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A kinase subdomain of transforming growth factor-β (TGF-β) type I receptor determines the TGF-β intracellular signaling specificity

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Transforming growth factor-β (TGF-β) signals through a heteromeric complex of related type I and type II serine/threonine kinase receptors. In Mv1Lu cells the type I receptor TβRI mediates TGF-β-induced gene expression and growth inhibition, while the closely related type I receptors Tsk7L and TSR1 are inactive in these responses. Using chimeras between TβRI and Tsk7L or TSR1, we have defined the structural requirements for TGF-β signaling by TβRI. The extracellular/transmembrane or cytoplasmic domains of TβRI and Tsk7L were functionally not equivalent. The juxtamembrane domain, including the GS motif, and most regions in the kinase domain can functionally substitute for each other, but the αC–β4–β5 region from kinase subdomains III to V conferred a distinct signaling ability. Replacement of this sequence in TβRI by the corresponding domain of Tsk7L inactivated TGF-β signaling, whereas its introduction into Tsk7L conferred TGF-β signaling. The differential signaling associated with this region was narrowed down to a sequence of eight amino acids, the L45 loop, which is exposed in the three-dimensional kinase structure and diverges highly between TβRI and Tsk7L or TSR1. Replacement of the L45 sequence in Tsk7L with that of TβRI conferred TGF-β responsiveness to the Tsk7L cytoplasmic domain in Mv1Lu cells. Thus, the L45 sequence between kinase subdomains IV and V specifies TGF-β responsiveness of the type I receptor.

Keywords: kinase/signaling/specificity/TGF-β/type I receptor

Introduction

Transforming growth factor-β (TGF-β) regulates cell growth and differentiation (for recent reviews, see Derynck, 1994a; Kingsley, 1994) by interacting with two types of receptors, the type I and type II TGF-β receptors, which are both essential for signal transduction (Wrana *et al.*, 1994; and reviews by Derynck, 1994b; Massagué, 1996). Both type I and type II receptors belong to an expanding family of transmembrane serine/threonine kinases. While the type II TGF-β receptor, TβRII, binds TGF-β1 by itself, the type I receptor only binds TGF-β1 in the presence of TβRII, consistent with a physical interaction of these two receptor types (Ventura *et al.*, 1994; Feng and Derynck, 1996; and reviews by Derynck, 1994b; Massagué, 1996). In contrast, TGF-β2 does not bind efficiently to either receptor type, but rather to a receptor complex consisting of both TβRI and TβRII (Rodriguez *et al.*, 1995).

Several type I receptors, e.g. TβRI/ALK-5, Tsk7L/ActRI and TSR1/SKR3, have been shown to bind TGF-β when co-expressed with TβRII (Attisano *et al.*, 1993; Ebner *et al.*, 1993a; Franzén *et al.*, 1993; ten Dijke *et al.*, 1994a). However, only TβRI mediates the induction of gene expression and growth inhibition in response to TGF-β in Mv1Lu epithelial cells (Franzén *et al.*, 1993; Bassing *et al.*, 1994b; Feng *et al.*, 1995), while Tsk7L plays a role in TGF-β-induced differentiation in another epithelial cell line NMuMG (Miettinen *et al.*, 1994). Several structural features distinguish type I from type II receptors. In particular, type I receptors have a highly conserved GS motif (SGSGSGLP) in the cytoplasmic juxtamembrane region immediately preceding the kinase domain (reviewed by Derynck, 1994b). Deletion of this juxtamembrane domain abolishes the ability of TβRI to undergo autophosphorylation and to mediate TGF-β-dependent intracellular responses (Feng *et al.*, 1995), and single amino acid substitutions in the GS motif cause loss-of-function or gain-of-function mutations of TβRI (Wieser *et al.*, 1995). These results suggest that the conserved GS domain plays an important role in activating the type I receptor kinase (Wieser *et al.*, 1995) and/or probably in recruiting intracellular signaling components (Saitoh *et al.*, 1996). Furthermore, the extensive sequence differences in the cytoplasmic juxtamembrane domains, but not in the rest of the cytoplasmic domain of the type I receptors, suggest that they play a role in conferring signaling specificity to different ligands, possibly by interacting with different cytoplasmic effector proteins (Saitoh *et al.*, 1996).

The signaling TGF-β receptor complex consists of two type I and and two type II receptors (Yamashita *et al.*, 1994; Luo and Lodish, 1996; Weis-Garcia and Massagué, 1996). In this complex, the cytoplasmic domains of the two receptor types have an inherent affinity for each other (Chen *et al.*, 1995; Feng and Derynck, 1996), and ligand binding stabilizes this heteromeric association. In response to TGF-β binding, the cytoplasmic domains of the type I receptors are phosphorylated by the constitutively active and autophosphorylated type II receptor kinase (Wrana *et al.*, 1994; Chen and Weinberg, 1995). This phosphorylation of the type I receptor cytoplasmic domain occurs primarily in the juxtamembrane and conserved GS domains (Wrana *et al.*, 1994; Chen and Weinberg, 1995; Souchelnytskyi *et al.*, 1996). Following activation of the type I receptor, the heteromeric receptor complex mediates the biological responses of TGF-β, such as growth inhibition and extracellular matrix production. Recent progress strongly suggests that mammalian homologs of *Drosophila*

Mad (mother against dpp) (Sekelsky *et al.*, 1995), recently renamed Smads, are intracellular effectors of TGF-β receptor signaling (Derynck and Zhang, 1996; Massagué, 1996). Smad2 and Smad3 have been shown to associate with the TβRI–TβRII receptor complex and to be phosphorylated (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). Furthermore, Smad2/3 associate with Smad4 and are translocated into the nucleus (Lagna *et al.*, 1996; Zhang *et al.*, 1996, 1997; Wu *et al.*, 1997). Ligand binding induces phosphorylation of Smads and nuclear translocation (Macias-Silva *et al.*, 1996). In the nucleus, Smads are thought to function as transcriptional activators for TGF-β-responsive genes (Liu *et al.*, 1996).

To gain insight into the role of TβRI in TGF-β-mediated responses, we characterized the structural requirements for the function and signaling specificity of type I receptors. As a model, we compared TβRI, which mediates TGF-β-induced growth inhibition and expression of plasminogen activator inhibitor type 1 (PAI-1) in Mv1Lu epithelial cells, with the related Tsk7L receptor, which does not induce these responses. We made chimeric receptor mutants between TβRI and Tsk7L and analyzed their ability to rescue TGF-β responsiveness in Mv1Lu-R1B cells which lack functional TβRI. In R1B cells, TβRI but not Tsk7L rescues TGF-β-induced growth inhibition and gene expression responses. Our results demonstrate that in the cytoplasmic domain, the juxtamembrane domain (including the GS motif) and most regions in the kinase domain are functionally equivalent in the two type I receptors. However, the αC–β4–β5 region, in particular the L45 loop between the β4 and β5 strands, determined the signaling capacity of TβRI. Significantly, mutation of this sequence of TβRI dramatically decreased the signaling capacity of TβRI, and introduction of the L45 sequence of TβRI into Tsk7L conferred TGF-β-induced signaling activity to this otherwise inactive receptor. Our results show that the L45 loop defines the signaling specificity of the type I receptor for TGF-β intracellular responses.

Results

The extracellular/transmembrane or cytoplasmic domains of type I receptors TβRI and Tsk7L are not functionally equivalent

The type I receptors TβRI, Tsk7L and TSR1 are structurally closely related in both the extracellular and cytoplasmic domains. The extracellular domains of type I receptors, which contain the conserved Cys-rich sequences, display 23% sequence identity between TβRI and Tsk7L, 23% between TβRI and TSR1, and 24% between Tsk7L and TSR1. The cytoplasmic domains of these receptors demonstrate a much higher sequence similarity and identity, consistent with the conserved sequence requirements of the large serine/threonine kinase domains. The sequences of the cytoplasmic domains of the three type I receptors are 73–83% similar (60–73% identical) (Figure 1). In spite of their sequence conservation and ability to bind TGF-β1, these type I receptors have different signaling functions and specificities. In Mv1Lu epithelial cells, the most frequently used TGF-β-responsive cell system, only TβRI transduces TGF-β-induced antiproliferative and gene expression responses. In contrast, Tsk7L and TSR1 do not signal these responses in Mv1Lu cells (Bassing *et al.*, 1994b; ten Dijke *et al.*, 1994a; Feng *et al.*, 1995).

To delineate the structural basis of the difference in ability of TβRI and Tsk7L to mediate the TGF-β responses, we made expression plasmids for chimeric receptors that exchange domains of Tsk7L and TβRI. As a first step, we constructed two chimeric type I receptors that combined the extracellular/transmembrane (ET) and cytoplasmic domains of TβRI and Tsk7L, thereby creating the receptor hybrids TβRI–Tsk7L and Tsk7L–TβRI (Figure 2A). These chimeric receptors, which were C-terminally FLAG tagged, were expressed with their expected molecular weights and at levels similar to the parental receptors in transfected R1B-L17 cells. Both chimeric receptors were able to bind [^{125}I]TGF-β when co-expressed with TβRII, although the ligand binding to the TβRI ET domain was stronger than to the Tsk7L ET domain (Figure 2B). Thus, the mutant receptors, like the wild-type receptors, were readily expressed at the cell surface and displayed normal ligand binding.

The signaling potential of these chimeric receptors was assessed in R1B cells (Laiho *et al.*, 1990, 1991) using both the cyclin A–luciferase and PAI-1–luciferase reporter assays (Feng *et al.*, 1995). Expression of TβRI, but not Tsk7L, restored responsiveness to TGF-β in transfected R1B cells (Figure 2C), consistent with previous findings that TβRI, but not Tsk7L, mediates the effect of TGF-β on transcription of both cyclin A and PAI-1 (Feng *et al.*, 1995). However, neither of the chimeric receptors conferred TGF-β responsiveness in R1B cells. This suggests that the ET domain of TβRI plays a role in TGF-β signal transduction which cannot be replaced by the Tsk7L ET domain. Furthermore, the inactivity of Tsk7L in TGF-β signaling is not only associated with its ET domain but also with its cytoplasmic domain, since TβRI–Tsk7L did not mediate TGF-β responses, despite its close structural similarity with TβRI.

We also determined the *in vitro* kinase activity of the receptors and their hybrids. As shown in Figure 3, TβRI had strong kinase activity (lane 2), while the kinase activity of Tsk7L was weak (lane 3). The replacement of the cytoplasmic domain in TβRI with the corresponding domain of Tsk7L resulted in a strong decrease in the kinase activity of the TβRI–Tsk7L chimera (lane 4), while Tsk7L–TβRI had a somewhat decreased kinase activity compared with TβRI (lane 5). Thus, the inactivity of the hybrid receptors in the biological assays did not correlate with the results from the kinase assays.

The cytoplasmic juxtamembrane domain and the kinase subdomains I–II of TβRI can be replaced by the corresponding sequences of Tsk7L

Our results with the two chimeras strongly suggest that the cytoplasmic domain of the type I receptor determines the signaling specificity of TGF-β receptors, since replacement of the cytoplasmic domain of TβRI with the closely related domain of Tsk7L abolishes TGF-β signaling. The cytoplasmic domains of TβRI and Tsk7L, as well as of TSR1, show extensive sequence identity over the 11 kinase subdomains (as defined by Hanks *et al.*, 1988) that comprise most of the cytoplasmic domain (Figure 1). Some sequence differences are scattered over the different

Fig. 1. Sequence alignment of the cytoplasmic domains of the three type I receptors TβRI, Tsk7L and TSR1. Multiple sequence alignment was performed using the Pileup and Prettybox programs of the Wisconsin Package (Genetics Computer Group). The single amino acid code is used. Ser163 in rat TβRI (corresponding to Ser165 in human TβRI) is indicated. Arrows indicate the junctions used in constructing swap receptors between type I receptors. Roman numerals below the sequences indicate the kinase subdomains as defined by Hanks *et al.* (1988). Secondary structure assignments for type I receptors were obtained from comparison with cAPK (Knighton *et al.*, 1991) and IRK (Hubbard *et al.*, 1994). Secondary structures are shown only in and around the subdomain II and V: α, α-helix; β, β-strands; L, loop.

subdomains, but the most notable differences are in the juxtamembrane domain immediately upstream from the conserved GS motif that defines the type I receptors. These juxtamembrane sequences of ~40 amino acids long have only minimal sequence similarity.

To determine which cytoplasmic sequences conferred TGF-β signaling, we made several chimeric receptors in which defined fragments of the cytoplasmic domain of TβRI were replaced by the corresponding sequences of Tsk7L. We first concentrated on the highly divergent juxtamembrane domains by making expression plasmids for three chimeric receptors, named Swap C1, C2 and C3 (Figure 4A). In these chimeras, the TβRI sequences starting at amino acid 149 and extending to residues 202, 234 and 284 for Swap C1, C2 and C3, respectively, were replaced by the corresponding Tsk7L sequences. These chimeric receptors were expressed at similar levels in transfected cells (Figure 3, lanes 6–8) but displayed different abilities to mediate TGF-β signaling. As shown in Figure 4B, Swap C1 and C2, but not Swap C3, restored TGF-β signaling in R1B cells as scored in both reporter assays, similarly to TβRI. These results demonstrate that the first 86 amino acids (i.e. amino acids 149–234 of TβRI) in the cytoplasmic domain are functionally indistinguishable between the two type I receptors. The replaceable sequences comprise the divergent juxtamembrane domain and the conserved GS domain that is a target for phosphorylation by TβRII (Wrana *et al.*, 1994; Chen and Weinberg, 1995; Souchelnytskyi *et al.*, 1996), as well as the kinase subdomains I–II. Thus, neither the juxtamembrane nor GS domains define the differential signaling abilities of the two type I receptors. In contrast, extending the replaced sequences into kinase subdomain V, as in TβRI–Tsk7L Swap C3, abolished the ability to mediate TGF-β signaling in R1B cells (Figure 4B), suggesting that the kinase subdomains III–V may play a role in defining the signaling specificity.

Since the chimeric receptors were expressed at similar levels, the differential signaling abilities could not be explained by inadequate expression of Swap C3 (Figure 3, lanes 6–8). Furthermore, the immunoprecipitated receptors were able to autophosphorylate *in vitro*, albeit with somewhat different efficiencies. The wild-type Tsk7L receptor and Swap C1 had very low *in vitro* kinase activity when compared with TβRI (Figure 3, lanes 2, 3 and 6, lower panel). Thus, the similar biological activities of TβRI and Swap C1 indicate a lack of correlation between the biological and kinase activities. Accordingly, the biological inactivity of Swap C3 did not correlate with an inability to autophosphorylate (Figure 3, lane 8, lower panel).

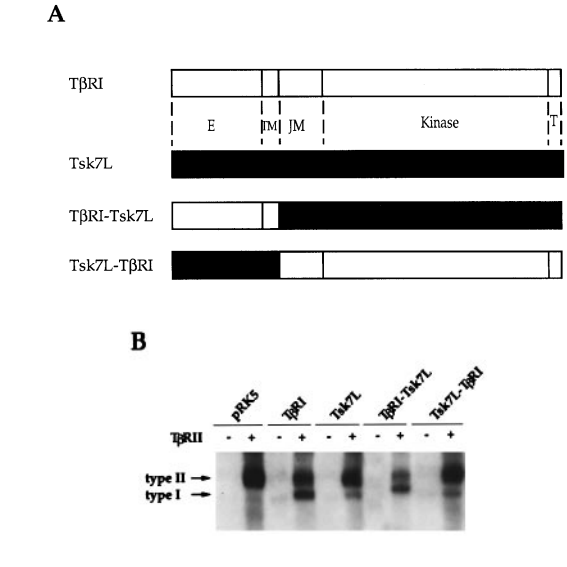

Fig. 2. Structure and activities of the type I receptors TβRI, Tsk7L and two complementary chimeric receptors. (**A**) Schematic representation of the wild-type and the chimeric receptors. E, extracellular domain; TM, transmembrane domain; JM, intracellular juxtamembrane domain; T, cytoplasmic tail following the kinase domain. (**B**) Ligand binding of the type I receptors expressed in COS-1 cells. COS-1 cells were transfected with expression plasmids expressing type I receptors in the presence or absence of type II receptor. Binding of [^{125}I]TGF-β to the cell surface receptors was carried out by chemical cross-linking followed by SDS–PAGE. Equal amounts of protein lysate were loaded in each lane, as determined by BCA assay (BioRad). Type I, type I receptor; type II, type II receptor. (**C**) Signaling abilities of type I receptors in R1B cells. Cells were transfected with reporter plasmids p800luc or pCAL2 and expression plasmids for type I receptors, as indicated on the *x*-axes, treated with TGF-β and lysed prior to performing the reporter assays. The data presented were obtained in two experiments, with each point determined in triplicate. The vertical bars show the standard deviations.

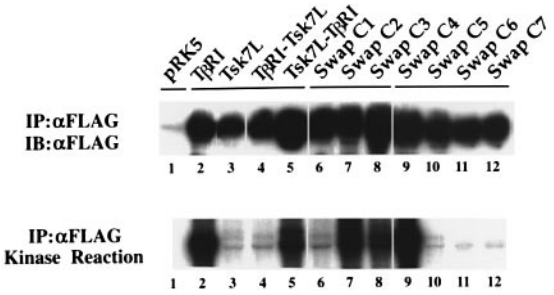

Fig. 3. Expression and *in vitro* kinase activity of type I receptors in R1B/L17 cells. L17 cells were transfected with receptor expression plasmids. The receptors were immunoprecipitated using anti-FLAG antibody and divided into two equal parts. One part was subject to SDS–PAGE and immunoblotting with anti-FLAG antibody to detect the expression level of the receptors (top panel). The other half of the immunoprecipitated proteins were incubated in *in vitro* kinase reactions using [γ-^{32}P]ATP prior to gel analysis (lower panel). The receptor chimeras shown in lanes 2–5, 6–8 and 9–12 are schematically represented in Figures 2A, 4A and 5A, respectively. pRK5, control vector.

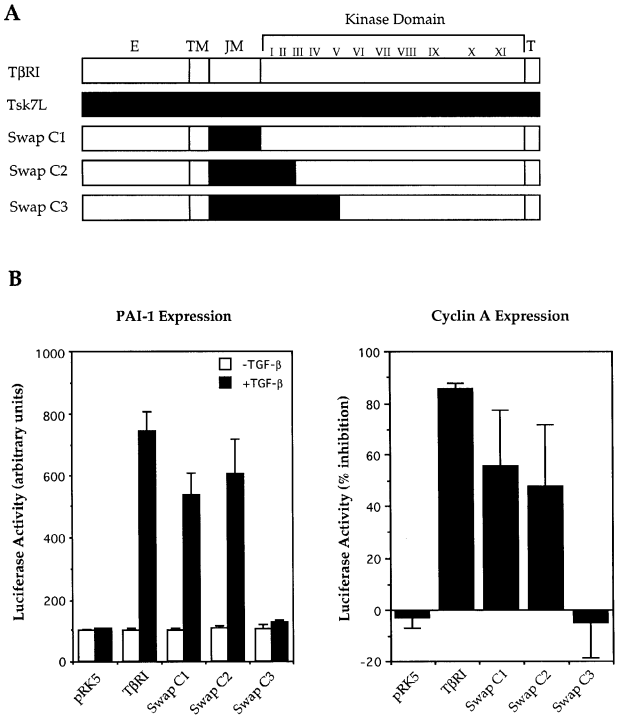

Fig. 4. Structure and signaling activities of the chimeric receptors Swap C1, C2 and C3. (**A**) Schematic representation of the chimeric receptors Swap C1, C2 and C3. The kinase subdomains are indicated above the diagram. Other abbreviations are the same as in Figure 2A. (**B**) Signaling activity of mutant receptors in R1B cells in both the PAI-1– and cyclin A–luciferase reporter assays, presented as in Figure 2C.

The C-terminal segment, i.e. subdomains VI–XI, of the cytoplasmic domain of TβRI can be replaced by Tsk7L sequences

To examine further the functions of C-terminal kinase subdomains of TβRI, we generated expression plasmids for additional receptor chimeras in which increasingly longer C-terminal segments of TβRI were replaced by Tsk7L sequences. As shown in Figure 5A, TβRI–Tsk7L Swap C4 and C5 had the C-terminal 119 and 217 amino acids, respectively, replaced by the corresponding Tsk7L sequences. In Swap C4, this substitution comprised the C-terminal tail and kinase subdomains IX, X and XI, while the replacement of the C-terminal segment in Swap C5 was extended into kinase subdomain V. The chimeric receptors, Swap C4 and C5, were as active as TβRI in both the PAI-1 and cyclin A assays in transfected R1B cells (Figure 5B). In contrast, two other chimeras, i.e. Swap C6 and C7, were unable to signal TGF-β responses in these assays (Figure 5B). These two chimeras contained larger C-terminal segments of Tsk7L, including part of

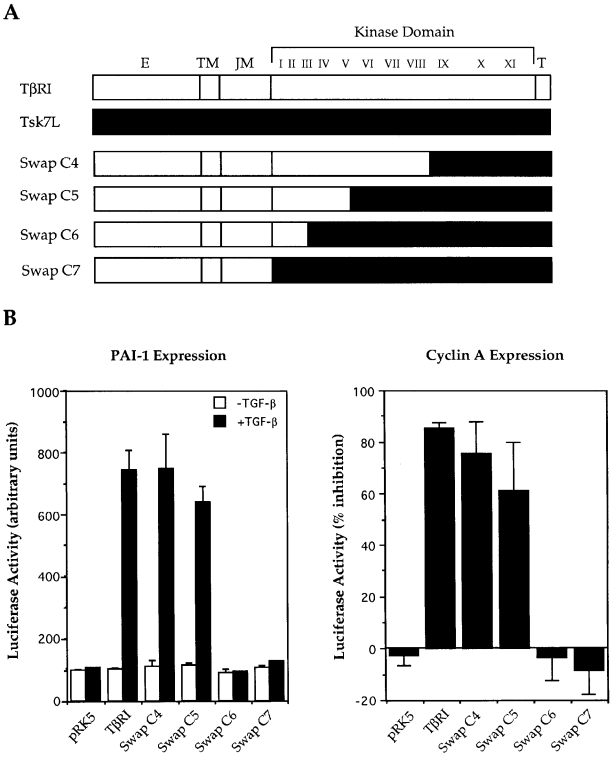

Fig. 5. Structure and signaling activities of the chimeric receptors Swap C4, C5, C6 and C7. (**A**) Schematic representation of the chimeric mutants. (**B**) Signaling activity of mutant receptors in R1B cells in both the PAI-1– and cyclin A–luciferase reporter assays, presented as in Figure 2C.

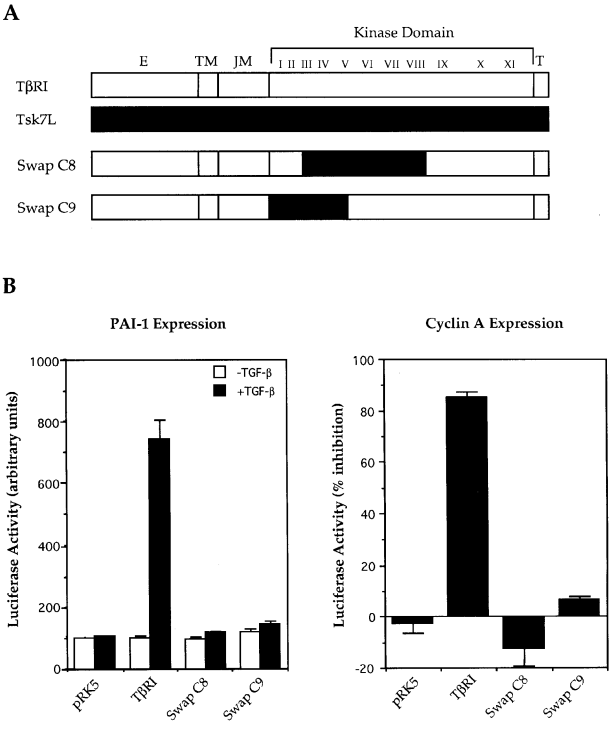

Fig. 6. Structure and signaling activities of the chimeric receptors Swap C8 and C9. (**A**) Schematic representation of the chimeric mutants. (**B**) Signaling activity of mutant receptors in R1B cells in both the PAI-1– and cyclin A–luciferase reporter assays, presented as in Figure 2C.

kinase subdomain III and all downstream sequences in the case of Swap C6, and the entire kinase domain of Tsk7L in the case of Swap C7. The abilities of Swap C4 and 5 to mediate TGF-β signaling and the inactivity of Swap C6 and C7 in these assays suggested an important role of kinase subdomains IV and V in mediating TGF-β signaling by TβRI. The kinase activity of the immunoprecipitated receptors was thus tested *in vitro*. As shown in Figure 3 (lower panel, lanes 9–12), only Swap C4 demonstrated a high autophosphorylation activity, while Swap C5, C6 and C7 had minimal activity in this assay. Thus, there was again no correlation between the signaling ability and kinase activity of these swap receptors.

The L45 sequence loop of the TβRI kinase domain is essential for signaling

Our results described above suggested the importance of amino acids 235–284 in defining the signaling activity of TβRI. This sequence is located within subdomains III, IV and V of the kinase and corresponds in the three-dimensional structure of cAMP-dependent protein kinase to a region containing two β strands (named the αC–β4–β5 region) (Knighton *et al.*, 1991). Based on our results, we predicted that replacement of kinase subdomains IV and V in TβRI with the corresponding sequences of Tsk7L would inactivate the signaling activity of TβRI. To test this prediction, we constructed two additional chimeric receptors, named Swap C9 and C8, which had a central region of the TβRI cytoplasmic domain replaced by Tsk7L sequences. TβRI–Tsk7L Swap C9 and Swap C8 had kinase subdomains I–V (amino acids 203–284) and III–VIII (amino acids 235–383), respectively, replaced by Tsk7L sequences (Figure 6A). Both Swap C8 and C9 were unable to mediate TGF-β responsiveness in transfected R1B cells (Figure 6B). This result is consistent with the requirement of kinase subdomains III–V (amino acids 235–284) of TβRI for TGF-β signaling (Figures 4 and 5).

The amino acid sequences of the αC–β4–β5 regions (amino acids 235–284 in TβRI) of TβRI and Tsk7L show 68% identity (Figure 1). Within this segment, a stretch of nine amino acids, which we will name the L45 sequence (amino acids 263–271 of TβRI), are different between the two receptors, with the exception of a conserved Asp (Figure 1). Furthermore, this short sequence in Tsk7L is nearly identical to the L45 sequence in the TSR1 type I receptor, which is, similarly to Tsk7L, also inactive in TGF-β transcriptional responses in Mv1Lu cells (Attisano *et al.*, 1993).

To investigate the role of the L45 sequence, we generated two TβRI mutants. In $T\beta RI^{Tsk7L(\alpha C\beta 45)}$, the αC–β4–β5 region of TβRI (amino acids 235–284) was substituted with the corresponding Tsk7L sequence and, in $T\beta RI^{Tsk7L(L45)}$, only the L45 loop of TβRI (amino acids 263–271) was mutated into the corresponding Tsk7L sequence (Figure 7A). In R1B cells, the signaling ability of both mutant receptors was only minimal when compared with TβRI. While TGF-β increased PAI-1–luciferase activity ~10-fold in TβRI-expressing R1B cells, this activity was only marginally (<2-fold) increased in cells that expressed $T\beta RI^{Tsk7L(\alpha C\beta 45)}$ or $T\beta RI^{Tsk7L(L45)}$ (Figure 7B, left panel). The ability of $T\beta RI^{Tsk7L(\alpha C\beta 45)}$ and $T\beta RI^{Tsk7L(L45)}$ to mediate TGF-β-induced inhibition of cyclin A–luciferase expression was also marginal (Figure

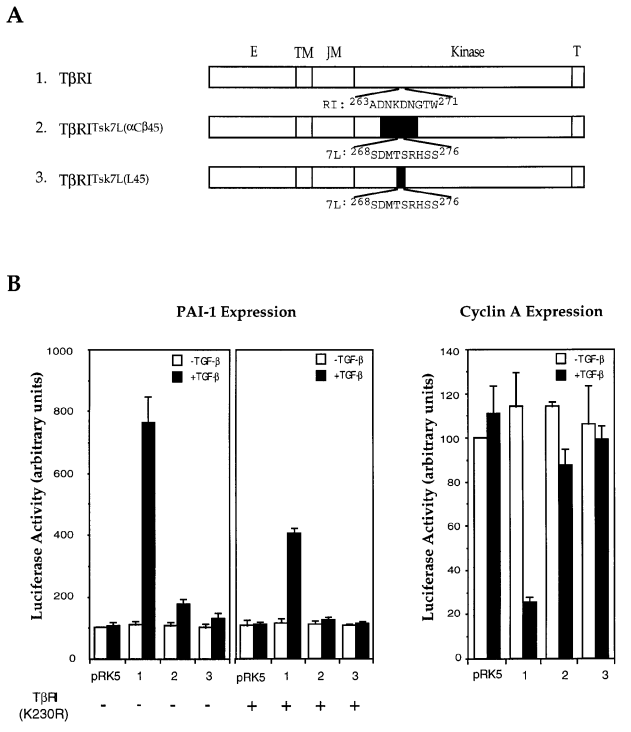

Fig. 7. Structure and signaling activities of TβRI mutant receptors. (**A**) Schematic representation of mutant type I receptors. These two TβRI derivatives were constructed by replacing the αC–β4–β5 or L45 region with the corresponding regions of Tsk7L. (**B**) Signaling activity of mutant receptors in R1B cells. Signaling activity of mutant receptors in R1B cells in both the PAI-1– and cyclin A–luciferase reporter assays, presented as in Figure 2C. Lane 1, TβRI; lane 2, $T\beta RI^{Tsk7L(\alpha C\beta 45)}$; lane 3, $T\beta RI^{Tsk7L(L45)}$.

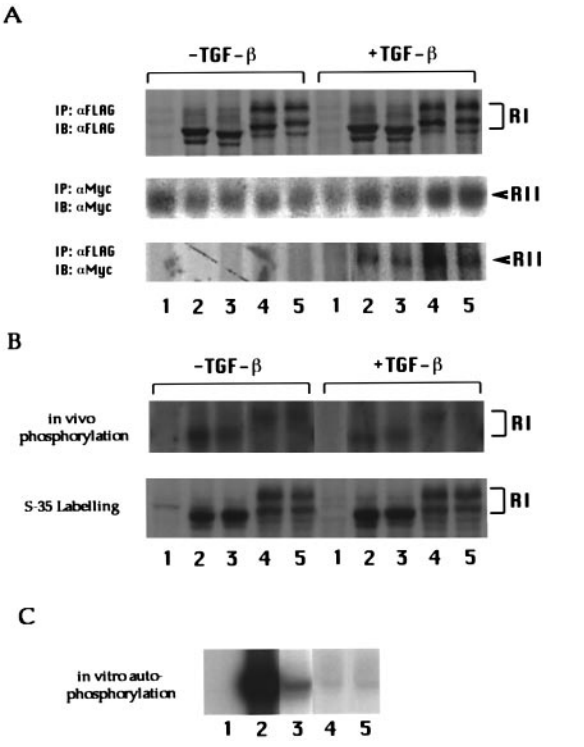

Fig. 8. TβRII association and phosphorylation of mutant type I receptors. (**A**) Ligand-induced association of type I receptors with TβRII. R1B/L17 cells were co-transfected with expression plasmids for FLAG-tagged type I receptors as indicated in lanes and for myc-tagged TβRII. Following metabolic ^{35}S-labeling of the proteins, receptor association was assessed by immunoprecipitation (IP) followed by immunoblotting (IB) with anti-FLAG or anti-myc as indicated. (**B**) *In vivo* phosphorylation of mutant type I receptors. R1B/L17 cells were transfected, metabolically labeled with [^{35}S]methionine or [^{32}P]orthophosphate, treated with TGF-β for 60 min, lysed in Flag lysis buffer (Feng *et al.*, 1995) and then immunoprecipitated with anti-FLAG antibody. Immunoprecipitated proteins were analyzed by SDS–PAGE. (**C**) *In vitro* kinase activity of type I receptors. R1B/L17 cells were transfected with the plasmids indicated, and the immunoprecipitated receptors were subjected to *in vitro* kinase assay using [γ-^{32}P]ATP and SDS–PAGE. Lane 1, pRK5, control vector; lane 2, TβRI; lane 3, $T\beta RI^{Tsk7L(L45)}$; lane 4, TβRI–Tsk7L; lane 5, TβRI–$Tsk7L^{T\beta RI(L45)}$ (see Figures 7A and 10A for diagrams of these receptors).

7B, right panel). In addition, co-transfection of these mutant receptors with a kinase-negative version of TβRI did not rescue TGF-β responsiveness (Figure 7B, left panel). Furthermore, the presence of the Tsk7L L45 sequence in TβRI did not affect its ligand-induced association with TβRII (Figure 8A, compare lanes 2 and 3) and its *in vivo* phosphorylation (Figure 8B, compare lanes 2 and 3), which is thought to be mediated largely by TβRII (Wrana *et al.*, 1994; Souchelnytskyi *et al.*, 1996). However, the *in vitro* kinase activities of $T\beta RI^{Tsk7L(L45)}$ and $T\beta RI^{Tsk7L(\alpha C\beta 45)}$ (data not shown) were considerably lower than that of TβRI (Figure 8C, compare lanes 2 and 3).

Inactive type I receptor cytoplasmic domains can be converted into TGF-β signal-transducers

Our experiments, described above, revealed that most regions in the cytoplasmic domains of the type I receptors, with the exception of the αC–β4–β5 region, in particular the L45 loop, are functionally equivalent for TGF-β signaling. This finding raised the possibility that a type I receptor that is inactive in the TGF-β signaling assays can be converted into an active receptor merely by replacing its αC–β4–β5 region with that of TβRI. We thus introduced the αC–β4–β5 region of TβRI (amino acids 235–284) into TβRI–Tsk7L, thereby generating the receptor chimera TβRI–$Tsk7L^{T\beta RI(\alpha C\beta 45)}$ (Figure 9A). Although the original chimera TβRI–Tsk7L was unable to restore the TGF-β-induced PAI-1 and cyclin A responses, TβRI–$Tsk7L^{T\beta RI(\alpha C\beta 45)}$ mediated TGF-β signaling to an extent that was comparable with TβRI (Figure 9B). Since this chimera TβRI–$Tsk7L^{T\beta RI(\alpha C\beta 45)}$ was active not only in the PAI-1 but also in the cyclin A reporter assay (data not shown), we expected that TGF-β treatment of transfected R1B cells expressing TβRI–$Tsk7L^{T\beta RI(\alpha C\beta 45)}$ would result in inhibition of DNA synthesis. Indeed, expression of either wild-type TβRI or TβRI-$Tsk7L^{T\beta RI(\alpha C\beta 45)}$ in R1B-L17 cells reduced the [^3H]thymidine incorporation by 36–40% in the presence of TGF-β. Considering the transfection efficiency of 40–60%, this would correspond to an inhibition of DNA synthesis of ~60–90% in response to TGF-β. In constrast, DNA synthesis was not inhibited in cells expressing TβRI–Tsk7L or in control-transfected cells lacking TβRI (Figure 9C).

To evaluate further the ability of the αC–β4–β5 region of TβRI to confer TGF-β signaling ability in our assays, we introduced this sequence into the cytoplasmic domain of TSR1/SKR3 (Attisano *et al.*, 1993), which like Tsk7L does not mediate TGF-β-induced gene expression and growth arrest in Mv1Lu cells (Attisano *et al.*, 1993; Bassing *et al.*, 1994b). Thus, amino acids 235–284 of

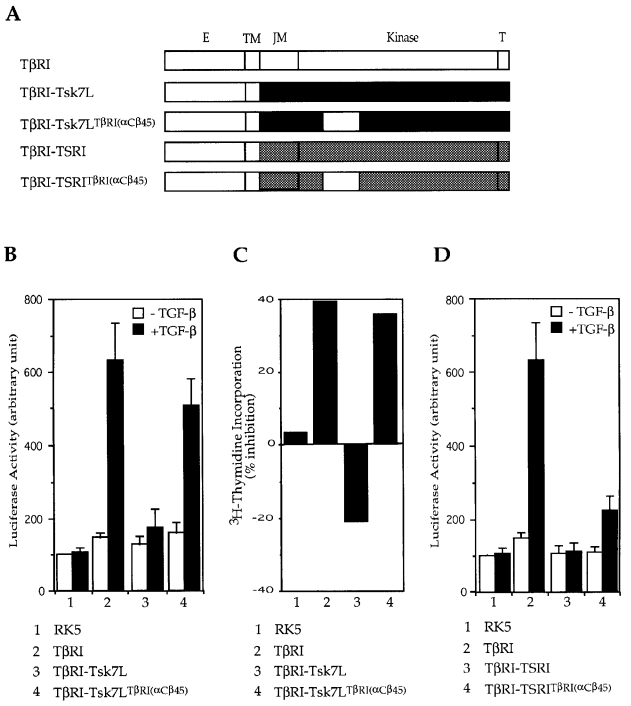

Fig. 9. Structure and signaling activities of mutant type I receptor hybrids with Tsk7L or TSR1 cytoplasmic domains. (**A**) Schematic representation of the chimeric TβRI–Tsk7L and TβRI–TSR1 receptors. (**B**) Signaling activity of mutant TβRI–Tsk7L type I receptors in R1B cells in the PAI-1–luciferase reporter assay, presented as in Figure 2C. (**C**) Ability of the receptor hybrids to inhibit [^{3}H]thymidine incorporation in R1B cells treated with TGF-β. (**D**) Signaling activity of mutant TβRI–TSR1 type I receptors in R1B cells in the PAI-1–luciferase reporter assay, presented as in Figure 2C.

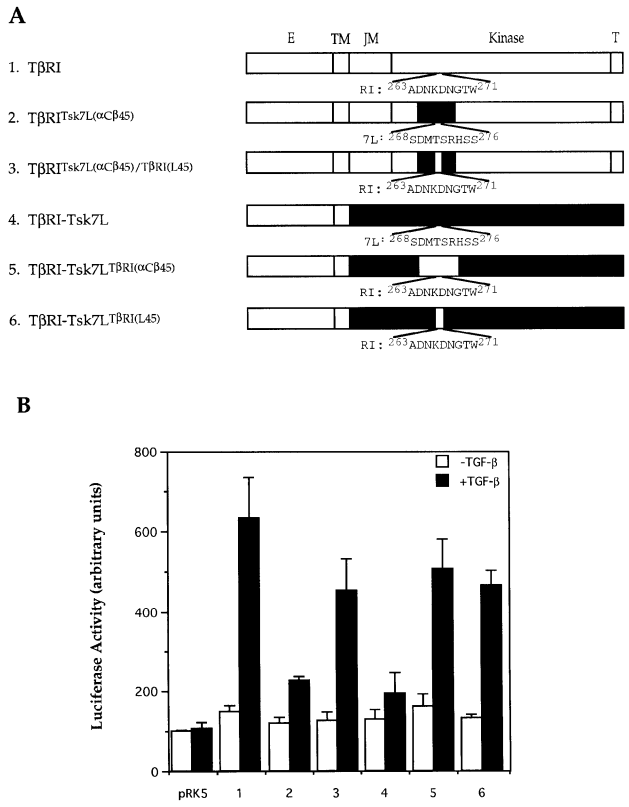

Fig. 10. Structure and signaling activities of mutant type I receptor hybrids with αC–β4–β5 and/or L45 sequence replacements. (**A**) Schematic representation of the mutant TβRI–Tsk7L chimeric receptors. (**B**) Signaling activity of mutant TβRI–Tsk7L type I receptors in R1B cells in the PAI-1–luciferase reporter assay, presented as in Figure 2C.

TβRI were introduced into the corresponding region of TβRI–TSR1, a hybrid receptor which contains the extracellular and transmembrane domain of TβRI and the cytoplasmic domain of TSR1, thereby generating chimera TβRI–TSR1$^{TβRI(αCβ45)}$ (Figure 9A). Similarly to TSR1, TβRI–TSR1 was completely inactive in mediating TGF-β-induced PAI-1 (Figure 9D) and cyclin A responses (data not shown) in R1B cells. In constrast, TβRI–TSR1$^{TβRI(αCβ45)}$ was able to mediate a PAI-1–luciferase response following treatment with TGF-β, albeit to a lesser extent than TβRI (Figure 9D). These results further emphasize the essential role of the αC–β4–β5 region of TβRI in TGF-β signaling and its ability to confer TGF-β signaling specificity to otherwise inactive type I receptors.

The L45 region of TβRI is sufficient to confer the ability of heterologous type I receptor cytoplasmic domains to transduce TGF-β signals

Our results suggested that the nine amino acid long L45 sequence of TβRI was essential for TGF-β signal transduction by TβRI and that this loop might be the motif that determines the intracellular signaling specificity. We therefore made derivatives from the receptor chimeras TβRI$^{Tsk7L(αCβ45)}$ and TβRI–Tsk7L, in which the L45 sequence of Tsk7L was mutated back into the corresponding TβRI sequence (Figure 10A, hybrids 3 and 6). These mutant receptors, named TβRI$^{Tsk7L(αCβ45)/TβRI(L45)}$ and TβRI-Tsk7L$^{TβRI(L45)}$, had signaling abilities in response to TGF-β that were similar to TβRI (Figure 10B). Thus, replacement of only eight amino acids in the L45 loop of inactive receptor chimeras with the corresponding TβRI sequence is sufficient to convert these into receptors capable of transducing TGF-β signals. Both the signaling-inactive TβRI–Tsk7L and -active TβRI–Tsk7L$^{TβRI(L45)}$ were able to form a ligand-induced complex with TβRII (Figure 8A, lanes 4 and 5) and were similarly phosphorylated *in vivo* (Figure 8B, lanes 4 and 5). However, their *in vitro* kinase activity was minimal (Figure 8C, lanes 4 and 5, data not shown). Therefore, introduction of the TβRI αC–β4–β5 or L45 into the Tsk7L kinase domain did not enhance its interaction with TβRII and phosphorylation either *in vivo* and *in vitro*.

Discussion

The receptors for TGF-β-related factors are transmembrane serine/theronine kinases, classified as type I and type II receptors based on their structural and functional characteristics. The type II TGF-β receptors form homo-oligomers in the absence of ligand and are constitutively autophosphorylated (Chen and Derynck, 1994; Henis *et al.*, 1994; Luo and Lodish, 1996). Type II receptor homo-oligomers interact with type I receptor homo-oligomers to form a hetero-oligomeric complex that is stabilized by ligand binding (Yamashita *et al.*, 1994; Weis-Garcia and Massague, 1996). The physical association of both receptor types, the phosphorylation of the type I receptor cytoplasmic domains by type II receptor kinases and the requirement of both receptor types for full responsiveness

to TGF-β strongly suggest that the heteromeric receptor complex represents the signaling unit for the multiple responses to TGF-β and related factors (Wrana *et al.*, 1992, 1994; Franzén *et al.*, 1993; Bassing *et al.*, 1994a; Okadome *et al.*, 1994; Chen and Weinberg, 1995; Feng *et al.*, 1995). In this signaling receptor complex, the type I receptor defines the intracellular signaling specificity (Cárcamo *et al.*, 1994), although the mechanism and structural basis for the signaling specificity of the type I receptors is not understood. In this study, we have evaluated the sequence requirements that determine the ability of TβRI to mediate TGF-β signaling. We have found that a nine amino acid sequence, the L45 loop, in the kinase domain of the type I receptor defines the TGF-β signaling specificity.

Most regions in the cytoplasmic domains of TβRI and Tsk7L are functionally exchangeable

To define the structural basis for the TGF-β signaling ability of TβRI, we exchanged corresponding domains between two related type I receptors, TβRI and Tsk7L, and tested the signaling of the resulting receptor hybrids in response to TGF-β. TβRI mediates TGF-β signaling in a variety of cell types, including Mv1Lu epithelial cells (Franzén *et al.*, 1993; Bassing *et al.*, 1994b; Feng *et al.*, 1995). In contrast, Tsk7L and the related TSR1 receptor do not mediate TGF-β responsiveness in Mv1Lu cells and cannot substitute for TβRI to transduce TGF-β signals in R1B cells (Attisano *et al.*, 1993; Bassing *et al.*, 1994b; Feng *et al.*, 1995). Nevertheless, both receptors bind TGF-β when co-expressed with TβRII (Attisano *et al.*, 1993; Ebner *et al.*, 1993b), although they also have the ability to bind activin (Attisano *et al.*, 1993; Ebner *et al.*, 1993b) and, in the case of Tsk7L, BMP-7 (ten Dijke *et al.*, 1994b). However, dominant-negative interference studies strongly suggest a role for Tsk7L in TGF-β-induced epithelial to mesenchymal transdifferentiation, but not in other TGF-β responses, of NMuMG mammary epithelial cells (Miettinen *et al.*, 1994). This further suggests that different type I receptors may mediate different TGF-β responses in various cell types.

The hybrid receptors, in which the entire ET or cytoplasmic domains were exchanged between TβRI and Tsk7L, were both unable to mediate TGF-β responsiveness. The inability of the Tsk7L–TβRI chimera to transduce TGF-β signals may be due to a conformational difference from wild-type TβRI which may be reflected in the weak binding of TGF-β to Tsk7L ligand-binding domain, while that of the reverse chimera TβRI–Tsk7L suggests the inability of the Tsk7L cytoplasmic domain to mediate TGF-β-dependent PAI-1 and cyclin A responses. Thus, the lack of TGF-β signaling of Tsk7L in these assays may be associated with properties of both its ET and cytoplasmic domains.

The differential ability of the TβRI and Tsk7L cytoplasmic domains to mediate TGF-β signaling allowed us to characterize the structural basis for this functional difference. Most subdomains in the cytoplasmic domain of TβRI can be replaced with the corresponding portions of Tsk7L. Most notable is our observation that the juxtamembrane and GS domains of both TβRI and Tsk7L receptors are functionally equivalent in TGF-β signaling. The GS domain is highly conserved and of critical importance for type I receptor function, and point mutations of this sequence in TβRI modify the signaling ability of the type I receptor (Brand and Schneider, 1995; Wieser *et al.*, 1995). The juxtamembrane domain of TβRI also contains a phosphorylation site at Ser165 (Souchelnytskyi *et al.*, 1996), which is absent in the corresponding Tsk7L and TSR1 region (Figure 1). Since the juxtamembrane domain is the only region of any length that is located outside the kinase domain, it has been proposed that this region represents a docking site for interacting cytoplasmic proteins that defines the signaling specificity (Saitoh *et al.*, 1996). This would then resemble the activation mechanism of tyrosine kinase receptors, with which cytoplasmic effector proteins associate at sequences outside the kinase subdomains (for a review, see Schlessinger and Ullrich, 1992), and would be consistent with the high degree of sequence divergence among the juxtamembrane domains of type I receptors. Furthermore, deletion of this juxtamembrane domain inactivated TGF-β signaling by TβRI in reporter assays (Feng *et al.*, 1995), and deletion of a shorter segment, i.e. amino acids 150–181 of TβRI, abolished TGF-β-induced growth inhibition but not gene expression in response to TGF-β (Saitoh *et al.*, 1996). The signaling phenotype of the latter deletion mutant is, however, consistent with an impaired signaling ability of the resulting receptor and with different thresholds of signaling required to transduce the gene expression and growth arrest responses (Feng *et al.*, 1995). Thus, a decreased signaling ability of the receptor is expected first to inactivate the growth inhibition response, as the induction of gene expression still occurs at lower levels of receptor activation (Feng *et al.*, 1995). Taken together, previous reports certainly emphasize the critical role of the juxtamembrane domain in receptor signaling, but our current findings strongly argue against a role for this segment in defining the signaling specificity and in recruiting intracellular proteins that mediate differential responses. Furthermore, the TβRII-induced phosphorylation at Ser165 which occurs in TβRI (Souchelnytskyi *et al.*, 1996), but not in Tsk7L and TSR1, may not be essential for the signaling activity.

Our functional analyses showed that most kinase subdomains of TβRI are functionally replaceable with the corresponding sequences of the Tsk7L receptor. However, replacement of the kinase subdomains IV and V of TβRI with those of Tsk7L inactivated TGF-β signaling, and substitution of those sequences in Tsk7L with the corresponding segment of TβRI conferred the ability to mediate TGF-β signaling. These sequences provide a defined region consisting of an α-helix followed by two β-strands, named the αC–β4–β5 region in the three-dimensional structure of kinases, as defined by crystallographic analysis of the cAMP-dependent protein kinase (Knighton *et al.*, 1991) and several other kinases (De Bondt *et al.*, 1993; Hubbard *et al.*, 1994). Thus, this sequence of ~50 amino acids in the kinase subdomains IV and V plays a critical role in TGF-β signaling by TβRI.

The L45 region in kinase subdomains IV and V as determinant of TGF-β signaling specificity

The αC–β4–β5 regions of TβRI and Tsk7L share 68% sequence identity. In this region of TβRI, amino acids 263–271, which form the L45 loop, are highly divergent

Fig. 11. Ribbon diagram of insulin receptor kinase to indicate the positions of the β4 and β5 strands and the L45 loop. N- and C-termini of the insulin receptor kinase are indicated. β-strands are represented by numbered ribbons with arrows, and α-helices by coiled ribbons. Only β-strands 4 and 5 are shown in the diagram. Considering the overall similarity in three-dimensional structures of protein kinases (Hanks and Hunter, 1995), the L45 of TβRI corresponds to the loop between the β-strands 4 and 5. The amino acid sequence of the L45 loop of TβRI is shown.

from the corresponding sequences in the Tsk7L and TSR1 receptors, strongly suggesting that this sequence might be critical in TGF-β signaling and responsible for the functional difference between TβRI and Tsk7L or TSR1. Indeed, the mutant TβRI receptors $T\beta RI^{Tsk7L(\alpha C\beta 45)}$ and $T\beta RI^{Tsk7L(L45)}$ in which the αC–β4–β5 and L45 sequence were replaced by Tsk7L sequences, respectively, were unable to mediate TGF-β responsiveness. In complementary experiments, introduction of the αC–β4–β5 region or even only the eight divergent amino acids of the L45 loop of TβRI into the Tsk7L cytoplasmic domain conferred TGF-β signaling activity to this otherwise inactive receptor, to a similar extent as wild-type TβRI. Similarly, the αC–β4–β5 region or only the L45 loop of TβRI also conferred an ability to mediate TGF-β signaling to the TSR1 cytoplasmic domain. Thus, the L45 loop of TβRI is essential and sufficient to confer the ability to mediate TGF-β signaling to another type I receptor cytoplasmic domain.

The mechanistic basis for this essential role of the αC–β4–β5 region, and in particular the L45 sequence, in TGF-β signaling is as yet unclear. However, the three-dimensional protrusion of the L45 loop carried by the β4 and β5 strands in the three-dimensional structure of the kinase strongly suggests that it is sterically accessible and structurally sensitive to alterations of a few amino acids (Figure 11). One possible mechanism would be that the ability to mediate TGF-β signaling correlates with the kinase activity of the type I receptors. This appears to be in agreement with our results that the *in vitro* kinase activity of the Tsk7L kinase is much lower than that of TβRI. However, several chimeras with high kinase activity were unable to mediate TGF-β signaling in our assays, as other chimeras that mediated TGF-β responsiveness in our assays had only a low level of kinase activity comparable with Tsk7L. Although replacement of the αC–β4–β5 region or only the L45 loop sequence of TβRI with the corresponding sequences of Tsk7L decreased the kinase activity *in vitro*, introduction of the αC–β4–β5 region of TβRI into the Tsk7L or TSR1 kinase domain did not enhance the *in vitro* kinase activity of chimeric receptors, yet conferred a significant TGF-β signaling activity. For example, the TβRI-$Tsk7L^{T\beta RI(L45)}$ receptor hybrid has a much lower kinase activity thanTβRI, yet its signaling activity was comparable with that of TβRI. We therefore conclude that the signaling activity of the different hybrids did not correlate with the kinase activity *in vitro*.

Another possibility would be that the differential ability of the type I receptor hybrids to mediate TGF-β signaling might be associated with differences in activation by the type II receptor kinase, whereby receptor chimeras lacking the L45 loop sequence of TβRI would be defective in activation. Activation-defective type I receptors are unable to mediate TGF-β signaling, similarly to kinase-defective mutants, but co-expression of these two types of inactive type I receptors results in their functional complementation, which is consistent with the presence of two type I receptors in the heteromeric receptor complex (Weis-Garcia and Massagué, 1996). However, co-expression of our TGF-β signaling-defective chimeras with the kinase-defective TβRI (K230R) mutant (Feng *et al.*, 1995) did not result in signaling activity due to complementation in R1B cells (Figure 7B), indicating that they are not activation-defective mutants. In addition, we did not observe any differences in TGF-β-induced association of the signaling-defective TβRI–Tsk7L and the active TβRI–$Tsk7L^{T\beta RI(L45)}$ with TβRII, when compared with TβRI, suggesting that the nature of the L45 does not interfere with the heteromeric receptor association. Furthermore, the nature of the L45 sequence did not detectably affect the *in vivo* phosphorylation of TβRI, which is thought to result from the constitutive kinase activity of TβRII. Therefore, it is unlikely that the L45 region of TβRI is involved in an interaction with and transphosphorylation and activation by TβRII.

Protein kinases display conservation in amino acid sequences clustered in several subdomains. In the catalytic core with its striking sequence conservation, most amino acids contribute directly to ATP binding or catalysis. However, the recognition and binding of substrate proteins involve many other sequences that are not necessarily conserved at the surface of the kinase. For example, a sequence in the kinase subdomain III of cAMP-dependent protein kinase, which corresponds to a loop between the β3 strand and helix C, interacts with the regulatory RI subunit (Cox and Taylor, 1994), whereas, in cyclin-dependent kinases, this loop is involved in binding of cyclins (Ducommun *et al.*, 1991; De Bondt *et al.*, 1993).

No function has as yet been attributed to the L45 loop in the αC–β4–β5 region of any kinases. However, the

location of the L45 loop on the surface of the protein kinase and its sequence divergence among different type I receptors raise the possibility that it may mediate interaction with another protein. Possible interacting proteins might be adaptors or substrates such as the closely related Mad homologs, Smad2 and Smad3, which mediate TGF-β signaling and are phosphorylated as a result of an association with the receptor complex (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). An involvement of the αC–β4–β5 region in protein–protein interactions does not rule out the possibility that proteins may interact with other cytoplasmic sequences of the type I receptor, including the juxtamembrane domain of the type I receptor, as demonstrated in the TβRI–FKBP12 interaction (Charng *et al.*, 1996; Okadome *et al.*, 1996; Wang *et al.*, 1996). However, the L45 sequence might play an essential role in the selectivity of interacting proteins and in the specificity of intracellular signaling. Thus, the L45 loop, which defines the ability to mediate TGF-β signaling of the type I receptor, may serve as a docking site for specific intracellular signaling mediators or substrates which associate with the heteromeric TGF-β receptor complex. Determination of the crystal structure of type I receptor kinases and a characterization of proteins that interact with this region should provide additional insights into the signaling mechanism of TGF-β receptors.

Materials and methods

Construction of chimeric and mutant type I receptors

Expression plasmids encoding the FLAG-tagged type I receptors TβRI and Tsk7L have been described previously (Feng *et al.*, 1995) and were used as parental plasmids to create the chimeric and mutant type I receptors. The domains of the receptors that were fused to create the chimeric receptors were obtained in most cases by amplification using PCR methodology and/or by restriction digestion of receptor cDNAs. In the latter case, we took advantage of the presence of several restriction sites, e.g. one *Bam*HI and two *Xba*I sites, in TβRI and the conservation in amino acid sequences derived from these sites of Tsk7L. Since no restriction sites were available or could be created without altering the amino acid sequence at the junction of the cytoplasmic juxtamembrane domain and the kinase domain, we developed a modified PCR method which we named 'Fusion PCR'. To construct TβRI–Tsk7L chimera C7 (see Figure 5A), we designed a primer, GTTCAGAGAACAATTGCGAGAACT*ATAACCCTGTTGGAGTGTGTC*, whose first half is from TβRI (amino acids 197–204) and second half (shown in italics) from Tsk7L (amino acids 208–214). Amplification of Tsk7L using this primer and a C-terminal reverse primer (GGCAGTCGACACAGTCAGTCTTCAATTTGTC; underlining indicates the incorporated *Sal*I site) gave a product that a contained a small portion of TβRI (amino acids 197–204) followed by Tsk7L (amino acids 208–509). Use of this product as the reverse primer and TAGAATTCATGGAGGCAGCATCGGC (ATG is the start codon; underlining indicates the incorporated *Eco*RI site) from TβRI (amino acids 1–6) as a forward primer to amplify TβRI template in a second PCR produced a chimeric product consisting of TβRI (amino acids 1–204) and Tsk7L (208–501) sequences. Cloning of this PCR product into the *Eco*RI and *Sal*I sites in pRK5F (Feng *et al.*, 1995) generated a FLAG-tagged TβRI–Tsk7L chimera, named Swap C7 (see Figure 5A).

All receptor constructs generated using PCR were analyzed for their sequence integrity using dideoxy sequencing methods. In addition, all receptors expressed from expression plasmids were expressed as C-terminally FLAG-tagged versions. Due to the large number and complexity of receptor expression plasmids, a detailed description has not been provided here, but can be obtained upon request.

Cell culture

COS-1 cells (CRL 1650, American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator and the medium was changed every 3 days. Mv1Lu mink lung epithelial cells (CCL-64, ATCC) and the derivative mutant line R1B and R1B/L17, provided by Dr J.Massagué, were maintained in MEM Eagle's with Earle's balanced salt solution supplemented with non-essential amino acids (NEAA) and 10% FBS at 37°C in a 5% CO_2 incubator.

Transfections, immunoprecipitations, in vitro kinase reactions and [^{125}I]TGF-β cross-linking of receptor proteins in COS-1 cells

COS-1 cells were transfected using LipofectAMINE (Gibco-BRL) according to the manufacturer's instruction. Forty eight hours after transfection, cells were starved for 1 h in methionine-free, cysteine-free DMEM and then metabolically labeled overnight in the same medium containing 0.5 mCi of Pro-mix (Amersham). Labeled cells were lysed in MLB lysis buffer (20 mM Tris–HCl, pH 8, 137 mM NaCl, 1% NP-40) for anti-myc antibody 9E10, or FLB lysis buffer (25 mM Tris–HCl, 300 mM NaCl, 1% Triton-X-100) for anti-FLAG antibody M2 (Kodak). Lysates were pre-cleared in a mixture of rabbit anti-mouse IgG (Jackson Laboratories) and protein A–Sepharose CL4B (Pharmacia), and immunoprecipitated using the monoclonal antibodies 9E10 (anti-myc) or M2 (anti-FLAG, Kodak). Immunoprecipitated proteins were subjected to electrophoresis on SDS–PAGE and visualized by autoradiography.

For *in vitro* kinase assays, immunoprecipitated proteins was incubated at room temperature for 30 min in 1× kinase buffer (10 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$ and 5 mM $CaCl_2$) containing 5 μCi of [γ-^{32}P]ATP (5000 μCi/mmol, Amersham). The reaction was then stopped by adding an equal volume of 2× SDS sample buffer (80 mM Tris, pH 6.8, 3.2% SDS, 16% glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue) prior to electrophoresis on SDS–PAGE.

COS-1 cells were transfected, with one or several expression plasmids for a single receptor, as described above for immunoprecipitation. Forty eight hours after transfection, COS-1 cells were cross-linked with ^{125}I-labeled TGF-β1 (Amersham) as described (Gazit *et al.*, 1993).

Functional assays

Plasmid p800Luc contains the luciferase reporter gene under the control of the TGF-β-responsive promoter for plasminogen activator inhibitor type I (PAI-1) (Keeton *et al.*, 1991) and was used to monitor TGF-β-induced gene expression. The cyclin A–luciferase reporter plasmid pCAL2 was used to assay for TGF-β-induced growth inhibition. Plasmid pRKβGal, which expresses β-galactosidase under the control of the cytomegalovirus promoter, was used to normalize for transfection efficiency. Plasmids pCAL2 and pRKβGal have been described elsewhere (Feng *et al.*, 1995).

The reporter assays were performed in transient transfection assays of Mv1Lu-R1B cells, whereby the receptor expression plasmids and reporter plasmids are co-transfected (Feng *et al.*, 1995). The luciferase expression levels were carried out using Analytic Luminescence Laboratory's assay reagents, and β-galactosidase was assayed in Galacto Light Plus kit (Tropix). Both luciferase and β-galactosidase activities were measured in a Luminometer Monolight 2010 (Analytic Luminescence Laboratory). The luciferase activities which reflect the promoter activity of cyclin A or PAI-1 were normalized to β-galactosidase expression.

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References

Attisano,L., Carcamo,J., Ventura,F., Weis,F.M., Massagué,J. and Wrana,J.L. (1993) Identification of human activin and TGFβ type I receptors that form heteromeric kinase complexes with type II receptors. *Cell*, **75**, 671–680.

Bassing,C.H., Howe,D.J., Segarini,P.R., Donahoe,P.K. and Wang,X.-F. (1994a) A single heteromeric receptor complex is sufficient to mediate biological effects of transforming growth factor-β ligands. *J. Biol. Chem.*, **269**, 14861–14864.

Bassing,C.H., Yingling,J.M., Howe,D.J., Wang,T., He,W.W., Gustafson,M.L., Shah,P., Donahoe,P.K. and Wang,X.-F. (1994b) A transforming growth factor β type I receptor that signals to activate gene expression. *Science*, **263**, 87–89.

Brand,T. and Schneider,M. (1995) Inactive type II and type I receptors for TGF-β are dominant inhibitors of TGF-β-dependent transcription. *J. Biol. Chem.*, **270**, 8274–8284.

Cárcamo,J., Weis,F.M., Ventura,F., Wieser,R., Wrana,J.L., Attisano,L. and Massagué,J. (1994) Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor β and activin. *Mol. Cell. Biol.*, **14**, 3810–3821.

Charng,M.-J., Kinnunen,P., Hawker,J., Brand,T. and Schneider,M. (1996) FKBP-12 recognition is dispensable for signal generation by type I transforming growth factor-β receptors. *J. Biol. Chem.*, **271**, 22941–22944.

Chen,F. and Weinberg,R.A. (1995) Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor β receptor kinases. *Proc. Natl Acad. Sci. USA*, **92**, 1565–1569.

Chen,R.-H. and Derynck,R. (1994) Homomeric interactions between the type II TGF-β receptors. *J. Biol. Chem.*, **269**, 22868–22874.

Chen,R.-H., Moses,H.L., Maruoka,E.M., Derynck,R. and Kawabata,M. (1995) Phosphorylation-dependent interaction of the cytoplasmic domains of the type I and type II TGF-β receptors. *J. Biol. Chem.*, **270**, 12235–12241.

Cox,S. and Taylor,S.S. (1994) Holoenzyme interaction sites in the cAMP-dependent protein kinase. Histidine 87 in the catalytic subunit complements serine 99 in the type I regulatory subunit. *J. Biol. Chem.*, **269**, 22614–22622.

De Bondt,H., Rosenblatt,J., Jancarik,J., Jones,H., Morgan,D. and Kim,S.-H. (1993) Crystal structure of cyclin-dependent kinase 2. *Nature*, **363**, 595–602.

Derynck,R. (1994a) Transforming growth factor-β. In Thomson,A.W. (ed.), *The Cytokine Handbook*. Academic Press, San Diego, CA, pp. 319–342.

Derynck,R. (1994b) TGF-β receptor-mediated signaling. *Trends Biochem. Sci.*, **19**, 548–553.

Derynck,R. and Zhang,Y. (1996) The Mad way to do it. *Curr. Biol.*, **6**, 1226–1229.

Ducommun,B., Brambilla,P., Félix,M.-A., Franza,J., Karsenti,E. and Draetta,G. (1991) cdc2 phosphorylation is required for its interaction with cyclin. *EMBO J.*, **10**, 3311–3319.

Ebner,R., Chen,R.-H., Lawler,S., Zioncheck,T. and Derynck,R. (1993a) Determination of type I receptor specificity by the type II receptors for TGF-β or activin. *Science*, **262**, 900–902.

Ebner,R., Chen,R.-H., Shum,L., Lawler,S., Zioncheck,T.F., Lee,A., Lopez,A.R. and Derynck,R. (1993b) Cloning of a type I TGF-β receptor and its effect on TGF-β binding to the type II receptor. *Science*, **260**, 1344–1348.

Feng,X.-H. and Derynck,R. (1996) Ligand-independent activation of transforming growth factor (TGF)-β signaling pathways by heteromeric cytoplasmic domains of TGF-β receptors. *J. Biol. Chem.*, **271**, 13123–13129.

Feng,X.-H., Filvaroff,E.H. and Derynck,R. (1995) Transforming growth factor-β (TGF-β)-induced down-regulation of cyclin A expression requires a functional TGF-β receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J. Biol. Chem.*, **270**, 24237–24245.

Franzén,P., ten Dijke,P., Ichijo,H., Yamashita,H., Schulz,P., Heldin,C.-H. and Miyazono,K. (1993) Cloning of a TGFβ type I receptor that forms a heteromeric complex with the TGFβ type II receptor. *Cell*, **75**, 681–692.

Gazit,D., Ebner,R., Kahn,A. and Derynck,R. (1993) Modulation of expression and cell surface binding of members of the transforming growth factor-β superfamily during retinoic acid-induced osteoblastic differentiation of multipotential messenchymal cells. *Mol. Endocrinol.*, **7**, 189–198.

Hanks,S. and Hunter,T. (1995) The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, **9**, 576–596.

Hanks,S., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

Henis,Y.I., Moustakas,A., Lin,H.Y. and Lodish,H.F. (1994) The type II and III transforming growth factor-β receptors form homo-oligomers. *J. Cell Biol.*, **126**, 139–154.

Hubbard,S., Wei,L., Ellis.L. and Hendrickson,W. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature*, **372**, 746–754.

Keeton,M.R., Curriden,S.A., van Zonneveld,A. and Loskutoff,D.J. (1991) Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta. *J. Biol. Chem.*, **266**, 23048–23052.

Kingsley,D.M. (1994) The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.*, **8**, 133–146.

Knighton,D., Zheng,J., ten Eyck,L., Ashford,V., Xuong,N.-H., Taylor,S. and Sowadski,J. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*, **253**, 407–414.

Lagna,G., Hata,A., Hemmati-Brivanlou,A. and Massagué,J. (1996) Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature*, **383**, 832–836.

Laiho,M., Weis,F.M. and Massagué,J. (1990) Concomitant loss of transforming growth factor (TGF)-β receptor types I and II in TGF-β-resistant cell mutants implicates both receptor types in signal transduction. *J. Biol. Chem.*, **265**, 18518–18524.

Laiho,M., Weis,F.M., Boyd,F.T., Ignotz,R.A. and Massagué,J. (1991) Responsiveness to transforming growth factor-β (TGF-β) restored by genetic complementation between cells defective in TGF-β receptors I and II. *J. Biol. Chem.*, **266**, 9108–9112.

Liu,F., Hata,A., Baker,J.C., Doody,J. Cárcamo,J., Harland,R.M. and Massagué,J. (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. *Nature*, **381**, 620–623.

Luo,K. and Lodish,H.F. (1996) Signaling by chimeric erythropoietin–TGF-β receptors: homodimerization of the cytoplasmic domain of the type I TGF-β receptor and heterodimerization with the type II receptor are both required for intracellular signal transduction. *EMBO J.*, **15**, 4485–4496.

Macias-Silva,M., Abdollah,S., Hoodless,P., Pirone,R., Attisano,L. and Wrana,J.L. (1996) MADR-2 is a substrate of the TGF-β receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, **87**, 1215–1224.

Massagué,J. (1996) TGFβ signaling: receptors, transducers, and Mad proteins. *Cell*, **85**, 947–950.

Miettinen,P.J., Ebner,R., Lopez,A.R. and Derynck,R. (1994) TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.*, **127**, 2021–2036.

Okadome,T., Yamashita,H., Franzen,P., Moren,A., Heldin,C.-H. and Miyazono,K. (1994) Distinct roles of the intracellular domains of transforming growth factor-β type I and type II receptors in signal transduction. *J. Biol. Chem.*, **269**, 30753–30756.

Okadome,T., Oeda,E., Saitoh,M., Ichijo,H., Moses,H.L., Miyazono,K. and Kawabata,M. (1996) Characterization of the interaction of FKBP12 with the transforming growth factor-β type I receptor *in vivo*. *J. Biol. Chem.*, **271**, 21687–21690.

Rodriguez,C., Chen,F., Weinberg,R.A. and Lodish,H.F. (1995) Cooperative binding of transforming growth factor (TGF)-β2 to the types I and II TGF-β receptors. *J. Biol. Chem.*, **270**, 15919–15922.

Saitoh,M., Nishitoh,H., Amagasa,T., Miyazono,K., Takagi,M. and Ichijo,H. (1996) Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-β. *J. Biol. Chem.*, **271**, 2769–2775.

Schlessinger,J. and Ullrich,A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron*, **9**, 383–391.

Sekelsky,J., Newfeld,S., Raftery,L., Chartoff,E. and Gelbart,W.M. (1995) Genetic characterization and cloning of *mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. *Genetics*, **139**, 1347–1358

Souchelnytskyi,S., ten Dijke,P., Miyazono,K. and Heldin,C.-H. (1996) Phosphorylation of Ser165 in TGF-β type I receptor modulates TGF-β1-induced cellular responses. *EMBO J.*, **15**, 6231–6240.

ten Dijke,P., Yamashita,H., Ichijo,H., Franzén,P., Laiho,M., Miyazono,K. and Heldin,C.-H. (1994a) Characterization of type I receptors for transforming growth factor-β and activin. *Science*, **264**, 101–104.

ten Dijke,P., Yamashita,H., Sammpath,T.K., Reddi,A.H., Estevez,M., Riddle,D.L., Ichojo,H., Heldin,C.-H. and Miyazono,K. (1994b) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J. Biol. Chem.*, **269**, 16985–16988.

Ventura,F., Doody,J., Liu,F., Wrana,J.L. and Massagué,J. (1994) Reconstitution and transphosphorylation of TGF-β receptor complexes. *EMBO J.*, **13**, 5581–5589.

Wang,T., Li,B.-Y., Danielson,P., Shah,P., Rockwell,S., Lechleider,R., Martin,J., Manganaro,T. and Donahoe,P. (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF-β family type I receptors. *Cell*, **86**, 435–444.

Weis-Garcia,F. and Massagué,J. (1996) Complementation between kinase-defective and activation-defective TGF-β receptors reveals a novel form of receptor cpoperativity essential for signaling. *EMBO J.*, **15**, 276–289.

Wieser,R., Wrana,J.L. and Massagué,J. (1995) GS domain mutations that constitutively activate TβRI, the downstream signaling component in the TGF-β receptor complex. *EMBO J.*, **14**, 2199–2208.

Wrana,J.L., Attisano,L., Cárcamo,J., Zentella,A., Doody,J., Laiho,M., Wang,X.F. and Massagué,J. (1992) TGF β signals through a heteromeric protein kinase receptor complex. *Cell*, **71**, 1003–1014.

Wrana,J.L., Attisano,L., Wieser,R., Ventura,F. and Massagué,J. (1994) Mechanism of activation of the TGF-β receptor. *Nature*, **370**, 341–347.

Wu,R.Y., Zhang,Y., Feng,X.-H. and Derynck,R. (1997) Homomeric and heteromeric interactions are required for signaling activity and functional cooperativity of Smad3 and 4. *Mol. Cell. Biol.*, **17**, 2521–2528.

Yamashita,H., ten Dijke,P., Franzen,P., Miyazono,K. and Heldin,C.-H. (1994) Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. *J. Biol. Chem.*, **269**, 20172–20178.

Zhang,Y., Feng,X.-H., Wu,R.-Y. and Derynck,R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature*, **383**, 168–172.

Zhang,Y., Musci,T. and Derynck,R. (1997) The tumor suppressor Smad4/DPC4 as a central mediator of Smad function. *Curr. Biol.*, **7**, 270–276.

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