We have previously proposed that transforming growth factor-β (TGF-β) receptor activation occurs via a relative rotation between the receptors. This model suggests that in the absence of the ligand the receptor extracellular domain negatively regulates the activation of the receptor complex. To investigate this proposition, four TGF-β type I and II receptor extracellular/transmembrane-cytoplasmic and extracellular-transmembrane/cytoplasmic chimeras, TβRII-I-I and TβRII-II-II as well as TβRII-II-I and TβRII-I-II, and two extracellular domain truncated receptors TβRI-SC and TβRI-STC were generated. In either mutant mink lung R1B (lacking functional type I receptor) or DR26 (where the type II receptor is nonfunctional) cells, coexpression of two chimeric receptors, which are complementary in extracellular and cytoplasmic domains, transduced TGF-β-induced signaling, as measured by the transcriptional activation of a p3TP-Lux reporter gene. Coexpression of this type of chimeric receptor with a wild-type receptor containing the opposite cytoplasmic domain exhibited a varied level of constitutive activity depending on the particular combination of the extracellular domains. In general, the type I-type I extracellular domain combination gave higher constitutive activity than the type I-type II or type II-type II combinations. Furthermore, coexpression of the extracellular domain truncated receptor with any receptor containing the opposite cytoplasmic domain always resulted in ligand independent receptor signaling. Immunoprecipitation studies showed that the formation of the receptor complexes paralleled the ligand independent activation of p3TP-Lux. Our results support the conclusion that the TGF-β receptor extracellular domain plays a negative regulatory role in receptor activation in the absence of ligand.

Secreted peptide growth factors have key roles in the development of multicellular organism. They exert biological effects by binding to their cell surface receptors. The binding of the growth factor to the extracellular domain of the receptor induces receptor dimerization/oligomerization, leading to activation of the intracellular kinase and subsequently, the intracellular signaling pathway. Although evidence has started to emerge that some receptor dimers/oligomers are preformed in the absence of ligand, the main function of the receptor extracellular domain has been thought to be ligand recognition and binding. Little has been published about other functions of motifs in the receptor extracellular domain, e.g. clustering motifs, trafficking signals, and proteosome activation.

Transforming growth factor-β (TGF-β) are important for intercellular communication (1–3): they regulate cell proliferation, differentiation, migration, organization, and death, as well as affecting a wide range of biological functions, such as embryonic development, hematopoiesis, and immune and inflammatory cell responses (reviewed in Ref. 4). Alterations in the activity of these growth factors and their receptors in humans have been implicated in fibrosis (4), immunosuppression (5), and cancer (6–8). Biological responses to TGF-β are mediated mainly by the type I and type II cell surface receptors, referred to as TβRI and TβRII, respectively (2, 9–13). Both of these receptors comprise a small extracellular region, a single transmembrane domain, and a cytoplasmic domain with serine/threonine kinase activity. There is almost 40% homology between the TβRI and TβRII kinase domains (1–3, 14–16). Genetic evidence from mutant cells resistant to TGF-β action suggests that both TβRI and TβRII are required for TGF-β signaling (12, 17–20). TβRII specifies growth inhibitory and transcriptional response (18) while TβRII determines ligand binding (19, 20). TβRII is a constitutively active kinase and is phosphorylated (21). While TGF-β binds directly to TβRII, TβRII only binds to TGF-β in the presence of TβRII and TβRII signals through TβRI. It has been proposed that TGF-β binds to TβRII, TβRI is then recruited into the complex and becomes phosphorylated by TβRII, and the phosphorylated TβRII then propagates the signal to downstream substrates (22–26).

However, subsequent studies on heteromeric and homomeric associations of TGF-β receptors in the absence of TGF-β suggest that some fundamental questions concerning the molecular mechanism of receptor activation have yet to be answered. Experiments using a yeast two-hybrid interaction assay and double immunoprecipitation analyses (27, 28) have shown that full-length TβRII and TβRII can form heteromeric complexes in the absence of TGF-β. Using an antibody-mediated immunofluorescence co-patching technique, a recent study (29) provided evidence in live cells that TβRII and TβRII can form heteromeric receptor complexes in the absence of ligand. Heteromeric complexes were also formed between the extracellular domains as well as between the cytoplasmic domains of TβRI and TβRII in the absence of ligand (27, 28). More importantly, these studies indicate that the TβRII-TβRII heteromeric recep-
tor complexes pre-exist in latent forms and TGF-β activates the complexes. Double immunoprecipitation analyses using lysates from metabolically labeled cells co-transfected with differentially epitope-tagged type II receptors have demonstrated that the type II receptors form a homomeric complex both in the presence and absence of TGF-β (30). Ligand-independent TβRII homomeric complex formation has also been demonstrated in live cells using an immunofluorescence co-patching technique (31). It has also been shown that the extracellular domains of TβRII are capable of interacting with itself in the absence of TGF-β (30, 32). Furthermore, it has been shown that after ligand binding, TβRII forms a heteromeric complex with TβR-2.1, a chimeric receptor containing the extracellular and transmembrane domains of type II receptor and the intracellular domain of type I receptor, but this complex fails to signal any TGF-β response in R1B cells, which lack functional type I receptor (33). Taken together, these results suggest that TGF-β is not required for the receptor oligomerization and that receptor oligomerization is not sufficient for TGF-β signaling.

Interestingly, coexpression of the cytoplasmic domains of TβRI and TβRII activates TGF-β signaling pathways in the absence of TGF-β (34). Clearly the cytoplasmic domains of TβRI and TβRII can physically and functionally interact with each other to form an active heteromeric complex (34). Furthermore, coexpression of a single cytoplasmic domain with its complementary full-length receptor also activates the signaling complex (34). Since expression of full-length receptors does not induce spontaneous signaling, these results indicate that the extracellular domains of TGF-β receptors may play roles, in addition to ligand binding, in the regulation of receptor activation and signaling.

Experimental Procedures

Construction of C-terminal-tagged Chimeric and Extracellular Domain-truncated TGF-β Receptors—Polymerase chain reaction (PCR) and human cDNAs ALK-5 (TβRII) (14) and H2-3FF (TβRII) (15) were used to generate chimeric and extracellular truncated TGF-β receptors. The primers used were reported previously (35). In addition, the following two primers were also used, where a single underline indicates the cDNAs of the type I receptor and a double underline indicates the type II receptor. Restriction sites are shown in lower case, RI-SCs (sense), cggggtttgaactgcacgtgctgtcatt and RIi (antisense), Gaattagaccactgacggtctgttctgcgctgtcatt.

The primers were designed to exchange the type I receptor extracellular domain (125 amino acids, 1–125) or the cytoplasmic domain (356 amino acids, 148–503) with the type II receptor extracellular domain (159 amino acids, 1–159) or the cytoplasmic domain (376 amino acids, 190–565). RI-SCs and RIi-Sa were designed to truncate the extracellular domains of TβRI and TβRII, respectively. The construction of TβR-I-M2, TβR-II-I-M2, TβR-II-HA3, TβR-II-III-HA3, and TβRILs-II-HA3 was described previously (35). PCR products of the extracellular (E), extracellular-transmembrane (E-T), transmembrane-cyttoplasmic (T-C), and cytoplasmic (C) domains of type I and II were obtained using a Perkin-Elmer DNA Thermal Cycler with T7q DNA polymerase (BIOTECH), ALK-5 (14), or H2-3FF (15) as templates, and primers as indicated previously (35). The PCR products were first ligated to a linearized PCR vector (Invitrogen). Because of the deletion of the signal nucleotides in the signal sequence region of TβRI (33, 35) using PCR amplification, primer RI-Sa was used with RI-1a or RI-2a to generate TβRI-E or TβRI-E,T, respectively. Two cDNA fragments, TβRI-E and TβR1-T, or TβRI-E,T and TβRI-C were ligated to pcDNA I/Amp (Invitrogen) at HindIII and SpeI sites to form TβRI-I-I-M2 or TβRI-II-I-M2 cDNA constructs. To create HA3-tagged TβR-I-II-HA3, TβR-II-II-HA3, and TβRILs-II-HA3 were described previously (35). PCR products of the respective domains (HA) coding sequence replaced the corresponding fragment in TβR-I-M2 or pcDNA I/Amp (35), then at its Smal and EcoRI sites, fragments TβRI-E and TβR1-T, or TβRI-E,T and TβRI-C were ligated to the modified TβRII-M2 or pcDNA I/Amp. As shown in Fig. 1, TβRII-I consists of the extracellular domain of TβRII (aa 1–159) and the transmembrane/cytoplasmic domains (TCDs) of TβRII (aa 159–503) and TβRII-II consists of the extracellular/transmembrane domains (E-TDs) of TβRII (aa 1–189) and the cytoplasmic domain (CD) of TβRII (aa 148–503). Similarly, TβRII-II contains the extracellular domain of TβRII (aa 1–125) and the transmembrane/cytoplasmic domains of TβRII (aa 160–565), and TβRII-I contains the E-TDs of TβRII (aa 1-147) and the CD of TβRII (aa 190–565). To generate TβRII-STCs-M2, the Smal-SpeI fragment in TβR-I-M2 was replaced by a PCR fragment using primers RI-SCs and RI-3a and ALK-5 as template. Replacement of HindIII-SpeI fragment in TβR-I-II-HA3 by a type II leader sequence coding fragment obtained by PCR using primers RIi-I-Sa and RI-2F as template generated TβRII-STC-HA3. Thus, TβRII-STC contains the leader sequence, transmembrane, and cytoplasmic domains of TβRII, with most of the extracellular domain (aa 31–123) being truncated. TβRII-STC is TβRII
with a truncation of the most of the extracellular domain (aa 30–159), consisting of the leader sequence, transmembrane, and cytoplasmic domains.

**Cell Culture and Transient Transfection—** COS-1 cells were obtained from the American Type Culture Collection. Mutant mink lung epithelial (Mv1Lu) cells R1B and DR26 (12) were gifts from A. B. Roberts (National Institutes of Health). The cells were grown in a 5% CO₂ atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum (CSL, Australia), 60 μg/ml penicillin, and 100 μg/ml streptomycin. Transient transfections were performed using a DEAE-dextran (12) or FuGENETM 6 (Roche Molecular Biochemicals) protocol and transfected cells were assayed 48 or 72 h later.

**Binding and Affinity Cross-linking—** ¹²⁵I-TGF-β was purchased from Amersham Pharmacia Biotech. Binding and affinity cross-linking assays using bis(sulfosuccinimidyl) suberate (BS³) (Pierce) were performed as described previously (27). Briefly, 2 days after transient transfection with TGF-β receptor constructs, COS-1 cells in 6-well plates were washed with binding buffer (phosphate-buffered saline, containing 0.9 mM CaCl₂, 0.49 mM MgCl₂, and 1 mg/ml bovine serum albumin) and incubated on ice for 10 min. After incubation, the cells were washed with the binding buffer without bovine serum albumin and cross-linked with 0.5 ml of 0.28 mM BS³ (in binding buffer without bovine serum albumin) for 15 min on ice. The cells were then washed with PBS and lysed in 100 μl of lysis buffer consisting of 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminoethane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100, and 1% Trasylol (Bayer). The cell lysates were immunoprecipitated using anti-M2 FLAG antibody-conjugated beads (IBI, Eastman Kodak Co.) followed by SDS-gel electrophoresis using 10% polyacrylamide and autoradiography.

**Luciferase Assay—** The p3TP-Lux (12) TGF-β inducible luciferase reporter construct, containing a region of the human plasmaglomin activator inhibitor-1 (PAI-1) gene promoter and three repeats of 12-O-tetradecanoylphorbol-13-acetate-responsive elements upstream of the luciferase gene (12) was obtained from A. B. Roberts (National Institutes of Health). p3TP-Lux (6 μg) was co-transfected into mutant Mv1Lu cells together with 6 μg of TGF-β receptor construct(s). The cells in a 10-cm dish were divided into six wells in 6-well plates 24 h after transfection. At 48 h post-transfection, the media were changed to Dulbecco’s modified Eagle’s medium, 0.2% bovine serum albumin and three wells of each transfected cell line were stimulated with TGF-β (2 ng/ml). Thereafter, cells were ice for 3 h and 0.4 μCi of ¹²⁵I-TGF-β/well in 200 μl of binding buffer. After incubation, the cells were washed with the binding buffer without bovine serum albumin and cross-linked with 0.5 ml of 0.28 mM BS³ (in binding buffer without bovine serum albumin) for 15 min on ice. The cells were then washed with PBS and lysed in 100 μl of lysis buffer consisting of 25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminoethane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100, and 1% Trasylol (Bayer). The cell lysates were immunoprecipitated using anti-M2 FLAG antibody-conjugated beads followed by SDS-gel electrophoresis using 10% polyacrylamide and autoradiography.

**RESULTS**

**Expression and Binding of Chimeric and Mutated TGF-β Receptors—** Our previous work demonstrated that the C-terminal M2-tagged TGF-β type I and C-terminal HA₃-tagged type II receptors are functional (35). The M2 and HA₃ tags facilitate the detection of each receptor type. Tagged type I and type II receptors, TβRII(M2) and TβRII(HA₃), were transfected into COS-1 cells and analyzed by SDS-PAGE and Western blot. Expression of receptors containing the type I cytoplasmic domain (Fig. 2A) and receptors containing the type II cytoplasmic domain (Fig. 2B) was confirmed. COS-1 cells transfected with chimeric receptor cDNAs were used to determine the ligand binding properties. Binding of ¹²⁵I-TGF-β to chimeric receptors was confirmed by affinity cross-linking (Fig. 2C). Both M2-tagged TβRII-I and TβRII-II bind TGF-β (Fig. 2C). The HA₃-tagged TβRII-I and TβRII-II are also present after cross-linking with ¹²⁵I-TGF-β and immunoprecipitation with anti-M2 (TβRII-I or TβRII-II) antibody (Fig. 2C), which confirms the formation of complementary extracellular/intracellular chimeric receptor complexes. These results indicate that these C-terminal tagged chimeric receptors are expressed, transported to the cell surface, bind TGF-β, and form receptor complexes.

**TGF-β-induced Signaling Properties of Extracellular/Cytosplasmic Chimeric Receptors—** TGF-β induces expression of PAI-1 (12). Consequently, the induction of PAI-1 can be used as a measure of TGF-β signaling activity (12). A reporter gene construct, p3TP-Lux (21) in which PAI-1 promoter drives expression of luciferase, and TGF-β receptor construct(s) were co-transfected into Mv1Lu mutant cells, R1B or DR26 cells (12). R1B cells express endogenous TβRII but lack functional TβRI and are not responsive to TGF-β stimulation while DR26 cells express endogenous TβRI but lack functional TβRII and do not transduce TGF-β induced signal (12). Thereby the luciferase activity correlates with the receptor activation. We demonstrated successful expression and TGF-β binding of TβRII-I and TβRII-II as well as TβRII-II and TβRII-I in COS-1 cells (Fig. 2). However, when each of the four extracellular/ cytoplasmic chimeric receptor constructs was transfected alone in either R1B or DR26 cells, the TGF-β responsiveness was not restored (Fig. 3), although the wild-type TβRI restored the responsiveness in R1B cells as did TβRII in DR26 cells (Fig. 3). This result is consistent with previous studies (33, 34). When the cDNAs of a pair of complementary chimeric receptors TβRI-I and TβRII-I were co-transfected in R1B cells, the TGF-β induced transcriptional activation of p3TP-Lux was observed (Fig. 3A). In addition, coexpression of TβRI-I and TβRII-II resulted in some constitutive activation, in the absence of ligand (Fig. 3A). The level of TGF-β induced transcriptional activation with the co-transfection of TβRI-I and TβRII-I
is much less than that induced with the transfection of TβRI into R1B cells. Similar results were observed in DR26 cells (Fig. 3B). TβRI-I-II and TβRII-I-I are closely related to the previous reported TGF-β chimeric receptor construct R1.2 and R2.1 (33) as well as RI-RII and RII-RI (34, 36). The above results are consistent with earlier reports (33, 34, 36), supporting the notion that both the extracellular and cytoplasmic domains of TβRI and TβRII must be present for TGF-β mediated signaling. To explore the basis of this notion further, we co-transfected different combinations of the extracellular/cytoplasmic chimeric receptors into the TβRI-deficient R1B and the TβRII-deficient DR26 cells. There were reciprocal type I and type II extracellular and cytoplasmic domains in each combination of the chimeric receptors. The transmembrane domains were not always reciprocal. As shown in Fig. 3, A and B, coexpression of these combined chimeric receptors resulted in the restoration of TGF-β responsiveness in both R1B and DR26 cells, further confirming the indispensable role of the extracellular and cytoplasmic domains in the ligand induced signaling. Again, some ligand independent activation of p3TP-Luc (Fig. 3) was obtained following the coexpression of two chimeric receptors.

Constitutive Activation Can Result from Coexpression of Chimeric TGF-β Receptors—As shown above, coexpression of two reciprocal chimeric extracellular/cytoplasmic receptors resulted in constitutive TGF-β signaling. Our earlier studies (35) on the TGF-β transmembrane chimeric receptors have led us to propose that the TGF-β receptor activation occurs via relative reorientational rotation (35) and that the interaction of the receptor extracellular domains may prevent the constitutive activation of the receptor. To explore the control factors of the receptor constitutive activation, we co-transfected different combinations of the wild-type and chimeric receptors into the mutant R1B and DR26 cells. The constitutive activation of the TGF-β receptors was apparent when both the type I and II cytoplasmic domains were expressed (Figs. 3 and 4). There was no significant receptor constitutive activation when only one type of cytoplasmic domain was expressed, even though the extracellular combination was heteromeric (data not shown), i.e. co-transfection of TβRII with TβRII-II-II or TβRII-I-II in R1B cells or co-transfection of TβRI with TβRII-I-I or TβRII-I-II-I in DR26 cells. Nevertheless, the constitutive activities were high when the wild-type TβRI was coexpressed with TβRII-II-II or TβRII-I-II (Fig. 4). Coexpression of TβRI and TβRII-II-II or TβRI and TβRII-I-II resulted in the type I homomorphic combination of the extracellular domains and type I and type II heteromeric combination of cytoplasmic domains. However, the constitutive activities were low (Fig. 4) when the wild-type TβRII was expressed with TβRII-I-I or TβRII-II-I. In this case the combination of the extracellular domains was homomorphic type II. The degree of constitutive activation when TβRII and TβRII-I-I or TβRII and TβRII-II-I were coexpressed was even lower than that when two extracellular/cytoplasmic chimeric receptors were expressed. These results are more marked in R1B cells than in DR26 cells, probably due to the fact that the R1B cells are more sensitive than DR26 cells in the transcriptional activation assay.

Signaling Activity of Extracellular Domain-truncated TGF-β Receptors—Our previous results (Fig. 4) suggested that the combination of extracellular domains of the TGF-β receptors...
may greatly affect the constitutive activation of the receptor. To explore further how the receptor extracellular domain regulates the receptor activation, we examined the signaling activity of the extracellular domain-truncated receptors, TβRI-RII and TβRII-STC. In particular, the activity of these truncated receptors in the absence of TGF-β was examined when they were coexpressed with a range of different receptors containing a reciprocal cytoplasmic domain. Neither TβRI-RI-STC nor TβRII-STC alone was able to activate p3TP-Lux transcription either in the presence or absence of TGF-β (Fig. 5, A and B). In the TβRII-deficient R1B cells, co-transfection of TβRII-RII with TβRI or TβRII-RI in the TβRIII-deficient DR26 cells. Again, TGF-β was not stimulating (Fig. 5B). These results are consistent with those reported early using only receptor cytoplasmic domains (34). Furthermore, in R1B cells, co-transfection of TβRI-RII with any of receptors containing the type II receptor cytoplasmic domain, TβRII-I, TβRII-III, TβRII-I, and TβRII-III (35), resulted in a high level of activation of the p3TP-Lux reporter in the absence of TGF-β (Fig. 5A). TβRIIIA-I is TβRII with Leu160 deletion in the transmembrane domain (35). Despite different extracellular and transmembrane domains attached to the type II cytoplasmic domain, the level of constitutive activation was similar. High levels of constitutive activation were also observed in DR26 cells following the introduction of TβRIII-RII together with a receptor containing the type I cytoplasmic domain, such as, TβRII-I, TβRII-I-I, or TβRII-II-I (Fig. 5B). In summary, coexpression of the extracellular domain-truncated receptor with any other receptor resulted in high ligand independent activation if the other receptor is complementary with the truncated receptor in the cytoplasmic domain.

**Complex Formation between TGF-β Receptors**—We previously reported that the amount of formation of the receptor complex parallels the receptor activation (35). In this study, we have shown that the receptor extracellular domains have a great influence on the constitutive activation of the receptor. We therefore investigated the effect of the extracellular domain on the formation of receptor complex using chimeric and truncated receptors. When the wild-type TβRII was co-transfected into COS cells with TβRII-I, only a small amount of TβRII co-immunoprecipitated out with the chimeric TβRII-II-I or TβRII-II-III (lanes 1 and 2, Fig. 6A), indicating weak complex association between the receptors. However, the association between the wild-type TβRII and TβRII-I-II or TβRII-II-III was strong, with an increased amount of receptor complexes detected in co-immunoprecipitation (lanes 3–6, Fig. 6A). As we have described previously (35), the increased complex formation observed here correlates with high constitutive activity and a small amount of complex also correlates with the low constitutive activity (Fig. 5). Co-transfection of various combinations of the chimeric receptors also resulted in receptor complex formation as shown in lanes 3–6 in Fig. 6. As shown in
FIG. 7. Schematic illustration of extracellular domain-regulated TGF-β receptor activation. A, the wild-type TβRI and TβRII form an inactive tetrameric receptor complex due to the unproductive interactions between the extracellular domains of the receptors. An equilibrium exists between the free receptors and the receptor complex, favoring TGF-β binding to the complex breaks up the unproductive extracellular interactions between TβRI and TβRII, enabling productive interactions between the cytoplasmic kinase domains of TβRI and TβRII to take place, forming an active receptor complex. Furthermore, in the absence of ligand, a small amount of active receptor complex may be formed due to the interactions between the extracellular domains of TβRI and TβRII, with the equilibrium favoring the inactive complex. However, ligand binding can stabilize the active complex, resulting in a high level of receptor activation. B, deletion of the extracellular domain of TβRI or TβRII or both impairs the interactions between the extracellular domains of TβRI and TβRII. The productive interactions between the extracellular domains of TβRI and TβRII become dominant, shifting the equilibrium to the active receptor complex and resulting in high constitutive activation.

lane 1 in Fig. 6B and reported previously (35), the complex between the full-length TβRI and TβRII can be formed but to a lesser extent than that between various chimeric receptors. Interestingly, truncation of the extracellular domain of one of the receptors results in an increase of receptor complex formation (lanes 2 and 3, Fig. 6B). Increased receptor complex formation was also observed between two receptors where both the extracellular domains were deleted (lane 4, Fig. 6B). Our previous data (35) showed a direct correlation between the amount of receptor complex formation and subsequent receptor activity. The data in the current experiments further support our previous findings, but in addition, demonstrate the role of the extracellular receptor domains in preventing such receptor complex formation.

DISCUSSION

Studies on the mechanism of TGF-β receptor activation, like those on other cytokine receptors, have mainly centered on the functional roles of the receptor intracellular domains. Little attention has been paid to the functions of the receptor extracellular domains aside from their ligand binding properties. In the present work, we identify an additional role of the extracellular domains of TGF-β receptors play a negative regulatory role in ligand independent receptor activation.

The ligand-induced receptor activation appeared to be clear several years ago after a series of publications (22–26). These reports led to the proposition that TGF-β binds to TβRII, recruits TβRI, forming a TβRI-TβRII receptor complex, resulting in activation of TβRI and downstream signaling (22–26). According to this model, the ligand-induced receptor complex formation is necessary and sufficient for receptor activation. Consistent with this model is the heteromeric association of TβRI and TβRII observed after TGF-β binding (22, 27–29). However, it has been well documented that TβRI and TβRII form latent heteromeric receptor complexes even in the absence of TGF-β (27–29, 35). Furthermore, in the presence of TGF-β, the formation of a complex between TβRII and TβRII-2.1, which contains the extracellular and transmembrane domains of TβRII and the intracellular domain of TβRI, did not result in activation of the receptor complex (33). We need to explain why the receptor complexes are latent in the absence of ligand, and how activation of the complexes are controlled by TGF-β.

Our previous work (35) on the receptor transmembrane domain has indicated that a rotation-activation model (Fig. 7A) can explain the molecular mechanism of the TGF-β receptor activation. In this model, in the absence of TGF-β, free TβRI and TβRII equilibrate with a latent TβRI-TβRII receptor complex which may equilibrate with an active receptor complex but the equilibrium favors the latent form (Fig. 7A). The difference between the non-active and active complexes is the relative orientation of receptors in the complexes. A relative rotation of the receptor is required to activate the latent receptor complex (Fig. 7A). TGF-β binding to the latent receptor complex forces the receptors to undergo a relative rotation, resulting in an orientation favorable for a productive alignment of receptor kinase domains and thereby signaling (Fig. 7A). This model provides insights into how a latent form of receptor complex is activated in the presence of TGF-β and explains many of the reported observations.

Detailed analysis of our results (Table I) reveals a consistent trend that the combination of the extracellular domains correlates the level of constitutive activation. The combination of the type I and type II or two type II extracellular domains is associated with lower levels of constitutive activation than the combination of two type I extracellular domains. In the absence of TGF-β, the association between the type I and type II and between the type II and type II extracellular domains has been previously demonstrated (27, 28, 30), indicating strong extra-
cellular interactions between TβRI and TβRII as well as between TβRII and TβRII. However, the type I extracellular domains showed no intrinsic association with each other (27, 28, 30, 31), indicating weak or no interactions between two type I extracellular domains. Thus, these findings together with our data demonstrate a consistent correlation between a strong extracellular interaction and latent state of the receptor complex. In the extreme instance, receptor extracellular domains may not interact due to the competition between the extracellular and intracellular interactions, with the predominance of the extracellular interactions resulting in the latency of the receptor complex, and results in more receptor complex formation and a high level of constitutive activation (35). We would also predict that by changing the extracellular domain of TβRII to that of TβRI, the extracellular interaction would be weakened, allowing the interaction of the kinase domains to dominate, resulting in a productive receptor complex formation and high constitutive activity. This is what we observe (Figs. 6A and 4). Furthermore, the total removal of one or both of the receptor extracellular domains would then diminish the counterproductive interaction between the extracellular domains, allowing more productive kinase interaction, more productive receptor complex formation (Fig. 6B), and a higher level of constitutive activation (Fig. 5) as observed. We conclude that the inhibitory effect of the receptor extracellular domain is due to the intrinsic interaction between the extracellular domains of TβRI and TβRII, which prevents the productive interaction between the receptor cytoplasmic kinase domains.

Ligand independent receptor oligomerization is not restricted to TGF-β receptors. When the full-length epidermal growth factor (EGF) receptor (EGFR) was transiently co-expressed in human 293 fibroblasts with a truncated receptor that lacks the extracellular domain, association of these receptors in the absence of ligand has been observed, in addition to constitutive kinase activity and tyrosine phosphorylation (37). A recent study (38) has shown that a common mutant EGF receptor ΔEGFR, which occurs frequently in cancers and lacks a portion of the extracellular ligand-binding domain due to genomic deletions, was constitutively phosphorylated. These observations suggest that the concept of a negative regulatory role of receptor extracellular domains may also be applicable to the EGF receptor system.

A recent crystallographic study has demonstrated that the dimers of extracellular domains of erythropoietin (Epo) receptor (EpoR) can be preformed in the absence of Epo (39). The formation of the dimer is attributed to a symmetric interaction between two extracellular domains (39). Importantly, this study together with an in vivo protein fragment complementation assay (40) have demonstrated that such an extracellular interaction results in the EpoR intracellular domains being too far apart to be phosphorylated and activated by JAK2 and therefore prevents a constitutive signaling. Comparison of structures of Epo liganded (41) and unliganded (39) EpoR dimers has suggested that binding of Epo results in the preformed dimers undergoing a process of re-orientation, bringing the intracellular domains close together to be activated (39, 40). These studies suggest that the concept of negative regulation of receptor extracellular domains may yet be applicable to Epo receptor signaling.

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Table I

| Co-transfection pairs | Combinations of extracellular domains | Change of constitutive activity |
|-----------------------|--------------------------------------|-------------------------------|
| TβRI                  | TβRII-1-II                           | I/I → I/I ↑ ↑                 |
| TβRI                  | TβRII-1-II                           | I/I → I/I ↑ ↑                 |
| TβRII-I                | TβRII-1-II                           | I/I → I/I ↑ ↑                 |
| TβRII-II               | TβRII-1-II                           | I/I → I/I ↑ ↑                 |
| TβRII-I                | TβRII-II-1                          | I/I → I/I ↑ ↑                 |
| TβRII-1-II             | TβRII-II-1                          | I/I → I/I ↑ ↑                 |
| TβRII-I                | TβRII-II-I                           | II/I → I/I ↑ ↑                |
| TβRII                  | TβRII-II-I                           | II/I → I/I ↑ ↑                |

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