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Signaling Activity of Transforming Growth Factor β Type II Receptors Lacking Specific Domains in the Cytoplasmic Region

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The transforming growth factor β (TGF-β) type II receptor (TβR-II) is a transmembrane serine/threonine kinase that contains two inserts in the kinase region and a serine/threonine-rich C-terminal extension. TβR-II is required for TGF-β binding to the type I receptor, with which it forms a heteromeric receptor complex, and its kinase activity is required for signaling by this complex. We investigated the role of various cytoplasmic regions in TβR-II by altering or deleting these regions and determining the signaling activity of the resulting products in cell lines made resistant to TGF-β by inactivation of the endogenous TβR-II. TGF-β binding to receptor I and responsiveness to TGF-β in these cells can be restored by transfection of wild-type TβR-II. Using this system, we show that the kinase insert 1 and the C-terminal tail of TβR-II, in contrast to the corresponding regions in most tyrosine kinase receptors, are not essential to specify ligand-induced responses. Insert 2 is necessary to support the catalytic activity of the receptor kinase, and its deletion yields a receptor that is unable to mediate any of the responses tested. However, substitution of this insert with insert 2 from the activin receptor, ActR-IIB, does not diminish the ability of TβR-II to elicit these responses. A truncated TβR-II lacking the cytoplasmic domain still binds TGF-β, supports ligand binding to receptor I, and forms a complex with this receptor. However, TGF-β binding to receptor I facilitated by this truncated TβR-II fails to inhibit cell proliferation, activate extracellular matrix protein production, or activate transcription from a promoter containing TGF-β-responsive elements. We conclude that the transcriptional and antiproliferative responses to TGF-β require both components of a heteromeric receptor complex that differs from tyrosine kinase receptors in its mode of signaling.

A large body of evidence has provided a detailed account of the initial events during activation of tyrosine kinase growth factor receptors (24, 27, 28). Upon ligand binding, these receptors dimerize and undergo autophosphorylation on tyrosine residues. Some of the resulting phosphotyrosine groups act as binding sites for cytoplasmic components that contain, or interact with, enzymatic structures that propagate specific signals. Most of the substrate-binding phosphotyrosines reside in the C-terminal region following the kinase domain and, in some cases, in a noncatalytic insert within the kinase domain (24). Substrate interactions with the C-terminal tail and the kinase insert are central to signaling by tyrosine kinase receptors.

This study investigates whether similar regions might be important for signaling by the emerging family of serine/threonine kinase receptors. This family includes receptors for transforming growth factor β (TGF-β)-related factors, i.e., the mammalian TGF-β type II receptor (TβR-II) (16), the activin type II receptors ActR-II and ActR-IIB from vertebrates (2, 21, 22), and Atr-II from *Drosophila melanogaster* (7), as well as the bone morphogenetic protein type II receptor daf-4 (9) and the orphan receptor daf-1 (10), both from *Caenorhabditis elegans*. The structural organization of these receptors is remarkably similar to that of the receptor tyrosine kinases. They contain a cysteine-rich extracellular domain followed by a single transmembrane region, a protein kinase domain with two characteristic short inserts and predicted specificity toward serine and threonine residues, and a C-terminal tail with several serine and threonine residues that might serve as autophosphorylation sites (15, 20). These observations raised the possibility that the mechanism of serine/threonine kinase receptor activation resembles that of tyrosine kinase receptors.

Little is known about the activation process of receptors in this family. Biochemical and genetic characterization of the TβR-II have revealed that it signals as part of a heteromeric receptor complex. This complex contains at least one additional ligand-binding subunit, called the type I receptor (5, 30). Evidence indicates that the type I receptor binds ligand only in the presence of receptor II (14, 30). Receptor II can bind ligand in the absence of receptor I; however, it requires receptor I to signal all responses tested (3, 30). Candidate receptor I molecules that bind TGF-β and activin have been cloned from mammalian (1, 8) and *Drosophila* (31) cells and shown to be transmembrane serine/threonine kinases distantly related to the type II receptors.

This study addresses two issues regarding the mechanism of TGF-β receptor activation. First, it shows that the kinase inserts and the C-terminal tail of TβR-II, in contrast to the corresponding regions in tyrosine kinase receptors, are not essential to specify various responses. Second, the results show that none of these domains is required for association of receptor II with receptor I. In fact, a truncated TβR-II lacking the entire kinase domain still binds TGF-β, supports TGF-β binding to receptor I, and forms a complex with this receptor. However, TGF-β binding to receptor I facilitated by the truncated TβR-II fails to induce antiproliferative or transcriptional responses, arguing that induction of these responses requires the activity of both receptor components.

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TABLE 1. Oligonucleotides

Name or purpose	Sequence[a]
201	5′-CCGCTCGAGTCGGTCTATGACGAGCAGCG-3′ (nt −29 to −10)
102	5′-CCGGAATTCGGGGCAGCCTCTTTGGACAT-3′ (nt 1745–1726)
Δi1	5′-ACCTCCACAGTGATCAC--ATCGTGCACAGGGACCT-3′ (nt 1079–1139)
Δi2	5′-GAAGGACAAC--GCTAGC--GGCATCCAGATGGTGTG-3′ (nt 1461–1541)
P498A	5′-CGAGGGCGAGCAGAAATTCCAAGCTTC-3′ (nt 1483–1509)
Δtail	5′-CGGGAATTCCTAGAGCCTGTCCAGATGCTC-3′ (noncoding, nt 1641–1624)
Δcyt	5′-CCGGGATCCTCATGAACTCAGCTTCTGCTG-3′ (noncoding, nt 597–579)
S502A	5′-AGGCCTGAAATTCCCGCTTTCTGGCTCAACCAC-3′ (coding, nt 1489–1521) and 5′-GAGCCAGAAAGCGGGAATTTCAGGCCTCCCTCGATCTCTCAA-3′ (noncoding, nt 1515–1474)
ActRi2	5′-AAGATGAGGCCCACGATTAAGGATCACTGGCTGAAACACCCG/ GGCATCCAGATGGTG-3′ (coding, nt 1375–1416 of ActRII-B1, nt 1525–1539 of TβR-II) and 5′-GTGATCCTTAATCGTGGGCCTCATCTTGTGGACAACCAC/ GTTGTCCTTCATGCT-3′ (noncoding, nt 1401–1360 of ActRII-B1, nt 1470–1456 of TβR-II)
Cloning of the cytoplasmic domain into pGEX2T	5′-CCGGGATCCAACCGGCAGCAGAAGCTGGAG-3′(nt 574–593)

[a] Numbering is based on +1 for the A of the start methionine codon (16). Restriction sites introduced to facilitate cloning are underlined. Unless otherwise indicated, the two internal primers were exactly overlapping, and only the sequence of the coding strand is shown. A *Nhe*I site (coding for Ala-Ser) was introduced instead of insert 2 in Δi2, and conservative base pair changes were introduced to generate *Hin*dIII and *Stu*I sites in P498A and S502A, respectively, to allow for easy detection of mutant versus wild-type clones. Dashes indicate the sites of deletions in Δi1 and Δi2. Base changes are shown in boldface.

MATERIALS AND METHODS

Mutant receptor constructs. Mutant forms of the human TβR-II were generated by the polymerase chain reaction (PCR), using an influenza virus hemagglutinin (HA) epitope-tagged human TβR-II cDNA (30) as the template. For construction of the deletion and missense mutants Δi1, Δi2, P498A, S502A, and ActRi2, two PCR fragments were generated. Primer 201 and the respective antisense mutagenic primer were used to amplify the upstream fragment, and primer 102 and the mutant sense primer were used for the downstream fragment. Reaction conditions were 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C, for 15 to 30 cycles. Gel-purified reaction products were then combined pairwise and reamplified with primers 201 and 102. Amplification conditions were as described above except that the annealing step was for 60 s. Products of these reactions were cloned into pBluescript/HA-TβR-II (30), using convenient internal restriction sites. For construction of the Δtail mutant, a single PCR fragment was generated by using primer 201 and a primer introducing a stop codon and an *Eco*RI site after nucleotide (nt) 1641. Likewise, Δcyt was constructed by introducing a stop codon and a *Bam*HI site after nt 597. Amplification conditions were as specified above. All constructs were sequenced (U.S. Biochemical Sequenase version 2 kit) and subcloned with *Kpn*I and *Bam*HI into pMEP4 (Invitrogen), a Zn^{2+}-inducible mammalian expression vector.

Glutathione *S*-transferase (GST) fusion proteins were generated by PCR amplification of the entire cytoplasmic domain of TβR-II, starting at amino acid 192 (nucleotide 574), and cloning it into pGEX2T (Pharmacia). The deletion of insert 2 was introduced into this construct by swapping the *Acc*I-*Eco*RI fragment of the respective pBluescript clone into the pGEX2T-TβR-II fusion plasmid.

The oligonucleotide primers used for PCR are described in Table 1.

Cell lines and transfections. The cell lines DR-26 and DR-27 were derived in earlier studies by chemical mutagenesis of the mink lung epithelial cell line Mv1Lu (CCL-64; American Type Culture Collection) and selection for resistance to TGF-β (3, 14). Cells were grown in minimal essential medium (MEM) containing nonessential amino acids (NEAA) and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. To generate cell lines that stably express the various mutant forms of TβR-II, cells were transfected by using Lipofectin (GIBCO-BRL) according to the manufacturer's instructions. Media for selection and maintenance of transfectants contained MEM, NEAA, 10% FBS, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 25 ng of amphotericin B (Fungizone; GIBCO-BRL) per ml, and 300 μg of hygromycin B (400 U/mg; Calbiochem) per ml.

Protein kinase assays. Overnight cultures of *Escherichia coli* cells containing the pGEX2T constructs were diluted 10-fold, grown for 2 h, and then induced with 100 μM isopropylthiogalactopyranoside (IPTG) for 2 h. Cell pellets were resuspended in lysis buffer (phosphate-buffered saline [PBS], 150 mM NaCl, 1% Triton X-100), sonicated for 20 s on ice, and centrifuged for 5 min. Glutathione-Sepharose beads (glutathione-Sepharose 4B; Pharmacia) in lysis buffer (1:5, vol/vol) were added to the supernatant, and adsorption of the fusion proteins to the beads was allowed to proceed for 10 min at room temperature. Beads were washed three times with PBS. One microgram of fusion protein was incubated in 50 mM Tris-HCl (pH 7.4)–10 mM $MgCl_2$–1 mM $CaCl_2$–20 μM cold ATP–1 μl of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) for 20 min at room temperature. Proteins were resolved on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and visualized by autoradiography.

TGF-β-responsive promoter assays. The 3TP promoter-luciferase construct used for transient transfections and luciferase assays was as described previously (30). Briefly, it contains, from 5′ to 3′, three copies of a tetradecanoyl phorbol acetate-responsive element from the human collagenase promoter region from −73 to −42, a TGF-β-responsive element of the human plasminogen activator inhibitor 1 (PAI-1) promoter (−740 to −636), and the adenovirus E4 promoter sequences from −38 to +38. These promoter elements were cloned into the luciferase expression vector pGL2 (Promega), to yield p3TP-Lux.

For p3TP-Lux expression assays, cells seeded at a density of 75,000 per well in six-well plates were grown for 2 days prior to transfection. After two washes with serum-free medium, cells were incubated for 2 min with 1.5 ml of 100 μM chloroquine per well in serum-free medium. A mixture

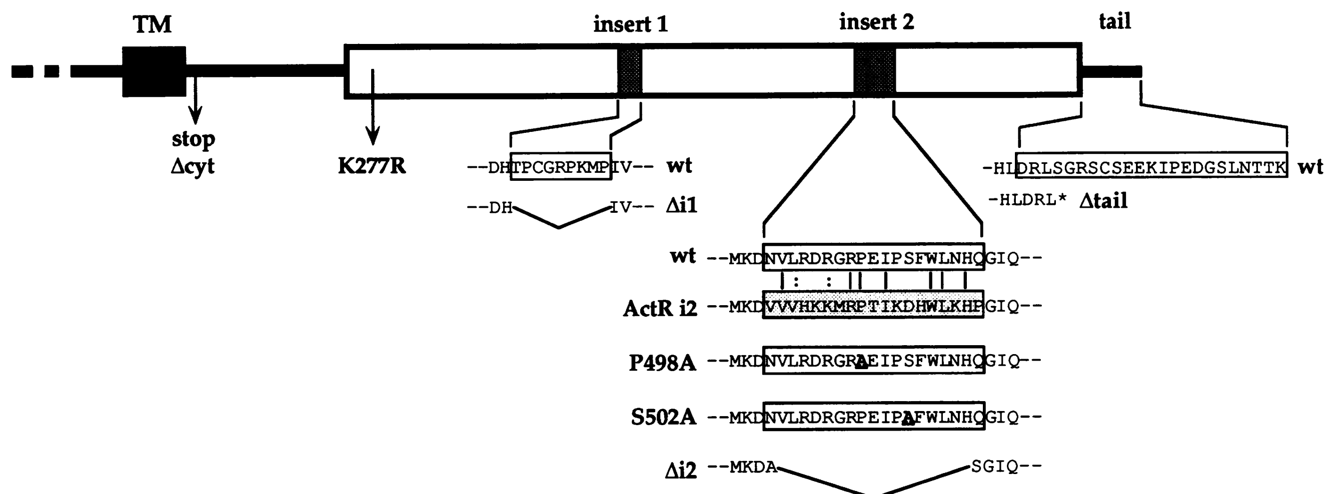

FIG. 1. Schematic representation of the intracellular region of TβR-II and its mutant derivatives. The kinase domain is indicated by an open box. The amino acid sequences of inserts 1 and 2 and the C-terminal tail (16) are boxed. Single amino acid mutations in insert 2 are underlined. ActRi2 shows the sequence of insert 2 from ActR-IIB (2). The identities (|) and similarities (:) between amino acids in the TβR-II and ActR-IIB insert 2 sequences are shown. The position of the stop codon that generates the truncated TβR-II(Δcyt) is shown, as is the position of the K277R mutation in the ATP-binding site (30). wt, wild type; TM, transmembrane domain.

containing the receptor/pMEP4 construct and p3TP-Lux in 0.75× PBS, 0.22% NaCl, and 0.5 mg of DEAE-dextran per ml in a final volume of 340 μl was added to each well. In experiments testing the ability of mutant receptors to rescue TGF-β responsiveness, 1.5 μg of receptor/pMEP4 construct and 6 μg of p3TP-Lux were used. To assay for the dominant negative effect of TβR-II(Δcyt), 0.5 μg of p3TP-Lux and increasing concentrations of TβR-II(Δcyt)/pMEP4 were added. The total amount of DNA added was kept constant by including appropriate amounts of pMEP4. Cells were incubated with DNA for 3 h at 37°C. After removal of the supernatant, cells were shocked with 10% dimethyl sulfoxide in PBS for 2 min, rinsed with medium containing 10% serum, and incubated in this medium overnight. Receptor expression was induced by incubation with MEM–NEAA–0.2% FBS–50 μM $ZnCl_2$ for 4 h prior to the addition of TGF-β1. After 20 h, cells were washed twice with PBS, and 120 μl of lysis buffer (luciferase assay system; Promega) was added. After 10 to 15 min, cells were scraped and debris was removed by centrifugation for 20 s. Luciferase activity was measured in the first 20 s after substrate addition, using a luminometer (Berthold Lumat LB 9501).

Other assays. For receptor affinity labeling, expression of the transfected receptors was induced by overnight incubation with $ZnCl_2$. Affinity labeling and immunoprecipitations were done as described previously (18, 30). Growth inhibition assays and measurement of extracellular matrix-associated proteins were carried out as described previously (30).

RESULTS

Mutant TβR-II molecules lacking kinase inserts and the C-terminal region. We investigated the role of various cytoplasmic regions in TβR-II by altering or deleting these regions and determining the signaling activity of the resulting products when transfected into the receptor-defective cell lines DR-26 and DR-27. These cell lines were derived from Mv1Lu cells by chemical mutagenesis (3, 14) and are resistant to TGF-β as a result of mutations in the transmembrane (DR-26) or extracellular (DR-27) domains of the endogenous TβR-II that truncate this receptor or inactivate its ligand-binding ability, respectively (30). TGF-β binding to receptor I and responsiveness to TGF-β in these cells can be rescued by transfection of wild-type TβR-II, whereas TβR-II(K277R), which contains a point mutation in its ATP-binding site and is inactive as a kinase, restores TGF-β binding to receptor I but not responsiveness to TGF-β (30).

The C-terminal region following the kinase domain of TβR-II and two characteristic inserts in the kinase region (Fig. 1) were of particular interest because the corresponding regions in tyrosine kinases receptors contain autophosphorylation sites that are critical for signaling. If any of these domains are essential in TβR-II, their deletion should prevent signaling. We deleted the C-terminal tail by inserting a stop codon at position 548 (counting the initiator methionine codon as 1) (16), yielding TβR-II(Δtail) (Fig. 1). This position was chosen for the truncation because it lies 20 amino acids downstream of the last conserved arginine in subdomain XI of the kinase, a distance that is thought to encompass the end of the kinase domain (11, 16). This truncation removed the last 20 amino acids of TβR-II, including four serines and two threonines. The most C-terminal potential phosphorylation site in TβR-II(Δtail) was a serine located 11 residues downstream of the conserved arginine in subdomain XI and was therefore considered still part of the kinase domain. Indeed, deletion of the last 29 amino acids from the C terminus resulted in a pronounced reduction in in vitro kinase activity, and truncation by 38 amino acids abolished any detectable in vitro kinase activity (data not shown). Insert 1 comprises amino acids 366 to 374 and is located between kinase subdomains VIa and VIb. Insert 2 comprises amino acids 490 to 508 and lies between kinase subdomains X and XI. We constructed receptor molecules TβR-II(Δi1) (lacking insert 1) and TβR-II(Δi2) (lacking insert 2) (Fig. 1).

The wild-type and mutant TβR-II constructs were engineered with an HA epitope (23, 29) in the N terminus, a modification that does not perturb the activity of TβR-II (30). These constructs were stably transfected into DR-26 and DR-27 cells, and their expression was tested by affinity labeling of cells with ^{125}I-TGF-β1. All of the mutant recep-

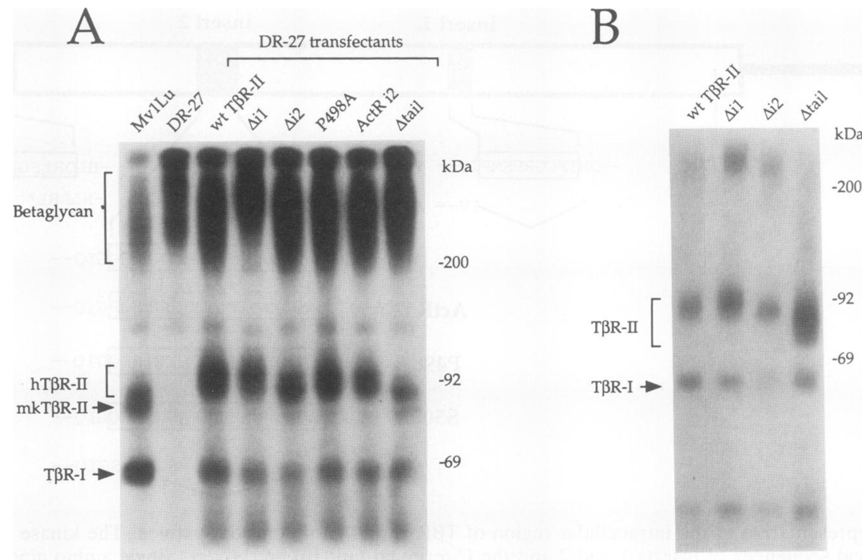

FIG. 2. Cell surface expression of TβR-II and its derivatives and association with receptor I. (A) Confluent monolayers of parental Mv1Lu cells, or DR-27 cells transfected with the indicated TβR-II constructs and activated with Zn^{2+}, were affinity labeled by sequential incubation with ^{125}I-TGF-β1 and disuccinimidyl suberate. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. The endogenous mink TβR-II (mkTβR-II) migrates slightly faster than the transfected full-length human TβR-II (hTβR-II). wt, wild type. (B) Affinity-labeled TβR-II was immunoprecipitated from the same cell lysates with a monoclonal antibody directed against the HA epitope engineered into the TβR-II sequence (30). Precipitated material visualized by PAGE and autoradiography shows the association of the rescued receptor I with TβR-II and its mutant derivatives.

tors were expressed on the cell surface and were able to bind ligand (Fig. 2A). In addition, all mutant receptors, like the wild-type TβR-II in previous experiments (30), were able to rescue ligand binding to the endogenous type I receptor (Fig. 2A). Furthermore, the mutant type II receptors were physically associated with receptor I, as determined by immunoprecipitating this receptor complex with anti-HA antibody (Fig. 2B). In the experiment shown in Fig. 2, the level of recovery of TβR-I varied between the different cell lines. However, given the intrinsic variability of receptor expression and coimmunoprecipitation, the significance of the observed differences is uncertain. Regardless, the results showed that none of the regions deleted in TβR-II were essential for transport to the cell surface, ligand binding, or interaction with receptor I.

Signaling activity of TβR-II lacking kinase inserts or C-terminal tail. The signaling activities of TβR-II and its derivatives were determined by testing their abilities to restore biological responses to TGF-β1 in DR-26 and DR-27 cells. Since the signaling pathways leading to different responses might depend on different domains of the receptor, we examined several TGF-β responses.

Elevated production of fibronectin and PAI-1 represent long-term and short-term effects of TGF-β on gene expression, respectively (12, 13, 19, 25). Fibronectin production was assayed by metabolic labeling of cells and isolation of secreted fibronectin with gelatin-Sepharose. DR-27 cells expressing TβR-II(Δi1) or TβR-II(Δtail) showed a fibronectin response to TGF-β1 whose concentration dependence was similar to that in DR-27 cells expressing wild-type TβR-II (Fig. 3). In contrast, deletion of insert 2 completely abolished the fibronectin response. Similar results were obtained with the effect of TGF-β1 on PAI-1 synthesis (Table 2).

The TGF-β-responsive construct, p3TP-Lux, contains a TGF-β-responsive region from the PAI-1 promoter and three tetradecanoyl phorbol acetate-responsive elements (TREs) driving expression of a luciferase reporter gene. DR-26 cells cotransfected with p3TP-Lux and TβR-II respond to TGF-β1 with a marked increase in luciferase activity (30). TβR-II(Δi1) and TβR-II(Δtail) were able to mediate this response, whereas TβR-II(Δi2) was not (Table 2). A similar pattern was obtained with these receptors in the ^{125}I-dU

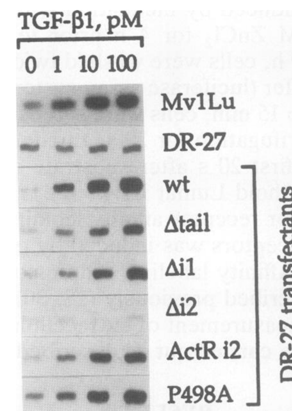

FIG. 3. Stimulation of fibronectin production through TβR-II and its mutant derivatives. Expression of transfected receptors in DR-27 cells was induced by incubation for 5 h with Zn^{2+}. Cells were then incubated with the indicated concentrations of TGF-β1 for 18 h in the same medium. Cells were washed with methionine-free medium and metabolically labeled with [^{35}S]methionine for 3 h. Media were collected; fibronectin was isolated by binding to gelatin-Sepharose and visualized by SDS-PAGE and fluorography. Only the relevant parts of the fluorograms are shown. Basal fibronectin levels varied somewhat between the various cell lines, but the relative elevation induced by TGF-β was similar in all responsive cell lines.

TABLE 2. Ligand-binding and signaling activities of TβR-II and its derivatives expressed in receptor-defective cell mutants

TβR-II construct	Activity					
	TGF-β binding[a]	Receptor I rescue[a]	Fibronectin response[b]	PAI-1 response[c]	3TP-Lux response[c]	Growth inhibition[c]
Wild type	+	+	+	+	+	+
Δtail	+	+	+	+	+	+
Δi1	+	+	+	+	+	+
Δi2	+	+	−	−	−	−
ActRi2	+	+	+	+	+	+
P498A	+	+	+	+	+	+/−[d]
S502A	+	+	+	+	+	+
Δcyt	+	+	−	−	−	−

[a] Assayed as shown in Fig. 2.
[b] Assayed as shown in Fig. 3.
[c] Assayed as shown in Fig. 6.
[d] Diminished response, as shown in Fig. 4.

incorporation assay used as a measure of the TGF-β antiproliferative effect (30). The TβR-II(Δi1) and TβR-II(Δtail) constructs mediated an antiproliferative effect comparable to that mediated by the wild-type receptor, whereas TβR-II(Δi2) was inactive (Table 2).

These results indicated that insert 1 and the C-terminal tail were dispensable for signaling various TGF-β responses, whereas insert 2 was essential. The importance of insert 2 was also suggested by the presence of a proline that is conserved in all identified members of the receptor serine/threonine kinase family and whose mutation in daf-1 leads to defective regulation of dauer larva formation in *C. elegans* (10). When this proline in TβR-II was mutated to alanine, the resulting construct, TβR-II(P498A), was able to convey TGF-β responses (Fig. 3; Table 2), however, the activity of this receptor was significantly less than that of wild-type TβR-II, at least as a mediator of growth inhibition (Fig. 4).

Insert 2 is essential for kinase activity. The inability of TβR-II(Δi2) to signal TGF-β responses, and the effect of the P498A mutation, raised the possibility that insert 2 contains an activating phosphorylation site or a site for interaction with signaling components. Alternatively, insert 2 might be essential for the catalytic activity of the receptor. To distinguish between these possibilities, we constructed TβR-II(ActRi2) by substituting insert 2 with its counterpart from ActR-IIB, which displays only limited sequence similarity with the TβR-II insert 2 (Fig. 1).

When transfected into DR-26 or DR-27 cells, this construct was effective as a mediator of the fibronectin, PAI-1, 3TP-Lux, and antiproliferative responses to TGF-β1 (Fig. 3; Table 2). Furthermore, mutation of the only serine residue present in insert 2 to alanine, yielding TβR-II(S502A) (Fig. 1), was without effect on the signaling activity of this receptor (Table 2). The ability to substitute the TβR-II insert 2 or mutate its potential phosphorylation site without altering the signaling capacity of the receptor suggested that insert 2 was not involved in interactions that specify TGF-β responses.

To determine whether insert 2 was essential for the catalytic activity of the receptor kinase, we expressed the cytoplasmic regions of TβR-II and TβR-II(Δi2) as GST fusion proteins in *E. coli* and tested their kinase activities in vitro. The wild-type kinase product became phosphorylated in this assay (Fig. 5). Phosphorylation was on serine and threonine, as previously reported (16), and tryptic phosphopeptide mapping of the resulting radiolabeled product yielded multiple labeled peptides (data not shown). The TβR-II(Δi2) intracellular region did not become phosphorylated in this assay (Fig. 5). Neither this product nor the TβR-II(K277R) kinase product, which is also inactive as a mediator of TGF-β responses (30), was phosphorylated when mixed with the wild-type TβR-II kinase (data not shown), suggesting that this in vitro assay measures receptor autophosphorylation rather than transphosphorylation. It therefore appears that insert 2 is required for the autophosphorylating activity of TβR-II.

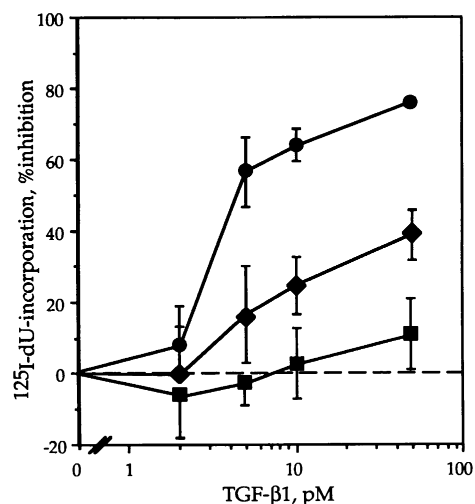

FIG. 4. Antiproliferative response to TGF-β1 in DR-27 cells transfected with wild-type TβR-II or the TβR-II(P498A) mutant. Cells were seeded sparsely into 24-well plates. Receptor expression was induced with Zn^{2+}, and cells were incubated with various concentrations of TGF-β1 for 18 h. ^{125}I-dU incorporation into DNA was then determined. Results are expressed as percent inhibition of ^{125}I-dU incorporation to cultures that did not receive TGF-β1. Data are averages of triplicate determinations ± standard deviations. The experiment was repeated three times with similar results. Squares, DR-27; circles, DR-27 expressing HA-TβR-II; diamonds, DR-27 expressing HA-TβR-II(P498A).

Activity of TβR-II lacking the cytoplasmic domain. The observation that the kinase-deficient TβR-II mutants TβR-

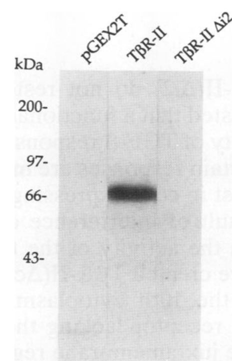

FIG. 5. Kinase activities of the TβR-II and TβR-II(Δi2) kinase products in vitro. Equal amounts of GST–TβR-II kinase and GST–TβR-II(Δi2) kinase fusion proteins produced in *E. coli* were incubated with [γ-^{32}P]ATP in kinase assay buffer. Samples were then subjected to SDS-PAGE and autoradiography.

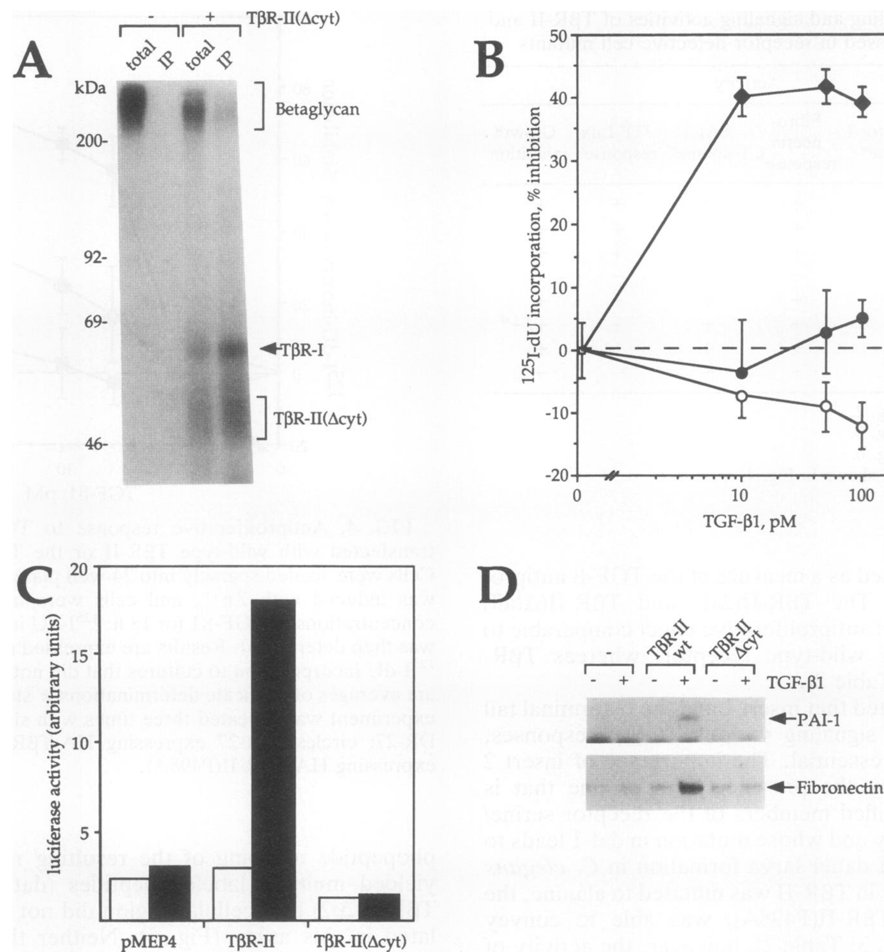

FIG. 6. TβR-II lacking the cytoplasmic domain supports ligand binding to receptor I but not signaling. (A) Control and TβR-II(Δcyt)-transfected DR-27 cells were affinity labeled with ^{125}I-TGF-β1. Cell lysates (total) and immunoprecipitates of these lysates with anti-HA antibody (IP) were subjected to SDS-PAGE and autoradiography. (B) Growth inhibition assay. The experiment was carried out as described in the legend to Fig. 4. Open circles, DR-27; closed circles, DR-27 expressing TβR-II(Δcyt); squares, DR-27 expressing TβR-II. (C) 3TP-Lux assay. DR-26 cells at 50% confluence were cotransfected with 6 μg of p3TP-Lux and 1.5 μg of the respective receptor construct in pMEP4 or of the empty vector. Receptor expression was induced for 4 h with Zn^{2+}, TGF-β1 was added at 100 pM, and cell extracts were prepared 20 h later. Luciferase activity was measured for the first 20 s after substrate addition in a luminometer. White bars, without TGF-β; black bars, with TGF-β. (D) PAI-1 and fibronectin assays. To test induction of PAI-1 protein, cells were incubated in low-serum medium with 50 μM $ZnCl_2$ overnight. TGF-β1 at 50 pM was added for 5 h, and cells were labeled with [^{35}S]methionine for the last 2 h of incubation. Extracellular matrix was prepared, and the proteins were separated on SDS–10% polyacrylamide gels. Only the relevant portion of the fluorogram is shown. Fibronectin assays were carried out as described in the legend to Fig. 3, but total medium samples were loaded onto the gel. In both assays, the same results were obtained with pools of transfectants and two independent clones.

II(K277R) and TβR-II(Δi2) do not restore any of the responses tested suggested that a functional TβR-II kinase was necessary for a variety of TGF-β responses. Alternatively, it was possible that certain responses are mediated by the type I receptor and are lost in cells expressing TβR-II(K277R) or TβR-II(Δi2) as a result of interference of the inert TβR-II kinase domains with the activity of the type I receptor. To address this point, we created TβR-II(Δcyt), which contains a stop codon after the 10th cytoplasmic codon and thus encodes a truncated receptor lacking the entire kinase domain and most of the juxtamembrane region (Fig. 1). DR-27 cells stably transfected with TβR-II(Δcyt) yielded a TGF-β affinity-labeled product of ~50 kDa (Fig. 6A), the predicted size of the truncated receptor. Furthermore, TβR-II(Δcyt) rescued TGF-β binding to receptor I and formed a complex with this receptor (Fig. 6A), indicating that the cytoplasmic domain of the TβR-II receptor was dispensable for ligand binding and interaction with receptor I.

The ability of TβR-II(Δcyt) to present ligand to receptor I allowed us to determine whether TGF-β binding to receptor I was sufficient to induce a response in DR-27 cells. Growth inhibition assays (Fig. 6B), 3TP promoter activation assays (Fig. 6C), and PAI-1 and fibronectin production assays (Fig. 6D) showed that ligand binding to receptor I supported by TβR-II(Δcyt) was not sufficient to mediate any of these responses.

The ability of TβR-II(Δcyt) to associate with receptor I generating an inactive receptor complex raised the possibility that TβR-II(Δcyt) could act in a dominant negative fashion over endogenous wild-type receptors in parental Mv1Lu cells, diminishing the response to TGF-β in these cells. Indeed, independent Mv1Lu cell clones stably trans-

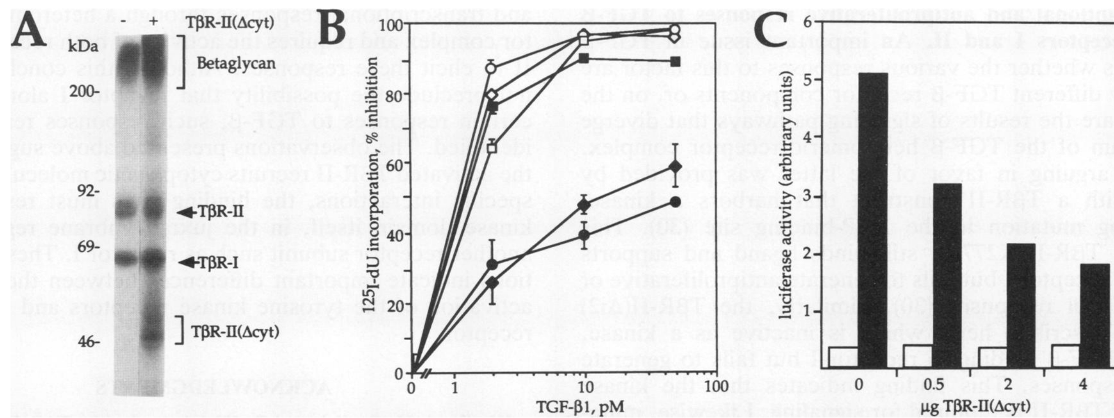

FIG. 7. Dominant negative effect of TβR-II(Δcyt) on antiproliferative and transcriptional responses in Mv1Lu cells. (A) Control and TβR-II(Δcyt)-transfected Mv1Lu cells were affinity labeled with ^{125}I-TGF-β1, and proteins were analysed by SDS-PAGE and autoradiography. (B) Growth inhibition assay. Conditions were the same as in Fig. 4 except that some assays were done in the absence of Zn^{2+} as an internal control. Open symbols, without Zn^{2+}; closed symbols, with Zn^{2+}; rectangles, Mv1Lu; diamonds and circles, two independent clones of Mv1Lu expressing HA-TβR-II(Δcyt). (C) 3TP-Lux assay. Mv1Lu cells at 50% confluence were cotransfected with 0.5 μg of p3TP-Lux, increasing amounts of TβR-II(Δcyt), and the appropriate amounts of empty pMEP4 plasmid to yield a constant total DNA concentration in all transfections. TβR-II(Δcyt) expression was induced with Zn^{2+}, and 100 pM TGF-β1 was added 5 h later. Extracts were prepared after 20 h, and luciferase activity was determined in a luminometer.

fected with TβR-II(Δcyt) (Fig. 7A) showed a marked reduction in the antiproliferative response to TGF-β1 (Fig. 7B) and in the PAI-1 response (data not shown) when TβR-II(Δcyt) expression was induced with Zn^{2+}.

To confirm that TβR-II(Δcyt) acted in a dominant negative fashion on transcriptional responses to TGF-β, Mv1Lu cells were transiently cotransfected with the 3TP-Lux reporter construct and increasing amounts of TβR-II(Δcyt) DNA. Total amounts of DNA in each transfection were kept constant by adding the appropriate amounts of empty vector. This assay showed that TβR-II(Δcyt) suppressed the 3TP-Lux response to TGF-β in a concentration-dependent manner without altering the basal level of luciferase activity (Fig. 7C). Similar results were obtained with TβR-II(K277R) (data not shown). Collectively, these results argued that the activities of both the type I and type II receptors are required for the mediation of antiproliferative and transcriptional responses to TGF-β in these cells.

DISCUSSION

In this study, we examined the structural requirements for signaling by TβR-II, a member of the serine/threonine kinase receptor family. To assess the functional role of specific regions in TβR-II, we used cell lines that are defective in endogenous receptor as a result of chemically induced mutations in the TβR-II gene (3, 14, 30). Transfection of wild-type TβR-II into these cells restores responsiveness to TGF-β, as determined by the recovery of various transcriptional and antiproliferative responses (30). To investigate whether specific regions in TβR-II are involved in signaling, receptor constructs containing mutations or deletions of interest were analyzed for the ability to restore responses to the receptor-defective cells.

The C-terminal tail and the kinase inserts of TβR-II do not specify TGF-β responses. Given the overall structural similarity between the serine/threonine kinase receptors and the tyrosine kinase receptors, we investigated whether the kinase inserts and C-terminal tail, which correspond to signal transducer-binding regions in tyrosine kinase receptors, are required for signaling by TβR-II. The responses analyzed, i.e., growth inhibition, gene expression, and extracellular matrix production, represent the principal classes of TGF-β responses in mammalian cells (17, 19, 25). The results show that the TβR-II C-terminal tail (16), which contains four serines and two threonines, is dispensable for signaling these responses since its deletion yields a TβR-II molecule that is as active as the wild-type receptor. Insert 1, which lies close to the catalytic center of the kinase, can also be deleted with no significant effect on the signaling activity of TβR-II. However, deletion of insert 2 renders the receptor unable to signal any of the responses tested.

Insert 2 is located between subdomains X and XI of the receptor kinase (11, 16), a region that in protein kinase A forms a loop with no assigned function (26). The importance of this region is underscored by the phenotype of the *C. elegans daf-1* mutant (10). The constitutive dauer larva phenotype of these animals is caused by mutation of proline 546 to serine in daf-1. Proline 546, located in insert 2, is conserved in all known members of the serine/threonine kinase receptor family (15, 20). We found that mutation of the corresponding proline in TβR-II (proline 498) to alanine reduced significantly the antiproliferative activity of TβR-II.

The loss of function in TβR-II lacking insert 2 or harboring the P498A mutation could reflect the presence of a critical phosphorylation site or a site for interaction with a signaling molecule. However, mutation of the only potential phosphorylation site in this region, serine 502, has no discernible effect on the signaling activity. In addition, a more invasive mutation, the entire substitution of this insert with insert 2 from the activin receptor ActR-IIB (2), is also without effect. Although we cannot rule out the possibility that both inserts can mediate interaction with the same substrates, the ability to exchange insert 2 in TβR-II without disrupting signaling suggests that the function of this insert is to support the catalytic activity of the receptor rather than to specify TGF-β responses. This conclusion is supported by the observation that the TβR-II kinase domain is inactive as a kinase in vitro when insert 2 is removed.

Transcriptional and antiproliferative responses to TGF-β require receptors I and II. An important issue in TGF-β signaling is whether the various responses to this factor are elicited by different TGF-β receptor components or, on the contrary, are the results of signaling pathways that diverge downstream of the TGF-β heteromeric receptor complex. Evidence arguing in favor of the latter was provided by studies with a TβR-II construct that harbors a kinase-inactivating mutation in the ATP-binding site (30). This construct, TβR-II(K277R), still binds ligand and supports binding to receptor I but fails to generate antiproliferative or transcriptional responses (30). Similarly, the TβR-II(Δi2) construct described here, which is inactive as a kinase, supports TGF-β binding to receptor I but fails to generate TGF-β responses. This finding indicates that the kinase activity of TβR-II is required for signaling. Likewise, mutations that prevent TGF-β binding to receptor I render cells resistant to the antiproliferative and transcriptional responses to TGF-β, suggesting that receptor I is required for these responses as well (3). These observations argue that both receptors are required for various classes of TGF-β responses.

A separate approach to examine these issues makes use of truncated TβR-II constructs as dominant negative receptors. The rationale of these experiments is that transfection of a truncated TβR-II should compete with endogenous TβR-II receptors in the formation of receptor complexes. As a result, the truncated receptor would diminish those responses that require the activity of TβR-II and leave intact any response that could be conveyed solely through the activity of receptor I. Using this approach, we find that TβR-II(Δcyt), a construct with a truncation that eliminates most of the cytoplasmic domain, acts in a dominant negative fashion when transfected into Mv1Lu cells, diminishing their antiproliferative and transcriptional responses to TGF-β. A similar construct diminishes transcriptional responses to TGF-β in neonatal rat cardiomyocytes (4).

In contrast to these observations, it has been recently reported that a truncated TβR-II construct lacking the cytoplasmic region diminishes the antiproliferative responses but not the transcriptional responses to TGF-β in Mv1Lu cells, leading to the suggestion that these two classes of responses are mediated by separate receptor combinations, with receptor I being sufficient to mediate transcriptional effects (6). The reason for this discrepancy with our results is not clear, since the cell line and TGF-β responses tested are the same in the two studies and the truncated receptor constructs are very similar.

To address the question with an independent approach, we used cells that are defective in endogenous receptor II and, as a result, fail to bind TGF-β through receptor I. TβR-II(Δcyt) transfected into these cells restores TGF-β binding to TβR-I and forms a complex with this receptor. Since TβR-II(Δcyt) is essentially devoid of cytoplasmic region, it should not sterically hinder any cytoplasmic activity of receptor I. Thus, if ligand binding to receptor I suffices to mediate certain responses without the involvement of the TβR-II kinase, expression of TβR-II(Δcyt) should suffice to allow TGF-β binding to receptor I and generation of such responses. However, the results obtained with this system clearly show that TGF-β binding to receptor I is not sufficient to generate any of the antiproliferative or transcriptional responses tested. We conclude that these responses require the activity of receptors I and II.

The evidence presented in this study and in previous studies (3, 30) indicates that TGF-β signals antiproliferative and transcriptional responses through a heteromeric receptor complex and requires the activity of both receptors I and II to elicit these responses. Although this conclusion does not preclude the possibility that receptor I alone conveys certain responses to TGF-β, such responses remain to be identified. The observations presented above suggest that if the activated TβR-II recruits cytoplasmic molecules through specific interactions, the binding sites must reside in the kinase domain itself, in the juxtamembrane region, or in another receptor subunit such as receptor I. These observations indicate important differences between the modes of activation of the tyrosine kinase receptors and the TGF-β receptor.

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REFERENCES

1. **Attisano, L., J. Cárcamo, F. Ventura, F. M. B. Weis, J. Massagué, and J. L. Wrana.** Identification of human activin and TGF-β type I receptors that form heteromeric kinase complexes with type II receptors. Cell, in press.
2. **Attisano, L., J. L. Wrana, S. Cheifetz, and J. Massagué.** 1992. Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell **68:**97–108.
3. **Boyd, F. T., and J. Massagué.** 1989. Growth inhibitory response to transforming growth factor-β linked to expression of a 53 kDa cell surface TGF-β receptor. J. Biol. Chem. **264:**2272–2278.
4. **Brand, T., W. R. MacLelland, and M. D. Schneider.** 1993. A dominant-negative receptor for type β transforming growth factor created by deletion of the kinase domain. J. Biol. Chem. **268:**11500–11503.
5. **Cheifetz, S., J. A. Weatherbee, M. L.-S. Tsang, J. K. Anderson, J. E. Mole, R. Lucas, and J. Massagué.** 1987. The transforming growth factor-β system, a complex pattern of crossreactive ligands and receptors. Cell **48:**409–415.
6. **Chen, R.-H., R. Ebner, and R. Derynck.** 1993. Inactivation of type II receptor reveals two receptor pathways for the diverse TGF-β activities. Science **260:**1335–1338.
7. **Childs, S. R., J. L. Wrana, L. Attisano, K. Arora, M. O'Connor, and J. Massagué.** Identification of a Drosophila activin receptor. Proc. Natl. Acad. Sci. USA, in press.
8. **Ebner, R., R.-H. Chen, L. Shum, S. Lawler, T. F. Zioncheck, A. Lee, A. R. Lopez, and R. Derynck.** 1993. Cloning of a type I TGF-β receptor and its effect on TGF-β binding to the type II receptor. Science **260:**1344–1348.
9. **Estevez, M., L. Attisano, J. L. Wrana, P. S. Albert, J. Massagué, and D. L. Riddle.** *daf-4*, a gene controlling *C. elegans* dauer larva development, encodes a BMP receptor. Nature (London), in press.
10. **Georgi, L. L., P. S. Albert, and D. L. Riddle.** 1990. *daf-1*, a C. elegans gene controlling dauer larva development, encodes a novel receptor protein kinase. Cell **61:**635–645.
11. **Hanks, S. E., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.
12. **Ignotz, R. A., and J. Massagué.** 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. **261:**4337–4345.
13. **Laiho, M., O. Saksela, and J. Keski-Oja.** 1987. Transforming growth factor-β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to urokinase. J.

Biol. Chem. **262:**17467–17474.

14. **Laiho, M., F. M. B. Weis, and J. Massagué.** 1990. Concomitant loss of transforming growth factor-β receptor types I and II in cell mutants resistant to TGF-β. J. Biol. Chem. **265:**18518–18524.
15. **Lin, H. Y., and H. F. Lodish.** 1993. Receptors for the TGF-β superfamily. Trends Cell Biol. **3:**14–19.
16. **Lin, H. Y., X.-F. Wang, E. Ng-Eaton, R. Weinberg, and H. Lodish.** 1992. Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell **68:** 775–785.
17. **Lyons, R. M., and H. L. Moses.** 1990. Transforming growth factors and the regulation of cell proliferation. Eur. J. Biochem. **187:**467–473.
18. **Massagué, J.** 1987. Identification of receptors of type β transforming growth factor. Methods Enzymol. **146:**174–195.
19. **Massagué, J.** 1990. The transforming growth factor-β family. Annu. Rev. Cell Biol. **6:**597–641.
20. **Massagué, J.** 1992. Receptors for the TGF-β family. Cell **69:** 1067–1070.
21. **Mathews, L. S., and W. W. Vale.** 1991. Expression cloning of an activin receptor, a predicted transmembrane kinase. Cell **65:** 973–982.
22. **Mathews, L. S., W. W. Vale, and C. R. Kintner.** 1992. Cloning of a second type of activin receptor and functional characterization in *Xenopus* embryos. Science **255:**1702–1705.
23. **Meloche, S., G. Pages, and J. Pouyssegur.** 1992. Functional expression and growth factor activation of an epitope-tagged p44 mitogen activated protein kinase, $p44^{mapk}$. Mol. Biol. Cell **3:**63–71.
24. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.
25. **Roberts, A. B., and M. B. Sporn.** 1990. The transforming growth factor-betas, p. 419–472. *In* M. B. Sporn and A. B. Roberts (ed.), Peptide growth factors and their receptors. Springer-Verlag, Heidelberg.
26. **Taylor, S. S., D. R. Knighton, J. Zheng, L. F. Ten Eyck, and J. M. Sowadski.** 1992. Structural framework for the protein kinase family. Annu. Rev. Cell Biol. **8:**429–462.
27. **Ullrich, A., and J. Schlessinger.** 1990. Signal transduction by receptors with tyrosine kinase activity. Cell **61:**203–212.
28. **Williams, L. T.** 1989. Signal transduction by the platelet-derived growth factor receptor. Science **243:**1564–1570.
29. **Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenson, M. L. Connolly, and R. A. Lerner.** 1984. The structure of an antigenic determinant in a protein. Cell **37:**767–778.
30. **Wrana, J. L., L. Attisano, J. Carcamo, A. Zentella, J. Doody, M. Laiho, X.-F. Wang, and J. Massagué.** 1992. TGF-β signals through a heteromeric protein kinase receptor complex. Cell **71:**1003–1014.
31. **Wrana, J. L., H. Tran, L. Attisano, K. Arora, S. R. Childs, J. Massagué, and M. B. O'Connor.** Submitted for publication.