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A Transforming Growth Factor β Type I Receptor That Signals to Activate Gene Expression

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Transforming growth factor beta (TGF-β) is a multifunctional factor that regulates many aspects of cellular functions. TGF-β signals through a heteromeric complex of the type I and type II TGF-β receptors. However, the molecular mechanism of signal transduction by this receptor complex remains unresolved. The type II receptor belongs to a transmembrane receptor serine-threonine kinase family. A new member of this receptor family (R4) was identified and shown to be a functional TGF-β type I receptor on the basis of its ability to restore a TGF-β–induced gene response in mutant cell lines lacking endogenous type I receptor. Both ligand binding and signaling of the R4 protein were dependent on the presence of a functional type II receptor. The type I receptor has an intrinsic serine-threonine kinase activity, which was essential for signal transduction.

The transforming growth factor betas (TGF-βs) are a family of multifunctional cytokines that regulate many aspects of cellular function, including cell proliferation, differentiation, adhesion, and migration (*1*). TGF-β signals through a heteromeric complex between the type I and type II receptors (*2*, *3*). The type II receptor can directly bind ligand, but is incapable of mediating TGF-β responses in the absence of a type I receptor (*3*, *4*). The type II TGF-β receptor has been cloned and is a member of the transmembrane receptor serine-threonine kinase family (*5*). Recently, a murine receptor serine-threonine kinase, Tsk 7L, was concluded to be a type I receptor for both TGF-β and activin because of its biochemical properties (*6*). However, the ability of the Tsk 7L protein to mediate biological responses to TGF-β was not shown.

Because the type II receptors for both TGF-β and activin belong to the same group of receptors (*7*), it was hypothesized that there may be a family of such receptors for the ligands of the TGF-β superfamily. To explore this possibility, we used a polymerase chain reaction cloning strategy to isolate other members of this receptor family. Four putative receptor serine-threonine kinases (R1 through R4) were isolated from the urogenital ridge of 14.5- to 15-day fetal Sprague-Dawley rats (*8*) (Fig. 1). Sequence comparison revealed that the R1 clone represents the rat homologue of Tsk 7L. Because clones R1 through R4 have a high degree of sequence similarity, we investigated the functional properties of all four proteins.

Mink lung epithelial (MvlLu) cells are highly responsive to the effects of TGF-β. Through chemical mutagenesis, several classes of TGF-β–resistant MvlLu cell lines have been generated (*4*, *9*). Mutants of MvlLu cells defective in either the type I (R mutants) or type II (DR mutants) TGF-β receptors lack TGF-β–induced gene expression and TGF-β–induced growth inhibition (*3*, *4*, *9*). A TGF-β reporter construct (p3TP-Lux) containing a luciferase gene

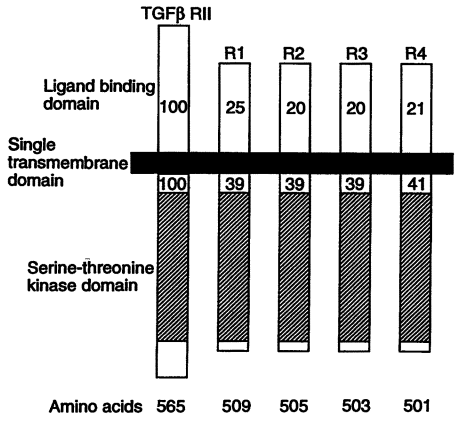

Fig. 1. Schematic diagram comparing the amino acid sequences of R1 through R4 to the TGF-β type II receptor. The percent amino acid similarity of the extracellular and kinase domains of each clone as compared to the type II receptor sequence is indicated. The sequence similarities were generated by the GAP program of the Genetics Computer Group. The nucleotide sequence of the R4 clone has been deposited to GenBank (accession number L26110). The number of amino acids in each protein is indicated.

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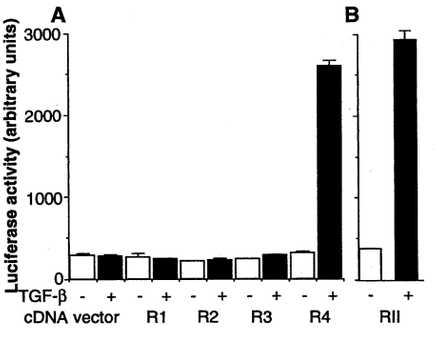

Fig. 2. Screen for the ability of R1 through R4 to restore TGF-β–dependent gene expression in the nonresponsive R1B mutant cell line. (**A**) R1B cells were transiently transfected with 6 μg of p3TP-Lux and 6 μg of either pCMV4, pCMV8-R1, pCMV4-R2, pCMV6-R3, or pCMV6-R4. Twelve hours after transfection, cells were placed in Dulbecco's modified Eagle's medium containing fetal bovine serum (0.2%) for 36 hours in the absence or presence of TGF-β1 (240 pM). Cells were lysed and the amount of luciferase activity in the extracts was assayed by integrating light emission over 30 s on a Berthold luminometer. All experiments with transfection and luciferase assays were done at least twice with triplicates in each experiment. The bars indicate the average values ± SEM of luciferase activity from triplicates in a representative experiment. (**B**) As a positive control, 6 μg of p3TP-Lux and 6 μg of the human type II receptor cDNA (pCMV8-RII) were transiently transfected into the DR-26 cell line. These cells were also treated with and without TGF-β1 for 36 hours and then assayed for luciferase activity.

controlled by a TGF-β–inducible promoter was generated to study TGF-β–induced changes in gene expression in MvlLu cell lines (*3*). In a transient assay, transfection of p3TP-Lux into MvlLu cells allows the measurement of TGF-β–dependent induction of luciferase activity (*3*). This response is not observed when the assay is done with either of the TGF-β nonresponsive mutant cell lines. Co-transfection of the human type II TGF-β receptor complementary DNA (cDNA) along with p3TP-Lux into the DR mutants restores TGF-β–inducible gene expression (*3*). Therefore, this assay allows detection of clones that can functionally rescue the mutant phenotype. We used this approach to screen the ability of R1 through R4 to restore a functional TGF-β response in a MvlLu mutant cell line that has lost expression of the endogenous type I TGF-β receptor.

Eukaryotic high-expression vectors (*10*) containing the cDNAs encoding R1 through R4 were transiently transfected with p3TP-Lux into R1B cells (*3*, *4*). Transfectants treated with TGF-β1 (240 pM) for 36 hours were lysed and assayed for luciferase activity. Only R1B cells transfected with the R4 cDNA showed TGF-β–dependent luciferase activity (Fig. 2A) (*11*). The amount of luciferase activity induced by the R4 clone was comparable to that of the DR mutant cells (DR-26) transfected with the type II receptor cDNA and p3TP-Lux (Fig. 2B). Furthermore, the magnitude of the induction of luciferase activity in R1B cells transfected with R4 was equivalent to that in wild-type MvlLu cells (*12*).

Both the type I and type II receptors are required for TGF-β signaling (*3*). We tested whether R4 induced a TGF-β response when transfected into the DR mutants, which lack expression of endogenous type II receptor (*3*). DR-26 cells transfected with R4 and p3TP-Lux showed no increase in luciferase activity in response to TGF-β (Fig. 3A). In contrast, transfection of the human type II receptor along with p3TP-Lux restored the TGF-β response in a ligand-dependent fashion (Fig. 3A). This result indicates that R4 requires the presence of a functional type II TGF-β receptor to signal and further establishes the molecular nature of R4 as a functional type I receptor.

A functional type II receptor kinase is essential for TGF-β signaling through both the gene activation and growth inhibitory pathways (*3*). We investigated whether the kinase activity of the type I receptor is also required for signal transduction. We used site-directed mutagenesis to generate a catalytically inactive R4 receptor (K230R), in which lysine-230 of the adenosine triphosphate (ATP)–binding site in the kinase domain was changed to an arginine residue (*13*, *14*). Transient transfection of p3TP-Lux and a vector containing the mutant R4 into R1B cells failed to restore TGF-β induction of luciferase activity (Fig. 3B). Thus, the kinase activity of the type I receptor is also essential for signaling.

Binding of TGF-β to the type I receptor requires the presence of the type II receptor, whereas binding to the type II receptor occurs independently (*3*). The capability of independent ligand binding and differences in molecular sizes of the two types of receptors have been used as functional criteria to classify members of the TGF-β family of receptors. We therefore examined the ligand binding properties of R4 in transfected cells. Because the MvlLu cells are poor recipients for DNA transfection, we performed ligand-binding and crosslinking assays on transiently transfected COS cells. Affinity labeling with ^{125}I-labeled TGF-β1 of COS cells transfected with only the R4 cDNA showed no additional crosslinked bands when compared to those from mock transfected COS cells (Fig. 4). The type II cDNA transfected alone gave rise to an increase in intensity of crosslinked protein as a diffuse band of 70 to 97 kD, larger than that of endogenous receptors. When R4 cDNA was transfected with the human type II receptor cDNA, an increase in labeled proteins migrating as 63- to 65-kD and 85- to 97-kD bands was observed. These bands correspond to the type I and type II TGF-β receptor proteins. Binding of TGF-β1 to these proteins was eliminated when the assay was done in the presence of an excess of unlabeled TGF-β1. Treatment of transfected COS cells with dithiothreitol (DTT) before ligand binding selectively abolished

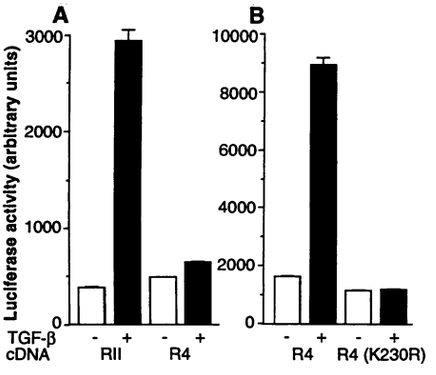

Fig. 3. Functional characterization of R4. (**A**) Dependence of TGF-β signaling by R4 on the presence of a functional type II receptor. DR-26 mutants were transiently transfected with 6 μg of p3TP-Lux and 6 μg of either pCMV8-RII or pCMV6-R4. Cells were either treated with or without TGF-β1 for 36 hours and then assayed for luciferase activity. (**B**) Requirement of kinase activity of R4 for signal transduction. R1B mutants were transiently transfected with 6 μg of p3TP-Lux and 6 μg of either pCMV6-R4 or pCMV6-R4(K230R). Cells were treated with and without TGF-β1 for 36 hours and then assayed for luciferase activity.

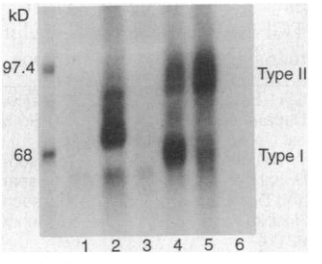

Fig. 4. Affinity crosslinking of ^{125}I-labeled TGF-β1 to transiently transfected COS cells. COS cells were transfected with 10 μg pCMV4 (lane 1), 10 μg pCMV8-RII (lane 2), 50 μg pCMV6-R4 (lane 3), or 5 μg pCMV8-RII and 50 μg pCMV6-R4 (lanes 4 to 6). Cells were grown to confluency and crosslinked with ^{125}I-labeled TGF-β (150 pM). Lane 5 represents cells treated with 1 mM DTT for 5 min before addition of ^{125}I-labeled TGF-β1. Lane 6 represents cells crosslinked with ^{125}I-labeled TGF-β in the presence of an excess (200 times the concentration of labeled TGF-β) of unlabeled TGF-β1. Each lane represents equal amounts of protein lysate, as determined by Coomassie blue staining. The migration of molecular size markers is indicated.

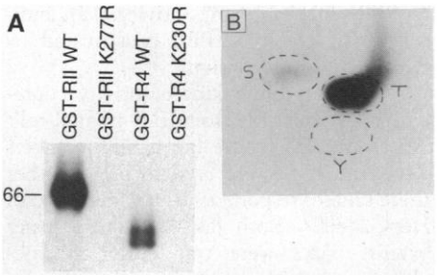

Fig. 5. Autophosphorylation of R4 in vitro. (**A**) In vitro autophosphorylation assay to compare the kinase activity of GST-RII and GST-R4. An equal amount of protein was loaded in each lane and separated by SDS–polyacrylamide gel electrophoresis (8% gel). Autoradiography was done at −80°C for 1.5 hours. (**B**) Phosphoamino acid analysis of in vitro autophosphorylated GST-R4.

binding of TGF-β1 to the 63- to 67-kD protein. These results demonstrate that R4 has the binding properties of the type I TGF-β receptor (*4*).

The type II TGF-β receptor is an active kinase capable of autophosphorylation in an in vitro kinase assay (Fig. 5A) (*5*). Because the cytoplasmic domain of R4 also contains a putative serine-threonine kinase domain, which has sequence similarity to the type II receptor kinase (41% identity), we tested whether R4 could act as a functional serine-threonine kinase in vitro. A fusion protein between glutathione-S-transferase and the type I receptor cytoplasmic domain was generated and used in an in vitro kinase assay (*15*). As a control, we constructed a similar fusion protein using the R4 cytoplasmic domain containing the K230R mutation. The wild-type R4 kinase was active whereas the fusion protein containing the K230R mutation did not autophosphorylate (Fig. 5A). The type II receptor fusion protein showed four times more activity than the R4 protein (Fig. 5A). We also assayed a GST-R1 construct for autophosphorylation activity and found its activity to be 1/100 that of the type II receptor (*12*). This result suggests that in vitro the R4 type I receptor, like the type II receptor, acts as a functional serine-threonine kinase. The GST-RII kinase is mainly a serine kinase with a small amount of activity toward threonine (*5*). Phosphoamino acid analysis of the autophosphorylated GST-R4 kinase revealed mainly phosphothreonine with a small amount of phosphoserine (Fig. 5B).

We conclude that R4 is a functional type I TGF-β receptor because it restored expression of a reporter gene in response to TGF-β in the nonresponsive R1B mutant cells, it required the presence of a functional type II receptor to bind ligand and to signal, its kinase activity was required for signaling, and the kinase domain was capable of serine-threonine autophosphorylation under in vitro conditions. R4 may represent only one of the functional type I receptors for TGF-β because the functional assay involving TGF-β induction of a specific promoter in a particular cell type may have limited our ability to test the functional nature of the other members of this receptor family. The diversity of biological responses elicited by TGF-β could be derived from the existence of multiple receptors that can functionally mediate various effects in different biological systems.

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Association of Transcription Factor APRF and Protein Kinase Jak1 with the Interleukin-6 Signal Transducer gp130

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Interleukin-6 (IL-6), leukemia inhibitory factor, oncostatin M, interleukin-11, and ciliary neurotrophic factor bind to receptor complexes that share the signal transducer gp130. Upon binding, the ligands rapidly activate DNA binding of acute-phase response factor (APRF), a protein antigenically related to the p91 subunit of the interferon-stimulated gene factor–3α (ISGF-3α). These cytokines caused tyrosine phosphorylation of APRF and ISGF-3α p91. Protein kinases of the Jak family were also rapidly tyrosine phosphorylated, and both APRF and Jak1 associated with gp130. These data indicate that Jak family protein kinases may participate in IL-6 signaling and that APRF may be activated in a complex with gp130.

Interleukin-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), interleukin-11 (IL-11), and ciliary neurotrophic factor (CNTF) are members of a family of cytokines and neuronal differentiation factors (*1*) or neurokines (*2*). These factors exert pleiotropic effects on multiple cell types and bind to composite receptors containing the