Transforming growth factor-β (TGF-β) inhibits the proliferation of epithelial cells by altering the expression or function of various components of the cell cycle machinery. Expression of one of these components, cyclin A, is inhibited by TGF-β treatment. We have identified a 760-base pair fragment of the human cyclin A gene promoter that is sufficient to confer TGF-β responsiveness. Using this promoter fragment, we have developed a cyclin A-based luciferase reporter assay that quantitates the growth inhibitory effect of TGF-β in transient transfection assays. This assay was used to determine which domains of the type I (RI) and type II (RII) receptors were required for the antiproliferative effect of TGF-β. In parallel, the functionality of chimeric receptors, between RI and RII (RI-RII or RII-RI), was tested for TGF-β effect on gene expression using a reporter assay based on the plasminogen activator inhibitor type 1 (PAI-1) promoter. We found that TGF-β-induced inhibition of cyclin A expression was absent in RI or RII-deficient Mv1Lu cells and that this response was restored by expression of wild-type type I or type II receptors in these cells. Furthermore, expression of a single chimeric receptor, either RI-RII or RII-RI, did not confer cyclin A regulation by TGF-β. However, expression of two reciprocal chimeras (RI-RII and RII-RI) resulted in growth inhibition, similarly to wild-type receptors. In addition, chimeric receptors as well as mutant receptors with a deleted cytoplasmic domain and kinase-negative receptors inhibited TGF-β responsiveness in the cyclin A reporter assay in a dominant negative fashion. Finally, in both receptor types, the juxtamembrane domain preceding the kinase domain was essential for receptor function but the cytoplasmic tail was dispensable. Our results suggest that a functional TGF-β receptor complex is required for TGF-β-dependent down-regulation of cyclin A gene expression and illustrate the identical receptor requirements for TGF-β-induced growth inhibition and gene expression.

Transforming growth factor-β (TGF-β) is a secreted protein that induces a range of cell responses which affect growth and differentiation (for recent reviews see Refs. 1 and 2). TGF-β treatment of epithelial cells results in cell cycle arrest in late G1, coincident with an inhibition of synthesis and/or activity of some cyclins and cyclin-dependent kinases (3–6). In mink lung epithelial cells, TGF-β decreases the activities of two G1 cyclin-dependent kinases, cdk2 and cdk4 (4, 6, 7). TGF-β down-regulates the synthesis of cdk4 and constitutive expression of cdk4 results in resistance to TGF-β-induced growth arrest, suggesting that cdk4 is a major downstream target in TGF-β-induced growth inhibition (4). The inhibition of cdk2 activity by TGF-β may involve the cdk inhibitor p27, which prevents complex formation of cyclin E/cdk2 as well as cyclin D/cdk 4 (10). Another cdk inhibitor p15, that is TGF-β-inducible, interferes with the cyclin D/cdk 4 complex in keratinocytes (11).

While down-regulation of cdk4 and activation of the cdk inhibitors correlates with TGF-β-induced cell cycle arrest, other components of the cell cycle machinery are also inhibited by TGF-β. Indeed, late G1 arrest by TGF-β is associated with decreased synthesis of cdc2 (12) and cyclin A (13–17). Cyclin A plays a critical role in cell cycle progression by complexing with and regulating the activities of cdc2 and cdk2. Cyclin A/cdk2 and cyclin A/cdk2 complexes also interact with other proteins involved in the regulation of the G1 to S transition, including pRB, the pRB-like proteins p107 and p130, and the transcription factor E2F (18–22). Inhibition of pRB hyperphosphorylation by cyclin A/cdk2 may be involved in control of G1 progression by TGF-β (23).

In addition to its antiproliferative effect, TGF-β induces the expression of many genes, including those for extracellular matrix proteins. In fact, the induction of plasminogen activator inhibitor type 1 (PAI-1) expression is often used as a biochemical marker for TGF-β responsiveness. Induction of PAI-1 expression can be easily measured using a reporter plasmid in which luciferase expression is driven from the PAI-1 promoter in a TGF-β-dependent manner (24, 25). This assay is often used in combination with transient transfection experiments to measure cellular responsiveness to TGF-β and the corresponding receptor requirements. In contrast, no similarly convenient reporter assay has been developed to measure the growth inhibitory response of TGF-β, even though considerable evidence suggests that TGF-β’s effects on growth inhibition and gene expression result from divergent signaling cascades (for review, see Ref. 26).

The early signaling events that lead to growth inhibition or extracellular matrix production by TGF-β are largely unknown. TGF-β interacts with a set of cell surface receptors (for review, see Refs. 26, 27), including the type I and type II receptors which are essential for signal transduction. Both receptor types belong to an expanding family of transmembrane serine/threonine kinases (26, 28–38). Several structural features distinguish type I from type II receptors. The extracellular domain of the type I receptor is shorter than that of the...
Down-regulation of Cyclin A Expression

...type II receptor. The type I receptor also has a highly conserved GS motif (SGGSGLP) in the juxtamembrane region preceding the kinase domain, which is absent in the type II receptor (for review, see Ref. 26). The type II receptor can bind TGF-β by itself, but the type I receptors bind TGF-β only when co-expressed with the type II receptor, presumably as a result of a physical interaction of these receptors. To date, only one type II receptor for TGF-β has been identified (32), while several type I receptors, i.e. R4/ALK5 (34), Tsk7L (33), TSR1 (31), have been shown to bind TGF-β when co-expressed with the type II TGF-β receptor. Despite the structural similarity, certain functional differences among the different type I receptors have been found. For instance, only R4/ALK-5 transduces TGF-β signals in Mv1Lu epithelial cells (34, 39), while Tsk7L mediates TGF-β signaling in another epithelial cell line (40).

The type I receptors (41, 42), and presumably also the type II receptors, constitutively homodimerize independent of ligand binding. In addition, these two receptor types interact with each other (25, 43, 44) and form a heteromeric (most likely tetrameric) complex which is stabilized by ligand binding (45). The cytoplasmic domain of the type II receptor is constitutively phosphorylated, independent of ligand binding, due to both autophosphorylation and the activity of cytoplasmic kinases (46, 47). In the heteromeric complex, the type II receptor kinase phosphorylates the type I receptor (46, 47) and the two types of cytoplastic domains associate with each other (48), further stabilizing the heteromeric interaction. This heteromeric complex is likely to be the signaling receptor unit which mediates the biological responses of TGF-β.

Epithelial Mv1Lu cells lacking either type I or type II receptor are completely unresponsive to TGF-β to induce growth inhibition and gene expression (49, 50). But the TGF-β responsiveness can be restored to these mutant cell lines by transfection with the missing receptor type (25, 39). These findings support the notion that the two receptors are needed for both autophosphorylation and the activity of cytoplasmic kinases. In the heteromeric complex, the type II receptor kinase phosphatase signals the type I receptor, and the two types of cytoplastic domains associate with each other (48), further stabilizing the heteromeric interaction. This heteromeric complex is likely to be the signaling receptor unit which mediates the biological responses of TGF-β.

Epithelial Mv1Lu cells lacking either type I or type II receptor are completely unresponsive to TGF-β to induce growth inhibition and gene expression (49, 50). But the TGF-β responsiveness can be restored to these mutant cell lines by transfection with the missing receptor type (25, 39). These findings support the notion that the two receptors are needed for both autophosphorylation and the activity of cytoplasmic kinases. In the heteromeric complex, the type II receptor kinase phosphatase signals the type I receptor, and the two types of cytoplastic domains associate with each other (48), further stabilizing the heteromeric interaction. This heteromeric complex is likely to be the signaling receptor unit which mediates the biological responses of TGF-β.

Detailed description of how they were made is not provided in this report. However, further details of the cloning strategies can be obtained upon request. A total of four chimeric receptors between RI and RII domains were cloned between the HindIII site of plasmid pGL (Promega). To amplify the RI extracellular/transmembrane (ET) domain: R4E3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2E3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

Construction of Mutant and Chimeric TGF-β Receptors—The cDNAs encoding the type II receptor (RII) (55) and type I receptor Tsk7L (designated RI) (33) were of mouse origin, whereas the type I receptor R4 (designated R4/A) cDNA was a rat cDNA (35). Specific domains of RI (36, 37), R4 (38, 39), and RII were amplified using PCR methodology. In PCR primers listed below, restriction sites were incorporated to allow subcloning and fusion of different receptor domains. The underlined sequences indicate incorporation restriction sites. In bold text, these are incorporated restriction sites and those in bold type are restriction sites that are not incorporated.

To amplify the RIi extracellular/transmembrane (ET) domain: R2I3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2I3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

Construction of Mutant and Chimeric TGF-β Receptors—The cDNAs encoding the type II receptor (RII) (55) and type I receptor Tsk7L (designated RI) (33) were of mouse origin, whereas the type I receptor R4 (designated R4/A) cDNA was a rat cDNA (35). Specific domains of RI (36, 37), R4 (38, 39), and RII were amplified using PCR methodology. In PCR primers listed below, restriction sites were incorporated to allow subcloning and fusion of different receptor domains. The underlined sequences indicate incorporation restriction sites. In bold text, these are incorporated restriction sites and those in bold type are restriction sites that are not incorporated.

To amplify the RIi extracellular/transmembrane (ET) domain: R2I3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2I3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

The cyclin A reporter assay with the PAI-1 reporter assay with the cyclin A-Luciferase Reporter Plasmid—Construction of the Cyclin A-Luciferase Reporter Plasmid—To gain insight into the role of the type I and II receptors in the growth inhibitory response of TGF-β, we constructed plasmid pRK5M. These expression vectors are derived from pRK5 (56) but contain the sequences for a myc (57) or FLAG epitope tag (58) between the cyclin A promoter and the epitope tag. Considering the large number of plasmids, a detailed description of how they were made is not provided in this report. However, further details of the cloning strategies can be obtained upon request. A total of four chimeric receptors between RI and RII domains were cloned between the HindIII site of plasmid pGL (Promega). To amplify the RI extracellular/transmembrane (ET) domain: R4E3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2E3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

Construction of Mutant and Chimeric TGF-β Receptors—The cDNAs encoding the type II receptor (RII) (55) and type I receptor Tsk7L (designated RI) (33) were of mouse origin, whereas the type I receptor R4 (designated R4/A) cDNA was a rat cDNA (35). Specific domains of RI (36, 37), R4 (38, 39), and RII were amplified using PCR methodology. In PCR primers listed below, restriction sites were incorporated to allow subcloning and fusion of different receptor domains. The underlined sequences indicate incorporation restriction sites. In bold text, these are incorporated restriction sites and those in bold type are restriction sites that are not incorporated.

To amplify the RIi extracellular/transmembrane (ET) domain: R2I3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2I3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

Construction of Mutant and Chimeric TGF-β Receptors—The cDNAs encoding the type II receptor (RII) (55) and type I receptor Tsk7L (designated RI) (33) were of mouse origin, whereas the type I receptor R4 (designated R4/A) cDNA was a rat cDNA (35). Specific domains of RI (36, 37), R4 (38, 39), and RII were amplified using PCR methodology. In PCR primers listed below, restriction sites were incorporated to allow subcloning and fusion of different receptor domains. The underlined sequences indicate incorporation restriction sites. In bold text, these are incorporated restriction sites and those in bold type are restriction sites that are not incorporated.

To amplify the RIi extracellular/transmembrane (ET) domain: R2I3, CACGCTATCCGACGAGCACATGAAATGCTTTAC; R2I3, TCACTGCCATGACGAGCACGAGCTTCTAGGATA; in the control of the cytomegalovirus promoter and can be included in all transfections as an internal standard to monitor transfection efficiency.

Construction of Mutant and Chimeric TGF-β Receptors—The cDNAs encoding the type II receptor (RII) (55) and type I receptor Tsk7L (designated RI) (33) were of mouse origin, whereas the type I receptor R4 (designated R4/A) cDNA was a rat cDNA (35). Specific domains of RI (36, 37), R4 (38, 39), and RII were amplified using PCR methodology. In PCR primers listed below, restriction sites were incorporated to allow subcloning and fusion of different receptor domains. The underlined sequences indicate incorporation restriction sites. In bold text, these are incorporated restriction sites and those in bold type are restriction sites that are not incorporated.
anti-myc antibody 9E10 (gift of J. M. Bishop), or FLB lysis buffer (25 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100) for anti-FLAG antibody M2 (Kodak). Lysates were precleared in a mixture of rabbit anti-mouse IgG (Jackson Laboratories) and protein A-Sepharose CL4B (Pharmacia), and immunoprecipitated using monoclonal antibodies 9E10 (antimyc) or M2 (anti-FLAG). Immunoprecipitated proteins were subjected to electrophoresis on SDS-PAGE and visualized by autoradiography.

For in vitro kinase assays, immunoprecipitated receptors with specific deletions in the cytoplasmic domains of RI \(^{\alpha+\beta}\) and RII were split into two halves. While half was directly subjected to SDS-PAGE, the other half was used in a kinase autophosphorylation assay. The kinase assay was carried out at room temperature for 30 min in 1 x kinase buffer (10 mM HEPES-KOH, pH 7.5, 5 mM MgCl\(_2\), and 5 mM CaCl\(_2\)) containing 5 \(\mu\)Ci of \(\gamma\)-\[^{32}\]P\]ATP (5000 \(\mu\)Cl/mmol, Amersham) and then stopped by adding equal volume of 2 x SDS sample buffer (80 mM Tris, pH 6.8, 3.2% SDS, 16% glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue).

TGF-\(\beta\) Cross-linking of Receptor Proteins—

COS-1 cells were transfected, with expression plasmids containing cDNAs of a single receptor or a combination of two receptors, as described above for immunoprecipitation. Forty-eight h after transfection, COS-1 cells were cross-linked with \[^{125}\]I-labeled TGF-\(\beta\)-receptor or a combination of two receptors, as described above for single receptor expression plasmid was transfected, 5 \(\mu\)g of each plasmid was used. After electroporation, cells were recovered for 4 hr in MEM Eagle’s with Earle’s balance salt solution supplemented with non-essential amino acids and 10% FBS. The cells were then treated with or without 10 ng/ml TGF-\(\beta\)-1 in the same medium but containing 0.2% FBS. After 24 hr (for PAI-1 assay) or 48 hr (for cyclin A assay), the cells were harvested and lysed in reporter lysis buffer (Promega), and cell lysates were assayed for luciferase and \(\beta\)-galactosidase activities. The luciferase assay was carried out using Analytic Luminescence Laboratory’s assay reagents and luminometer monolight 2010. \(\beta\)-Galactosidase was assayed in an assay buffer (Promega), and the activity was measured at 420 nm in a spectrophotometer. The luciferase activity which reflects the promoter activity of cyclin A or PAI-1 was normalized to \(\beta\)-galactosidase to account for transfection efficiency.

R1B-L7 cells, a highly transflectable line of R1B cells were provided by Dr. D. Loskutoff. Transfection of L7 cells was carried out using the DEAE-dextran method as described (46) at 40% confluence in 6-well plates. For each transfection, 1 \(\mu\)g of receptor expression plasmid or vector control and 3 \(\mu\)g of reporter constructs (1.5 \(\mu\)g of pRK\(\beta\)Gal + 1.5 \(\mu\)g of luciferase expression plasmid) were used.

RESULTS

Down-regulation of Cyclin A Expression by TGF-\(\beta\)-1 in Mv1Lu Cells—

Transcription of the PAI-1 gene has frequently been used as a marker for TGF-\(\beta\) responsiveness. A reporter system in which the TGF-\(\beta\)-inducible PAI-1 promoter drives luciferase expression has allowed the rapid evaluation of TGF-\(\beta\) receptor function in transient transfection assays (25). However, TGF-\(\beta\) is often primarily studied for its growth inhibitory effect, and considerable evidence suggests that the effects on growth inhibition and gene expression result from different signaling cascades. Unfortunately, no reporter assay has been available to monitor the antiproliferative effect of TGF-\(\beta\). Since TGF-\(\beta\) arrests epithelial cell proliferation in late G1 phase, thereby preventing the induction of cyclin A gene expression (17), we developed a cyclin A promoter-based reporter system to monitor the TGF-\(\beta\)-induced growth inhibition response in transient transfection assays.

As a first step in developing this assay, we identified the 5’ promoter fragment of the cyclin A gene that mediates down-regulation of cyclin A gene expression in response to TGF-\(\beta\). The upstream region of the human cyclin A gene corresponding to nucleotides \(-516\) to \(+245\) contains many potential regulatory elements (54) and was isolated by PCR-based amplification from human genomic DNA. We linked this promoter region to a luciferase reporter plasmid pCAL2 and pCD1, together with a \(\beta\)-galactosidase expression plasmid pRK\(\beta\)Gal, were transfected into Mv1Lu cells. Four h after transfection, TGF-\(\beta\) was added at 400 pM to the media for the defined times. Luciferase and \(\beta\)-galactosidase activity were determined as described under "Materials and Methods." Data are from two experiments with each point determined in triplicates. The vertical bars show the standard deviations. pCAL2 and pCD1 contain the human cyclin A promoter sequence from nucleotide \(-516\) to \(+245\) and from \(-7300\) to \(+245\), respectively. B, regulation of cyclin A-luciferase activity by TGF-\(\beta\)-1 in wild-type and mutant Mv1Lu cells. Untransfected cells or DR26 cells transfected with RII or R1B cells transfected with RII \(^{A+\beta}\) were treated (+) or untreated (−) with TGF-\(\beta\). Transfections, TGF-\(\beta\)-1 treatment (48 h), and reporter assays were carried out as in panel A. RII, type II TGF-\(\beta\)-receptor; R1B, type I TGF-\(\beta\)-receptor; Mv1Lu, wild-type Mv1Lu cells; DR26, RII-deficient cells; R1B, RII\(^{A+\beta}\)-deficient cells.

![Fig. 1. A, cyclin A-luciferase reporter assay.](image-url)
To evaluate the TGF-β responsiveness of the cyclin A promoter, both luciferase plasmids were transfected into Mv1Lu cells, and the normalized luciferase activity was calculated at defined time points after initiation of TGF-β treatment. In Mv1Lu cells transfected with pCAL2, the luciferase activity was down-regulated by TGF-β (Fig. 1A) in a manner similar to that in pCD1-transfected cells. This decreased expression from the cyclin A promoter parallels the inhibition of cyclin A expression by TGF-β in Mv1Lu (15-17) and other epithelial cell lines (13). The TGF-β-induced inhibition of luciferase expression was most evident when TGF-β treatment was initiated within 6 h after release from contact inhibition (data not shown), and the cyclin A promoter response was maximal when the cells were exposed to TGF-β for 48 h (Fig. 1A). These results indicate that the 760-bp cyclin A promoter fragment in pCAL2 contains the TGF-β-responsive element(s) and that this cyclin A-luciferase assay can be used as a transcriptional reporter system to measure the antiproliferative effect of TGF-β in transient transfection assays.

Down-regulation of Cyclin A Expression by TGF-β Requires Both Type I and Type II TGF-β Receptors—As shown in Fig. 1A, cyclin A transcriptional down-regulation by TGF-β was apparent in wild-type Mv1Lu cells, which possess functional type I (RI) and type II (RII) receptors. To further characterize the requirement of receptors in TGF-β-dependent cyclin A expression, we investigated whether the luciferase activity driven from the cyclin A promoter in pCAL2 is down-regulated by TGF-β treatment in Mv1Lu mutant cell lines lacking functional receptors (49, 50). In both the RII-deficient DR26 cells and RI(R4), RII(R4), and RII(R4)-deficient R1B cells, the cyclin A promoter-driven luciferase expression was unchanged upon TGF-β treatment (Fig. 1B). In contrast, reintroduction of RI in DR26 cells or RI(R4), but not RI(R4), in R1B cells restored the response to TGF-β in the cyclin A-luciferase assay (Fig. 1B). These results suggest that the TGF-β-induced growth inhibition requires both RI(R4) and RII receptors.

The Cytoplasmic Domains of Both Receptors Are Required for TGF-β Responsiveness in the Cyclin A-Luciferase Assay—To further examine the role of specific domains of TGF-β receptors in TGF-β-dependent down-regulation of cyclin A expression, we generated chimeras between the extracellular/transmembrane (ET) and cytoplasmic (C) domains of RI and RII(R4) or RII(R7) as well as mutant receptors with cytoplasmic truncations of RI(R4), RI(R7), and RII, as shown in Fig. 2. These mutant receptors were engineered to incorporate a C-terminal myc- or FLAG-epitope tag which allows immunoprecipitation with tag-specific antisera. We first examined whether these chimeric and truncated receptors were efficiently expressed in COS-1 or 293 cells. Following metabolic 35S-labeling of the cells, the receptors were immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography. All truncated and chimeric receptors were expressed at significant levels and had the expected molecular weights, although degradation products were often apparent (Fig. 3A). According to the mobility on SDS-PAGE gels, RII had the expected size of ~80 kDa, and the R4 and Tsk7L type I receptors, expressed by plasmids RI(R4) and RI(R7), respectively, had the expected size of ~70 kDa. Transfection of the plasmids for the truncated receptors lacking the C domain generated a polypeptide of ~35 kDa for RII(ET) and ~30 kDa for both RI(R4)(ET) and RI(R7)(ET). The hybrids between RI (either RI(R4) or RI(R7)) and RII conigrated with a size of ~80 kDa for the RII(ET)-RI(R7)(C) chimeras and ~75 kDa for RI(ET)-RII(C) chimeras. The ability of the mutant receptors to bind TGF-β at the cell surface was determined by binding and cross-linking of 125I-TGF-β. As shown in Fig. 3B, both RII and RII(ET)-RII(C) chimeras with their type II extracellular domain bound 125I-TGF-β.
The receptors were immunoprecipitated from 125I-labeled cell lysates using antibodies specific for the FLAG (F) or myc (M) epitope as indicated. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. The positions of the receptors and molecular weight markers are shown on the side. Lane 1, pRK5; lane 2, RI1; lane 3, RI4; lane 4, RI2; lane 5, RI1(ET)-RI4(C); lane 6, RI1(ET)-RI2(C); lane 7, RI4(ET)-RI2(C); lane 8, RI2(ET)-RI4(C); lane 9, RI1(ET); lane 10, RI4(ET); lane 11, RI2(ET). B, ligand binding of mutant receptors expressed in COS-1 cells. Binding of 125I-TGF-β to COS-1 cells transfected with plasmids expressing wild-type or mutant receptors was examined. Cross-linked complexes were subjected to SDS-PAGE and autoradiography. Each lane represents an equal amount of protein lysate as determined by BCA assay (Bio-Rad). Migration of the receptors are indicated using the same abbreviations for receptors as in Fig. 2A. Lane 1, pRK5; lane 2, RI1; lane 3, RI4; lane 4, RI2 and RI1; lane 5, RI2; lane 6, RI4 and RI1; lane 7, RI1(ET)-RI4(C); lane 8, RI2(ET)-RI4(C); lane 9, RI1(ET)-RI2(C); lane 10, RI4(ET)-RI2(C); lane 11, RI2(ET)-RI4(C); lane 12, RI4(ET)-RI2(C) and RI1; lane 13, RI1(ET); lane 14, RI4(ET); lane 15, RI2(ET) and RI1; lane 16, RI4(ET); lane 17, RI2(ET) and RI1.

Signaling activity of chimeric receptors in mutant Mv1Lu cells using TGF-β-dependent cyclin A-luciferase reporter assay. Receptor expression plasmids were transfected in DR26 or R1B cells together with the cyclin A-luciferase plasmid pCAL2. Cyclin A-luciferase data are shown as the percentage decrease in luciferase activity relative to cells that did not receive TGF-β. In the cases when the cyclin A activity was higher after TGF-β treatment, a negative value for the percentage inhibition was observed. The vertical bars show the standard deviations. Receptors are abbreviated as in Fig. 2 except that hR111 represents wild-type human R1I.

Table 1: Signaling activity of wild-type and mutant receptors in Mv1Lu cells in response to TGF-β.

| Receptor          | Inhibition of cyclin A transcription | Induction of PAI-1 transcription |
|-------------------|-------------------------------------|---------------------------------|
| Wild-type Mv1Lu   | +++                                 | +++                             |
| RI4(ET)           | ++                                   | ++                              |
| RI4(ET)-RI1(C)    | ++                                   | ++                              |
| RI4(KR)           | +/−                                  | +/−                             |
| RI7(ET)           | +++                                 | +++                             |
| RI7(ET)-RI1(C)    | +++                                 | +++                             |
| RI1(ET)           | +/−                                  | +/−                             |
| RI1(ET)-RI4(C)    | +/−                                  | +/−                             |
| RI1(ET)-RI7(C)    | +/−                                  | +/−                             |
| RI1KR             | +/−                                  | +/−                             |
| R1B cells         |                                     |                                 |
| RI4               | +++                                 | +++                             |
| RI4(KR)           | +/−                                  | +/−                             |
| RI4(ET)-RI1(C)    | +/−                                  | +/−                             |
| RI1(ET)           | +/−                                  | +/−                             |
| RI1(ET)-RI4(C)    | +/−                                  | +/−                             |
| RI1(ET)-RI7(C)    | +/−                                  | +/−                             |
| RI1(ET)-RI7(C)    | +/−                                  | +/−                             |
| R111              |                                     |                                 |
| RI1(ET)-RI4(C)    | +/−                                  | +/−                             |
| RI1(ET)-RI7(C)    | +/−                                  | +/−                             |
| R1(ET)            | +/−                                  | +/−                             |
| R1(ET)-RI4(C)     | +/−                                  | +/−                             |
| R1(ET)-RI7(C)     | +/−                                  | +/−                             |
| R111              | +/−                                  | +/−                             |
| R111              | +/−                                  | +/−                             |
| R111              | +/−                                  | +/−                             |
| R111              | +/−                                  | +/−                             |

*+/−, diminished response.

Figure 4: Signaling activity of chimeric receptors in mutant Mv1Lu cells using TGF-β-dependent cyclin A-luciferase reporter assay. Receptor expression plasmids were transfected in DR26 or R1B cells together with the cyclin A-luciferase plasmid pCAL2. Cyclin A-luciferase data are shown as the percentage decrease in luciferase activity relative to cells that did not receive TGF-β. In the cases when the cyclin A activity was higher after TGF-β treatment, a negative value for the percentage inhibition was observed. The vertical bars show the standard deviations. Receptors are abbreviated as in Fig. 2 except that hR111 represents wild-type human R1I.

Because for ligand-independent signaling and that the two receptor types have an intrinsic heterodimeric affinity for each other which is consistent with previous findings (48). In contrast to the chimeric receptors derived from RI4, cotransfection of RI1(ET)-RI4(C) with RI7(ET)-RI1(C) did not lead to TGF-β responsiveness in the cyclin A-luciferase assay, consistent with the inactivity of the Tsk7L type I receptor in Mv1Lu cells (31, 39).

Dominant Negative Effect of Chimeric and Truncated Receptors on TGF-β-dependent Down-regulation of Cyclin A Expression—We further characterized the role of RI and R1I in TGF-β-induced down-regulation of cyclin A expression by examining the function of the chimeras in wild-type Mv1Lu cells which contain endogenous RI and R1I. Because of the TGF-β responsiveness associated with endogenous receptors, overexpression of wild-type RI or R1I did not alter cyclin A transcription. In contrast, expression of chimeric receptors RI4(ET)-RI1(C), RI1(ET)-RI4(C) or RI1(ET)-RI7(C), but not RI7(ET)-RI1(C), abolished TGF-β-dependent inhibition of cyclin A transcription (Fig. 5, left panel), indicating that chimeras with either RI4, or R1I-derived ET domains inhibited the activities of the endogenous receptors in a dominant negative fashion. Furthermore, in transfusions with the RI1(ET)-RI4(C) or RI1(ET)-RI7(C) chimeric receptors, the cyclin A activity was even higher after TGF-β treatment (resulting in the negative value for the percentage inhibition, Fig. 5). Dominant negative inhibition was also observed with the kinase-deficient point mutants R1I(KR) and R1I4(KR), in which the Lys in the ATP-binding sites of RI and RI4 was replaced by Arg (Fig. 5, middle panel). The
dominant negative effect of these mutant receptors in wild type Mv1Lu cells was also apparent in the PAI-1-luciferase assay (data not shown; Table I).

Using the TGF-β-responsive wild-type Mv1Lu cells and the cyclin A-luciferase assay, we also evaluated the effect of expression of truncated receptors lacking the cytoplasmic domain (Fig. 5, right panel). Consistent with the results using a truncated human type II receptor (51, 60), expression of the mouse RII lacking its cytoplasmic domain, RII(ET), also blocked TGF-β-dependent inhibition of cyclin A transcription in a dominant negative manner. Overexpression of the truncated RIIKR (ET) type I receptor lacking its cytoplasmic domain, but not RIKR(ET), in Mv1Lu cells also abolished TGF-β responsiveness (Fig. 5, right panel). This effect was also seen in the PAI-1 assay (Table I). However, the inhibitory effect of RIKR(ET) was less effective than RII(ET) in the PAI-1 assay (Table I, data not shown).

Juxtamembrane Domains of Type I and Type II TGF-β Receptors Are Required for TGF-β Responsiveness—Finally, we evaluated the signaling ability of RII and RIKR, receptors in which the juxtamembrane segment (between the transmembrane and the kinase domain) or the C-terminal tail following the kinase domain, or both, were deleted. We also included kinase-deficient point mutants of RII and RIKR, i.e. RIIKR and RIKR, for comparison. Expression plasmids for the RII mutants were transfected into DR26 cells and plasmids for the RIKR mutants into R1B cells. These receptors were then tested for their ability to restore TGF-β responsiveness using both cyclin A- and PAI-1-luciferase assays (Fig. 6; Table I). Both RIKR and RII with the deletion of the cytoplasmic tail (amino acids 491–501 in RI and 546–567 in RII) conferred TGF-β responsiveness to the corresponding mutant cell lines. In contrast, deletion of the juxtamembrane domains inactivated the signaling activity of both receptor types in the two assays, as shown by transfecting the mutant receptors RIKR and RII in R1B cells or RI and RII in DR26 cells. Accordingly, deletion of both the juxtamembrane and the cytoplasmic tail in both receptors also inactivated both receptor types. As expected, RIKR and RIIKR were also biologically inactive (Fig. 6, Table I).

When expression plasmids for these mutant receptors were transfected into COS-1 cells and the kinase activity of the immunoprecipitated receptors was tested in vitro, we observed a correlation between the receptor kinase activity and their biological functions (Fig. 7). While RIKR and RIIKR maintained their kinase and signaling activities similarly to wild-type receptors, RIKR and RIIKR, like the kinase-negative mutants RIKR and RIIKR, were also kinase inactive and unable to autophosphorylate. Thus, mutant receptors which were inactive in the biological assays were also inactive in the kinase assay.

DISCUSSION

Roles of TGF-β Receptors in TGF-β-dependent Down-regulation of Cyclin A Transcription—During the cell cycle, cyclin A transcription is suppressed in late G1 phase and activated during S phase (16, 17). Thus, cyclin A expression is considered to be an indicator of the cell growth status. Inhibition of cyclin A expression is sufficient to cause cell cycle arrest before G1/S transition (61, 62), and deregulated cyclin A expression is ap-
Down-regulation of Cyclin A Expression

Cyclin A expression is down-regulated by many factors, including TGF-β (13-17) and contact inhibition (64), which both arrest cell growth in late G1. The TGF-β-dependent down-regulation may play a critical role in TGF-β-induced growth arrest (17). To generate a convenient and rapid reporter system that allows a measurement of growth inhibition by TGF-β and other factors, we created a plasmid in which luciferase expression is driven by a 760-bp segment of the cyclin A promoter. Treatment of Mv1Lu cells with TGF-β resulted in a rapid inhibition of luciferase expression, indicating that this short sequence confers TGF-β responsiveness. Several potential regulatory sequence elements, including four Sp1 sites, one CAAT-responsive element (CRE), two Y1 sites, two E2F-binding sites, one AP1 site, and one p53 binding site, are contained within this short promoter sequence (54). Although the CRE sequence (also called activating transcription factor site, or ATF site) is critical in the down-regulation of cyclin A expression during contact inhibition (64), the promoter elements which mediate TGF-β-induced inhibition of cyclin A expression in cultured cells are not known. However, contact inhibition and TGF-β-mediated growth arrest have several features in common, e.g., p27, an inhibitor of cdk2 and cdk4, is activated in both contact-inhibited and TGF-β-inhibited cells (9), implicating a similar mechanistic basis for down-regulation of cyclin A expression under both conditions.

Based on the TGF-β responsiveness of the 760-bp cyclin A promoter element, we have developed a functional assay to monitor cyclin A regulation in response to TGF-β. This assay can be used in transient transfection assays to allow a functional evaluation of TGF-β receptors and other signaling proteins involved in the antiproliferative effect of TGF-β. In spite of the many TGF-β-induced responses, transcriptional activation of the PAI-1 gene is frequently used as the only indicator of TGF-β signaling and receptor function, primarily because of the availability of a highly sensitive and convenient reporter assay that measures luciferase expression from a TGF-β-responsive promoter element (25). Before this study, no facile reporter assay has been available to measure the antiproliferative effect of TGF-β, even though considerable evidence suggests that the growth inhibition and the induction of gene expression by TGF-β result from divergent signaling cascades. The availability of a cyclin A luciferase reporter system allowed us to characterize the role of specific domains of TGF-β receptors during TGF-β-mediated growth inhibition using transient transfection assays.

Our results demonstrate that inhibition of cyclin A transcript by TGF-β requires both RII and R4 receptors. The cyclin A transcription is inhibited by TGF-β in Mv1Lu cells which contain endogenous R4 and R4 receptors but not in the two mutant cell lines lacking either R4 or RII. Introduction of wild-type R4 into RI-deficient cells or RII into RI-deficient cells restores TGF-β-dependent cyclin A transcription. In contrast, various mutant receptors including the chimeric receptors RII(ET)-R4(C), R4(ET)-RII(C), R4(ET)-R4(ET)(L7), and R4(ET)-RII(C) (as well as the kinase-negative point mutants R4(ET)-R4, R4(ET)-RII, and R4(ET)-R4(ET)-RII(C) with the kinase-negative point mutants R4(ET)KR, and RIIKR, and receptors lacking the juxtamembrane domain R4(ET)-M and RII-M, are unable to rescue the ability of mutant cells to respond to TGF-β. Therefore, coexpression of RII(ET)-R4(C) and R4(ET)-RII(C), which allows a heteromeric interaction of both extracellular and intracellular domains (48), restores TGF-β-dependent down-regulation of cyclin A expression in either mutant cell line. In wild-type Mv1Lu cells, individual expression of these mutant receptors inhibits TGF-β-induced cyclin A down-regulation in a dominant negative fashion. These results suggest that cyclin A expression and, thus, the growth inhibitory effect of TGF-β need the cooperative interaction between type I and type II TGF-β receptors.

Functional Analysis of TGF-β Receptor Domains Using Both Cyclin A and PAI-1 Assays—Several recent studies have addressed the functions of the two receptor types (25, 34, 39) and their cytoplasmic domains in TGF-β signaling (46, 66). Transfection of RII receptor in DR26 cells or R4ALK-5 type I receptor (termed R4 in this study) in R1B cells restores TGF-β responsiveness, suggesting that both receptor types are required for TGF-β responsiveness (25, 34, 39). The ability of the two receptor types to undergo heteromeric interactions and the phosphorylation of the type I receptor cytoplasmic domain by the type II receptor kinase further reinforces the assumption that both cytoplasmic domains are required for TGF-β responsiveness (46, 47). Thus a heteromeric complex is thought to be the signaling unit for the multiple responses to TGF-β (25, 44).

In our study, we tested in parallel the TGF-β-dependent cyclin A response and PAI-1 transcriptional response and did not observe an uncoupling of these responses (see Table I), which is consistent with the proposed requirement of both cytoplasmic domains for full TGF-β responsiveness. More specifically, homomerization of cytoplasmic domains, resulting from expression of the RII(ET)-R4(C) chimera in DR26 cells or the R4(ET)-RII(C) chimera in R1B cells, is not sufficient for TGF-β-induced regulation of cyclin A and PAI-1 expression, suggesting that a functional interaction of the two types of cytoplasmic domains is a prerequisite for TGF-β responsiveness. In addition, despite the heteromeric combination of both cytoplasmic domains, homomeric combination of the extracellular/transmembrane domains of type I or type II receptors also fails to restore TGF-β responsiveness. In the case of the R4(ET)-RII(C) chimera in DR26 cells which express endogenous R4, this lack of signaling might be due to the inability of the R4 extracellular domains to bind ligand in the absence of RII. However, expression of the RII(ET)-R4(C) chimera in R1B cells which have endogenous RII yet lack functional R4 also does not activate TGF-β responsiveness even though these receptors bind TGF-β efficiently. This suggests an active function of the R4 extracellular domain in TGF-β signaling.

Expression of the RII(ET)-R4(C) and R4(ET)-RII(C) chimeras in DR26 or R1B cells results in TGF-β responsiveness in both reporter assays, suggesting that the coexistence of both extracellular and cytoplasmic domains in trans provides a functional TGF-β receptor unit. TGF-β is likely to induce a conformational change in the heteromeric complex of these chimeras in a manner similar to the complex of wild-type R4 and RII receptors. The resulting activation promotes the interaction of these receptors with downstream effectors. Our data are in agreement with the recent findings of Okada et al. (66), who showed the requirement of both cytoplasmic domains for TGF-β-induced PAI-1 transcription. We extended their findings by using the cyclin A reporter assay and by examining the effects of chimeric receptors in wild-type Mv1Lu cells.

In wild-type Mv1Lu cells, expression of the R4(ET)-RII(C) or RII(ET)-R4(C) chimera inhibits TGF-β-induced regulation of cyclin A and PAI-1 expression in a dominant negative manner. This inhibition is likely due to the fact that overexpression of the R4(ET)-RII(C) or RII(ET)-R4(C) chimera sequesters endogenous RII and/or R4 away from functional R4-RII receptor complexes. The resulting homomeric complexes of chimeric receptors and the heteromeric complexes between endogenous receptors and the chimeras are then unable to transduce the TGF-β signals. This dominant negative interference resembles the inhibitory effect of truncated receptors lacking their cytoplasmic domain and the kinase-negative mutant receptors. The inhibition of TGF-β responsiveness by RII(ET) is in accord-
ance with previous results (51, 60, 67). A dominant negative inhibition, albeit to a lesser extent, was also apparent with overexpression of RIa4 (ET) in Mv1Lu cells, but not with RI2*ET even though the truncated form of Tsk71 inhibited TGF-β-induced mesenchymal transdifferentiation in NMuMG cells (40). Thus, overexpression of chimeric, truncated, or kinase-defective receptors derived from RII or RIa4 results in dominant negative interference with TGF-β receptor signaling, most likely by interfering with the assembly of functional homo- and heteromeric complexes. After the submission of this manuscript, Vivien et al. (68) and Brand and Schneider (69) reported dominant negative inhibition of TGF-β signaling by chimeric and kinase-defective receptors, using assays for TGF-β-induced gene expression. Their conclusions and our findings based on cyclin A and PAI-1 assays are in agreement; moreover, our cyclin A assay results further extend their observations to TGF-β-induced growth inhibition.

Finally, we characterized the function of the RIa4 and RII receptor cytoplasmic domains containing defined deletions. Deletion of the C-terminal tail of either receptor did not affect the kinase and signaling activity of RIa4 and RII in both cyclin A and PAI-1 reporter assays. A similar result has been observed in the case of RII using the PAI-1 reporter assay (60). We also demonstrated that deletion of the juxtamembrane domain inactivates the signaling function of both receptor types. This correlates with a loss of kinase activity as measured by autophosphorylation. In the case of the type I receptor, the juxtamembrane domain contains a conserved SSQSSGLP sequence. This sequence is a major site of phosphorylation by the type II receptor, and its deletion has been shown to inactivate TGF-β signaling (46). Thus, our results indicate the requirement of the juxtamembrane domains for the function and kinase activity of both receptors. These domains may also represent binding sites for cytoplasmic effector proteins.

Coincident Responsiveness in the Cyclin A and PAI-1 Reporter Assays—In this study we did not observe a divergence of the pathways leading to growth inhibition and gene expression using the cyclin A and PAI-1 assays. This supports the conclusions of Wrana et al. (25) and Bassing et al. (44) who showed that the activities of both RIa4 and RII are required for TGF-β-induced gene expression and growth inhibition. Solely based on these studies, we would conclude that cytoplasmic domains of both RIa4 and RII are required for both types of TGF-β responses. However, uncoupling of the two types of responses has been observed in several systems. We have previously shown that stable expression of a truncated RII in Mv1Lu cells abolished the antiproliferative effect of TGF-β but not its induction of gene expression (51). Also, a Phe to Ala mutation at position 498 in RII does not affect the induction of transcription by TGF-β, yet reduces significantly its antiproliferative effect (60). In addition, several cell lines lacking detectable levels of RII expression or with a defective RII display TGF-β responsiveness in gene expression assays but are not growth inhibited by TGF-β (53, 70). Furthermore, various tumor cell lines also lack a growth inhibitory response to TGF-β yet respond to TGF-β by the induction of gene expression (52). Finally, differentiating osteoblasts undergo alterations in RI and RII expression and ligand binding and concomitantly show phenotypic changes in their responsiveness to TGF-β (71). Thus, these studies suggest that both signaling pathways should be considered as distinct and that in some cases, but not all, uncoupling can occur at the receptor level and that specific changes in functionality of TGF-β receptors correlate with induction of specific downstream signaling pathways. How all these observations can be reconciled in a simple model is unclear. However, the apparent discrepancy between both sets of observations might be explained by a threshold model. In such a model, both receptor types are required but the TGF-β-induced growth arrest requires full activity of endogenous RI (and maybe RII receptor), whereas changes in TGF-β-induced gene expression can be mediated at a much reduced level of receptor activity, i.e., at a low threshold level. For example, overexpression of a truncated RII in Mv1Lu cells (51) strongly interferes with endogenous RII, but the remaining activity might still suffice to confer the transcriptional response to TGF-β but not growth inhibition. This threshold model could also explain the diminished activity of RII defective in N-glycosylation (53) and RII mutated at position 498 (60) in the antiproliferative response but not in TGF-β-induced gene expression. Accordingly, we have observed that some chimeric and kinase-negative mutants derived from RIa4 and RII completely abolished TGF-β-dependent cyclin A regulation but only reduced TGF-β-induced PAI-1 transcription (data not shown). Thus, changes in expression or activity of receptors could possibly determine which downstream pathways are activated. Recently, a threshold model has also been proposed for the response of PC12 cells to receptor tyrosine kinase activation (for review see Ref. 72). In PC12 cells, transient versus sustained ERK activation by growth factors has very different consequences for nuclear translocation of ERK, gene expression, and subsequent proliferation or differentiation (72). An additional complexity in the current models for TGF-β receptor signaling is imposed by evidence that ligand binding to RI and RII is differentially regulated. This is apparent from the altered receptor binding in differentiating osteoblasts (71) as well as the observation that a mutated, monomeric TGF-β binds much less efficiently to type II than type I receptor. This mutated TGF-β is still 20% as active as native TGF-β1 in PA1-1 induction, but it is less than 1% potent as native TGF-β1 in growth inhibition in Mv1Lu cells (73), which may be consistent with the threshold model. Further assessment of receptor expression patterns, characterization of the cytoplasmic domains of the TGF-β receptors, and their interactions as well as the identification of interacting cytoplasmic proteins should bring insight into the mechanism of TGF-β receptor signaling.

Acknowledgments—We thank Drs. P. Donahoe for the rat R4 DNA and X.-F. Wang for the CDNAs encoding kinase-negative mutants of R4 and human type II receptor, Drs. J. Massague and H. Lodish for mutant Mv1Lu cells, Dr. D. J. Laskutoff for the PAI-1-luciferase reporter plasmid and Dr. C. Bréchot for plasmid pcD1. We also thank Dr. J. M. Bishop for providing the ant-myc antibody 9E10 and Dr. D. Littman for the use of his luminescence reader for luciferase assays. We are grateful to Drs. Ruey-Hwa Chen, Irene Griswiß-Prenner, Lisa Choy, and other members of the laboratory for helpful discussion and critical review of the manuscript.

REFERENCES

1. Derynck, R. (1994) In The Cytokine Handbook (Thompson, A. W., ed) pp. 319–342, Academic Press, San Diego.
2. Kingsley, D. M. (1994) Genes & Dev. 8, 133–146.
3. Egan, S. E., and Weinberg, R. A. (1993) Cell 75, 781–783.
4. Ewen, M. E., Sluss, H. K., Whitehouse, L. L., and Livingston, D. M. (1993) Cell 74, 1099–1102.
5. Geacintov, N., and Weinberg, R. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10315–10319.
6. Kdrt, A., Ohtuki, M., Pidjak, K., Roberts, J. M., and Massagué, J. (1993) Science 260, 536–539.
7. Silinger, J., Hengst, L., Pan, C.-H., Alexander, D., Stampfer, M., and Reed, S. (1994) Mol. Cell. Biol. 14, 3683–3694.
8. Pidjak, K., Lee, M.-H., Erdjument-Bromage, H., Kdrt, A., Roberts, J. M., Tempst, P., and Massagué, J. (1994) Cell 78, 59–66.
9. Pidjak, K., Kato, J., Solomon, M. J., Sherr, C. J., Massagué, J., Roberts, J. M., and Kdrt, A. (1994) Genes & Dev. 8, 9–22.
10. Toyoshima, H., and Hunter, T. (1990) Cell 78, 67–74.
11. Hannon, G. J., and Beach, D. (1994) Nature 371, 257–260.
12. Ebens, S. T., Fatsch, M. P., Burnette, R. J., Joshi, P., and Leof, E. B. (1994) Cell Growth Differ. 5, 109–116.
13. Landesman, Y., Pagano, M., Draetta, G., Rotter, V., Fusenig, N. E., and Kimchi, A. (1992) Oncogene 7, 1661–1665.
14. Barfart, I., Fequet, D., Bréchot, C., Henglein, B., Dupuy, d. A., Vie, A., and Blanchard, J. M. (1993) Cell Growth Differ. 4, 101–113.
15. Ralph, D., McEiIland, M., and Welch, J. (1993) Proc. Natl. Acad. Sci. U. S. A.
17. Satterwhite, D. J., Aakre, M. E., Gorska, A. E., and Moses, H. L. (1994) Cell Growth Diff. 5, 789–799
18. Mudryj, M., Devoto, S. H., Hibbert, S. W., Hunter, T., Pines, J., and Nevins, J. R. (1991) Cell 65, 1243–1253
19. Devoto, S. H., Mudryj, M., Pines, J., Hunter, T., and Nevins, J. R. (1992) Cell 68, 167–176
20. Shridar, E., Emun, M., DeCaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. (1992) Cell 68, 157–166
21. Schwarz, J. K., Devoto, S. H., Smith, E. J., Chellappan, S. P., jakol, L., and Nevins, J. R. (1993) EMBO J. 12, 1013–1020
22. Zhu, L., Enders, G., Lees, J. A., Beijersbergen, R. L., Bernard, R., and Harlow, E. (1995) EMBO J. 14, 1904–1913
23. Laiho, M., DeCaprio, J. A., Ludlow, J. W., and Livingston, D. M. (1990) Cell 62, 175–185
24. Keeton, M. R., Curriden, S. A., van, Z. A., and Laskutof, D. J. (1991) J. Biol. Chem. 266, 23048–23052
25. Wrana, J. L., Attisano, L., Caùcino, A., Zentella, A., Dody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) Cell 71, 1003–1014
26. Derynck, R. (1994) Trends Biochem. Sci. 19, 548–553
27. Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
28. Mathews, L. S., and Vale, W. W. (1991) Cell 65, 973–982
29. Attisano, L., Wrana, J. L., Cheifetz, S., and Massague, J. (1992) Cell 68, 97–108
30. Attisano, L., Caùcino, J., Ventura, F., Weis, F. M., Massague, J., and Wrana, J. L. (1993) Cell 75, 671–680
31. Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992) Cell 76, 753–765
32. Ebner, R., Chen, R.-H., Shum, L., Lawler, S., Zoncheck, T. F., Lee, A., Lopez, A. R., and Derynck, R. (1993) Science 260, 1344–1348
33. Franzeùn, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.-H., and Miyazono, K. (1993) Cell 75, 681–692
34. He, W., Gustafson, M., Hirobe, S., and Donahoe, P. (1993) Dev. Dynam. 196, 113–132
35. ten Dijke, P., Ichijo, H., Françzé, P., Schulz, P., Saraz, J., Yoshimura, H., Heldin, C.-H., and Miyazono, K. (1993) Oncogene 8, 2879–2887
36. ten Dijke, P., Yamashita, H., Ichijo, H., Franzeùn, P., Laiho, M., Miyazono, K., and Heldin, C.-H. (1994) Science 264, 101–104
37. ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C.-H., and Miyazono, K. (1994) J. Biol. Chem. 269, 16999–16988
38. Bassing, C. H., Yingling, J. M., Howe, D. J., Wang, T., He, W. W., Gustafson, M. L., Shah, P., Donahoe, P. K., and Wang, X.-F. (1994) Science 263, 87–89
39. Miettinen, P. J., Ebner, R., Lopez, A. R., and Derynck, R. (1994) J. Biol. Chem. 269, 2021–2036
40. Chen, R.-H., and Derynck, R. (1994) J. Biol. Chem. 269, 22868–22874
41. Henis, Y. I., Moustaques, A., Lin, H. Y., and Lodish, H. F. (1994) J. Biol. Chem. 269, 139–154
42. Ebner, R., Chen, R.-H., Lawler, S., Zoncheck, T., and Derynck, R. (1993) Science 262, 900–902
43. Brand, T., MacLellan, W. R., and Schneider, M. D. (1993) J. Biol. Chem. 268, 11500–11503
44. Vijayan, M., Pepperkok, R., Deve, F., Anserige, W., and Draetta, G. (1992) EMBO J. 11, 961–971
45. Fahe, B., Ewen, M., Tsai, L., Livingston, D., and Harlow, E. (1992) Science 255, 87–90
46. Yoshizumi, M., Hisie, C.-M., Zhou, F., Tsai, J.-C., Patterson, C., Perrél, M., and Lee, M.-E. (1995) Cell 15, 3266–3272
47. Wrana, J. L., Carùcino, J., Attisano, L., Cheifetz, S., Zentella, A., Lopez, C. F., and Massague, J. (1992) Cold Spring Harb. Symp. Quant. Biol. 57, 81–86
48. Okadame, T., Yamashita, H., Françzé, P., Morén, A., Heldin, C.-H., and Miyazono, K. (1994) J. Biol. Chem. 269, 30753–30756
49. Brand, T., MacLellan, W. R., and Schneider, M. D. (1993) J. Biol. Chem. 268, 11500–11503
50. Vivian, D., Attisano, L., Wrana, J. L., and Massague, J. (1995) J. Biol. Chem. 270, 7134–7141
51. Brand, T., and Schneider, M. (1995) J. Biol. Chem. 270, 8274–8284
52. Wrana, J. L., Lopez, A. R., Elfman, F., Ebner, R., Damsky, C. H., and Derynck, R. (1994) J. Biol. Chem. 269, 7134–7141
53. Carùcino, M., Kim, J., Phang, T. S., Casconado, J., Rosen, V., Wozney, J., and McCarthy, T. (1995) Mol. Cell. Biol. 15, 3273–3281
54. Marshall, C. (1995) Cell 80, 179–185
55. Amatayakul-Chantler, S., Gakenheimer, K., Bottinger, E. P., Roberts, A. B., and Sporn, M. B. (1994) J. Biol. Chem. 269, 27687–27691