A Single Heteromeric Receptor Complex Is Sufficient to Mediate Biological Effects of Transforming Growth Factor-β Ligands*

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Transforming growth factor β (TGF-β), a multifunctional cytokine that regulates a variety of biological functions, signals through a heteromeric receptor complex of the type I and type II TGF-β receptors. The type II receptor, a transmembrane serine-threonine kinase, was cloned based on its ability to directly bind TGF-β. Recently, a number of candidate type I TGF-β receptors have been isolated. Although only one of these transmembrane kinases (R4) has been shown to mediate TGF-β-dependent gene activation, others bind TGF-β when overexpressed in COS cells. Consequently, it has been postulated that the diversity of TGF-β responses is generated through the association of distinct type I receptors with the type II TGF-β receptor, thus creating receptor complexes of differential signaling capacities. In contrast to this model, we demonstrate that stable expression of only the R4 type I TGF-β receptor in a mutant cell line lacking endogenous type I TGF-β receptor was able to complex with the endogenous type II TGF-β receptor and restore the effects of TGF-β on inhibition of cell proliferation and activation of specific genes, regardless of which of the three mammalian isoforms of TGF-β was used as the ligand. Therefore, R4 acts as a fully functional type I TGF-β receptor, and the differential effects of TGF-β are likely mediated by a single receptor complex consisting of R4 and the type II receptor.

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**The abbreviations used are: TGF-β, transforming growth factor β; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

Moses, 1990; Massague, 1990). TGF-β signals through a heteromeric complex of the type I and II receptors (Wrana et al., 1992; Massague, 1992; Lin and Lodish, 1993). The type II TGF-β receptor, a transmembrane serine-threonine kinase, was cloned based on its ability to directly bind ligand (Lin et al., 1992). The kinase activity of the type II receptor is essential for signal transduction; however, it is incapable of mediating TGF-β responses in the absence of a type I receptor (Boyd and Massague, 1989; Wrana et al., 1992).

Recently, a number of novel transmembrane serine-threonine kinases (R1 to R4, ALK-3, and ALK-6) have been isolated (Attisano et al., 1993; Ebner et al., 1993a; Fransen et al., 1993; He et al., 1993; Matsuzaki et al., 1993; ten Dijke et al., 1993, 1994; Tsuichida et al., 1993; Yamazaki et al., 1993). Two of these molecules, R1 (Tsk7L) and R3 (TSR1), were classified as type I TGF-β receptors based primarily on their ability to bind TGF-β when co-expressed with the type II TGF-β receptor in COS cells (Attisano et al., 1993; Ebner et al., 1993a). When co-expressed with the activin type II receptor, these receptors also bound activin (Attisano et al., 1993; Ebner et al., 1993b; Tsuichida et al., 1993). These observations support a model in which the diverse responses of TGF-β may be mediated by a repertoire of receptor complexes consisting of the type II TGF-β receptor and distinct type I receptor subunits with different signaling capabilities. In this model, the type II receptor determines ligand specificity, while the type I receptors share members of the TGF-β superfamily and modulate the signals that the type II receptors generate. This model is analogous to the cytokine receptor system in which the cytokines bind distinct receptors yet share a common subunit required for signal transduction (Kishimoto et al., 1994).

Although R1 and R3 bind TGF-β, only R4 (ALK-5) has been shown to mediate TGF-β-dependent signal transduction (Fransen et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994). R4/ALK-5 was identified as a type I TGF-β receptor based on its ability to restore TGF-β induction of the plasminogen activator inhibitor I promoter in a mutant mink lung epithelial cell line (R1B) lacking endogenous type I TGF-β receptors by forming a signaling complex with the endogenous type II TGF-β receptors. The ability of R4 to bind TGF-β is dependent upon the binding of TGF-β to the type II TGF-β receptor (Bassing et al., 1994). Considering the high degree of homology among members of the type I serine-threonine receptor family, it is possible that the type I receptors for other ligands of the TGF-β superfamily bind TGF-β when overexpressed along with the type II TGF-β receptor in COS cells. Thus, the generation of diverse TGF-β responses could be mediated through a single heteromeric receptor complex of the R4 type I receptor and the type II TGF-β receptor. This model parallels the signaling of cyclic AMP and its sole associated kinase in which the differential signals are mediated by different intracellular effectors (Bourne and DeFranco, 1989). To distinguish between these conflicting models, we tested whether R4 could act as a fully functional type I TGF-β receptor with the capacity of mediating the various biological effects of TGF-β.

EXPERIMENTAL PROCEDURES

Luciferase Assays—Cells were plated into 6-well plates at a density of 250,000 cells/well. Twenty-four hours later, cells were transfected with 6 μg of the p3TP-Lux plasmid using DEAE-dextran as described (Wrana et al., 1992). Twelve hours after transfection cells were placed in Dul-
becco's modified Eagle's medium (DMEM) containing 0.2% fetal bovine serum (FBS). Expression of R4 was induced by adding ZnCl₂ (50 μM). Five hours later cells were incubated for an additional 24 h in the presence or absence of TGF-β1 (240 pM). Cells were lysed, and the amount of luciferase activity in the lysates was assayed by integrating total light emission over 30 s using a Berthold luminometer. The MvlLu and R1B control cells were not treated with ZnCl₂.

**Ligand Binding Assays**—Confluent 10-cm plate cultures of R1B-R4 no. 26 were placed in DMEM containing fetal bovine serum (0.2%) and grown overnight, and then placed in DMEM containing fetal bovine serum (0.2%). Expression of R4 was induced with 50 μM ZnCl₂ for 5 h. Cells were then incubated with [125I]-labeled TGF-β1 (150 pM) for 3.5 h at 4 °C. After incubation, the cells were washed and cross-linked with 60 μg/ml disuccinimidyldiisocyanate (DIDC). The cells were lysed, and protein lysates were analyzed by SDS-polyacrylamide gel electrophoresis and then subjected to autoradiography. The MvlLu and R1B control cells were not treated with ZnCl₂.

**Growth Inhibition Assays**—R1B-R4 no. 25 and no. 26 cells were plated into 12-well plates at a density of 20,000 cells/well, grown overnight, and then placed in DMEM containing fetal bovine serum (0.2%). Expression of R4 was induced with 50 μM ZnCl₂ for 5 h. Cells were then incubated with TGF-β1 for 18 h, during the last 2 h of which 4 μCi/ml [³H]thymidine was added to the medium. Cells were lysed and the amount of [³H]thymidine incorporation into the DNA was quantitated using a scintillation counter. The MvlLu and R1B control cells were not treated with ZnCl₂.

**Fibronectin Assay**—R1B-R4 no. 25 and no. 26 cells were plated into 6-well plates at a density of 300,000 cells/well and grown for 24 h in DMEM containing 10% FBS. Cultures were then placed in DMEM containing 0.2% FBS, and expression of R4 was induced for 5 h by the addition of 50 μM ZnCl₂. Cells were incubated for 12 h in the presence or absence of 100 μM TGF-β and then labeled with 50 μCi/ml [³H]thymidine for the last 2 h. To isolate synthesized fibronectin, 0.5-ml aliquots of the labeled media were incubated with gelatin-Sepharose beads and 0.05% Triton X-100 for 12 h. The beads were washed with Tris-buffered saline, and protein was eluted by boiling for 5 min in electrophoresis sample buffer. The amount of newly synthesized fibronectin was visualized by autoradiography of the protein eluted from the gelatin-Sepharose. The MvlLu and R1B control cells were not treated with ZnCl₂.

**RESULTS AND DISCUSSION**

Since constitutive expression of R4 proved toxic, R1B mink lung epithelial mutant cells were stably transfected with the R4 cDNA under the control of the metallothionein promoter in an expression vector pMEP4. Luciferase assays performed on R1B cells transiently transfected with pMEP4-R4 demonstrated that expression of R4 was dependent upon the presence of Zn²⁺. Transfected R1B cells were selected in growth medium containing 300 μg/ml hygromycin B, and drug-resistant colonies were amplified and screened for their ability to express R4 in a Zn²⁺-inducible manner.

To identify positive transfectants, the TGF-β-responsive luciferase reporter gene (p3TP-lux) was transiently transfected into hygromycin-resistant clones. Transfectants treated with TGF-β1 (240 pM) in the presence of 50 μM ZnCl₂ for 20–24 h were lysed and assayed for luciferase activity. Several clones were identified in which the fold induction of luciferase activity was comparable with that of wild-type MvlLu cells transfected with p3TP-lux (Fig. 1A). Two of the clones, R1B:pMEP4-R4-25 and -26 (nos. 25 and 26), showed Zn²⁺-dependent induction of luciferase activity and were chosen for further studies.

To confirm that the nos. 25 and 26 clones were expressing R4 in a Zn²⁺-dependent manner, we performed ligand binding and cross-linking assays with [125I]-labeled TGF-β1. Affinity labeling of the nos. 25 and 26 cells in the absence of Zn²⁺ treatment only showed labeled protein migrating as 80–85 kDa (Fig. 1B). These bands correspond to the molecular weight of the endogenous type II TGF-β receptors. Furthermore, no additional cross-linked type II TGF-β receptor was observed when compared with the control of R1B cells lacking endogenous type I TGF-β receptors (Fig. 1B). When the two clones were treated with 100 μM ZnCl₂ for 12 h prior to affinity labeling, an increase in labeled protein migrating as 63–65 kDa was observed (Fig. 1B). This band corresponds to the molecular weight of the type I TGF-β receptor. Therefore, this result confirms that R1B:pMEP4-R4-25 and -26 cells express R4 in a Zn²⁺-dependent fashion.

When treated with TGF-β, [³H]thymidine incorporation into the DNA of wild-type mink lung epithelial cells is typically inhibited to approximately 90%. In contrast, TGF-β has no effect upon the incorporation of [³H]thymidine into the DNA of R1B cells. To evaluate whether R4 is capable of mediating the TGF-β growth inhibitory response, we performed DNA synthesis assays on the cells of nos. 25 and 26 clones. When these cells were treated with TGF-β in the presence of 50 μM ZnCl₂, incorporation of [³H]thymidine into DNA was inhibited by 50–70% (Fig. 2). This magnitude of growth inhibition is comparable with that observed with DR-27 mutant mink lung epithelial cells stably expressing the human type II TGF-β receptor cDNA in the same vector pMEP4 (Wrana et al., 1992). In the absence of Zn²⁺, no inhibition of [³H]thymidine incorporation was observed (Fig. 2). Therefore, R4 is able to mediate the TGF-β growth inhibitory signal. Furthermore, the growth inhibitory effect of TGF-β was not restored in R1B cells stably expressing a kinase-defective R4 type I receptor² (R4-K230R) (Bassing et al., 1994). Therefore, the intrinsic kinase activity of the type I TGF-β receptor is necessary to signal TGF-β-dependent growth.

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² C. H. Bassing, D. J. Howe, and X.-F. Wang, unpublished results.
A Single Receptor Complex Signals TGF-β Responses

**Fig. 2.** Assay for the restoration of TGF-β-dependent growth inhibition. The amount of[^H]thymidine incorporation into the DNA of Mv1Lu, R1B, and R1B:pMEP4-R4 nos. 25 and 26 cells was quantitated. R1B:pMEP4-R4 nos. 25 and 26 cells were treated both with and without ZnCl2 as indicated. Data is plotted as the percentage decrease of[^H]thymidine incorporation relative to cultures that were not incubated with TGF-β. R1B:pMEP4-R4 no. 25 cells were treated with 100 pM TGF-β2; all other cells were treated with 100 pM TGF-β1.

**Fig. 3.** Assay for the induction of fibronectin synthesis. Mv1Lu, R1B, and R1B:pMEP4-R4 nos. 25 and 26 cells were treated with and without 240 pM TGF-β1. R1B:pMEP4-R4 nos. 25 and 26 cells were incubated with 50 μM ZnCl2 to induce expression of R4. The amount of newly synthesized fibronectin was visualized by fluorography of the protein eluted from the gelatin-Sepharose. A longer exposure for the stable clones is also shown.

inhibition as well as TGF-β-dependent gene responses (Bassing et al., 1994).

The ability of R4 to signal TGF-β-dependent gene responses has thus far been limited to the TGF-β-dependent induction of the plasminogen activator inhibitor-1 promoter (Franzen et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994). If R4 is a fully functional type I TGF-β receptor, it should be able to regulate expression of additional endogenous genes. To address this issue, we assayed the ability of TGF-β1 to induce synthesis of fibronectin in the two stable clones. Synthesis of fibronectin in wild-type mink lung epithelial cells is increased upon treatment with 100 pM TGF-β1 (Fig. 3). In contrast, R1B cells fail to show induction of fibronectin synthesis in response to TGF-β1.

**Fig. 4.** Assay for the mediation of signal for the three mammalian isoforms of TGF-β. A, assay for the gene activation response to TGF-β1, -β2, and -β3. Luciferase assays were performed on R1B: pMEP4-R4 nos. 25 and 26 cells incubated with 50 μM ZnCl2 and either untreated or treated with 240 pM TGF-β1, -β2, or -β3. The luciferase activity was normalized for the amount of protein in the lysates. The bars indicate the average values ± S.E. of luciferase activity from triplicates of a representative experiment. B, assay for growth inhibition response to TGF-β1, -β2, and -β3. Cells were treated with the indicated concentrations of TGF-β1 (○), -β2 (■), or -β3 (▲) for 16 h and then labeled with[^H]thymidine for 2 h, all in the presence of 50 μM ZnCl2. Data is plotted as the percentage decrease of[^H]thymidine incorporation relative to cultures that were not incubated with TGF-β.
ALK-5 was able to restore TGF-P-dependent activation of the TGF-β1 receptor when overexpressed in COS cells, whereas only R4/R4/ALK-5 were able to immunoprecipitate endogenous type TGF-β in various cell types are likely to be signaled through a lineage (ten Dijke TGF-β receptor and endoglin. Consistent with this model, all used as the ligand. These results demonstrate that a single heteromeric TGF-β receptor complex consisting of R4 and the type I1 receptor is sufficient for mediating the diverse effects of the TGF-β isoforms may derive from modulations by three mammalian isoforms of TGF-β.

Six cloned type I-like receptors have been shown to bind TGF-β2 and TGF-β3. TGF-β2 and TGF-β3 isoforms of TGF-β, we assayed both TGF-β-induced gene activation and growth inhibition responses for all three TGF-β isoforms (Franzen et al., 1995). Therefore, R4 is capable of mediating responses for all three mammalian isoforms of TGF-β.

We have shown here that stable expression of only the R4 type I TGF-β receptor in a mutant cell line lacking endogenous type I TGF-β receptor was able to restore the effects of TGF-β on inhibition of cell proliferation and activation of specific genes, regardless of which of the three isoforms of TGF-β was used as the ligand. These results demonstrate that a single heteromeric TGF-β receptor complex consisting of R4 and the type II receptor is sufficient for mediating the diverse responses of TGF-β, suggesting that the biological effects of TGF-β in various cell types are likely to be signaled through a single heteromeric receptor complex, whereas the differential effects of the TGF-β isoforms may derive from modulations by other cell surface TGF-β binding proteins, such as the type III TGF-β receptor and endoglin. Consistent with this model, all six cloned type I-like receptors have been shown to bind TGF-β1 when overexpressed in COS cells, whereas only R4/ALK-5 was able to restore TGF-β-dependent activation of the plasminogen activator inhibitor-1 gene in the R1B mutant cell line (ten Dijke et al., 1994). In addition, only antisera specific to R4/ALK-5 were able to immunoprecipitate endogenous type I and type II TGF-β receptor complexes from a number of TGF-β-responsive cell types, suggesting that R4/ALK-5 functions as the type I TGF-β receptor in a variety of cell types (ten Dijke et al., 1994).

Taken together, these results suggest that R4/ALK-5 is likely the only physiological type I TGF-β receptor identified so far. The other isolated type I-like receptors most likely represent type I receptors for other ligands of the TGF-β superfamily. It is possible, therefore, that a single receptor complex is responsible for mediating TGF-β signals across the membrane, and the differential TGF-β effects are mediated by diverse intracellular effectors distributed in various cell types. With the further characterization of the type I TGF-β receptor, the mechanism by which multiple TGF-β responses are transduced may now be adequately addressed at the molecular level.

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