

Chimeric extracellular domain of type II transforming growth factor (TGF)- β receptor fused to the Fc region of human immunoglobulin as a TGF- β antagonist

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Transforming growth factor- β (TGF- β) type-I and type-II receptors form a ligand-dependent heteromeric signalling complexes, in which transforming growth factor- β receptor type II (T β RII) trends to act as the primary receptor. In the present study, we used a chimeric soluble type-II receptor fused with the Fc regions of human immunoglobulin (T β RIIs-Fc) in order to obtain a putative TGF- β antagonist. Biochemical studies revealed that T β RIIs-Fc shared the same properties as the wild-type receptor. The T β RIIs-Fc receptor displayed an affinity of 1370 ± 363 pM which was similar to those of the wild-type T β RII when expressed alone in Cos-1 cells (1122 ± 413 pM). Furthermore, the chimeric receptor showed the same selectivity for TGF- β isoforms as the native receptor. Although both TGF- β 1 and TGF- β 3 were able to bind T β RIIs-Fc, TGF- β 2 could not compete with the binding of TGF- β 1 to T β RIIs-Fc. It was noted that this type of fused Fc receptor could be used in FlashPlate screening for potent agonism and antagonism of TGF β . Moreover, biological activities of the chimeric receptor showed it to be a potent TGF- β 1-antiproliferative and TGF- β 1-extracellular matrix transcriptional inhibitor on responses in Mv1Lu cells. To conclude, our results clearly show that the T β RIIs-Fc chimeric receptor could be used as a potent TGF- β antagonist. These data raised the possibility that this T β RIIs-Fc construct might act successfully as an antagonist of both TGF- β 1 and TGF- β 3 *in vivo*.

Keywords: human transforming growth factor β ; transforming growth factor β receptor; chimeric receptor; transforming growth factor β antagonist.

Transforming growth factor β 1, β 2 and β 3 (TGF- β s) are multifunctional cytokines involved in the regulation of cell proliferation, differentiation and extracellular matrix production [1–3]. Although many cultured cells will equally respond to the different TGF- β isoforms, several differences in the biological potencies of these isoforms have been reported. For example, growth of endothelial cells [4, 5] and hematopoietic cells [6, 7] is inhibited by TGF- β 1 and TGF- β 3 but not by TGF- β 2.

The recent creation of transgenic mice lacking the functional gene for TGF- β 1 [8, 9] have greatly aided our understanding of the role that TGF- β 1 plays in immunity and inflammation. Targeted disruption of the mouse TGF- β 1 gene causes defective hematopoiesis and vasculogenesis [10, 11], autoimmune [12] and excessive inflammatory responses, leading to early death. In contrast, TGF- β 1 has been shown to stimulate wound healing [13]; when applied locally to chronic or defective wounds, it accelerated their healing [14]. Recently, studies of human diseases and animal models have provided evidence for an involvement of TGF- β 1 in the pathogenesis of liver, kidney and lung diseases [15, 16], chronic inflammatory disorders such as cirrho-

sis, glomerulonephritis [17], chronic rejection [18–20] and pulmonary fibrosis [21]. Overexpression of TGF- β 1 in pancreatic cells [22], livers [23] of transgenic mice or after transfer of TGF- β 1 cDNA to rat lung [24], resulted in massive fibrosis of the targeted organs.

In mammalian cells, responses to TGF- β are mediated by type-I and type-II cell-surface receptors, which are expressed in most cell types and tissues [25]. TGF- β receptors types I and II (T β RI and T β RII, respectively) have estimated molecular masses of 53 kDa and 75 kDa, respectively, and are both endowed with serine/threonine kinase activities. Both types of receptors have been cloned [26–28]. Furthermore, these two receptor types can also coexist with other TGF- β -binding proteins such as TGF- β receptor type III (T β RIII) or betaglycan. T β RIII is the most abundant cell surface TGF- β -binding site, which does not seem to be able to transmit signals alone but is probably involved in the presentation of TGF- β to T β RI and T β RII for signalling [29–32]. Genetic evidence from TGF- β -resistant cell mutants suggests that T β RII can directly bind the ligand but is incapable of mediating TGF- β responses in the absence of the T β RI [33, 34]. TGF- β binds directly to T β RII, allowing this receptor to associate with and phosphorylate T β RI, which then propagates the signal [35] through activation of Smad2 and Smad4 heteromeric complexes [36, 37].

A better understanding of this mechanism of activation of the TGF- β receptor complexes may represent a step towards the development of TGF- β antagonist or agonist. So far, no clinically useful TGF- β agonists and/or antagonists are available. In this respect, a soluble version of the TGF- β receptor may prevent TGF- β interaction with cell-surface receptors [38, 39],

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Abbreviations. TGF- β , transforming growth factor- β ; T β RI, transforming growth factor- β receptor type I; T β RII, transforming growth factor- β receptor type II; T β RIII, transforming growth factor- β receptor type III; hT β RIIs-Fc, human soluble transforming growth factor- β type-II receptor fused with the Fc regions of human immunoglobulin; hT β RIIs, human soluble transforming growth factor- β type-II receptor.

thereby neutralising TGF- β activity. Indeed, it has been shown that the soluble extracellular domains of T β R β II [40] and T β R β III [41] are able to neutralise the proliferation inhibiting activity of TGF- β *in vitro* and that the TGF- β -binding protein, decorin, is able to antagonise the action of TGF- β in a fibrotic disease model [42, 43]. As a step towards developing such TGF- β agonist or antagonist drugs, we have investigated the possibility of using the soluble extracellular domain of T β R β II in a non-separation microplate receptor-binding assay. To facilitate this approach and increase the probability of success, we constructed a chimeric soluble receptor by fusing the extracellular domain of T β R β II to the Fc regions of human immunoglobulin (T β R β IIs-Fc). Through the Fc region, the chimeric receptor, T β R β IIs-Fc, expressed in a transiently transfected Cos-7 cell line can be easily purified by one-step protein A affinity chromatography and coated with high efficiency to a protein-A-coated microplate for a binding assay. This protein was biochemically characterised, then used to study its ability to bind ^{125} I-labelled TGF- β 1. Additionally, we successfully investigated the possibility of using the T β R β IIs-Fc protein as a TGF- β 1 antagonist for both TGF- β 1 proliferative and transcriptional activities.

EXPERIMENTAL PROCEDURES

Construction of vector. The cDNA encoding the extracellular domain of human T β R β II was amplified by PCR from the plasmid pBlueScript (Stratagene) which contained a full-length cDNA of the receptor (with minimal 5' and 3' untranslated regions) [31]. The sense primer was 5'-CCGGAATTCTCGGTC-TATGACGAGCAGCG-3' corresponding to an *Eco*R1 site (underlined) and to nucleotides 1–20 of the cDNA preceding the start codon. The antisense primer had the sequence 5'-CCGGATCCACTTACCTGTGTCAGGATTGCTGGTGTAT-3', representing nucleotides 458–477, which encoded residues Thr155–Asp159 immediately preceded by a splice donor site (bold) and a *Bam*H1 site (underlined). The standard PCR conditions, using *Pfu* DNA polymerase (Stratagene), were as follows: 92°C for 1 min, 55°C for 1 min, 72°C for 1 min for 30 cycles with a final extension at 72°C for 7 min. The resulting amplified PCR product was digested with *Eco*R1 and *Bam*H1 and ligated into the respective sites in the polylinker of the vector pIg-Tail (R&D System). The pIg-Tail expression system enables the mammalian production of fusion proteins with a C-terminal Fc tail. The truncated cDNA which was derived from PCR was sequenced [44] to confirm the fidelity of the reaction.

Transient transfection of Cos-7 cells. Cos-7 cells (American Type culture collection CRL 1651) were cultured in AIMV medium (Gibco BRL Life Technologies). All transfection into Cos-7 were performed by the calcium-phosphate precipitation method [45]. In brief, cells were seeded into 175-cm² flask at a density of 10⁶ cells/flask and transfected the following day with 80 μ g recombinant pIg-Tail vector containing the extracellular domain of the human T β R β II. The recombinant protein was expressed in the transfected cells and secreted into the medium.

Protein purification and analysis. The human recombinant protein T β R β IIs-Fc was purified by one-step protein A affinity chromatography. The tissue culture medium collected from transfected cells 4-days after transfection was centrifuged for 5 min at 3500 $\times$ g in order to recover cell-free supernatant. The supernatant was then adjusted to pH 8.2 by the addition of 1 M Tris base and applied to a HiTrap protein A column (Pharmacia Biotech) previously equilibrated with 10 mM sodium phosphate, 0.150 M NaCl, pH 7.2 (NaCl/P_i). After protein loading, the column was washed with NaCl/P_i to remove non-specifically bound proteins and the soluble hT β R β IIs-Fc was eluted by a decrease in

the pH of the elution buffer (0.1 M citric acid, pH 3). As a safety measure to preserve the activity of purified protein the pH was immediately neutralised by the addition of a 1/10 vol. 1 M Tris/HCl, pH 9.0, to the collected fractions. The eluted protein was dialysed overnight against 0.1 $\times$ NaCl/P_i and lyophilised. This protein was then analysed by 10% SDS/PAGE using cast gels (Novex) followed by silver staining (Silver Staining Kit, Novex). The amount of protein was quantified using Bio-Rad Protein Assay (microtiter plate format).

Western-blotting analysis. Recombinant protein eluted from the HiTrap protein A column was separated by 10% SDS/PAGE, then transferred to polyvinylidene difluoride (PVDF) transfer membrane (Scheicher & Schuell). After transfer, the membrane was blocked for 30 min with 10% dry fat milk in NaCl/P_i supplemented with 0.1% Tween 20. The membrane was incubated with specific polyclonal antibodies; goat anti-human T β R β II (R&D System) or goat anti-human Fc-specific IgG, then with swine anti-goat IgG conjugated to horseradish peroxidase and adsorbed onto human immunoglobulins (Caltag). The chemiluminescence immunoassay was performed with Renaissance Western-blot chemiluminescence reagent (NEN, Life Sciences Products) following the procedure as stated by the manufacturer.

Protein A FlashPlate binding assay. The FlashPlate (NEN, Life Sciences Products) is a white 96-well polystyrene microtiter plate having plastic scintillator-coated wells. The protein A FlashPlates were coated with protein A and allowed to immobilise the chimeric protein (100 μ l affinity-purified protein at 2.5 μ g/ml in pH 7.2 NaCl/P_i) by the Fc portion for 2 hours at room temperature. After two washes with 128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.3 mM CaCl₂, 50 mM Hepes, pH 7.6 (binding buffer), the wells were treated for 2 hours at room temperature with blocking solution consisting of binding buffer containing 5% BSA. After incubation, the wells were washed three times with further binding buffer containing 1% BSA. For the binding studies, ^{125} I-labelled TGF- β 1 (specific activity 3000–4500 Ci/mmol, NEN, Life Sciences Products) was diluted in binding buffer just prior to its addition to the wells. The final concentrations of ^{125} I-labelled TGF- β 1 ranged over 0.05–2.5 nM. The plates were incubated at room temperature for 2 hours, then sealed and radioactivity determined on a Packard Top Count microplate scintillation counter. A 1-min counting period was used. All determinations were carried out at least in duplicate. Non-specific binding was determined with an excess of unlabelled TGF- β 1 (100-fold). The counting efficiency was determined using a ^{125} I-labelled IgG (Amersham) directly coated into protein A FlashPlates. The radioactivity obtained at equilibrium divided by the number of disintegrations added to the well gave a measure of the efficiency evaluated at 5%. For the competition binding assay, the final concentration of radiolabelled ligand was 500 pM (specific activity 300–450 Ci/mmol), with final concentrations ranging over 0.05–200 nM for TGF- β 1, TGF- β 2 and TGF- β 3.

Binding of ^{125} I-labelled TGF β 1 to cells. The Cos-1 cell line (American Type Culture Collection CRL1650) was maintained in minimal essential medium (Gibco BRL Life Technologies) supplemented with 10% foetal calf serum. Transfection with recombinant pCMV5 vector containing T β R β I or T β R β II cDNA into Cos-1 were performed as described above. While ligand binding of cell monolayers with ^{125} I-labelled TGF- β 1 (0.02–1 nM) were carried out as previously described [46], then plotted with values obtained after γ scintillation counting. Scatchard experiments were performed on Cos-1 cells transiently transfected either with T β R β II (pCMV5 T β R β II) or with both T β R β I and T β R β II containing plasmids (pCMV5 T β R β I + pCMV5 T β R β II) and compared to

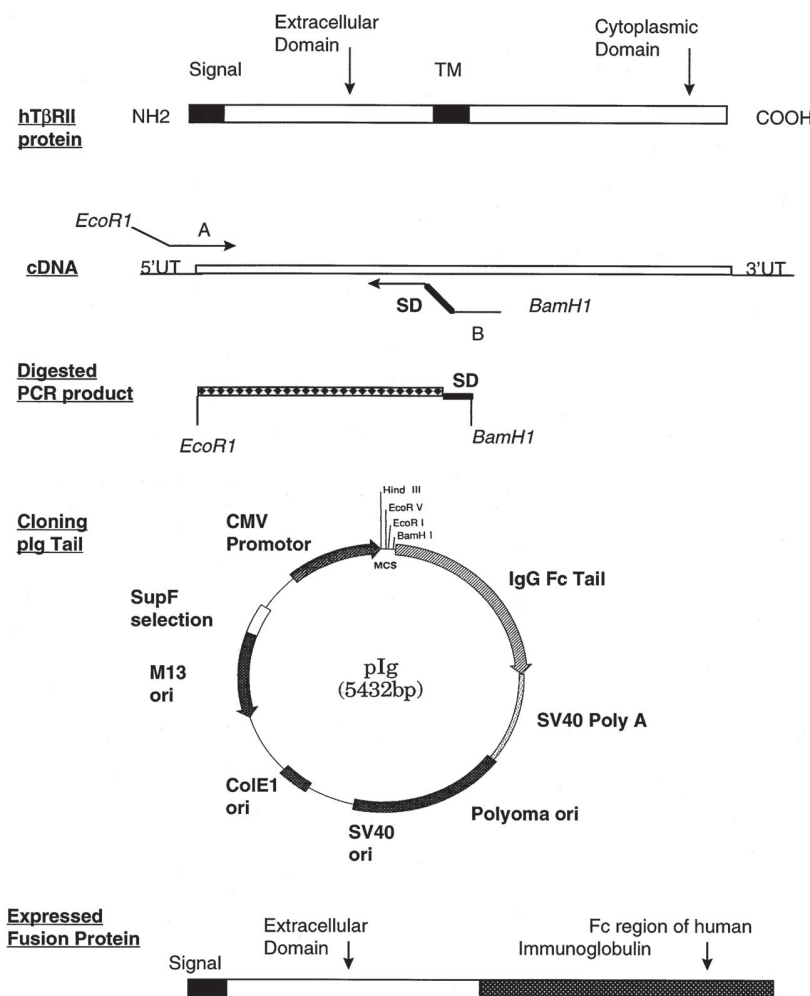


Fig. 1. Strategy for construction of a truncated cDNA encoding the extracellular domain of the soluble hTβRII. Synthetic PCR primers A and B for both sense and antisense, respectively, were used to amplify a portion of the human TβRII extracellular domain from the full-length cDNA [31] (Experimental Procedures). The amplified PCR product was digested with *EcoR1* and *BamH1*, and the digested fragment was ligated with the cloning site of the expression vector (pIg Tail R&D System). The last encoded residue of this soluble hTβRII is Asp159, which immediately precedes the first amino acid of the Fc region of human immunoglobulin G encoded by the genomic DNA cloned in the vector. Signal, hydrophobic signal sequence; TM, transmembrane domain; NH2, N-terminus; COOH, C-terminus; UT, untranslated region.

Cos-1 cells transfected with the empty pCMV5 (Invitrogen) in order to determine the non-specific binding.

Growth inhibition assay. The Mv1Lu cell line (American Type culture collection CCL-64) was maintained in minimum essential medium supplemented with 10% foetal calf serum. Assays were performed with the Mv1Lu cell line in the following manner. 10^4 cells/well were seeded into 96-well plates in a low serum medium (1% foetal calf serum) and incubated at 37°C. After 4 hours, TGF-β1 (50 pg/ml) and varying concentrations of TβRIIs and TβRIIs-Fc (0.001–1 μg/ml) were added for a further 24 hours. 6 hours before the end of this period, cells were pulsed with 1 μCi/well [^{3} H]thymidine (specific activity 16.3 Ci/mmol NEN, Life Sciences Products). Cells were harvested in a Filtermate 196 Packart Harvester and radioactivity determined on a Packard Top Count microplate scintillation counter. The DNA synthesis was then determined by measuring the incorporation of [^{3} H]thymidine.

Transient transfections and luciferase assay. Mv1Lu cells were transiently transfected with a TGF-β reporter construct

(p3TP-lux) containing a TGF-β-inducible promoter that controlled luciferase gene responses [31]. In a transient assay, transfection of p3TP-lux into Mv1Lu cells allowed the measurement of TGF-β-dependent induction of the luciferase activity. In this respect, exponentially growing Mv1Lu cells, at 40–60% confluence, were transfected 24 hours after plating in six-well plates with 4 μg/ml reporter vector (p3TP-lux) using 48 μg/ml DMRIE-C (Life Technologies). After a further 12 hours, the cells were plated into 96-well plates (5×10^4 cells/well) using media containing 1% foetal calf serum. 4 hours later TGF-β1 (300 pg/ml) and varying concentrations of TβRIIs and TβRIIs-Fc (0.001–1 μg/ml) were added to the medium. Cells were then harvested 20 hours later and assayed for luciferase activity using the Luciferase assay system (Promega) as described by the manufacturer. Total light emission was measured using a Packard Top Count microplate luminescence counter.

Data analysis. Data shown are from representative experiments. Each experiment has been repeated at least three times with different preparations of recombinant hTβRIIs-Fc. Data are

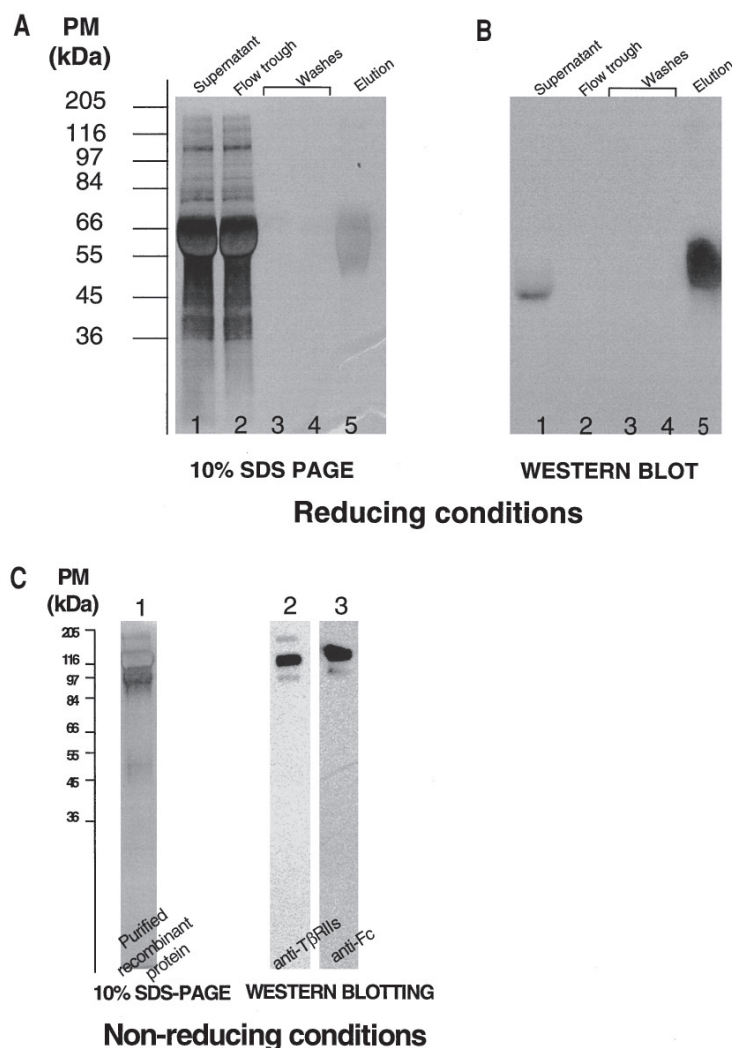


Fig. 2. SDS/PAGE and Western-blotting analysis of the recombinant soluble chimeric hT β RIIs-Fc. (A) 10% SDS/PAGE of protein fractions eluted from the HiTrap protein A column. Lane 1, supernatant of transiently transfected Cos-7 cells; lane 2, flow through; lanes 3 and 4, wash fractions; lane 5, elution fraction. The gel was silver stained and the position of molecular-mass standards (in kilodalton) is indicated on the left. (B) The same samples presented in (A) were immunoblotted with a goat anti-human T β R II Ig. (C) Samples of purified recombinant protein were separated under non-reducing conditions on 10% SDS/PAGE and visualized after silver staining (lane 1) and immunoblotted both with a goat anti-human T β R II (lane 2) and a goat anti-human Fc-specific IgG (lane 3). The position of molecular-mass standards (in kilodalton) is indicated on the left.

expressed as means $\pm$ SD. Statistical analysis was performed using the Wilcoxon two-sample test and the difference was considered statistically significant when $P < 0.05$.

RESULTS

A soluble type-II receptor fused with the Fc region of human IgG is secreted by Cos-7 cells. Cos-7 cells were transfected with the expression plasmid pIg-Tail containing the cDNA encoding for the truncated TGF- β type-II receptor (amino acid residues 1–159) as described (Fig. 1). After one-step purification by affinity chromatography on protein-A–Sepharose, 5–10 μ g pure soluble chimeric protein was recovered from the media of 10^6 transiently transfected cells with recombinant pIg-Tail (Fig. 2A, lane 5) while no protein was purified from the media of 10^6 transiently transfected cells with empty pIg-Tail vector (data not show).

Analysis of the purified chimeric receptor by 10% SDS/PAGE was performed under reducing conditions (2-mercaptoethanol) and stained using silver stain (Fig. 2A, lane 5). A product of molecular mass around 45–65 kDa was obtained, which corresponded to the purified recombinant protein. Furthermore, electrophoresis analysis of the purified fraction performed without reducing agent showed, following silver staining, a product of molecular mass around 90–130 kDa (Fig. 2C, lane 1). The Western-blot analysis (Fig. 2B and C, lanes 2 and 3) showed that the secreted recombinant protein was recognised both by a polyclonal antibody raised against T β R II (Fig. 2B and C, lane 2) and a polyclonal antibody specifically raised against the human Fc fragment (Fig. 2C, lane 3). These results indicated that this recombinant purified protein corresponded to the extracellular domain of T β R II fused with the Fc fragment of human IgG. The different molecular masses observed for the recombinant purified protein after analysis under reducing and non-reducing

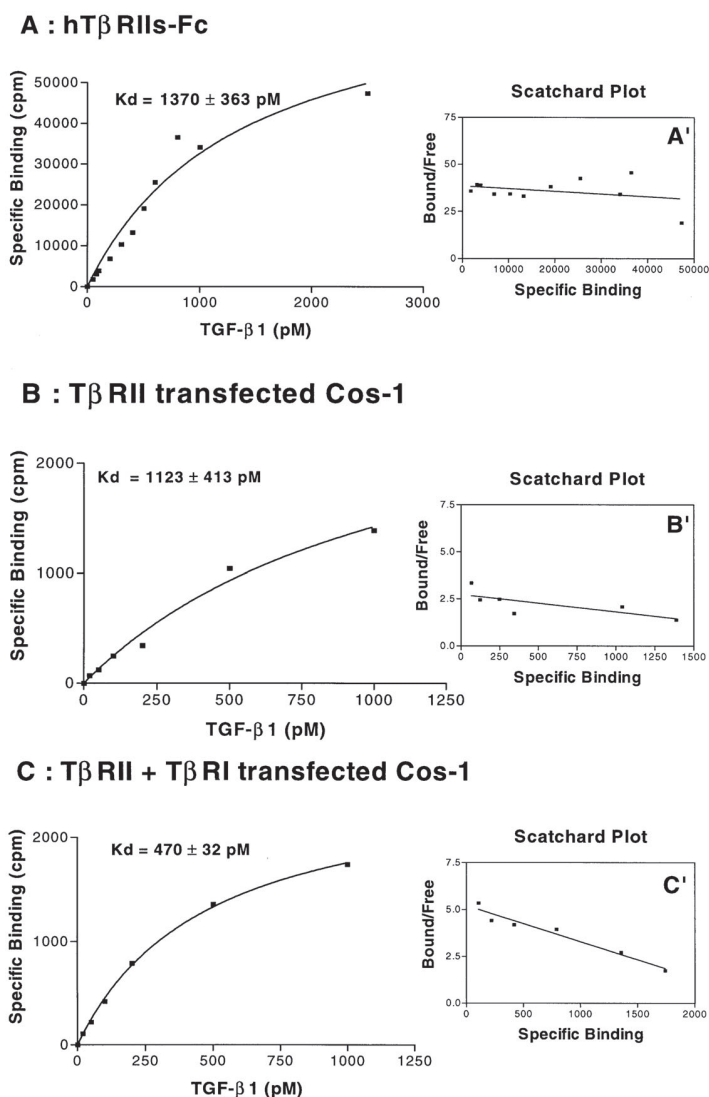


Fig. 3. Equilibrium binding of ^{125}I labelled TGF- β 1 to recombinant soluble chimeric hT β RIIs-Fc and to cell-surface transfected receptor complex. The recombinant hT β RIIs-Fc from transfected Cos-7 cell line was tested using a protein A FlashPlate binding assay in the presence of increasing concentration of [^{125}I]TGF- β 1 (Experimental Procedures). (A) Specific binding curve. (A') Scatchard analysis of the binding data in (A). The cell surface receptor complex from Cos-1 was assayed following published procedure [46]. (B and C) Specific binding curve obtained from Cos-1 transfected with either the cDNA of T β RII alone or in association with the cDNA of T β RI respectively. (B' and C') Scatchard analysis of the binding data obtained in (B) and (C). Data in (A) (B) and (C) are from representative experiments for which the calculated K_d values were 1370 ± 363 , 1123 ± 413 and 470 ± 32 pM, respectively. The K_d values were determined with GraphPad Prism 2.0 program.

conditions probably indicated a homodimeric form of secreted chimeric hT β RIIs-Fc due to the presence of the Fc fragment. In addition, the predicted molecular mass for the chimeric protein raised was approximately 40–45 kDa, which corresponded to the molecular mass seen by 10% SDS/PAGE. The presence of multiple bands with molecular masses greater than predicted by the cDNA sequence could be the result of varying amounts of glycosylation.

Comparative studies on binding of ^{125}I -labelled TGF- β 1 to human T β RIIs-Fc and wild-type T β RII. Ligand-binding activity of the recombinant hT β RIIs-Fc receptor was tested in a protein A FlashPlate binding assay. Protein A coated plastic surfaces within the wells were coated with the Fc portion of the affinity-purified recombinant chimeric receptor. The background

level was measured in non-coated wells of protein A FlashPlate and shown to represent 50% of the non-specific binding obtained upon T β RIIs-Fc coating. This non-specific binding was determined for each condition with 200 nM TGF- β 1 and evaluated at 20–30% of the total binding. The soluble chimeric receptor secreted by Cos-7 cells was able to effectively bind ^{125}I -labelled TGF- β 1, which (Fig. 3A) showed a typical saturation curve of TGF- β 1 binding to hT β RIIs-Fc in the protein A FlashPlate assay. The kinetics of ^{125}I -labelled TGF- β 1 binding, performed at room temperature, were relatively rapid since equilibrium binding was achieved usually after 2–3 hours of incubation (data not show).

Meaningful values for binding affinity constants could be derived from the obtained binding data. Scatchard analysis (Fig. 3A') revealed a single class of binding sites, giving a K_d

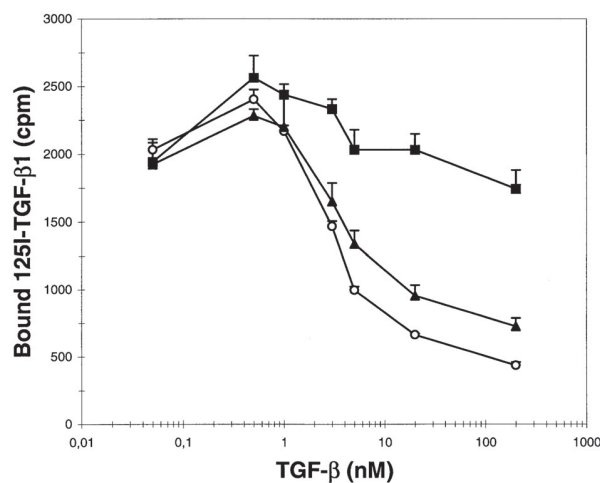


Fig. 4. Competition of ^{125}I -labelled TGF- β 1 binding in protein A FlashPlate assay by three different isoforms of TGF- β . ^{125}I -labelled TGF- β 1 (final concentration 500 pM) was mixed with serial dilutions of TGF- β 1 (○), TGF- β 2 (■) or TGF- β 3 (▲). These mixtures (0.05–200 nM) were added without prior incubation to the wells of hT β RIIs-Fc-coated protein A FlashPlate. Binding of ^{125}I -labelled TGF- β 1 to hT β RIIs-Fc immobilized on the plate was measured as described in Experimental Procedures and is expressed as the mean $\pm$ SD of triplicates from a representative experiment.

value of 1370 ± 363 pM. The data obtained from the Scatchard analysis indicated that approximately 180–200 fmoles of recombinant hT β RIIs-Fc were immobilised in each coated well.

In order to understand the relevance of our results, the K_d value of the hT β RIIs-Fc for TGF- β 1 was compared with the K_d value obtained with recombinant hT β RII expressed in the Cos-1 cell line. Previous data showed that hT β RI was able to enhance the affinity of complexes formed with hT β RII in comparison to T β RII alone [47, 48]. Here, we have investigated the affinity of Cos-1 cells expressing either T β RII alone or with both T β RI and T β RII. Although the relative affinity of Cos-1 cells for ^{125}I -labelled TGF- β 1 was low (K_d 1122 ± 412 pM) when T β RII was expressed alone (Fig. 3B and B'), co-expression of both T β RI and T β RII (Fig. 3C and C') converted the system to a higher affinity model (470 ± 32 pM).

Differential binding affinity of TGF- β isoforms for soluble chimeric receptor T β RIIs-Fc. It has previously been shown that TGF- β 1 and TGF- β 3 bind with much higher affinities than TGF- β 2 to T β RII [38]. To identify the selectivity of hT β RIIs-Fc, TGF- β isoforms were compared in competition experiments with ^{125}I -labelled TGF- β 1 binding in the protein A FlashPlate assay, where ^{125}I -labelled TGF- β 1 (final concentration 500 pM) was mixed with serial dilution of recombinant TGF- β isoforms. The relative inhibitory concentration was determined as the TGF- β concentration which inhibited 50% of ^{125}I -labelled TGF- β 1 binding (Fig. 4). The inhibitory concentration values obtained in this test were 3.3 ± 0.06 nM and 3.9 ± 0.09 nM for TGF- β 1 and TGF- β 3, respectively, whereas TGF- β 2 was unable to compete, even at concentration of 200 nM, with the binding of 500 pM of ^{125}I -labelled TGF- β 1 to recombinant T β RIIs-Fc. These results indicated that the selectivity for binding TGF- β isoforms was maintained by this chimeric recombinant receptor.

Blocking effect of chimeric soluble hT β RIIs-Fc on two different functional assays. In order to test whether the recombinant chimeric hT β RIIs-Fc was capable of acting as a TGF- β 1

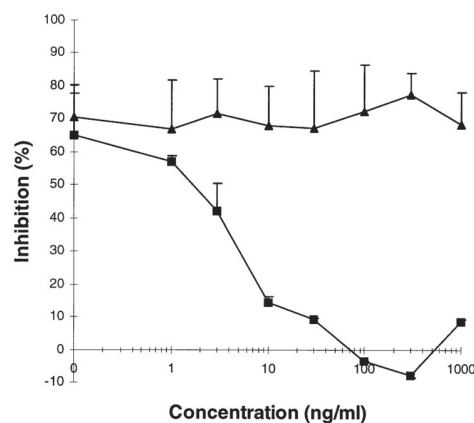


Fig. 5. Recombinant soluble chimeric hT β RIIs-Fc can act as an antagonist of the bioactivity of TGF- β 1. Increasing doses of hT β RIIs-Fc expressed in Cos-7 were able to reverse the proliferation-inhibiting activity of TGF- β as measured using a Mv1Lu growth-inhibition assay. TGF- β 1 was incubated with serial dilution of recombinant soluble hT β RIIs-Fc (0.001–1 $\mu\text{g}/\text{ml}$) to the cells to be assayed for inhibition of proliferation. In the experiment shown, incorporation of ^3H -labelled thymidine in the absence of TGF- β 1 gave counts of 15863 ± 2638 cpm. 50 pg/ml TGF- β 1 reduced this incorporation level to 6143 ± 1407 cpm (61% growth inhibition). The inhibitory activity of TGF- β 1 was completely reversed by the highest concentration of hT β RIIs-Fc (■) but unaffected even by 1 $\mu\text{g}/\text{ml}$ recombinant hT β RII (R&D System) (▲). Increasing levels of hT β RIIs-Fc and hT β RIIs without TGF- β 1 showed no reversal of inhibition (data not show). These results indicated that hT β RIIs-Fc has a much higher affinity for TGF- β 1 than hT β RIIs. The relative effective dose for hT β RIIs-Fc was determined as 5 ng/ml. Data are expressed as the mean $\pm$ SD of quadruplicates from a representative experiment.

antagonist by competing with membrane-bound TGF- β receptors, its ability was tested to reverse the growth inhibition of Mv1Lu cells induced by TGF- β 1 and its ability to inhibit expression of the TGF β -responsive luciferase reporter gene. In the growth inhibition assay, 50 pg/ml TGF- β 1, which results in 65–75% inhibition of [^3H]thymidine incorporation in Mv1Lu cells (data not show), was added to the cells together with increasing amounts of soluble hT β RIIs-Fc (0.001–1 $\mu\text{g}/\text{ml}$). The recombinant chimeric receptor hT β RIIs-Fc was able to reverse the inhibitory effect of TGF- β 1 (Fig. 5). The relative effective dose value was determined as 5 ng/ml hT β RIIs-Fc. Moreover, the soluble hT β RII (R&D System) at the same concentration was unable to inhibit TGF- β 1 activity in this assay. In the TGF β -responsive luciferase reporter gene assay, chimeric hT β RIIs-Fc also demonstrated TGF- β antagonist activity (Fig. 6). After treatment of the transiently transfected Mv1Lu with 300 pg/ml TGF- β 1 and 0.2 $\mu\text{g}/\text{ml}$ hT β RIIs-Fc or hT β RIIs, the luciferase activity was significantly decreased ($P < 0.03$) compared to treatments with 300 pg/ml TGF- β 1 only (Fig. 6A). The relative effective dose value for T β RIIs-Fc was determined as 50 ng/ml (Fig. 6B) while hT β RIIs was unable, even at the highest concentration, to entirely inhibit TGF- β 1 activity. These results showed the significant ability of hT β RIIs-Fc to act as an TGF- β 1 antagonist.

DISCUSSION

In this publication, we have described the molecular basis for the generation of a soluble chimeric form of T β RII; T β RIIs-Fc. This T β RIIs-Fc molecule, constructed by linkage of the T β RII ectodomain to a human IgG heavy chain, was produced in the Cos-7 cell line. The IgG tail presents major advantages:

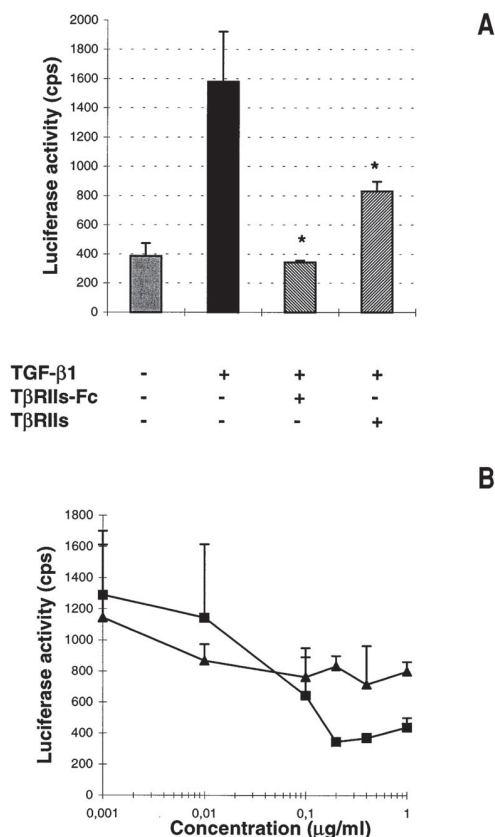


Fig. 6. Effects of soluble chimeric TβRIIs-Fc on TGF-β-responsive luciferase reporter gene assay. (A) Mv1Lu cell lines transiently transfected with the reporter vector p3TP-lux [31] were incubated overnight in the presence (plus) or absence (minus) of 300 pg/ml TGF-β1 and 0.2 μg/ml hTβRIIs-Fc or recombinant soluble hTβRII (R&D System). (B) Transiently transfected Mv1Lu cells were incubated overnight in the presence of 300 pg/ml TGF-β1 and various concentrations (0.001–1 μg/ml) of hTβRIIs-Fc (■) and hTβRIIs (▲). Cells were harvested and luciferase activity measured as described in Experimental Procedures. Luciferase activity was determined in a luminometer and expressed as photon/s. The TGF-β1 response was entirely inhibited by 0.2 μg/ml hTβRIIs-Fc. The relative effective dose for TβRIIs-Fc was determined as 50 ng/ml. The recombinant soluble hTβRII was unable, even at 1 μg/ml, to entirely inhibit TGF-β1 activity. Results were expressed as the means ± SD of triplicates from a representative experiment, * indicate $P < 0.03$.

it facilitates purification and immobilisation of the chimeric receptor. Moreover, transient expression in Cos-7 cells yielded a protein with a molecular mass of approximately 45–65 kDa when analysed under reducing conditions, while the molecular mass was doubled under non-reducing conditions, indicating that this fusion protein could be bivalent and has an antibody-like structure.

The binding properties of recombinant hTβRIIs-Fc for TGF-β1 was analysed using a non-separation microplate receptor-binding assay. For our experiments, the hTβRIIs-Fc component was coated to the wells of protein A FlashPlates and incubated with ^{125} I-labelled TGF-β1. Only the bound radioactive product was sufficiently close to excite scintillant, while the more distant unbound ligand was not detected. The key feature of this protein A FlashPlate binding assay, as described here, is its simplicity; it comprises of only the addition of the ^{125} I-labelled TGF-β1, incubation and measurement. No crosslinking, no filtration or

washing steps are involved. Unlike separation-based assays, this assay does not disturb the equilibrium while, the radioactivity obtained was directly correlated to the bound ^{125} I-labelled TGF-β1. In our assay, we determined a counting efficiency of about 5%; this result could be explained by the increased distance between ^{125} I-labelled TGF-β1 and solid scintillant due to the coating of protein A and hTβRIIs-Fc onto the wells of the Flash-Plate. Nevertheless, despite the poor counting efficiency obtained, the assay is accurate and its correlation with a conventional approach suggests that it is suitable for hTβRIIs-Fc-binding studies.

The affinity obtained for our chimeric recombinant protein (1370 ± 363 pM), as determined in the protein A FlashPlate binding assay, is higher than the apparent K_d of 120 nM estimated for hTβRIIs (R&D System) using surface plasmon resonance [49]. A previous study reported an affinity in the 200-pM range for the binding of TGF-β1 to the TβRII ectodomain [38] but this result was obtained with non-purified protein and the estimate has been carried out indirectly using a conditioned medium from transfected Cos-7 cells, cross-linking, then immunoprecipitation. Currently, we have not been able to determine the K_d of the hTβRIIs in our binding assay. Firstly, we are unable to coat this protein efficiently onto the protein A FlashPlate and, secondly, if we use non-coated FlashPlate, the adsorption of this protein would be quite different; i.e. there would be no orientation of the binding site compared to hTβRIIs-Fc with which the coating occurs via the Fc region. The higher affinity measured for hTβRIIs-Fc compared to rhTβRIIs may be due to the dimerization of the chimeric protein. Thus, each of the two receptor subunits in a homodimer could bind one TGF-β monomer, increasing the binding energy and thus lowering the dissociation constant for binding of TGF-β. Indeed, a recent article reported that TβRIIs is expressed as a monomer but ligand binding induced its homodimerization [50]. Other experiments demonstrated that recruitment of TβRI by the TβRII ligand complex is able to induce high affinity interaction in the complex form [35]. In our study, we compared the affinity of hTβRIIs-Fc (1370 ± 363 pM) to the affinity of Cos-1 cells expressing either TβRII alone (1122 ± 413 pM) or with both TβRI and TβRII (470 ± 32 pM). In these experiments, the affinity of our chimeric soluble receptor for TGF-β1 was threefold lower than the affinity of the heterodimer receptor complex (TβRI and TβRII) on the cell surface, but was very similar to that of TβRII when expressed alone in the Cos-1 cell line. Several recent papers have reported that the affinity of TGF-β1 in Mv1Lu cell line was around 250 pM [47, 48]. Thus, the affinity observed in the present study in Cos-1 cell takes place in the same range.

One significant result is that the chimeric protein shows the same selectivity for different TGF-β isoforms as the native receptor [51], either in glycosylated [38] or unglycosylated hTβRIIs forms [39]. The fusion with the Fc region does not disturb this selectivity. TGF-β2 is not able to compete with the binding of ^{125} I-labelled TGF-β1, although TGF-β1 and TGF-β3 show both a relative inhibitory concentration in the 3–4-nM range. Previous data have shown that TβRII binds TGF-β2 with low affinity [27, 31, 47] and co-expression of TβRIII is required [38].

We have also tested the ability *in vitro* of the soluble hTβRIIs-Fc to act as an antagonist for TGF-β1 using two different functional assays. Our results show that hTβRIIs-Fc has sufficient affinity for TGF-β1 to compete with a membrane-bound form of the receptor. hTβRIIs-Fc is able to reverse not only the inhibitory effect of TGF-β1 on Mv1Lu cells with a relative effective dose of 5 ng/ml, but also inhibit the TGF-β1 response in a TGF-β-responsive luciferase reporter gene assay (relative effective dosage, 50 ng/ml). The two different values

of relative effective dosage obtained in these experiments are due to the different concentration of TGF- β 1 used for both assay on Mv1Lu/TGF- β -induced growth inhibition and TGF- β -induced luciferase response (50 pg/ml and 300 pg/ml respectively).

Recent studies have shown that hT β RIIs has a blocking effect on TGF- β activity under favourable circumstances. hT β RIIs is able to reverse the proliferation-inhibiting activity of TGF- β using HT2 cells proliferating in response to interleukin 4 [52]. However, HT2 cells have extremely low levels of receptors for TGF- β and with other cell types such as Mv1Lu as used in these studies which show much higher numbers of TGF- β receptors, the inhibition of TGF- β activity was only partial, even at the highest level of added hT β RIIs [40]. However, comparison of hT β RIIs and hT β RIIs-Fc under the same assay conditions, showed that our recombinant hT β RIIs-Fc had a better antagonist activity than hT β RIIs, which had no effect on Mv1Lu growth inhibition, while hT β RIIs-Fc inhibited with a relative effective concentration of 5 ng/ml. Nevertheless, hT β RIIs-Fc and hT β RIIs are both able to block the TGF- β 1 response in a luciferase reporter gene assay, showing the functionality of hT β RIIs. However, in this test, the hT β RIIs-Fc antagonist activity was better, probably due to the dimerization of the chimeric protein. Moreover, TGF- β 1-extracellular matrix transcriptional responses in Mv1Lu cells seemed to be more sensible to TGF- β 1 antagonist than TGF- β 1-antiproliferative responses.

In conclusion, our results show that hT β RIIs-Fc has a high inhibitory activity on TGF- β 1. *In vitro* binding assays also demonstrate that hT β RIIs-Fc is able to bind TGF- β 1 with a similar affinity to that of wild-type hT β RII when overexpressed alone in Cos-1 cells, and can be used accordingly in the FlashPlate screening test to search for TGF- β agonist and antagonist compounds. The selectivity of the soluble chimeric receptor T β RIIs-Fc for TGF- β 1 and TGF- β 3 associated with its TGF- β 1 antagonist properties may be used for studying pathogenic mechanisms in which TGF- β s are involved. Moreover, recombinant T β RIIs-Fc could be successfully used as a TGF- β 1 and TGF- β 3 antagonist both *in vitro* and *in vivo*.

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Note added in proof. A similar chimeric receptor is now commercially available by R&D System. Nevertheless, the authors have initiated this work and characterised this reagent prior to its commercialisation.