-IIR and to investigate further the endogenous receptor complexes in a variety of cells. Moreover, the N-terminal (anti-TGF-β-IIR-NT) antibody exhibited the unique property of blocking TGF-β binding to TGF-β-IR without disrupting the binding of TGF-β to TGF-β-IIR, thus identifying a putative interaction domain and a strategic mechanism by which cellular responsiveness to TGF-β can be altered.

EXPERIMENTAL

Cell cultures

Human MG-63 and TE-85 osteosarcoma cells and human WI-38 diploid fibroblasts were obtained from the American Type Culture Collection and maintained as monolayers in exponential-phase growth in RPMI 1640 media supplemented with 10 % fetal calf serum. Primary cultures of differentiated rabbit articular chondrocytes and secondary cultures of retinoic acid (RA)-modulated rabbit chondrocytes were prepared as described previously [19]. RA-modulated chondrocytes no longer express the differentiated chondrocyte phenotype characterized by type-II collagen synthesis, but re-express this phenotype in response to TGF-β1 under serum-free conditions.

Reagents

TGF-β1 and ^{125}I-TGF-β1 (152 μCi/μg) were obtained from R & D Systems (Minneapolis, MN, U.S.A.) and DuPont–NEN (Boston, MA, U.S.A.) respectively. Digitonin was purchased from Sigma (St. Louis, MO, U.S.A.), dissolved at 10 mg/ml in water, heated at 55 °C for 30 min, cooled on ice for 2 h, and centrifuged at 10000 ***g*** at 4 °C for 5 min before use of the clarified supernatant at an assumed concentration of 10 mg/ml.

TGF-β-IIR-specific antibodies

Two different rabbit polyclonal antibodies selective for TGF-β-IIR were utilized in these studies. The anti-TGF-β-IIR-NT antibody is directed against a 28-residue synthetic peptide (IPPHVQKSVNNDMIVTDNNGAVKFPQLC) corresponding to amino acids 24–51 contained within the N-terminal (extracellular) domain of mature (proteolytically processed) TGF-β-IIR [12]. The anti-TGF-β-IIR-CT antibody is directed against a 16-residue synthetic peptide (CSEEKIPEDGSLNTTK) corresponding to sequences 550–565 of the deduced primary structure [12]. The synthetic peptides were coupled to activated keyhole limpet haemocyanin via terminal cysteine residues before immunization and production of anti-peptide antibodies in rabbits. IgG fractions of high-titre sera (ELISA) were prepared by Protein A chromatography; affinity-purified antibodies were prepared by column chromatography, utilizing the immunizing peptide coupled to Affigel-10 (Bio-Rad) as an affinity matrix.

Metabolic labelling and immunochemical methods

For ^{35}S protein labelling, cultured TE-85 and MG-63 osteosarcoma cells were washed twice with prewarmed labelling medium [methionine-free cysteine-free Dulbecco's modified Eagle's medium (DMEM); ICN]. After a 30 min incubation in the labelling medium, [^{35}S]Met/Cys (Tran ^{35}S-label; ICN) was added to the medium ($\sim$ 200 μCi/ml) and incubation was continued for 4 h before cell lysis [20], immunoprecipitation, SDS/PAGE, impregnation of the gel with autoradiographic enhancers (Amersham) for 30 min and analysis by autoradiography at $-$70 °C. Western-blot analysis of detergent lysates, specific immunoprecipitations and *in vitro* kinase assays were performed essentially as described previously [20].

Affinity cross-linking of ^{125}I-TGF-β

Affinity cross-linking of ^{125}I-TGF-β to TGF-β receptors with disuccinimidyl suberate (DSS; Pierce) was performed as described [21], with the modification that cytosol was extracted by digitonin permeabilization before membrane solubilization. Briefly, confluent cultures of TE-85 cells, differentiated chondrocytes and RA-modulated chondrocytes were washed in unsupplemented DMEM for 30–60 min at 37 °C to decrease endogenous TGF-β, and then briefly washed at 4 °C with binding buffer 2 (BB2: DMEM, high glucose, 25 mM Hepes, pH 7.5, without bicarbonate, osmolality adjusted with NaCl/0.2 % BSA) before a 3 h incubation at 4 °C with 40 pM ^{125}I-TGF-β in BB2. Specificity of binding was demonstrated by a 3 h preincubation with a 100-fold excess of unlabelled TGF-β. Cells were washed twice with binding buffer 1 (BB1: 128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM Hepes, pH 7.5, 0.2 % BSA) and twice with BB1 ($-$BSA) before cross-linking with 0.27 mM DSS in BB1 ($-$BSA) for 15 min at 4 °C. Cross-linking was quenched by washing twice for 5 min with ethanolamine in CSK buffer (10 mM Hepes, pH 6.8, 3 mM $MgCl_2$, 150 mM NaCl and 1 mM EGTA). Cells were permeabilized and extracted twice for 5 min each with CSK buffer containing 200 μg/ml digitonin and the following protease inhibitor mixture (10 μg/ml leupeptin, 50 μg/ml aprotinin, 100 μg/ml soyabean trypsin inhibitor, 100 μg/ml benzamidine hydrochloride and 300 μM PMSF). Dishes were washed and cell fragments collected by scraping into CSK buffer containing protease inhibitors. After centrifugation at 1310 ***g*** for 2 min, membrane proteins and TGF-β receptors

were solubilized from the pellets by extraction for 20 min at 4 °C with 200 μl of 0.5% Triton X-100 in CSK buffer containing protease inhibitors. Extracts were microfuged at 16000 ***g*** for 10 min and the supernatants were used for immunoprecipitation or analysed directly by adjustment to 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, boiling for 5 min and SDS/PAGE on 7% gels.

Immunoblocking and precipitation of affinity-labelled receptors

In vitro tests for binding of anti-TGF-β-IIR-NT antibody were performed by preincubating cultured cells in BB2 containing 40 μl of anti-TGF-β-IIR-NT serum for 3 h at 4 °C before addition of ^{125}I-TGF-β and affinity cross-linking. Identification of TGF-β-IR by sensitivity to reduction [13,22] was performed by dissolving 50 mM dithiothreitol (DTT) in freshly degassed BB1, diluting to 1 mM in degassed BB1 previously equilibrated to 37 °C, and preincubating cultures in this solution for 5 min at 37 °C before washing at 4 °C with BB2 and incubation with ^{125}I-TGF-β. For immunoprecipitation, Protein A–Sepharose beads were washed in 0.1 M borate, pH 8.2, preloaded with CT, NT, or non-immune serum for 30 min at room temperature (100 μl/200 μl of beads in 500 μl of borate buffer) and washed free of antibody in borate buffer. Loaded Protein A–Sepharose beads were then washed and held for 1 h in 1% polyvinyl pyrrolidone 40/0.1% Tween 20 in Tris-buffered saline, held for 30 min in CSK buffer containing 0.5% Triton X-100, and pelleted before addition of Triton extracts. Each extract (200 μl) was immunoprecipitated by rotation for 18 h at 4 °C with 30 μl of packed preloaded beads. Supernatants containing unbound material were adjusted to SDS/PAGE conditions (as above) and denatured. Immunoprecipitates were washed three times with CSK buffer containing 0.15% Triton X-100 and protease inhibitors, suspended in 200 μl of SDS sample buffer, and boiled for 2 min before SDS/PAGE (7% gels).

Assay of casein kinase activity

The casein kinase activity of anti-TGF-β-IIR-NT and -CT antibody immunoprecipitates from protein lysates of TE-85 cells at 80% confluence was assayed as described previously [20,23] using dephosphocasein (100 μg/ml) as the substrate.

Measurement of TGF-β activity by PAI-1-promoter-activated luciferase

Mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to firefly luciferase as a reporter gene respond to the presence of TGF-β1 at pg/ml concentrations by increasing the activity of luciferase in a dose-dependent manner [24]. These cells were plated at 1.6×10^4 cells per well of a 96-well plate and allowed to attach for 3 h in the presence of 10% fetal bovine serum in DMEM. The serum-containing medium was then removed and the cells washed with DMEM. A series of standard TGF-β1 concentrations from 5 to 500 pg/ml diluted in DMEM were then added to the cells and incubated for 20 h. The cells were then lysed and luciferase activity in the lysates was measured using a kit obtained from Analytical Luminescence Laboratories, Ann Arbor, MI, U.S.A. using a scintillation spectrometer equipped with a single-photon monitor.

Measurement of [^{3}H]thymidine uptake into DNA in cultured cells

Mink lung epithelial cells were plated in 24-well plates at 1×10^5–2×10^5 cells per well and grown in DMEM in the presence of [^{3}H]thymidine (1 μCi/well) for 6–8 h. The unincorporated thymidine was removed with two washes of PBS. The cells were then washed three times in ice-cold 5% trichloroacetic acid and lysed with 1 M NaOH. Radioactivity in the cell lysates was measured after neutralization with 1 M acetic acid using a scintillation spectrometer.

RESULTS

Characterization and application of anti-TGF-β-IIR antibodies

Initial characterization of the affinity-purified antisera by Western blotting of detergent lysates obtained from human osteosarcoma cells, Ewing's sarcoma cells and normal diploid fibroblasts (Figure 1) revealed multiple forms of TGF-β-IIR (about 75–80 kDa), which differed slightly in electrophoretic mobility. As shown in Figure 1(C), cross-reactivity with the rabbit type-II receptor(s) was observed with both the N-terminal (anti-TGF-β-IIR-NT)- and C-terminal (anti-TGF-β-IIR-CT)-specific antibodies. The detection of multiple receptor bands in both species and by antibodies recognizing two distinct domains suggests that multiple forms of the type-II receptor are present [22]. Immunoreactivity toward native receptors was demonstrated for both the TGF-β-IIR-NT and TGF-β-IIR-CT antibodies by immunoprecipitation of detergent lysates from TE-85 osteosarcoma cells metabolically labelled with [^{35}S]methionine. A prominent band of the expected size for the type-II receptor was detected by fluorography following SDS/PAGE (Figure 2A). Analysis of the anti-TGF-β-IIR-NT immune complexes by protein staining enabled the visualization of a ~75 kDa protein that was specifically precipitated by the primary antibodies (Figure 2B) and was subsequently confirmed by Western blotting to represent the type-II receptor. In addition to p75 TGF-β-IIR, two other proteins of ~110 kDa and ~250 kDa were co-precipitated with the type-II receptor (see Figure 2B), which may represent the

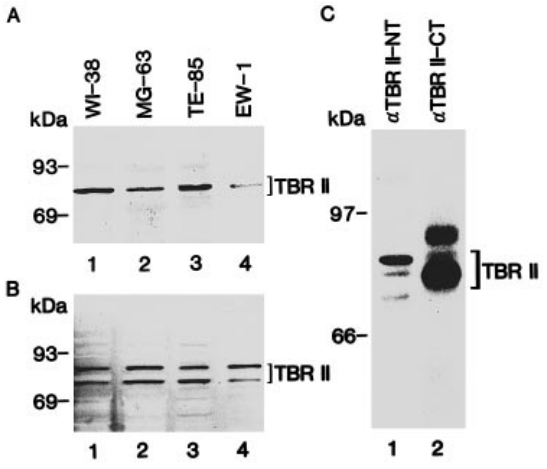

Figure 1 Western blotting of human (A and B) and rodent (C) TGF-β-IIR (TBR II) with affinity-purified anti-TGF-β-IIR antibodies directed against the N-terminal extracellular domain (αTBR II-NT) (A) or the C-terminal cytoplasmic tail (αTBR II-CT) (B) of the mature (proteolytically processed) receptor respectively in detergent lysates of human fibroblastic (WI-38), osteoblastic (MG-63 and TE-85) or primitive neuroectodermal (EW-1) cell derivatives

The gel in (C) demonstrates cross-reactivity of both antibodies with rodent (rabbit) TGF-β-IIR (TBR II). Note that several electrophoretic isoforms of the receptor are revealed by these antibodies.

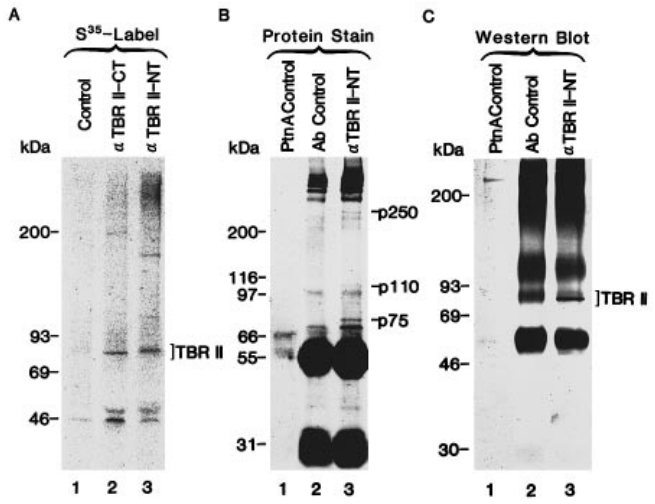

Figure 2 Immunoprecipitation of native TGF-β-IIR by anti-TGF-β-IIR-NT (αTBR II-NT) and anti-TGF-β-IIR-CT (αTBR II-CT) antibodies

(**A**) Both the αTBR II-CT and αTBR II-NT antibodies specifically immunoprecipitate the 75 kDa TGF-β-IIR from [^{35}S]methionine-labelled cell lysates. (**B**) Coomassie-Blue-stained immunoprecipitates from lysates of TE-85 cells demonstrating the specific appearance of TGF-β-IIR and two associated proteins, p100 and p250. (**C**) Western blot of the immunoprecipitate from (**B**) confirming the identity of TGF-β-IIR (TBR II); the high background that partially obscures the immunoreactive band in this blot is due to reactivity with the original immunoprecipitating IgGs. Ptn A Control, Protein A control; Ab Control, a preabsorbed antibody control.

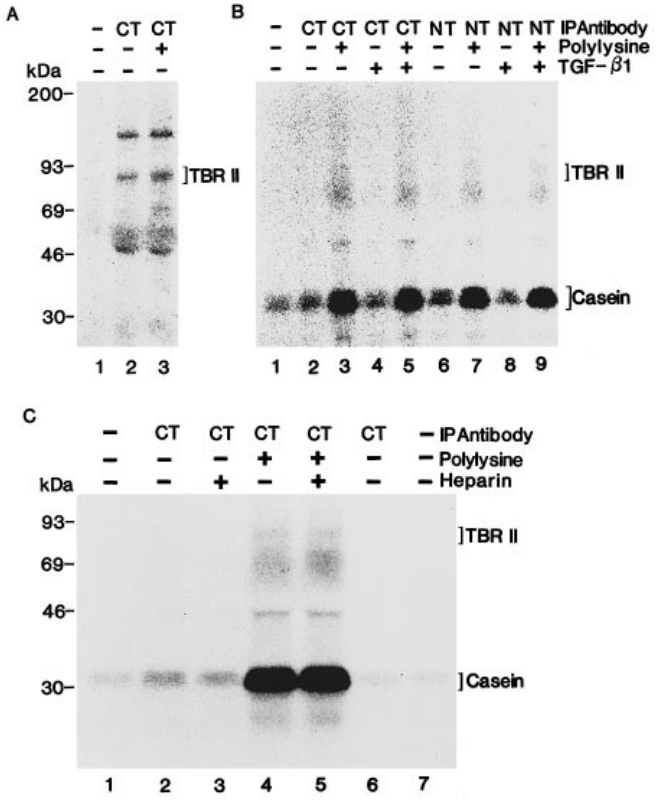

Figure 3 Autophosphorylation and phosphotransferase activity of immunoprecipitated TGF-β-IIR

(**A**) Immunoprecipitates with TGF-β-IIR-CT antibody demonstrate a 75 kDa TGF-β-IIR (TBR II) autophosphorylated band, autophosphorylation of which is somewhat enhanced by the presence of polylysine (50 μg/ml). (**B**) Phosphotransferase activity detected with dephosphocasein (50 μg/ml) as substrate: immunoprecipitates using both TGF-β-IIR-CT and TGF-β-IIR-NT antibodies were exposed to kinase assay conditions in the presence of polylysine (50 μg/ml) and/or TGF-β1 (20 μg/ml) and detected by SDS/PAGE and autoradiography. Phosphotransferase activity toward casein was markedly enhanced in the presence of polylysine. (**C**) Casein kinase assays as in (**B**) conducted in the presence of heparin (50 μg/ml) as a classical casein kinase II inhibitor. Heparin did not inhibit either basal or polylysine-stimulated casein kinase activity.

core protein and the modified (betaglycan) form of the type-III receptor.

Detection of receptor autophosphorylation and intrinsic casein kinase activity

Immunoprecipitation experiments followed by *in vitro* kinase assays demonstrated low but appreciable levels of receptor autophosphorylation, as determined by SDS/PAGE and autoradiography (Figure 3A). The kinase activity associated with the immunoprecipitated type-II receptor was capable of utilizing casein as a substrate and was found to be stimulated by polylysine (Figure 3B). The polylysine-stimulated casein kinase activity was specifically precipitated by both anti-TGF-β-IIR-CT and anti-TGF-β-IIR-NT antibodies, further demonstrating that the observed kinase activity was intrinsic to and/or associated with TGF-β-IIR. TGF-β-dependent stimulation of casein kinase activity *in vitro* was not observed in either anti-TGF-β-IIR-CT or anti-TGF-β-IIR-NT immune complexes under these conditions. As shown in Figure 3(C), the intrinsic casein kinase activity associated with the precipitated type-II receptor was not inhibited by heparin at concentrations (50 μg/ml) that effectively abolished cellular casein kinase II activity, as assayed in control reactions. Moreover, the polylysine-stimulated activity was not inhibited by heparin, as has been reported for the TGF-β type-V receptor [25,26].

Characterization of endogenous receptor complexes by affinity cross-linking and immunoprecipitation

A demonstration that the TGF-β-IIR-CT antibodies bind to native TGF-β-IIR was provided by immunoprecipitation of ^{125}I-TGF-β affinity-cross-linked receptors (Figure 4). In this study, differentiated rabbit chondrocytes in primary culture were labelled at 4 °C for 3 h with 40 pM ^{125}I-TGF-β, cross-linked with 0.27 mM DSS, quenched with ethanolamine, permeabilized with digitonin-containing protease inhibitors, scraped off, and the membrane fraction solubilized with 0.5% Triton X-100 for 30 min before SDS/PAGE on 7% gels. Direct analysis of the Triton lysates demonstrates the presence of the three major receptors, type I, II, III/betaglycan with appropriate mobility (Figure 4A, lane 2). Specificity of labelling is demonstrated by the absence of labelled bands from control samples pretreated for 3 h at 4 °C with a 100-fold excess of unlabelled TGF-β (Figure 4A, lane 1). Immunoprecipitation for 18 h at 4 °C with the cytoplasmic domain TGF-β-IIR-CT antibody prebound to Protein A–Sepharose resulted in essentially complete recovery of receptors I and II in the bound fraction and depletion from the free (unbound) fraction (Figure 4A, lanes 4 and 3 respectively). Specificity of the labelling and the immunoprecipitation was further demonstrated by the absence of bands in immunoprecipitates from cells pretreated with unlabelled TGF-β (Figure 4A, lane 5). In contrast with these results with TGF-β-IIR-CT, immunoprecipitations of rabbit chondrocytes with the extracellular domain TGF-β-IIR-NT antibody, did not contain affinity-labelled receptors, as the cross-linked receptors remained

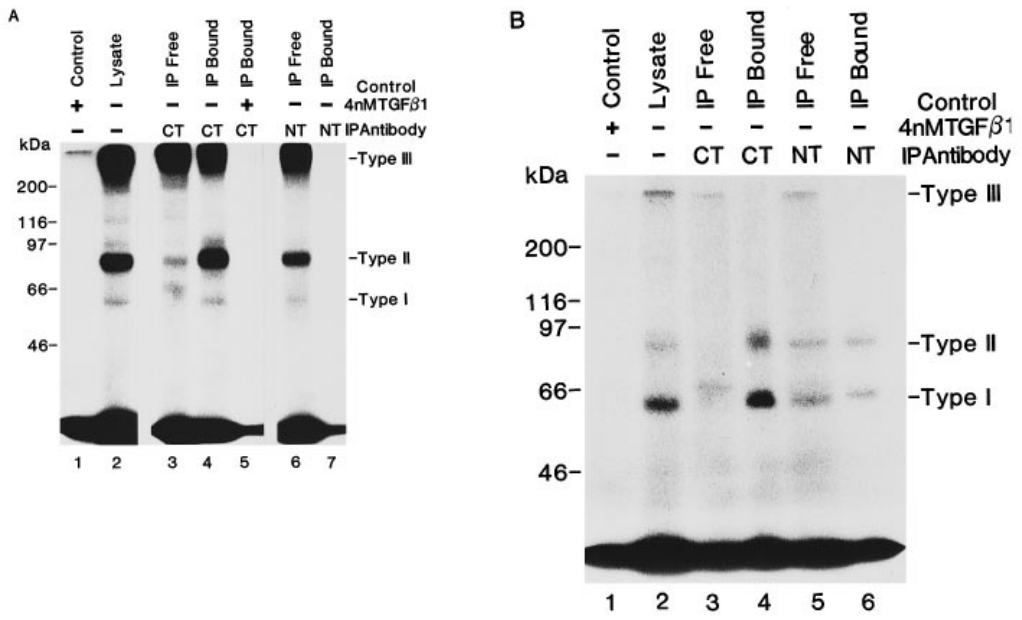

Figure 4 Immunoprecipitation of ^{125}I-TGF-β-affinity-cross-linked receptors from differentiated (RA-modulated) rabbit chondrocytes (A) and human osteosarcoma cells, TE-85 (B)

Confluent cells were pretreated with a 100-fold excess of unlabelled TGF-β1 (control) or incubated directly with 40 pM ^{125}I-TGF-β1 for 3 h at 4 °C before affinity-cross-linking with 0.27 mM DSS. Quenched, washed and digitonin-permeabilized cells were scraped off, and receptors were solubilized in the presence of protease inhibitors with 0.5% CSK buffer (lysate), before immunoprecipitation with Protein A–Sepharose beads preloaded with the C-terminal TGF-β-IIR-CT antibody or the N-terminal TGF-βIIR-NT antibody. Equal fractions of unbound material (IP Free) and bound material (IP Bound) were fractionated by SDS/PAGE (7% gels) and submitted to autoradiography for 6 days.

in the free fraction (lane 6). This was the same result as obtained with immunoprecipitation using non-immune antisera (not shown), and demonstrates that no affinity-labelled receptor is non-specifically bound during these immunoprecipitations. The lack of immunoprecipitation of affinity-labelled receptors by the TGF-β-IIR-NT antibody is due to either (1) a decreased spectrum of epitopes in the rabbit receptor resulting from differences in the primary sequence or (2) decreased epitope reactivity in general caused by amino group modification during cross-linking with DSS.

We have further clarified these findings by affinity labelling TGF-β receptors in the human osteosarcoma cell line HOS, TE-85 (Figure 4B). The results are comparable with those for rabbit chondrocytes with the exception that TGF-β-IIR-NT was capable of partial immunoprecipitation of TGF-β-IR and -IIR species from human osteosarcoma cells (Figure 4B, lanes 5 and 6). Whereas TGF-β-IIR-CT antibody efficiently precipitates native TGF-β-IIR and its associated proteins from both human and rabbit cells (compare Figures 4A and 4B), the TGF-β-IIR-NT antibody appears to immunoprecipitate only the human cross-linked receptors. Thus some uncross-linked epitopes appear to be present in the human TGF-β-IIR that are missing from the rabbit TGF-β-IIR. Immunoprecipitation of TGF-β-IR and -IIIR with antibodies raised against two distinct domains of TGF-β-IIR requires their direct or indirect physical association with the type-II receptor. These interactions must be strong enough to resist dissociation with 0.5% Triton X-100. The immunoprecipitation of all three receptors by antibodies to epitope-tagged expressed recombinant type-II receptor has previously been explained by the existence of separate binary complexes of receptors I and II and III and II, with receptor II being common to both. Although the present data are consistent with the existence of a ternary complex, it does not provide definitive support for this level of organization.

Inhibition of type-I receptor affinity labelling by preincubation of cultured cells with anti-TGF-β-IIR-NT antibodies

We then used the N-terminal extracellular domain antibody, anti-TGF-β-IIR-NT, to investigate further the ligand binding of the type-II receptor and the nature of this receptor's complexes with the type-I and -III receptors. RA-modulated chondrocytes were incubated for 3 h at 4 °C in the presence or absence of anti-TGF-β-IIR-NT, and then ^{125}I-TGF-β was added for a further 3 h in preparation for affinity cross-linking as described above. Autoradiographs of SDS/polyacrylamide gels revealing the cross-linked receptors are presented in Figure 5. When added in subsequent immunoprecipitations, anti-TGF-β-IIR-CT bound the native type-II receptor in the absence of potential steric hindrance by TGF-β ligand and in the absence of possible cross-linking-dependent modification of antigenic determinants. Only after equilibrium binding of TGF-β-IIR-NT were ^{125}I-TGF-β and DSS introduced to the cells. Direct analysis of Triton lysates demonstrated the presence of all three affinity-labelled receptors (Figure 5, lane 2). When chondrocytes were pretreated with the anti-TGF-β-IIR-NT antibody, lysate analysis showed a marked reduction in the proportion of labelling of the type-I receptor, and a consistent increase in the proportion of labelling of the type-III receptor, in relation to the labelling of the type-II receptor. These data indicate that binding of anti-TGF-β-IIR-NT to the N-terminal domain of the type-II receptor does not block the TGF-β-binding site of the receptor. Moreover, anti-TGF-β-IIR-NT binding prevents subsequent binding of TGF-β to the type-I receptor. Since TGF-β binding to the type-I receptor

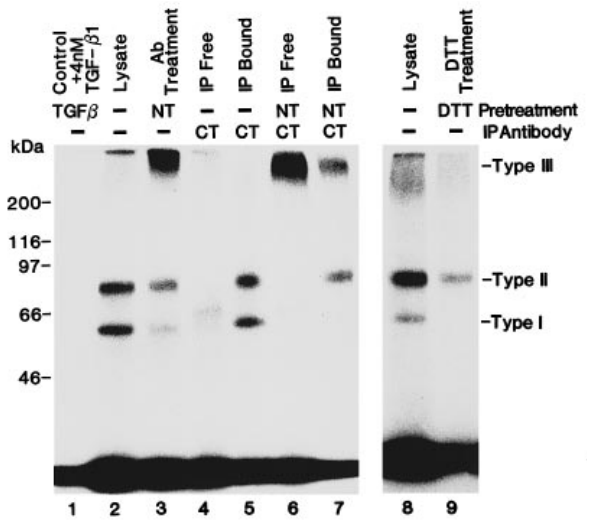

Figure 5 Blocking of TGF-β1 binding to the type-I receptor with the N-terminal-specific antibody to the type-II receptor (NT)

RA-modulated chondrocytes were pretreated for 3 h at 4 °C with TGF-β-IIR-NT antibody (NT) before affinity labelling with ^{125}I-TGF-β and cross-linking. In some instances lysates were immunoprecipitated with the C-terminal TGF-β-IIR-CT antibody. The identity of the type-I receptor band was confirmed by its sensitivity to brief reduction with DTT before ligand exposure. Pretreatment of cultured chondrocytes with anti-TGF-β-IIR-NT (extracellular domain) antibodies before ^{125}I-TGF-β exposure and cross-linking selectively blocked the ability of the type-I receptor to bind TGF-β, increased labelling of the type-III receptor and slightly reduced binding of radiolabelled TGF-β to the type-II receptor (lanes 3 and 7 versus lanes 2 and 5).

requires the receptor's interaction with a ligand-bound type-II receptor [16], the most likely explanation of the results is one of the following: (i) that TGF-β-IIR-NT binding sterically blocks the domain of the type-II receptor that is necessary for interaction with the type-I receptor; (ii) that TGF-β-IIR-NT binding distorts the conformation of such an interaction domain; (iii) that TGF-β-IIR-NT sterically blocks the type-I receptor from binding to part of the type-II receptor-bound TGF-β ligand.

Immunoprecipitation of the Triton lysates with the cytoplasmic domain antibody, anti-TGF-β-IIR-CT, efficiently precipitated receptors I and II (Figure 5, lane 5). When anti-TGF-β-IIR-NT was bound before affinity labelling and immunoprecipitation with anti-TGF-β-IIR-CT, immunoprecipitates showed essentially the same changes as observed in the whole cell lysates (Figure 5, lane 7). Interestingly, the increased binding to the type-III receptor is detected in both the free (unbound) fraction and the type-II receptor immune complexes. The simultaneous increase in type-III receptor labelling along with the observed decrease in type-I receptor labelling in the anti-TGF-β-IIR-CT immunoprecipitates suggests that the type-III receptor may present TGF-β ligand to the type-I/type-II receptor complexes as well as to the type-II receptors. Thus blocking the ability of receptor I to bind receptor II with TGF-β-IIR-NT may leave the TGF-β destined for the type-I receptor associated with the type-III–II complexes.

TGF-β-IIR-NT antibodies block TGF-β1 activation of PAI-1 promoter

TGF-β1 activated the PAI-1 promoter in mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene [24] at concentrations of TGF-β1 as low as 5 pg/ml (Figure

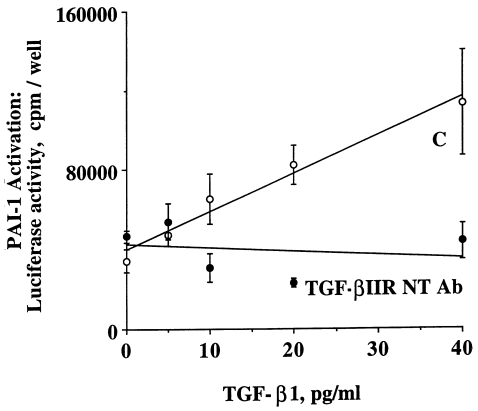

Figure 6 Blocking effects of TGF-β-IIR NT blocking antibodies (Ab) on PAI-1 promoter–luciferase activation by TGF-β1 in mink lung epithelial cells

TGF-β-IIR-NT blocking antibodies completely inhibited PAI-1 promoter luciferase activation by TGF-β1 in mink lung epithelial cells stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase gene.

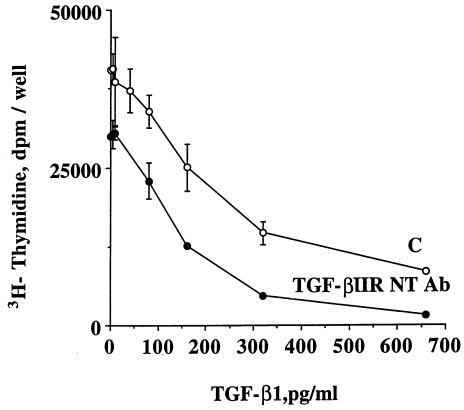

Figure 7 TGF-β-IIR-NT antibodies do not inhibit the negative effects of TGF-β1 on thymidine incorporation into DNA by mink lung epithelial cells

Although the resulting dose–response curves are somewhat offset, TGF-β1 inhibited the amount of thymidine incorporated into DNA in both the presence and absence of TGF-β-IIR-NT antibodies.

6). Moreover, the activation curve was linear up to 500 pg/ml TGF-β1 (not shown). Preincubation of the mink lung epithelial cells in the presence of 20 μg/ml affinity-purified TGF-β-IIR-NT antibodies for 1 h completely blocked activation of the PAI-1 promoter by TGF-β1 up to 40 pg/ml, whereas preincubation with non-immune IgG had no effect on PAI-1 promoter activation by TGF-β1.

TGF-β-IIR-NT antibodies do not block TGF-β1 inhibition of thymidine incorporation into DNA

TGF-β1 inhibited the amount of thymidine incorporated into DNA of mink lung epithelial cells by 75 % at a concentration of 650 pg/ml (IC_{50}, 175 pg/ml). Preincubation of mink lung epithelial cells in the presence of 20 μg/ml affinity-purified TGF-β-IIR-NT antibodies for 1 h resulted in an appreciable shift of the concentration–effect curve origin, reducing thymidine incorporation into DNA of mink lung epithelial cells by 25 % (Figure

7). However, the IC_{50} for TGF-β1 was 175 pg/ml in both the presence and absence of TGF-β-IIR-NT antibodies, whereas addition of TGF-β1 in the presence of anti-TGF-β-IIR-NT blocking antibodies resulted in more than 95% reduction in thymidine incorporation into DNA at 650 pg/ml. Preincubation with non-immune IgG had no effect on thymidine incorporation into DNA.

DISCUSSION

The cloning and expression of recombinant TGF-β receptors in mammalian somatic cells has provided major advances in our understanding of receptor interactions and signalling requirements, demonstrating co-operativity of TGF-β-IIIR–TGF-β-IIR complexes [14,15,27–29], the requirement for TGF-β-IIR for TGF-β-IR function [16,30–33] and the importance of intrinsic kinase domains within the signalling receptors [34–36]. Interpretations of receptor overexpression studies in physiological systems, however, are tempered by the likelihood of consumption of co-operating receptors, the titration of receptor-associated proteins and/or the impairment of receptor transport to the cell surface [37]. The present study demonstrates the formation of hetero-oligomeric receptor complexes in normal as well as neoplastic mesenchymal cells and provides additional insights into the biochemistry of the TGF-β-IIR.

The multiple forms of the type-II receptor observed here may reflect specific covalent modifications, proteolytic processing and/or expression of closely related isoforms that merit further characterization. The identification of TGF-β-IIR intrinsic casein kinase activity and the demonstration that TGF-β-IIR-associated casein kinase activity is stimulated by polyanions is interesting in that these are among the biochemical properties ascribed to the newly identified TGF-β type-V receptor [25,26]. However, in contrast with the type-V receptor, no stimulation of either TGF-β-IIR autophosphorylation or casein phosphorylation in the presence of added TGF-β was observed under our assay conditions. Nevertheless, the activation of TGF-β-IIR-associated casein kinase activity, as well as the identification of an exogenous substrate, should facilitate the biochemical characterization of this important signalling receptor/kinase.

TGF-β-mediated transcriptional and antiproliferative responses are mediated through heteromeric signalling complexes comprised of two TGF-β type-1 and -II receptors in each signalling complex of four interacting receptors [38,39]. TGF-β1 and β2 ligands bind to these receptors in different ways: TGF-β1 binds directly to TGF-β-IIR, whereas TGF-β2 binds co-operatively to TGF-β-IIR, having first bound either TGF-β-IR or -IIIR [33]. Transphosphorylation of TGF-β-IR by the TGF-β-IIR serine/threonine kinase results in TGF-β-IR activation which activates signalling [36]. Phosphorylation of serines in the GS domain of TGF-βIR is particularly important in this signalling mechanism [40]; a point proline-525 to leucine mutation in the TGF-β-IIR kinase subdomain results in a receptor that binds TGF-β ligand normally, but fails to recruit and activate TGF-β-IR in the signalling complex. Differential activation of transcriptional and growth-inhibitory responses to TGF-β ligands may then be specified by which TGF-β-IR is present in the signalling complex, such as the R4 subtype [41–44].

On the other hand, in cells expressing truncated kinase-deficient dominant negative TGF-β-IIR mutants, two separate TGF-β signalling pathways have been identified [18]. In the second system, TGF-β-IIR, possibly in conjunction with TGF-β-IR, mediates growth inhibition and hypophosphorylation of the retinoblastoma gene product, whereas TGF-β-IR is responsible for effects on extracellular matrix, such as the induction of the PAI-1 and fibronectin genes [18]. The results reported here support the latter concept: an anti-TGF-β-IIR-NT antibody differentially blocked PAI-1 promoter activation but not the negative regulation of thymidine incorporation in response to TGF-β1 peptide ligand signalling. Moreover, radioligand-binding studies in the presence of this antibody specifically displaced ligand from TGF-β-IR but not from TGFβ-IIR or -IIIR. However, further studies will be needed to determine definitively the effects of the TGF-β-IIR-NT antibody on the assembly of TGF-β signalling complexes.

Using double-receptor mutants of the Mv1Lu mink lung epithelial cell line, which exhibit no binding to either TGF-β-IR or TGF-β-IIR, Wrana et al. [16] demonstrated the rescue of endogenous TGF-β-IR binding by expression of epitope-tagged TGF-β-IIR. In addition, mutant cells expressing epitope-tagged TGF-β-IIR were capable of TGF-β-dependent growth inhibition, matrix synthesis and gene expression, whereas those expressing a kinase-defective construct of TGF-β-IIR were unable to do so, even though ligand binding was demonstrably restored to the endogenous TGF-β-IR. Thus in this cell system, interaction with functional TGF-β-IIR is required for TGF-β binding to TGF-β-IR, and signalling for all tested effects required the intrinsic kinase activity of TGF-β-IIR. In contrast with these results are those derived from stable transfection of Mv1Lu cells with a dominant negative TGF-β-IIR mutant lacking the cytoplasmic kinase domain which results in the down-regulation or elimination of endogenous TGF-β-IIR kinase activity without alteration of ligand binding to TGF-β-IR. Under these conditions, there is a nearly complete loss of the characteristic growth inhibition. However, TGF-β-dependent stimulation of fibronectin, PAI-1 and oncogene expression were not attenuated [18]. Thus, under some circumstances, receptor-complex-specific signalling occurs, with growth inhibition attributable primarily to TGF-β-IIR and matrix synthesis/gene expression associated with TGF-β-IR.

In the light of our present findings this latter concept is particularly germane. For, in addition to demonstrating that endogenous TGF-β-IIR associates in stable complexes with both TGF-β-IR and TGF-β-IIIR, the antibody directed against a particular extracellular segment of TGF-β-IIR effectively disrupted the physical association of TGF-β-IIR with TGF-β-IR without disrupting the ability of TGF-β to bind to (and presumably activate) the type-II receptor. This property of the antibody provides additional support for the emerging concept that molecular signalling through TGF-β-IR requires physical interaction with TGF-β-IIR [14,16]. Moreover, the ability of this particular antibody [strategically designed to recognize an epitope(s) remote from the TGF-β-IIR ligand-binding domain] to selectively block TGF-β binding to and activation of TGF-β-IR, without blocking TGF-β-IIR itself, provides a powerful pharmacological approach to the investigation of mechanisms of receptor–receptor interaction and the exploration of the differential signal-transduction pathways emanating from the different 'signalling' receptor complexes. The present study demonstrates the potential utility of anti-TGF-β-IIR-NT antibodies as a pharmacological tool for modulating specific TGF-β–receptor complexes and as a potential method of altering cellular responses. The molecular mechanism of action of these antibodies is currently unknown (steric hindrance or identification of a specific binding domain, for example). Future studies will address these questions and will further address the usefulness of strategic immunochemicals in modulating the TGF-β–receptor complexes that mediate cellular signalling responses.

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