

Type III TGF- β receptor-independent signalling of TGF- β 2 via T β RII-B, an alternatively spliced TGF- β type II receptor

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Transforming growth factor- β (TGF- β) signals through membrane-bound serine/threonine kinase receptors, which upon stimulation phosphorylate Smad proteins and thereby trigger their nuclear translocation and transcriptional activity. Although the three mammalian isoforms of TGF- β are highly homologous at the level of sequence, analysis of their *in vivo* function by gene knockouts revealed striking differences, suggesting no significant functional redundancy between TGF- β 1, -2 and -3. While signal transduction by TGF- β 1 has been well characterized, receptor binding and activation by the TGF- β 2 isoform is less well understood. Here, we show that T β RII-B, an alternatively spliced variant of the TGF- β type II receptor, is a TGF- β 2 binding receptor, which mediates signalling via the Smad pathway in the absence of any TGF- β type III receptor (T β RIII). L6 cells lacking endogenous T β RIII as well as T β RII-B do not respond to TGF- β 2. Transfection of these cells with T β RII-B restores TGF- β 2 sensitivity. The expression of T β RII-B is restricted to cells originating from tissues such as bone where the isoform TGF- β 2 has a predominant role. This reflects the importance of this receptor in TGF- β isoform-specific signalling.

Keywords: alternative splicing/osteoblast TGF- β /TGF- β receptor

Introduction

Transforming growth factor- β (TGF- β) is a member of a large family of structurally related cytokines. The family consists of >30 ligand proteins regulating a wide variety of biological processes, such as proliferation, differentiation and cell death (Roberts and Sporn, 1993). The phenotypes resulting from the knockout of the three mammalian TGF- β isoforms TGF- β 1, TGF- β 2 and TGF- β 3 are very distinct and not overlapping. TGF- β 1 null mice have an autoimmune-like inflammatory disease (Schull *et al.*, 1992; Kulkarni and Karlsson, 1993; Diebold *et al.*, 1995), TGF- β 2 knockout mice exhibit perinatal mortality and severe developmental defects (Sanford *et al.*, 1997) and TGF- β 3-deficient mice have cleft palate and are defective in lung development (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). This indicates that these ligands

have isoform-specific activities that cannot be compensated by other family members.

Signalling via TGF- β 1 is initiated by binding of TGF- β 1 to the constitutive active serine/threonine kinase receptor T β RII (TGF- β type II receptor). Upon ligand binding, the TGF- β type I receptor (T β RI) is recruited into the hetero-oligomeric signalling complex and subsequently T β RII activates T β RI by transphosphorylation at its cytoplasmic GS box (Wrana *et al.*, 1994a). Activated T β RI transiently associates with cytoplasmic effectors, the Smad proteins, which become phosphorylated at their C-terminus and dissociate from the receptor. Upon complex formation with Smad4, these hetero-oligomeric Smad complexes are translocated into the nucleus to regulate transcription (Piek *et al.*, 1999; Massagué and Chen, 2000).

In contrast to TGF- β 1, signalling by TGF- β 2 seems to have a different mode of receptor activation, since T β RII has a low intrinsic affinity to this isoform (Cheifetz and Massagué, 1991; Lin *et al.*, 1995). The requirement of the type III receptor (T β RIII) for responsiveness to TGF- β 2 has been described in different cell types (Lopez-Casillas *et al.*, 1993; Sankar *et al.*, 1995; Brown *et al.*, 1999). T β RIII binds the ligand TGF- β 2 and presents it to T β RII upon oligomerization of both receptor types (Lopez-Casillas *et al.*, 1993). However, it is still unclear why direct binding of TGF- β 1 to T β RII does not have the same effect. Therefore, it was proposed that TGF- β 2 alters the composition or activity of T β RII–T β RI complexes in order to activate a unique set of downstream signalling molecules that result in specific TGF- β 2 effects (Brown *et al.*, 1999).

Here we describe and functionally characterize an isoform of the type II receptor, T β RII-B, which binds and signals directly via the TGF- β 2 isoform without the requirement for T β RIII. T β RII-B is an alternatively spliced variant of T β RII resulting in N-terminal alterations of the mature receptor. Unlike T β RII this splicing variant shows a restricted expression pattern. The site of predominant expression includes osteoblasts and mesenchymal precursor cells, which correlates with the unique expression of TGF- β 2 in chondrocytes and osteocytes (Pelton *et al.*, 1991).

Results

Isolation of the T β RII-B cDNA clone

RT-PCR was used to screen for variants of the TGF- β type II receptor showing alterations in the extracellular domain. Upon amplification of cDNA from the human hepatoma cell line Hep3B, an additional PCR product with lower mobility was detected (Figure 6A, lane 9). Sequence analysis revealed that this PCR product is identical to T β RII-B, an alternatively spliced variant of T β RII

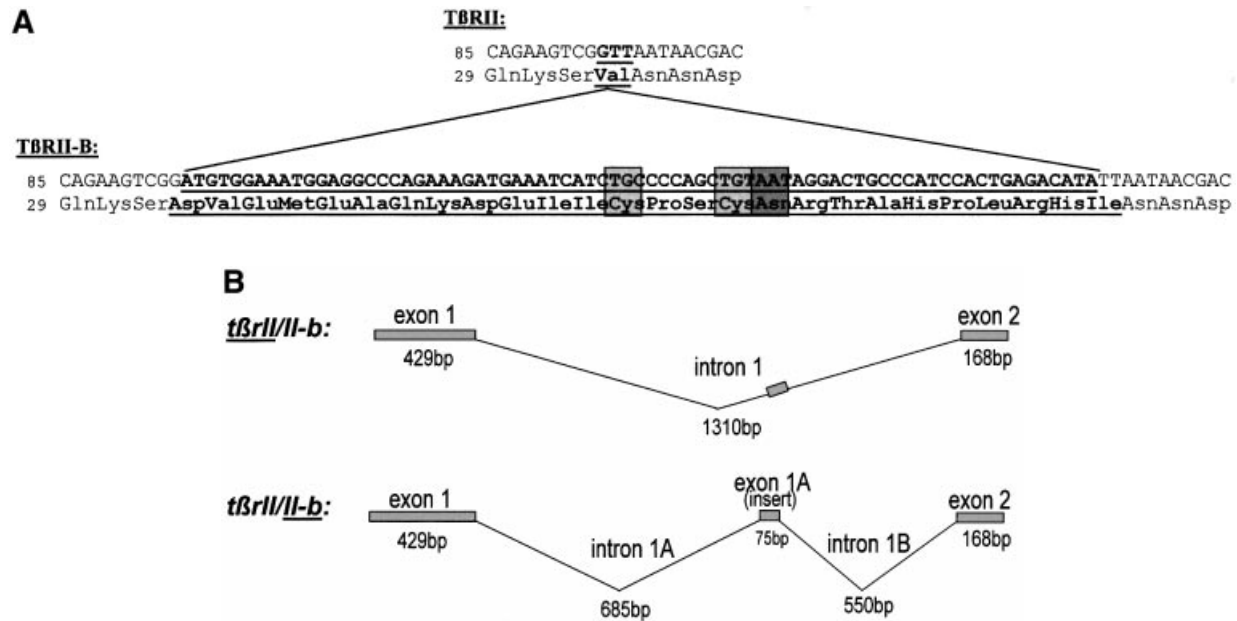


Fig. 1. TGF β type II-B receptor is an alternatively spliced form of T β RII. (A) The amino acid sequence of T β RII-B compared with T β RII contains an insert of 26 amino acids after Ser31, replacing Val32 of T β RII. The insertion sequence of human T β RII-B is underlined. A potential N-linked glycosylation site (Asn48) and two Cys residues (Cys44, Cys47) are shown in shaded boxes. (B) Schematic outline of alternative splicing of the *t β RII*-intron 1 resulting in an additional exon, exon 1A.

described previously (Nikawa, 1994; Hirai and Fujita, 1996). The alternative splicing causes an insertion of 26 amino acids at the N-terminus of the mature receptor, replacing Val32 (Figure 1A).

In order to analyse the exon–intron structure of *t β RII-b*, PCR analysis of genomic DNA from human placenta was performed using insert-specific primers (data not shown). We were able to localize the insert as an additional exon (exon 1A) within intron 1 (Figure 1B).

Unlike T β RII, T β RII-B binds all three TGF- β isoforms

T β RII is known to bind the isoforms TGF- β 1 and TGF- β 3. Binding of these ligands causes recruitment of the type I receptor (T β RI) into a signalling receptor complex followed by activation of T β RI through transphosphorylation (Wrana *et al.*, 1992; Moustakas *et al.*, 1993; Wrana *et al.*, 1994a; ten Dijke *et al.*, 1996; Wells *et al.*, 1999). The isoform TGF- β 2, however, does not follow this mode of receptor binding and oligomerization, at least not by using these receptors. T β RII does not bind the isoform TGF- β 2 when expressed alone (Lin *et al.*, 1995).

To study binding of different TGF- β isoforms to T β RII-B we performed binding and crosslinking analysis of radiolabelled ligands on COS-7 cells transfected with either T β RII or T β RII-B. The receptors were immunoprecipitated from cell lysates using the antiserum α -CR11, which detects both type II receptors (Figure 2, lanes 1–7 and 9). Both receptors bind the isoforms TGF- β 1 and TGF- β 3 indistinguishably. However, binding of the β 2 isoform is strikingly different. T β RII-B binds TGF- β 2 even in the absence of T β RI or T β RIII (Figure 2, lane 4), which suggests distinct binding properties of TGF- β 2.

This is different to the cooperative binding mode postulated for TGF- β 2 via preformed complexes of T β RII with T β RI or T β RIII (Rodriguez *et al.*, 1995; Massagué, 1998). Accordingly, other studies have shown that the majority of the type I and type II receptors for TGF- β exist as homodimers and not hetero-oligomers at the cell surface in the absence of ligand (Gilboa *et al.*, 1998; Wells *et al.*, 1999).

T β RII-B forms complexes with T β RI, T β RII and T β RIII

It has been shown before that addition of ligand induces hetero-oligomeric complexes of the known TGF- β receptors (T β RI–T β RII, T β RIII–T β RII) (Wrana *et al.*, 1992, 1994a; Henis *et al.*, 1994; Gilboa *et al.*, 1998; Wells *et al.*, 1999). In order to analyse complex formation of T β RII-B with the known TGF- β receptors at the cell surface, we performed ligand binding and crosslinking experiments in transiently transfected COS-7 cells expressing various combinations of TGF- β receptors. T β RII-B interacts with T β RI in the presence of each of the three TGF- β isoforms (Figure 3A, lanes 2, 4 and 6). The interaction of T β RII-B with T β RIII through TGF- β 1 and TGF- β 2 is shown in Figure 3B (lanes 2 and 6). Even though T β RII-B is not dependent on complexes with T β RIII for its binding of TGF- β 2, hetero-oligomers of both receptor types are detected. In contrast, T β RII binds TGF- β 2 only when co-expressed with T β RIII (compare lanes 3 and 5). This is observed as well in cells expressing endogenous TGF- β receptors. The cell line R1b/L17 lacks endogenous T β RI (Laiho *et al.*, 1990) and, as we show later, also T β RII-B. Binding of TGF- β 2 to T β RII (Figure 3C, lane 2) results from complex formation with T β RIII. These complexes

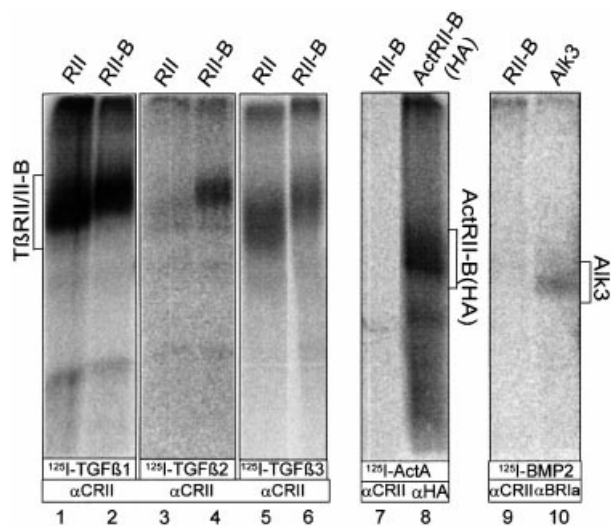


Fig. 2. All three TGF β isoforms bind T β RII-B. COS-7 cells transfected with T β RII or T β RII-B were affinity labelled with [125 I]TGF- β 1 (lanes 1 and 2), [125 I]TGF- β 2 (lanes 3 and 4) or [125 I]TGF- β 3 (lanes 5 and 6), crosslinked and immunoprecipitated with α -CRII, an antibody raised against the C-terminus of both type II receptors. Unlike T β RII, T β RII-B binds the isoform TGF- β 2, when expressed singly in COS-7 cells (lanes 4 and 3). In contrast, iodinated activin A (lane 7) or BMP-2 (lane 9) does not bind to T β RII-B, but do bind to their respective high-affinity receptors ActRII-B (lane 8) and ALK3 (lane 10).

are essential for TGF- β 2 binding to T β RII. Since Mv1Lu cells do not express any T β RII-B (Figure 6A and B, lanes 13 and 14) the existence of T β RIII in these cells seems to be absolutely necessary for binding and signalling via TGF- β 2.

To study the oligomerization of the two TGF- β type II receptors T β RII and T β RII-B, we used HA-epitope-tagged T β RII cotransfected with untagged T β RII-B. Each of these receptors carry in addition to the common epitope (detected by α -CRII) at least one specific epitope (recognized by α -hRIIB or by α -RIIB for T β RII-B, and by α -HA for HA-T β RII). Sequential immunoprecipitations from cell lysates were performed after binding and crosslinking with the indicated iodinated ligands to show that T β RII and T β RII-B form complexes in the presence of either isoform (Figure 4, lanes 4, 7 and 8). While heteromeric complexes were detected using first α -hRIIB and then α -HA, we were unable to detect these with the reverse experimental set-up (first α -HA, second α -hRIIB; see Figure 4, lane 3). One possible explanation is that the antibody α -hRIIB does not recognize its epitope under the conditions used in the second immunoprecipitation (Figure 4, lanes 5 and 6). There is no cross-reactivity of the antisera α -hRIIB or α -RIIB with T β RII, as tested by immunoprecipitations of affinity-labelled T β RII (data not shown).

We also showed that T β RII/T β RII-B heteromers bind TGF- β 2 (Figure 4, lane 7). In this case one T β RII-B receptor chain is enough to facilitate binding of TGF- β 2 to both T β RII and T β RII-B, whereas the homomeric form of T β RII is not.

In conclusion, we have demonstrated that T β RII-B interacts with T β RI, T β RII and T β RIII at the cell surface via TGF- β 1 and TGF- β 2.

Neither alternative disulfide bond formation nor N-glycosylation influences binding properties of T β RII-B to TGF- β 2

As illustrated in the sequence of the T β RII-B insert (Figure 1A), two additional cysteines (Cys44 and Cys47) are present in the extracellular domain of T β RII-B. This might enable additional or alternative disulfide bond formation. We have mutated the cysteines to alanines by PCR mutagenesis either individually or both (T β RII-B^{C44A}, T β RII-B^{C47A}, T β RII-B^{C44AC47A}). All constructs were expressed in COS-7 cells and tested for their binding properties. No difference between the mutants and the wild-type T β RII-B was seen with respect to binding of TGF- β 2 (Figure 5A, lanes 1–4) or TGF- β 1 and to interaction with T β RI (data not shown).

In addition, the sequence of the insert in T β RII-B shows a potential N-glycosylation site at Asn48 (Figure 1A). Deglycosylation of T β RII by tunicamycin treatment of transfected COS-7 cells has been shown not to affect binding of this receptor to TGF- β 1 (Wells *et al.*, 1997). To exclude potential glycosylation at Asn48 of T β RII-B, which might cause binding of TGF- β 2 to this receptor, we mutated this residue to alanine, resulting in the mutant T β RII-B^{N48A}. No difference was seen in binding TGF- β 2 compared with the wild-type receptor (Figure 5A, lanes 1 and 5). Similar results were obtained using tunicamycin-treated COS-7 cells transfected with the T β RII-B construct (data not shown).

Next, we tagged T β RII-B N-terminal of the insertion with an HA-epitope and examined whether this modification alters ligand binding or whether ligand binding interferes with recognition by the α -HA antibody. Figure 5B (lanes 5 and 6) shows that addition of the epitope does not inhibit ligand binding, but bound and crosslinked TGF- β 1 interferes with the accessibility of the epitope for the α -HA antibody (Figure 5B, lane 4). This is not the case for T β RII, if an epitope tag is added also to the very N-terminus (Figure 5B, lane 7). Controls without the ligand (Figure 5B, lanes 1–3) show that the HA-epitope (lane 2) as well as the insert epitope (lane 3) at the N-terminus of T β RII-B are equally accessible to their antibodies. This suggests that the N-terminus of T β RII-B makes major contributions to the binding pocket of TGF- β isoforms.

T β RII-B displays a restricted expression pattern

In order to study the expression of T β RII-B at the RNA and protein level, RT-PCR and binding experiments were performed in cell lines established from different tissues. Surprisingly, depending on the cell type, different scenarios for the expression of T β RII-B were observed: (i) no alternative splicing in Mv1Lu and L6 cells and therefore no T β RII-B expression (Figure 6A, lanes 13–16 and B, lanes 13–16); (ii) alternative splicing but no detectable expression of T β RII-B at the cell surface of Hep3B and IMR32 cells (Figure 6A, lanes 9–12 and B, lanes 9–12); (iii) alternative splicing and expression of T β RII-B at the cell surface of murine mesenchymal precursor cells (MC3T3 and C2C12 cells), human fetal osteoblast (hFOB) and the human osteosarcoma cell line U2OS (Figure 6A, lanes 1–8 and 17–22, B, lanes 1–8 and C).

While T β RII is almost ubiquitously expressed on cells, T β RII-B shows a distinct and specific expression pattern

mainly in bone-related cells, such as osteoblasts and mesenchymal precursor cells. The mesenchymal precursor cell line C2C12 can form myotubes when cultivated for 3–5 days in low serum [0.2% fetal calf serum (FCS)]. The addition of 40 nM bone morphogenetic protein (BMP)-2 converts the differentiation of C2C12 cells into the

osteoblast lineage (Katagiri *et al.*, 1994). As shown in Figure 6C, T β RII-B is expressed early in the precursor cell line (lanes 1 and 4), but is upregulated during differentiation into myoblasts (lanes 2 and 5) and even more strongly in osteoblasts (lanes 3 and 6).

Taken together, the data here show the restriction of expression of T β RII-B to cells such as osteoblasts, where the TGF- β 2 isoform has a specific biological role. In other cell lines such as human hepatoma cells and neuroblastoma cells, the alternative splicing does not result in detectable expression of the receptor at the cell surface. No alternative splicing occurs in a third subset of cells, suggesting a tissue-specific mechanism for splicing.

T β RII-B is a signalling receptor

In order to study signalling of TGF- β 2 via the endogenously expressed T β RII-B receptor, we investigated ligand-induced phosphorylation of Smad2, a TGF- β pathway-restricted Smad, which is phosphorylated by activated T β R1 (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). Two different cell lines have been used, which differ in the composition of their TGF- β receptors. The human osteosarcoma cell line U2OS expresses T β R1, T β RII and T β RII-B (Figure 6A and B), but lacks T β RIII (data not shown). The rat myoblast cell line L6 lacks T β RIII (Wang *et al.*, 1991; Lopez-Casillas *et al.*, 1993) and T β RII-B (Figure 6A and B), while it expresses T β R1 and T β RII. It has been shown previously that T β RIII binds all three isoforms with high affinity and is essential for the presentation of TGF- β 2 to the signalling complex, i.e. T β RII and T β R1 (Wang *et al.*, 1991; Sankar *et al.*, 1995; Brown *et al.*, 1999). Both cell lines were treated with either TGF- β 1 or TGF- β 2 for 30 min and cell lysates were analysed by western blotting using PS2 antiserum, which recognizes specifically the phosphorylated form of Smad2 (Ishisaki *et al.*, 1999). In L6 cells Smad2 is highly phosphorylated upon stimulation with TGF- β 1 (Figure 7A, lane 5) whereas it is phosphorylated to a lesser extent with TGF- β 2 (Figure 7A, lane 6). In U2OS cells, however, the additional expression of T β RII-B results in strong phosphorylation of Smad2 after TGF β 2 treatment. This is independent of T β RIII expression (Figure 7A, lane 3).

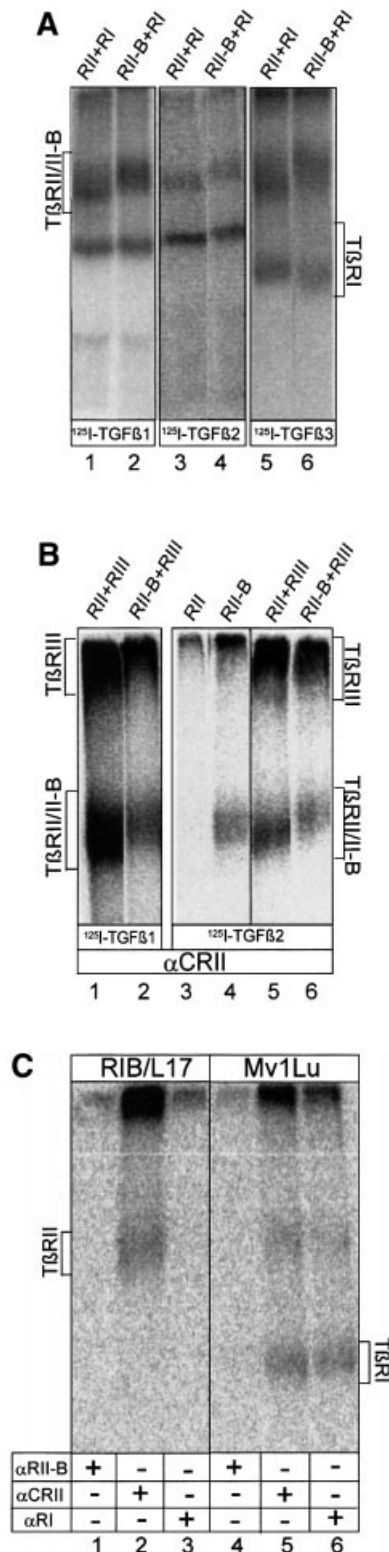


Fig. 3. Binding and complex formation of T β RII-B upon co-expression with T β R1 or T β RIII. (A) Receptor complexes containing T β RII and T β R1 or T β RII-B and T β R1 were detected after binding and cross-linking of [125 I]TGF- β 1 (lanes 1 and 2), [125 I]TGF- β 2 (lanes 3 and 4) and [125 I]TGF- β 3 (lanes 5 and 6) by immunoprecipitation with α -CRII. Receptor combinations are indicated above each lane. (B) COS-7 cells were transiently transfected with plasmids encoding T β RII or T β RII-B alone, or cotransfected with T β RIII (indicated above each lane). After affinity labelling with [125 I]TGF- β 1 (lanes 1 and 2) or [125 I]TGF- β 2 (lanes 3–6) receptors were detected by immunoprecipitation with α -CRII. The positions of ligand-bound T β RII, T β RII-B and T β RIII are indicated. Both type II receptors interact with T β RIII in the presence of TGF- β 1 (lanes 1 and 2) or TGF- β 2 (lanes 5 and 6). T β RII can bind to TGF- β 2 only if co-expressed with T β RIII (lane 5), but not without any associated receptor (lane 3). TGF- β 2 binding to T β RII-B is not dependent on the formation of receptor complexes (lane 4). (C) Binding of [125 I]TGF- β 2 to TGF- β receptors at the cell surface of Mv1Lu and R1b/L17 cells. Immunoprecipitations were performed using the T β RII-B-specific antibody, α -RII-B (lanes 1 and 4), the RII/RII-B antibody, α -CRII (lanes 2 and 5) and the type I receptor antibody, α -RI (lanes 3 and 6). R1b/L17 cells lack T β R1, whereas Mv1Lu-cells do not (lanes 3 and 6).

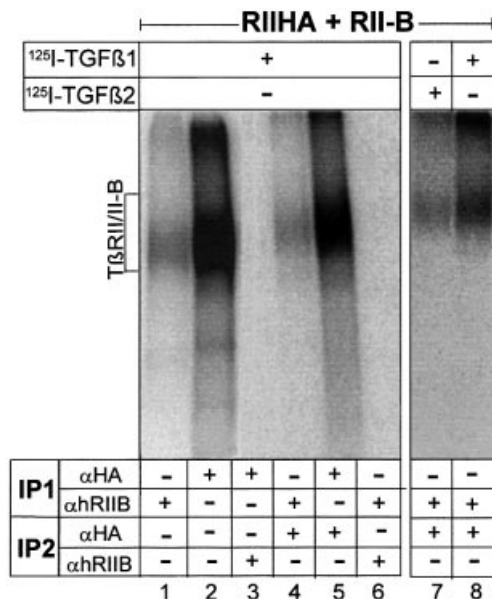


Fig. 4. Type II/II-B receptor hetero-oligomers are detected at the cell surface after ligand binding. COS-7 cells were cotransfected with HA-epitope-tagged TβRII and non-tagged TβRII-B. Binding and crosslinking were performed with [¹²⁵I]TGF-β1 (lanes 1–6 and 8) and [¹²⁵I]TGF-β2 (lane 7). The heteromeric complex of TβRII and TβRII-B was detected by sequential immunoprecipitations (IPs) using the human TβRII-B-specific antibody α-hRIIB in the first IP and the α-HA antibody in second IP (lanes 4, 7 and 8).

Next, signalling via both TGF-β isoforms was analysed in reporter gene assays. First, the induction of the TGF-β-responsive reporter gene p3TP-luc was tested in U2OS cells, where TGF-β1 as well as TGF-β2 showed a 2-fold increase in luciferase activity (Figure 7B). Secondly, we analysed L6 cells for their responsiveness to both TGF-β isoforms. The parental cell line does respond to the TGF-β1 isoform, but shows only weak induction by the TGF-β2 isoform (Figure 7C, columns 1–5). This indicates that even though preformed complexes of TβRII and TβRII that could bind the ligand TGF-β2 might exist (Rodriguez *et al.*, 1995; Gilboa *et al.*, 1998; Wells *et al.*, 1999), these complexes induce only minor responsiveness to TGF-β2 in the p3TP-luc reporter gene assay. Interestingly, transfection not only of TβRIII (Figure 7C, columns 19 and 20) but also of TβRII-B (Figure 7C, columns 14 and 15) leads to TGF-β2 response of these cells. Transfection of TβRII (Figure 7C, columns 6–10) shows no increase in responsiveness to TGF-β2. These data, together with the results from U2OS cells (Figure 7A and B), demonstrate for the first time signalling of TGF-β2 independently of the TβRIII.

Cells such as the Mv1Lu cells, which express a high amount of TβRIII, facilitate TGF-β2 signalling through this receptor. DR26 cells, which lack functional TβRII, were transiently transfected with either TβRII or TβRII-B. The TGF-β-responsive reporter p3TP-luc (Wrana *et al.*, 1992) was used to measure luciferase activity after TGF-β1 or -β2 addition. Figure 7D shows that there is no significant difference between signalling via the two TGF-β isoforms in these cells. This can be explained by the presence of TβRIII in Mv1Lu cells and derivative cell

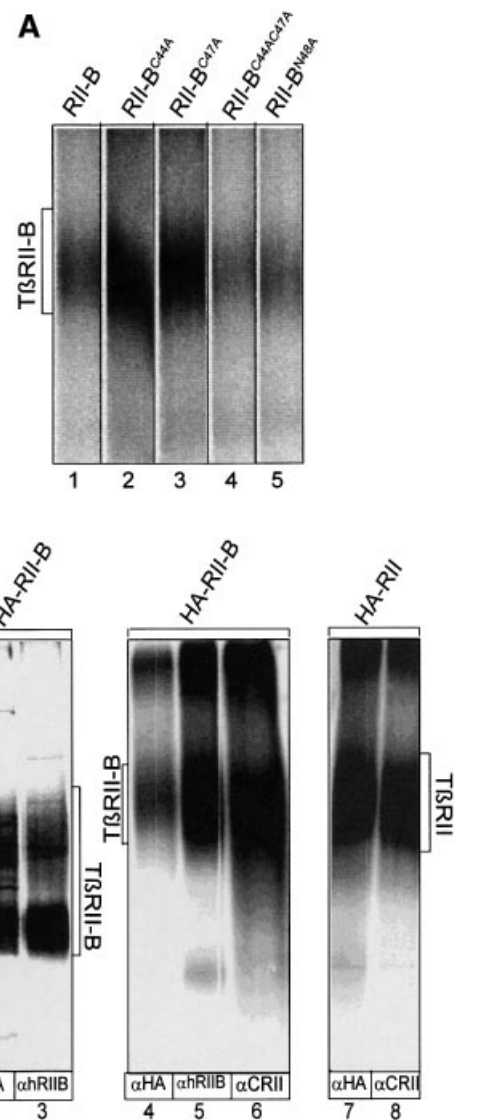


Fig. 5. Neither alternative disulfide bond formation nor N-glycosylation, but addition of N-terminal epitope tags, influences ligand binding to TβRII-B. (A) COS-7 cells were transiently transfected with the wild-type TβRII-B (lane 1) and mutant forms of TβRII-B, where Cys44 (lane 2), Cys47 (lane 3) or Cys44 and Cys47 (lane 4) or Asn48 (lane 5) were mutated to alanine. After binding and crosslinking with [¹²⁵I]TGF-β2, receptors were immunoprecipitated with α-CRII. The position of ligand-bound TβRII-B is indicated. All four mutants of TβRII-B are able to bind TGF-β2. (B) COS-7 cells were transfected with HA-tagged TβRII or TβRII-B. After metabolic labelling with [³⁵S]cysteine/methionine (lanes 1–3) or binding and crosslinking using [¹²⁵I]TGF-β1 (lanes 4–8), the receptors were immunoprecipitated using antibodies as indicated. Control immunoprecipitations were carried out with α-hRIIB (lane 5) and α-CRII (lanes 6 and 8).

lines, which compensates for the lack of TGF-β2 binding to the TβRII by presenting the ligand.

TβRII-B interacts with all known type I receptors (ALK1–7) after binding TGF-β1 (data not shown). To investigate signalling via these receptor complexes we have performed reporter gene assays in R1b/L17 cells. Different type I receptor constructs were expressed in R1b/L17 cells either in the presence or absence of TβRII-B. Transcriptional activation of the reporter plasmids

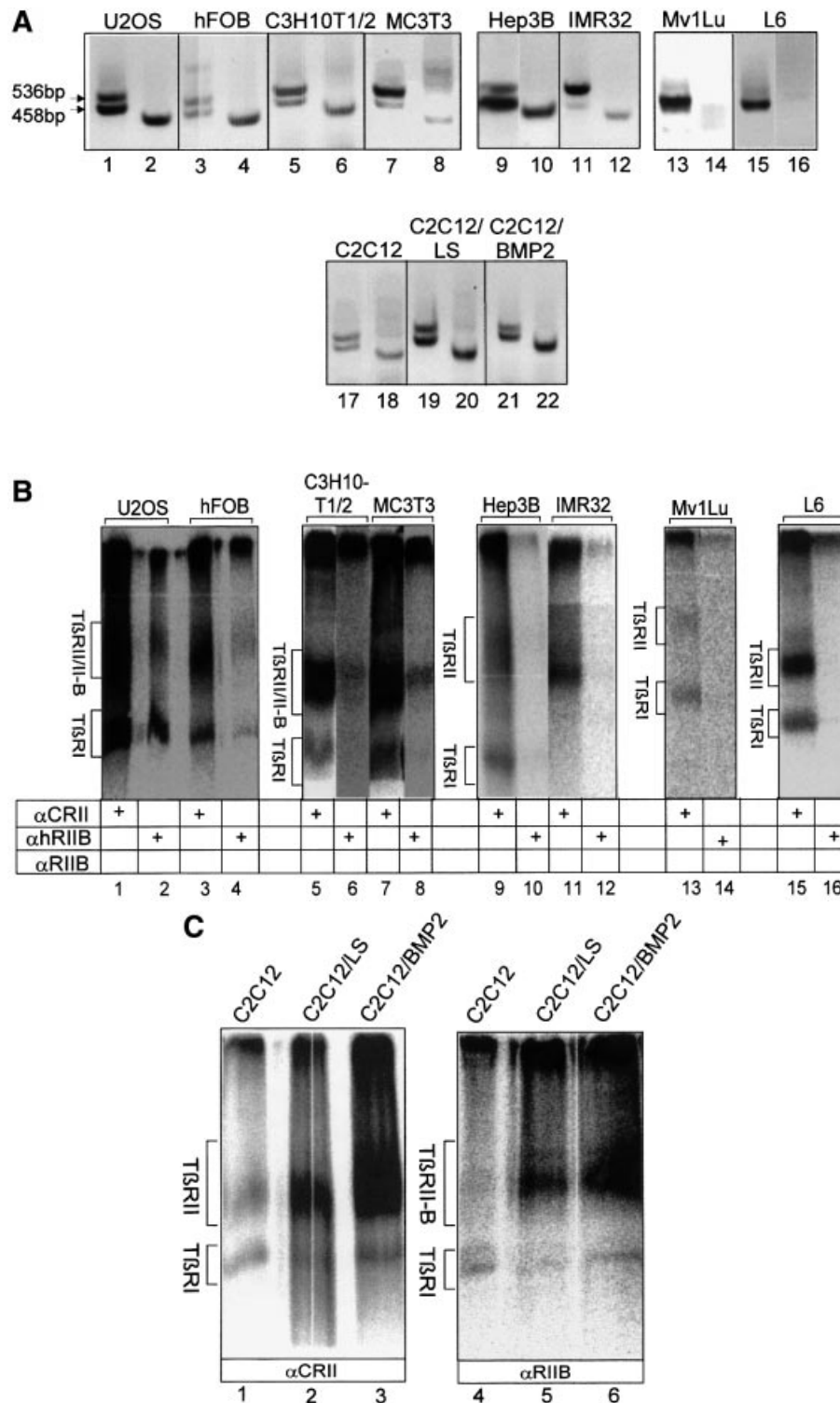


Fig. 6. Restricted expression pattern of T β R II -B. (A) RT-PCR analysis of T β R II -B mRNA in different cell lines. The cDNAs were prepared from human osteosarcoma cells (U2OS), human fetal osteoblasts (hFOB), murine mesenchymal precursor cells MC3T3, C3H10T1/2 cells and C2C12 myoblasts, the human hepatoma cell line Hep3B, human neuroblastoma cells (IMR32), Mv1Lu cells and rat myoblasts (L6). PCR products were obtained using the primers P1 and P5 (odd lane numbers) or the T β R II -B-specific primers Pins and P5 (even lane numbers). Two PCR products using P1/P5 (for example, lane 1) as well as a single PCR product using Pins/P5 (for example, lane 2) indicate the presence of T β R II -B mRNA. A single PCR product using P1/P5 (lanes 13 and 15) and no PCR product using Pins/P5 (lanes 14 and 16) indicate the expression of only T β R II mRNA. C2C12 cells were analysed either undifferentiated (lanes 17 and 18) or after differentiation in low serum (LS; lanes 19 and 20) or in LS containing 40 nM BMP-2 (lanes 21 and 22). (B) Endogenous expression of T β R II and T β R II -B at the cell surface of different cell lines was detected by affinity labelling with [125 I]TGF- β 1. Cell lysates were immunoprecipitated either with α -CRII (odd lane numbers), the antibody specific for the human T β R II -B, α -hRIIB (lanes 2, 4, 10 and 12) or α -RIIB, which recognizes also the murine T β R II -B (lanes 6, 8, 14 and 16). Hep3B, IMR32, Mv1Lu and L6 cells do not show any T β R II -B protein at the cell surface. (C) Upregulation of TGF- β receptors during differentiation of C2C12 cells. Cell surface expression of TGF- β type II receptors (lanes 1-3) and specifically T β R II -B (lanes 4-6) was determined after affinity labelling using iodinated TGF- β 1 on C2C12 cells, which are either undifferentiated (lanes 1 and 4), differentiated into multinucleated myotubes (lanes 2 and 5) or differentiated into the osteoblast lineage by BMP-2 (lanes 3 and 6).

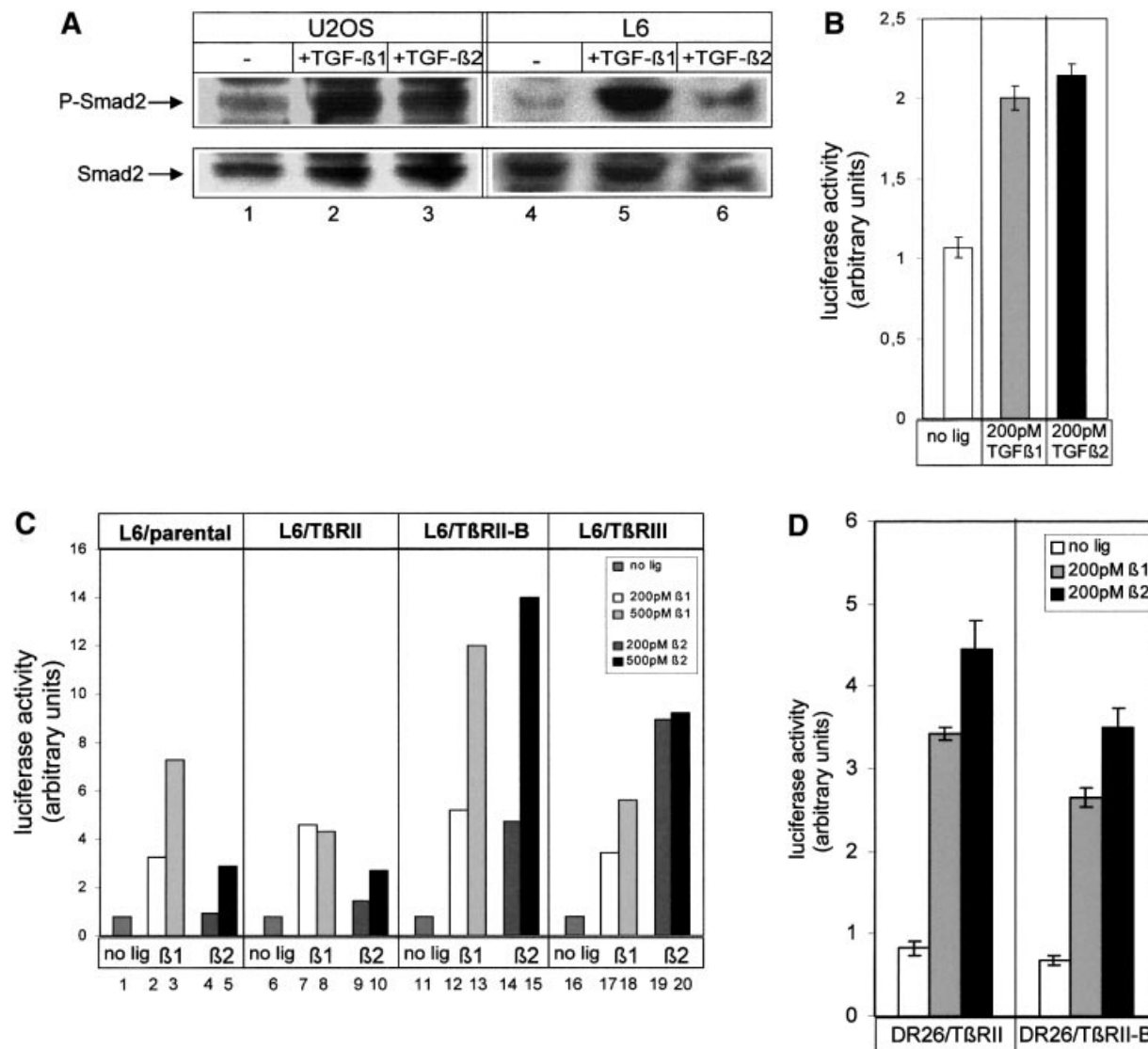


Fig. 7. TβRII-B transduces TGF-β2 signals via Smad2 independently of TβRIII. (A) U2OS cells (lanes 1–3) and L6 cells (lanes 4–6) were treated with 200 pM TGF-β1 (lanes 2 and 5) or 200 pM TGF-β2 (lanes 3 and 6). Total cell lysates were used for western blotting with PS2 antiserum (upper panel). Equal loading was confirmed using α-Smad2 (α-SED) antiserum (lower panel). (B) U2OS cells were transfected with the TGF-β-sensitive reporter plasmid p3TP-luc and pRL-TK for reference. After stimulation with 200 pM TGF-β1 or 200 pM TGF-β2, luciferase activity was measured. Data were normalized to pRL-TK activity to control for transfection efficiency. (C) L6 cells were transfected with the receptors indicated, p3TP-luc and pRL-TK, and then incubated with 200 pM (white), 500 pM TGF-β1 (grey), 200 pM TGF-β2 (dark grey) or 500 pM TGF-β2 (black). Data were normalized to pRL-TK activity and represent the mean of three independent experiments. (D) DR26 cells were transfected with p3TP-luc and pRL-TK together with TβRII-B or TβRII constructs. Luciferase activity was determined as described above.

p3TP-luc (Wrana *et al.*, 1992) and pSBE-luc (Jonk *et al.*, 1998; data not shown) was determined for both TGF-β1 and TGF-β2. In the case of p3TP-luc, only expression of ALK5 showed induction of the reporter gene; the co-expression of TβRII-B even results in ligand-independent activation (Figure 8). ALK4, the activin type Ib receptor, showed activation of the reporter by the ligand TGF-β1 only when TβRII-B (or TβRII, data not shown) was expressed. Therefore, signalling of TβRII-B via the Smad2/3 pathway is induced primarily through activation of ALK5 (TβRI).

Taken together, our results demonstrate that TβRII-B is a signalling receptor for the TGF-β2 isoform. Direct binding of this isoform induces TβRIII-independent signalling. This is of particular interest in cells and tissues

that lack TβRIII and in which TGF-β2 has a distinct function. In addition to the β2 isoform, TβRII-B also binds and triggers signals from TGF-β1.

Discussion

The three mammalian isoforms TGF-β1, -β2 and -β3 share 71–76% sequence identity and the proteolytically cleaved mature forms are highly conserved between different species. This suggests that during evolution there has been pressure to keep certain differences as well as similarities of these isoforms. This is also reflected in gene targeting experiments that revealed specific phenotypes (Schull *et al.*, 1992; Kulkarni and Karlsson, 1993; Diebold *et al.*, 1995; Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995; Sanford

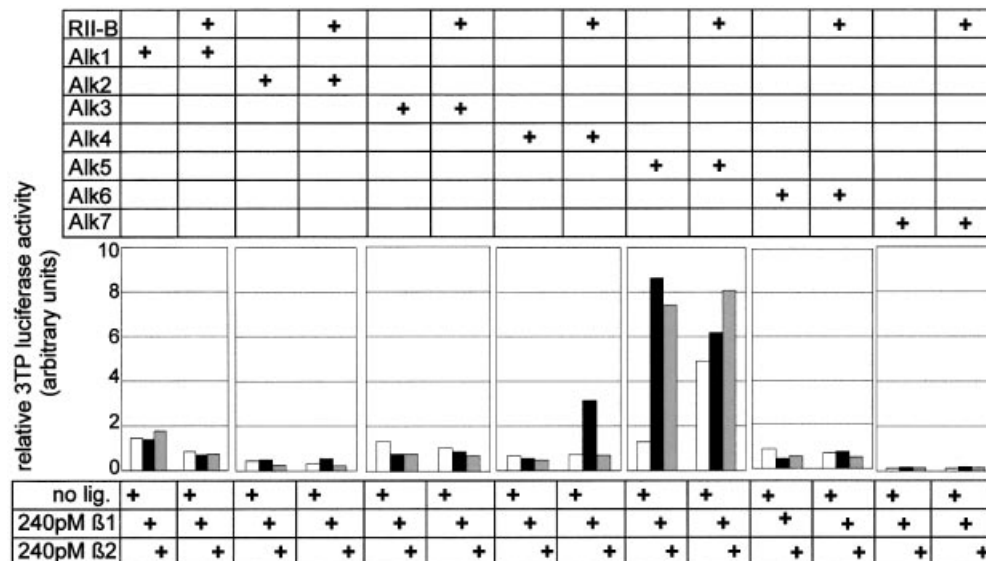


Fig. 8. T β RII-B signals via T β RI (ALK5) to the reporter p3TP-luc. R1b/L17 cells were transiently transfected with ALK1–7 in the absence or presence of T β RII-B (as indicated). Reporter gene activity was measured on p3TP-luc after treatment with either TGF- β 1 (black bars) or TGF- β 2 (grey bars). Luciferase activity was determined as described.

et al., 1997). Even though all isoforms are very similar in sequence and structure, their function *in vivo* is quite distinct.

While the initial steps of signal transduction by TGF- β 1 are well studied, signalling by the TGF- β 2 isoform is still less clear. TGF- β 1 follows the ‘sequential binding mode’ in order to form the signalling receptor complex at the cell surface (Wrana *et al.*, 1994a; Massagué, 1998). It first binds to the T β RII, causing the recruitment of T β RI into the signalling complex and its subsequent activation by transphosphorylation. Since T β RII does not bind the TGF- β 2 isoform (Lin *et al.*, 1995), it was proposed that signalling via TGF- β 2 requires the co-expression of T β RIII. T β RIII binds TGF- β 2 with high affinity and presents the ligand to T β RII by receptor oligomerization (Lopez-Casillas *et al.*, 1993; Henis *et al.*, 1994). This suggested that cells lacking T β RIII will not respond to TGF- β 2 (Sankar *et al.*, 1995), which has been confirmed recently in endocardial cells requiring functional T β RIII for transformation in the heart (Brown *et al.*, 1999). However, it is still unclear why TGF- β 1, which would directly activate the T β RII–T β RI complex, does not result in transformation of these ventricular endothelial cells.

Here we describe T β RII-B, an alternatively spliced T β RII, as a receptor that not only binds TGF- β 2 directly (Figure 2), but also activates the Smad pathway (Figure 7). Binding and signalling are independent of the co-expression of T β RIII (Figures 2 and 7).

The alternative splicing results in an insertion of 26 amino acids in exchange for Val32 at the extracellular domain of the receptor. This structural alteration leads to a new binding site for TGF- β 2 without abolishing binding to the other isoforms, TGF- β 1 and - β 3 (Figure 2). We have also shown that binding of antibodies close to this new binding site interferes with TGF- β 1 binding, probably due to steric hindrance (Figure 5B). Amino acids potentially important for structural characteristics favouring this new binding site are two cysteines in the human T β RII-B (one

in the murine T β RII-B) and Asn48, as a potential N-glycosylation site. Mutations of these residues to alanine, however, led to no significant differences in binding of the TGF- β 2 isoform compared with the wild-type T β RII-B (Figure 5A).

Co-immunoprecipitation studies revealed that T β RII-B interacts with all known TGF- β receptors (T β RI, T β RII and T β RIII, see Figures 3 and 4) in the presence of ligand. We even show that hetero-oligomeric complexes of T β RII/T β RII-B enable T β RII to bind the TGF- β 2 isoform without any T β RIII, suggesting that one subunit of T β RII-B is enough to get any binding of TGF- β 2 to T β RII. In cells that endogenously express both T β RII and T β RII-B, the functional significance of T β RII/T β RII-B hetero-oligomers is unknown. Homodimers of TGF- β isoforms are abundant, but TGF- β 1.2 and TGF- β 2.3 heterodimers have been identified *in vivo*. In view of the existence of heterodimeric ligands, it is conceivable that two different type II receptors constitute a signalling receptor complex, thereby creating combinatorial signalling (Cheifetz *et al.*, 1987; Ogawa *et al.*, 1992). It is of interest to investigate further the signals originating from T β RII and T β RII-B homo-oligomers versus T β RII/T β RII-B hetero-oligomers.

Previous publications on T β RII-B did not address in detail binding and signalling via different TGF- β isoforms (Hirai and Fijita, 1996). With our studies it became clear that competition with an excess of cold TGF- β 2 in a TGF- β 1 binding assay does not prove the inability of this receptor to bind TGF- β 2. We have shown direct binding of TGF- β 2 and this further implies that TGF- β 2, in contrast to TGF- β 1, uses a different binding site at the type II receptor. We, as well as Hirai and Fijita (1996), have shown that in DR26 cells overexpression of either T β RII-B or T β RII does not show any difference (Figure 7D). This is due to the presence of T β RIII, which binds TGF- β 2 and presents it to T β RII (Lopez-Casillas *et al.*, 1993). From our data it is evident now that it

is not possible to distinguish between TGF- β 2 signalling via T β RIII/T β RII, T β RIII/T β RII-B or directly via T β RII-B, if the reporter gene p3TP-luc is used (Figure 7D).

Therefore, we decided to analyse TGF- β 2 signalling in cells lacking T β RIII. Indeed it had been demonstrated before that cells not expressing T β RIII, such as various vascular endothelial and haematopoietic progenitor cell lines, show a relative resistance to TGF- β 2 (Ohta *et al.*, 1987; Ottmann and Pelus, 1988; Sankar *et al.*, 1995). Various muscle myoblast cell lines including L6 express T β RII and T β RI but lack T β RIII. We showed that there is no expression of T β RII-B in these cells (Figure 6A and B). For this reason the L6 cell line is the ideal cell system to compare signalling via T β RIII and T β RII-B. In transfection experiments we have shown that signalling via TGF- β 2 is restored in these cells in the presence of either T β RIII or T β RII-B (Figure 7C). However, T β RII does not induce TGF- β 2 sensitivity. To analyse TGF- β 2 signalling through endogenous T β RII-B, independently of T β RIII, we performed the same assays in the osteosarcoma cell line U2OS, which lacks T β RIII but still expresses T β RII-B. These cells respond to TGF- β 2, as we show by Smad2-phosphorylation experiments (Figure 7A) and reporter gene assays (Figure 7B). This signalling is facilitated via T β RI and not via ALK1-4 or ALK6 and ALK7 (Figure 8).

Besides the TGF- β type II receptor, other members of the receptor family exist as alternative forms. The type II receptor for MIS (AMHRII) shows alternative splicing, resulting in a 61-amino-acid insertion at the same site as T β RII (di Clemente *et al.*, 1994), the Dpp receptor Tkv has two alternative N-termini (Brummel *et al.*, 1994; Penton *et al.*, 1994) and there are two alternative extracellular juxtamembrane regions in ATR-I (Wrana *et al.*, 1994b). Moreover, four alternative splicing variants of ActRII-B (Attisano *et al.*, 1992) and a splicing variant of BMP-RII exist (Kawabata *et al.*, 1995; Nohno *et al.*, 1995; Rosenzweig *et al.*, 1995). Although the functional significance of these variants is still unknown, it is of interest that insertions at the N-terminus of the receptors are seen only in ligand-binding subunits (T β RII, AMHRII, Tkv), suggesting that the N-terminus of the ligand binding subunit is important for ligand specificity. Scanning deletion analysis of the extracellular domain of T β RII has revealed that amino acids N-terminal to the first cysteine are not important for binding to TGF- β 1 (Pepin *et al.*, 1996); the insertion in T β RII-B, however, seems to add binding properties for an additional isoform.

Of special interest is the expression and signalling capability of T β RII-B in osteoblasts and osteosarcoma cells. Osteoblasts are of mesenchymal origin and synthesize bone matrix, and osteoclasts derived from the haematopoietic system resorb bone. It was shown that TGF- β 2 increases osteoblastic activity by the induction of extracellular matrix secretion, inhibition of matrix mineralization and modulation of osteoprogenitor cell proliferation (Centrella *et al.*, 1994; Rosen *et al.*, 1994). Increased expression of TGF- β 2 in osteoblasts results in an osteoporosis-like phenotype (Erlebacher and Derynck, 1996). Inhibition of TGF- β receptor signalling in osteoblasts by transgenic expression of cytoplasmically truncated T β RII has recently been shown to result in decreased bone remodelling, which is probably due to decreased bone

resorption by osteoclasts (Filvaroff *et al.*, 1999). This is of special importance during metastasis of breast cancer cells to the skeleton, where TGF- β released from the bone matrix by the action of osteoclasts promotes metastatic growth (Yin *et al.*, 1999). While TGF- β stimulates bone resorption by differentiated osteoclasts it inhibits osteoclast differentiation from bone marrow monocytes.

The specific expression of the T β RII-B as a TGF- β 2 receptor in osteoblasts suggests a distinct function of this receptor in the process of bone remodelling. Signalling of TGF- β 2 or TGF- β 1 via this receptor in comparison with signalling via T β RII and T β RIII needs to be further investigated in order to understand the isoform-specific effects of TGF- β .

Materials and methods

Cell culture

COS-7, Mv1Lu, L6, C3H10T1/2, C2C12 and Hep3B cells were obtained from ATCC, MC3T3-E1 cells from P.ten Dijke (Uppsala), IMR 32 cells from K.Unsicker (Heidelberg) and U2OS cells from J.Hoppe (Würzburg). R1b/L17 and DR26 cells were obtained from J.Massagué (New York).

Isolation of the human T β RII-B clone, RT-PCR and mutagenesis

RNA was extracted from different cell lines as described (Chomczynski and Sacchi, 1987) and reverse transcribed using Superscript II (Gibco-BRL) according to the manufacturer's instructions. For subsequent PCR, *Pfu* polymerase (Invitrogen) and specific oligonucleotides corresponding to the extracellular domain of T β RII (P1, nucleotides -23 to -4; P5, nucleotides 435-417; Lin *et al.*, 1992) and for T β RII-B the specific primer Pins (nucleotides 106-132) were used in combination with P5. For the isolation of the T β RII-B clone, the T β RII-B-specific fragment was cut by *Hind*III and *Bgl*II to replace the corresponding fragment in T β RII (H20) (Knaus *et al.*, 1996).

All mutations were generated by PCR mutagenesis. The cysteine residues Cys44 and Cys47 in T β RII-B were mutated to alanine individually or in combination. The glycosylation mutant was generated by replacing Asn48 by alanine. The HA-epitope was introduced after Pro26.

Expression plasmids for ALK1-6 were kindly provided by C.H.Heldin (Uppsala), the expression construct for ALK7 by C.Ibanez (Stockholm).

Ligands

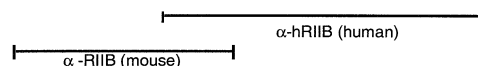
Recombinant TGF- β 1, TGF- β 2, TGF- β 3 and activin A were purchased from R&D Systems. Recombinant human BMP-2 was prepared as described (Ruppert *et al.*, 1996).

Antibodies

Polyclonal antibodies directed against two different peptides of the T β RII-B insert were raised in rabbits and are either specific for the mouse and human (α -RIIB) or the human (α -hRIIB) T β RII-B.

mouse KSDVEMEAQKIDASIHLSNRTIHPKHF

human KSDVEMEAQKDEIICPSCNRTAHPLRHI



The polyclonal antiserum against a peptide corresponding to the C-terminal sequence of the human T β RII (α -CRII) and the polyclonal antiserum specific for a cytoplasmic peptide in human ALK5 (α -RI) have been described previously (Moustakas *et al.*, 1995). The anti-phospho-Smad2 antiserum was kindly provided to us by P.ten Dijke and C.H.Heldin (Uppsala). The 12CA5 antibody against the HA-tag was purchased from BabCo. The polyclonal anti-peptide antibody against the BMP-type Ia receptor (BR1a) was described earlier (Gilboa *et al.*, 2000).

Transient transfections

COS-7 cells were transfected with plasmids encoding receptor cDNAs using the DEAE-dextran method (Aruffo and Seed, 1987). Forty-eight hours after transfection, binding and crosslinking were performed as described below. Aliquots of cell lysates were subjected to immunoprecipitation.

DR26 cells were transfected using DEAE-dextran, L6, R1b/L17 and U2OS cells using Lipofectamine (Life Technologies) according to the manufacturer's protocol. Cells were lysed 36–48 h after transfection to measure luciferase activity.

Ligand binding and crosslinking

TGF- β 1, - β 2, - β 3 and activin A were iodinated and crosslinked as described in Lin *et al.* (1992), BMP-2 as described in Gilboa *et al.* (2000).

Receptor immunoprecipitation

After binding and crosslinking, COS-7 cells were solubilized in lysis buffer (phosphate-buffered saline pH 7.4 containing 1.0% Triton X-100, 1 mM EDTA and including protease inhibitors) at 4°C for 40 min. Receptors were immunoprecipitated from cell extracts by 12CA5 monoclonal antibodies (α -HA) or by using specific rabbit anti-peptide antisera (α -hRIIB, α -RIIB and α -CRII) together with protein A-Sepharose for at least 4 h at 4°C. For single immunoprecipitations, the bound protein was eluted by heating the beads in SDS-PAGE sample buffer containing β -mercaptoethanol (5 min, 95°C). For sequential immunoprecipitations, the bound protein was eluted from the Sepharose beads in 1% SDS, 50 mM dithiothreitol, 10% β -mercaptoethanol (5 min, 95°C). The supernatant was diluted with lysis buffer to a final SDS concentration of <0.1% and the appropriate antibodies were added for the second immunoprecipitation. TGF- β receptors were analysed by 7.5–10% SDS-PAGE followed by exposure to a phosphorimager screen.

Metabolic labelling

COS-7 cells were starved in serum-free Dulbecco's modified Eagle's medium (DMEM) minus cysteine and methionine for 90 min at 37°C. The medium was then replaced with fresh medium supplemented with 0.2 mM oxidized glutathione (Boehringer) and 0.5 mCi/ml of [³⁵S]methionine and [³⁵S]cysteine (Dupont) and incubated for 2–3 h at 37°C. Cells were solubilized as described above. Lysates were immunoprecipitated with appropriate antibodies and proteins analysed by SDS-PAGE.

Reporter gene assays

Cells were starved for 12–24 h after transfection in 0.2% FCS for 4–6 h followed by the addition of 200 or 500 pM TGF- β 1 or TGF- β 2 for 18–24 h. Cells were lysed, and luciferase activity determined by the Dual Luciferase Assay system (Promega).

Smad2 phosphorylation assay

U2OS or L6 cells (5×10^5) were plated on 6 cm Petri dishes. Starvation was performed for 4 h in DMEM containing 0.2% FCS. TGF- β 1 and - β 2 (200 pM) were added for 30 min. Cells were lysed in cold TNE buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) including protease inhibitors and phosphatase inhibitors. Aliquots of the cleared lysate were submitted to SDS-PAGE followed by immunoblotting. C-terminally phosphorylated Smad2 was detected by a α -phospho-Smad2 (α -PS2) antibody (Ishisaki *et al.* 1999) and visualized using the ECL detection system. To show equal loading, the antibodies were removed by incubating the nitrocellulose membrane in stripping buffer (5 mM phosphate buffer, 2% SDS and 0.014% β -mercaptoethanol) for 30 min at 60°C. Smad2 protein was detected using an α -Smad2 (α -SED) antibody (Nakao *et al.*, 1997).

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