Heteromeric and Homomeric Transforming Growth Factor-β Receptors Show Distinct Signaling and Endocytic Responses in Epithelial Cells*

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Jules J. E. Doré, Jr., Maryanne Edens, Nandor Garamszegi, and Edward B. Leof‡

From the Thoracic Disease Research Unit and Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905

Transforming growth factor-β (TGF-β) induces distinct responses dependent upon the cellular context. It is unclear whether the initial receptor interactions identified in one cell type will be operative in another. Utilizing a chimeric receptor strategy we have examined the signaling and endocytic activity of both heteromeric (type I/type II) and homomeric (type I/type I) TGF-βR interactions in Mv1Lu epithelial cells. In agreement with that observed in mesenchymal cells, all TGF-βR signaling in Mv1Lu cells required the formation of a heteromeric type I-type II receptor complex. However, the initial endocytic response to TGF-βR oligomerization was distinctly regulated in the two cell types. While heteromeric TGF-β receptors were internalized and down-regulated, homomeric TGF-βR interactions showed diminished endocytic activity in Mv1Lu cells. This contrasts to that observed in mesenchymal cultures where ligand bound to TGF-βR homomers was internalized, yet the receptors were not down-regulated. Moreover, while previous reports have suggested that mutations at serine 172 or threonine 176 in the type II receptor down-regulation (17, 18). These studies showed that while signaling competent heteromeric TGF-β receptors were internalized and down-regulated, signaling incompetent homomeric receptor complexes appeared to be recycled back to the cell surface following initial internalization. This was not a secondary response due to an inability of homomeric receptors to signal (19), but reflected an inherent difference in the endocytic activity of TGF-βR heteromers and homomers.

The present article investigates both the TGF-βR complex required for epithelial cell signaling and the initial endocytic response of ligand-activated receptors. Consistent with previous reports in fibroblasts (18) and Ba/F3 cells (20, 21), chimeric TGF-βR signaling in Mv1Lu epithelial cells required the formation of an heteromeric type I-type II receptor complex. Homomeric type I-type I or type II-type II TGF-βR interactions were unable to induce the expression of a TGF-β-responsive reporter gene or generate a growth inhibitory signal. However, when effects on receptor trafficking were examined, both similarities and differences were observed from that seen in mesenchymal cells. For instance, although heteromeric receptors were internalized and down-regulated in both cell types, homo-

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‡ To whom correspondence should be addressed: Guggenheim 6, Mayo Clinic, Rochester, MN 55905. Tel.: 507-284-5717; Fax: 507-284-4521; E-mail: leof.edward@mayo.edu.

The abbreviations used are: TGF-β, transforming growth factor-β; GM-CSF, granulocyte macrophage-colony stimulating factor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; FACS, fluorescence-activated cell sorter.

TGF-βR signaling is regulated by both positive and negative acting sites in the type I and type II receptors (10). The type I TGF-βR has been shown to undergo serine and threonine phosphorylation in the GS domain (7, 11) and to be phosphorylated at serine 165 (12). Although type I receptor phosphorylation is required for TGF-βR signaling, the number of GS domain phosphorylations, and not their particular location, appears to be the primary determinant of type I receptor activity (10, 11). Similarly, autophosphorylation of the type II TGF-βR at serines 213 and 409 are necessary to signal growth inhibitory responses while serine 416 phosphorylation acts as a negative regulator of TGF-β action (13). In addition to being regulated by phosphorylation, a number of other sites have been shown to modulate TGF-βR activity (10). For instance, while there are no published reports of threonines 200 or 204 in the type I receptor being phosphorylated, their mutation to valine or aspartic acid generates a signaling incompetent or activated receptor complex, respectively (11).

The endocytic mechanisms controlling TGF-βR membrane expression have not been extensively characterized. Early reports were contradictory and found either no significant receptor down-regulation (14, 15) or an approximate 50% decrease in surface binding following addition of ligand (16). We recently addressed this question in mesenchymal AKR-2B cells growth stimulated by TGF-β (17). By using chimeric receptors consisting of the ligand-binding domain of the GM-CSF α or β receptor fused to the transmembrane and cytoplasmic domain of the type I or type II TGF-βR, the signaling and endocytic activity of heteromeric (i.e. type I/type II) or homomeric (type I/type I or type II/type II) TGF-βR cytoplasmic interactions could be evaluated (17, 18). These studies showed that while signaling competent heteromeric TGF-β receptors were internalized and down-regulated, signaling incompetent homomeric receptor complexes appeared to be recycled back to the cell surface following initial internalization. This was not a secondary response due to an inability of homomeric receptors to signal (19), but reflected an inherent difference in the endocytic activity of TGF-βR heteromers and homomers.

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meric receptor ligation resulted in a differential endocytic response.

**Experimental Procedures**

**Materials**—Recombinant human granulocyte/macrophage-colony stimulating factor (GM-CSF) was a generous gift from DNAx Research Institute (Palo Alto, CA) while recombinant human TGF-β2 was purchased from Austral Biologicals (San Ramon, CA) or R&D Systems (Minneapolis, MN). Cell culture media, horse serum, and Geneticin (G418 sulfate) were purchased from Life Technologies Inc. (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Summit (Fort Collins, CO) and hygromycin B was purchased from Boehringer Mannheim (Indianapolis, IN). All culture dishes were from Corning (Corning, NY).

**Cell Culture**—Parental mink lung epithelial cells, Mv1Lu, were maintained in DMEM supplemented with 10% (v/v) FBS. Chimeric receptor expressing clones were constructed in a two-step process utilizing the cDNAs described previously (18). Initially, either the βII or βII chimeric receptor was transfected into Mv1Lu cells. The designation βII, for example, refers to the extracellular domain of the human GM-CSF receptor β subunit coupled to the transmembrane and cytoplasmic domains of the TGF-β type I receptor. Mv1Lu cells were plated at 10⁵ cells per well in 6-well dishes 24 h prior to transfection. Cells were washed with serum-free DMEM and incubated in 2 ml of DMEM for 10 min prior to transfection with a mixture of 2 μg of plasmid and 4 μl of TransIT-LT2 (Mirus Corp., Madison, WI) in a final volume of 100 μl of Opti-MEM for 8 h at 37 °C. Medium was replaced with 10% FBS/DMEM for 16 h to allow the cells to recover and then changed to selection medium (10% FBS/DMEM supplemented with 300 μg/ml hygromycin B) for 24 h prior to trypsinization and replating at 1:40 dilution by surface area. Ten days post-transfection isolated colonies were expanded and screened by fluorescence-activated cell sorting (FACS) for membrane expression of the chimeric receptor as described previously (18). Two representative epithelial cell clones were chosen for the second transfection based upon high expression of the chimeric βI (clone MB102-2) or βI (clone MB202-2) receptor (determined by FACS analysis) and normal (~80%) inhibitory response to endogenous TGF-βR activation (data not shown).

To generate a high affinity ligand binding chimeric receptor complex a second transfection was performed with the αII and αII chimeric constructs. The designation αII, for example, refers to the α GM-CSF receptor ligand-binding domain coupled to the TGF-β type II transmembrane and cytoplasmic domains. Clones MB102 and MB202 were plated at 10⁴ cells in 6-well plates and transfected with 2 μg of α or αII plasmid in TransIT-LT2 and Opti-MEM as described. Following 16 h recovery in 10% FBS/DMEM, cells were placed in selection medium (10% FBS/DMEM supplemented with 500 μg/ml Geneticin and 250 μg/ml hygromycin B) for 24 h prior to 1:40 dilution. Medium was changed after 5 days and stable clones isolated and expanded 10 days post-transfection. Stabile chimeric expressing clones were maintained in 10% FBS/DMEM supplemented with 200 μg/ml Geneticin and 100 μg/ml hygromycin B. Two to three representative clones from each transfection group were then chosen for further analysis. The heteromeric expressing cell lines consisted of clones MB102-18 and MB202-9, type I homomeric receptor expressing clones were designated MB102-16 and MB102-35, and lines expressing only a chimeric type II homomeric receptor were designated MB202-2 and MB202-11. When the comparison of epithelial to mesenchymal cell internalization was performed in Fig. 4, the heteromeric AKR-2B clones were A105 and A110, the type I homomeric clones were A120 and A137, and the type II homomeric clones were A122 and A139 (18).

**Effects on Cell Growth**—Treatment of epithelial cells with TGF-β results in a characteristic inhibition of DNA synthesis and subsequent mitosis. Chimeric receptor expressing clones were plated in 10% FBS/DMEM at 4 × 10⁴ cells per well (1 ml) into 24-well culture dishes. Following overnight 37 °C incubation GM-CSF or TGF-β2 was directly added to a final concentration of 10 ng/ml for 20 h. The cultures were then pulsed for 2 h with 1 μCi/ml [³H]thymidine and trichloroacetic acid precipitable counts determined (22). To determine the chimeric TGF-βR complex modulating long-term proliferative responses, clones were plated at 10⁴ cells into 33-mm Petri dishes in DMEM containing 10% FBS alone or supplemented with 10 ng/ml GM-CSF or TGF-β2. On each of days 3, 5, and 7 (with medium being replaced on day 5) three dishes were trypsinized and cell number determined by a hemocytometer.

**Receptor Function and Expression**—Receptor binding assays were performed essentially as described previously (18). Briefly, clones were plated at 2 × 10⁴ cells/well in 6-well dishes in 10% FBS/DMEM 24 h prior to the experiment. Binding of radioactive ligand (100 μCi/μg GM-CSF, NEN Life Science Products Inc., Boston, MA) was performed at 4 °C in binding buffer (DMEM containing 0.2 M HEPES, pH 7.4, 2.5% (w/v) bovine serum albumin) for 2 h with or without the presence of 25-fold molar excess of unlabeled GM-CSF, to determine specific binding. Following the 2-h incubation, wells were washed twice with a solution of 75% horse serum, 25% binding buffer and cells solubilized with 0.2 M NaOH, 40 μg/ml sheared single stranded salmon sperm DNA. Clones which had 1,500 cpm or greater were analyzed by FACS to document the presence of both α and β receptors on the cell surface. Functional analysis involved the inhibition of thymidine uptake and the induction of transiently transfected plasmogen activating inhibitor-1 luciferase (3TP-Lux) reporter gene (18), by both GM-CSF through the αII receptor or TGF-β2 through the endothelial receptor pathway.

**Site-directed Mutagenesis of Chimeric cDNA**—The chimeric α-I receptor cDNA was mutated at serine 172 to alanine (S172A) and threonine 176 to valine (T176V) using the QuickChange™ Mutagenesis kit (Stratagene, La Jolla, CA), Mutagenic primers for S172A were: CCGCCCTTTATATgCAGAGGGTACTACG and CGTAGTACCCTCTGcAATATAAAΑGGGCG and for T176V, TCAGAGGGTACTTgGTTGAAAGACTTA-
ATT and AATTAAGTCTTTCAACacAGTACCCTCTGAA. The lowercase letters indicate the base changes necessary to introduce the appropriate amino acid change. Mutant constructs in pGEM-3Z were verified by automated DNA sequencing and ligated into the eukaryotic expression vector pNA at the SalI and HindIII sites. The S172A and T176V constructs were transfected into the MB202 parental cell line to generate an heteromeric TGF-βR complex expressing the appropriate type I receptor mutation.

Endocytosis—We previously documented that the endocytic response of heteromeric and homomeric TGFβ receptors is distinctly regulated in fibroblasts (17). Similar methods were utilized to determine whether epithelial cells internalize ligand and undergo receptor-mediated down-regulation. Internalization assays were performed as described previously with minor modifications, 2 × 10^5 cells/well were plated and internalization occurred in 10% FBS/DMEM (in contrast to binding buffer). Down-regulation of chimeric receptors was determined as described previously in fibroblasts.

Scatchard Analysis—Scatchard analysis was performed to determine whether observed differences in biological responses were due to changes in receptor number or high affinity binding. Briefly, 2 × 10^5 cells/well were plated into 6-well plates 24 h prior to the experiment. Cells were washed with binding buffer and 500 pM 125I-GM-CSF was added to triplicate wells with increasing amounts of unlabeled GM-CSF (5 pM to 50 nM) and the binding allowed to proceed at 4 °C to equilibrium (3 h). Wells were washed 3 times with 75% horse serum, 25% binding buffer and the cells lysed as described for binding assays. Aliquots of the total labeled GM-CSF added to each well were counted and included in the computer analysis for a two-site receptor model (23). Only the properties of high affinity α/β receptor complexes were compared since low affinity α subunit alone binding does not stimulate any biological response (18, 24, 25).

RESULTS

Expression of Chimeric Receptors and Biological Function—Chimeric GM-CSF/TGF-β receptor constructs were stably transfected into mink lung epithelial cells, Mv1Lu, as described previously for mouse fibroblasts (18) with minor modifications. Mv1Lu cells were initially transformed with a β1 or β2 receptor construct (designations described under “Experimental Procedures”) and two clones designated MB-102 (β1) and MB-202 (β2) selected based upon chimeric receptor membrane expression and growth inhibitory response to endogenous TGF-βR activation (data not shown).

MB-102 and MB-202 were then used as recipient cell lines into which the complementary α receptor chimeric receptors were transfected. Stable clones were obtained by dual selection in hygromycin and Geneticin (G418 sulfate) and clones obtained expressing homomeric (α1β1 and α2β2) and heteromeric (α1β2 and α2β1) TGF-βR interactions. Plasma membrane expression of both the α and β chimeric receptors was confirmed in the computer analysis for a two-site receptor model (23). Only the properties of high affinity α/β receptor complexes were compared since low affinity α subunit alone binding does not stimulate any biological response (18, 24, 25).

FIG. 2. Regulation of replication by chimeric receptors relative to endogenous TGF-β response. Panel A, thymidine incorporation was performed on proliferating control Mv1Lu cells, chimeric receptor heteromers (clones MB102–9 and -18), type I/type I homomers (clones MB102–16 and -35), and type II/type II homomers (clones MB202–2 and -11) following addition of 5 ng/ml TGF-β2 (black bars) or 5 ng/ml GM-CSF (striped bars) for 24 h. Percentage incorporation is relative to the incorporation of untreated cycling cells. Each value represents the mean ± S.E. of three separate experiments of two independent clones performed in duplicate. Panels B-D 7-day growth effects on type I/type II receptor heteromers (Panel B), type I/type I receptor homomers (Panel C), and type II/type II receptor homomers (Panel D) following treatment with 10% FBS/DMEM (●) or 10% FBS/DMEM supplemented with 5 ng/ml TGF-β2 (▲) or 5 ng/ml GM-CSF (●). Values represent the mean ± S.D. of duplicate experiments done in triplicate on two independent clones as described under “Experimental Procedures.”

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by FACS analysis and ligand binding assays (Fig. 1 and data not shown).

We have previously reported that (in fibroblasts) only heteromeric TGF-βR interactions are signaling competent following ligand binding (18). Since the biological response to TGF-β of epithelial and mesenchymal cells is so distinct (i.e. growth inhibition versus growth stimulation, respectively), the chimeric receptor expressing epithelial clones were assayed for their ability to reproduce typical TGF-β regulated responses following GM-CSF stimulation (Figs. 2 and 3). As shown in Fig. 2A, addition of TGF-β to cycling epithelial cells resulted in an approximate 90% inhibition in thymidine incorporation. However, while GM-CSF treatment had no significant effect on either the parental Mv1Lu cells or clones expressing chimeric type I or type II homomeric receptors, a similar growth inhibitory response to that seen following addition of TGF-β2 was observed when heteromeric type I/type II TGF-β receptors were activated by treatment with GM-CSF. Furthermore, to address the possibility that a single time point of mitotic inhibition does not adequately represent a growth inhibitory response, clones were grown in the presence of 5 ng/ml TGF-β2 or GM-CSF and cell counts performed over a 1-week period (Fig. 2, B-D). Consistent with the thymidine incorporation data, GM-CSF significantly inhibited mitosis only in the heteromeric receptor expressing clones (Fig. 2B). Moreover, the absence of any inhibitory response in the homomorphic receptor expressing clones was not simply a reflection of a signaling anergy since treatment with TGF-β2 to activate endogenous TGF-β receptors similarly resulted in an 80–90% inhibition of growth (Fig. 2).

Previous reports have suggested that different signaling pathways and receptor complexes are activated by TGF-β in Mv1Lu cells to stimulate extracellular matrix protein expression and regulate mitosis (26, 27). To determine whether stable expression and activation of either type I/type I or type II/type II homomeric TGF-β receptors would effect plasminogen activator inhibitor-1 gene activity, the studies shown in Fig. 3 were performed. While TGF-β stimulates luciferase activity through endogenous TGF-β receptors in all clones examined, chimeric receptor activation by GM-CSF could only occur following the formation of an heteromeric type I-type II TGF-βR complex. For instance, GM-CSF stimulated luciferase expression over 100-fold in the heteromeric receptor expressing clones, but <2-fold in clones expressing type I or type II homomeric TGF-β receptors. Thus, Figs. 2 and 3 document that homomeric type I or type II chimeric TGF-βR complexes are unable to signal in Mv1Lu epithelial cells.

**Chimeric Receptor Endocytosis—**Our previous studies documented that in fibroblasts homomeric and heteromeric TGF-βR complexes similarly internalized bound ligand (17). Whether a similar endocytic response would be observed in epithelial cells where TGF-β acts as a growth inhibitor was unknown. To address that question, the internalization of labeled GM-CSF was determined over a 60-min, 37 °C, treatment in Mv1Lu epithelial cells expressing chimeric TGF-βR heteromers and homomers. As shown in Fig. 4A, the mean endocytic rate constant, k−1 (defined as the probability of an occupied receptor being internalized in 1 min at 37 °C) (28), of type I/type I and type II/type II receptor homomers were 0.025/min ± 0.002 and 0.030/min ± 0.001, respectively. However, unlike that observed in fibroblasts, heteromeric TGF-βR expressing epithelial cell clones showed both an increased level of ligand internalization and a mean k−1 (0.084/min ± 0.014) approximately 3-fold greater than that of the receptor homomers. The heteromeric receptor k−1 is similar to that previously described for the epidermal growth factor and growth hormone receptors (29–31). These results contrast to that seen in AKR-2B fibroblasts which show a heteromeric receptor k−1 of 0.050/min ± 0.004 and homomeric rates of 0.038 ± 0.001 (type I/type I) and 0.036 ± 0.005 (type II/type II) (Fig. 4B). The finding of distinct responses following ligand binding to heteromeric and homomeric receptors suggests a TGF-β receptor subtype-specific trafficking mechanism in epithelial cells (relative to mesenchymal cells) for internalization.

In general, the internalization of bound ligand is followed by a decrease in cell surface receptor binding referred to as receptor down-regulation. In that regard, our finding of distinct internalization rates for chimeric TGF-βR homomers and heteromers (Fig. 4) suggested the action of an initial sorting event(s) whereby heteromeric TGF-β receptors were more rapidly recognized and removed from the cell surface than receptor homomers. To determine the fate of these internalized receptors we performed the down-regulation assays shown in Fig. 5. As expected, the heteromeric type I/type II chimeric receptors were rapidly removed from the cell surface. Following a 2-h ligand treatment surface binding was reduced to 23% of initial levels. In contrast to this, cell surface receptor binding was only reduced to 46 and 70% of initial levels for type II and type I receptor homomers, respectively. Although there was no difference between any of the chimeric receptors within the first 10 min of treatment, by 60 min surface binding was significantly different between all chimeric receptor types. This difference in receptor down-regulation is consistent with the hypothesis that an endosomal sorting mechanism differentiates not only TGF-βR homomers from heteromers, but also between homomeric interactions of type I and type II TGF-βR complexes (Figs. 4 and 5).

**Point Mutations Attenuate TGF-β Responses—**Studies in which single or multiple amino acids have been altered in the type I TGF-βR have attempted to determine regulatory sites within and in proximity to the GS domain (10, 11). Of particular interest was the finding that Ser-172 and Thr-176 were reported to separate matrix protein induction from cell cycle regulation in response to TGF-β (27). These results indicated a potential role(s) for these sites in modulating initial membrane events. As such, we wished to determine whether Ser-172 or Thr-176 might have a regulatory role in TGF-β trafficking.

Heteromeric receptor expressing clones were generated containing a wild type II TGF-βR and a type I TGF-βR mutated at Ser-172 (S172A series) or Thr-176 (T176V series).
Mean (±S.E.) of two independent clones assayed in duplicate from three separate experiments. Panel B, internalization assays as described in panel A were performed on AKR-2B heteromeric type I/type II TGF-βR expressing clones (●), type I/type I homomeric clones (▲), and type II/type II homomeric clones (■). Each curve represents the mean (±S.E.) of two independent clones assayed in duplicate from two separate experiments.

Fig. 5. Chimeric receptors show distinct kinetics of receptor down-regulation. Surface receptors of two individual clones of chimeric heteromers (●), type I/type I homomers (▲), and type II/type II homomers (■) were treated with 10 ng/ml GM-CSF at 37 °C for the indicated times. GM-CSF bound to the surface was removed and surface receptor binding determined by incubation with 100 pM 125I-GM-CSF for 2 h at 4 °C. The data represent the mean (±S.E.) of two independent clones assayed in duplicate from two separate experiments.

However, when screening clones of the S172A series prior to analyzing their endocytic activity, while FACS analysis showed no significant difference (data not shown), we noted a gradation in ligand-induced thymidine inhibition which correlated to specific 125I-ligand binding. To address this issue more thoroughly, we separated these clones into high (S172A-H; 5,000–10,000 cpm) and low (S172A-L; 1,500–3,000 cpm) binding groups.

We have previously determined that in fibroblasts the endocytic response to TGF-βR increases in cell surface receptor expression.

The preceding data indicate potential pitfalls in generating paradigms based upon 1 or 2 cell clones harboring a particular mutation. Similarly, the clonal variation observed between the parental Mv1Lu cells and the isolated clones depicted in Fig. 6A emphasizes the need for caution when small, 2–4-fold, changes in luciferase activity are considered significant. To ascertain if the S172A mutation changed ligand-receptor interactions such that a larger number of receptors could compensate for a decrease in receptor affinity, Scatchard analysis was performed on heteromeric clones expressing wild type chimeric receptors and a representative S172A-L and S172A-H expressing cell line. As shown in Table I, no significant difference was observed in the affinity of ligand binding for any of the tested cell lines. However, in agreement with the original binding data, there was a 4.5-fold difference in the binding capacity between the signaling impaired S172A-8 clone and the signaling competent S172A-27 line. Thus, the attenuation of TGF-βR signaling by mutation of Ser-172 (Fig. 6) can be overcome by an increase in cell surface receptor expression.

Mutant Receptor Down-regulation—We have previously determined that in fibroblasts the endocytic response to TGF-βR...
activation is regulated independent of receptor signaling (19).
For instance, while an heteromeric receptor complex consisting of a wild type type II receptor and a kinase inactive type I TGF-βR was signaling incompetent, there was no apparent effect on either the kinetics (or extent) of internalization or receptor down-regulation. Thus, while the preceding data indicated that the S172A and T176V mutations provided variable or no effect, respectively, on TGF-βR signaling, it was still to be determined whether these mutations might modulate initial events in receptor trafficking. To address that question in more detail, the studies shown in Fig. 7 were performed. The results document no appreciable difference in either the rate or extent of receptor down-regulation in the S172A or T176V series of mutated clones when compared with wild type receptor activity. In all cases there was a 55–70% decrease in receptor binding within 1-h ligand treatment and a maximal decrease of 70–80% by 2–4 h. Similar effects were seen when ligand internalization was analyzed (data not shown).

**DISCUSSION**

The diverse and sometimes opposing biological effects on different cell types has been a paradox of TGF-β physiology. Moreover, the presence of various heteromeric and homomeric cell surface TGF-βR interactions has made studies investigating the early endocytic response to receptor activation problematic. For instance, ligand bound to type II/type II TGF-βR homomers might be processed differently from ligand associated with type I/type II heteromeric TGF-β receptors. As such, in order to determine whether the endocytic machinery could distinguish these various receptor complexes, we designed a chimeric receptor strategy to specifically examine the fate of heteromeric (type I/type II) and homomeric (type I/type I and type II/type II) TGF-βR interactions (17, 18, 21). This system takes advantage of the modular aspect of receptor activity by fusing the extracellular ligand-binding domains of the human GM-CSF α or β receptors to the transmembrane and cytoplasmic domain of the type I or type II TGF-βR. Since high affinity GM-CSF binding requires the formation of an α/β complex (24, 25), we can independently examine the endocytic and signaling properties of specific TGF-βR oligomers. The present study demonstrated that in Mv1Lu epithelial cells chimeric heteromeric complexes of TGF-β type I and II receptors are required for signal transduction, and while homomeric complexes may form on the cell surface (8, 9), they have no direct role in signaling (Figs. 2 and 3). This is consistent with our previous data in which we showed that only heteromeric complexes signal in fibroblasts (18) and supports the receptor model originally proposed by Wrana et al. (6, 7). Although heteromeric receptor oligomerization is required to initiate receptor signaling, it is presently unclear whether the receptor docking complex for downstream effectors consists of a single or multisubunit complex of activated type I receptors (10, 32, 33).

The cytoplasmic domains of TGF-β receptors have been shown to interact in 2-hybrid screens and overexpressing COS cells (9, 34). Whether any of the signaling or endocytic activity of the chimeric receptors is reflecting an association with endogenous TGF-βR receptors is presently unclear. The low levels of receptor expression and absence of high quality precipitating antibodies has made it difficult to obtain consistent results.2 We have, however, addressed this question from a functional perspective. Since kinase inactive TGF-β receptors have been shown to act as dominant/negatives in vitro and in vivo (35–40), we reasoned that an interaction of a kinase inactive chimeric receptor with an endogenous TGF-βR should prevent signaling from the endogenous receptor when stimulated with TGF-β. Although the kinase inactive chimeric receptors were unable to signal, no evidence was observed for any dominant/negative activity (19). Although these studies do not directly document the specific receptor interactions formed, they are consistent with the hypothesis that, in vivo, TGF-βR receptor associations are primarily regulated through the extracellular ligand-binding (not cytoplasmic) domains (20).

There have been few studies investigating the endocytic response of TGF-β receptors and none which have specifically addressed the fate of heteromeric and homomeric TGF-βR complexes in epithelial cells. Although we have recently addressed

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this question in mesenchymal cells which are growth stimulated by TGFβ, it was important to determine whether a similar endocytic mechanism was utilized in epithelial cultures where TGF-β acts as a growth inhibitor. In that regard, while the type I-type II heteromeric receptor signaling complex was internalized and down-regulated in both mesenchymal (17) and epithelial cell lines (Figs. 4, A and 5), the initial endocytic response to type I or type II homomorphic receptor oligomerization was distinctly regulated in each cell type. For instance, while homomorphic TGF-βR complexes in mesenchymal cells were proposed to be recycled following initial ligand-mediated internalization, no evidence for such a response was observed in Mv1Lu epithelial cell cultures where both homomorphic receptor internalization and down-regulation were diminished (Figs. 4 and 5). Although it is presently unclear whether this differential response to receptor activation can be extended to help account for the distinct effect TGF-β has on epithelial and mesenchymal cell growth, it does indicate that the variety of cellular phenotypes to TGF-β can be initially regulated at an early event in receptor processing.

The endocytic rate constant over the first 30 min for heteromeric TGF-β receptors was calculated to be 0.084/min in Mv1Lu epithelial cells (17). It should now be noted that this region arose from recent studies which showed this region to be phosphorylated on serine and threonine residues when complexed with a type II TGF-βR (7, 11). Although single amino acid changes within the GS domain (amino acids 185–192) were subsequently shown to have no detectable effect on cellular signaling, multiple mutations resulted in a dose-dependent loss in receptor signaling capacity. Additional studies have identified other regions in the type I TGF-βR capable of modulating TGF-β action. For instance, mutation of threonine 200 to valine generates a signaling inactive receptor (11), mutation of serine 165 results in a type I receptor with an attenuated ability to signal (12), and mutation of serine 172 or threonine 176 results in a type I receptor capable of stimulating matrix gene expression but unable to promote growth inhibition (27). This dissociation of transcriptional from growth responses was of particular interest since it suggested a role for these sites in directing initial events in receptor processing. To address this hypothesis we expressed chimeric type I TGF-β receptors with alanine and valine mutations at serine 172 and threonine 176, respectively, with a wild type chimeric type II TGF-βR to directly test the effect (if any) these sites would have on endocytosis and receptor signaling. As shown in Fig. 7 (and data not shown) we observed no significant role for Ser-172 or Thr-176 in receptor down-regulation or internalization. More recently, while all Mv1Lu clones expressing a chimeric type I receptor mutated at Thr-176 showed similar inhibition (80%) through activation of chimeric or endogenous TGF-β receptors, two distinct families of Ser-172 mutants were identified by their ability to bind ligand (Fig. 6 and Table I). While the low binding family showed essentially no difference in GM-CSF signaling relative to parental untransfected Mv1Lu cells, the high binding clones induced both activities similar to clones expressing wild type chimeric receptors (Fig. 6). The lack of signaling in the S172A-L clones is not a reflection of the absolute number of chimeric receptors, per se, since the A105 line and members of the T176V family showed similar binding capacity (Table I and data not shown). Thus, although mutation at Ser-172 attenuated type I TGF-βR signaling, it results support the general conclusions that 1) the rate and extent of heteromeric TGF-βR internalization in Mv1Lu epithelial cells is greater than that observed in AKR-2B mesenchymal cells; 2) ligand bound to heteromeric TGF-βR receptors is internalized to a greater degree than ligand bound to homomorphic TGF-βR receptors in Mv1Lu cells; and 3) there is no significant difference in heteromeric or homomorphic receptor internalization in AKR-2B cells. Thus, the distinct phenotypes induced by TGF-β in epithelial and mesenchymal cells can be similarly observed in the initial endocytic response to receptor activation.

The type I TGF-βR contains a highly conserved region of 30 amino acids referred to as the GS domain (41, 42). Interest in this region arose from recent studies which showed this region to be phosphorylated on serine and threonine residues when complexed with a type II TGF-βR (7, 11). Although single amino acid changes within the GS domain (amino acids 185–192) were subsequently shown to have no detectable effect on cellular signaling, multiple mutations resulted in a dose-dependent loss in receptor signaling capacity. Additional studies have identified other regions in the type I TGF-βR capable of modulating TGF-β action. For instance, mutation of threonine 200 to valine generates a signaling inactive receptor (11), mutation of serine 165 results in a type I receptor with an attenuated ability to signal (12), and mutation of serine 172 or threonine 176 results in a type I receptor capable of stimulating matrix gene expression but unable to promote growth inhibition (27). This dissociation of transcriptional from growth responses was of particular interest since it suggested a role for these sites in directing initial events in receptor processing. To address this hypothesis we expressed chimeric type I TGF-β receptors with alanine and valine mutations at serine 172 and threonine 176, respectively, with a wild type chimeric type II TGF-βR to directly test the effect (if any) these sites would have on endocytosis and receptor signaling. As shown in Fig. 7 (and data not shown) we observed no significant role for Ser-172 or Thr-176 in receptor down-regulation or internalization. Moreover, no dissociation of transcriptional and growth responses was seen following mutation of Ser-172 or Thr-176 in receptor down-regulation or internalization. However, while all Mv1Lu clones expressing a chimeric type I receptor mutated at Thr-176 gave a similar induction of plasminogen activator inhibitor-luciferase (i.e. 80-fold) and growth inhibition (i.e. 90%) through activation of chimeric or endogenous TGF-β receptors, two distinct families of Ser-172 mutants were identified by their ability to bind ligand (Fig. 6). While the low binding family showed essentially no difference in GM-CSF signaling relative to parental untransfected Mv1Lu cells, the high binding clones induced both activities similar to clones expressing wild type chimeric receptors (Fig. 6). The lack of signaling in the S172A-L clones is not a reflection of the absolute number of chimeric receptors, per se, since the A105 line and members of the T176V family showed similar binding capacity (Table I and data not shown). Thus, although mutation at Ser-172 attenuated type I TGF-βR signaling, it

| Receptor | Heteromeric (low binding) | Heteromeric (high binding) | A105 |
|----------|--------------------------|--------------------------|------|
| Affinity (pM) | 46.5 ± 19.9 | 38.6 ± 8.5 | 41.7 ± 14.9 | 41.8 ± 4.1 |
| Capacity (pM) | 3.32 ± 0.49 | 0.43 ± 0.09 | 1.93 ± 1.08 | 0.99 ± 0.20 |

FIG. 7. Point mutations at Ser-172 and Thr-176 in chimeric type I TGF-β receptors does not effect receptor down-regulation. Receptor down-regulation was determined from triplicate (wild type) and duplicate (S172A and T176V series) experiments. Changes in specific binding (± S.E.) for heteromeric wild type (C), clones MB102-9 and -18, S172A-L ( ), clones MB202/172–8, -14, S172A-H ( ), clones MB202/172–27, -28, and T176V (Δ, clones MB202/176-1, 2-40) were plotted relative to binding at time 0.

This table shows the Scatchard analysis of various chimeric receptor expressing clones.

| Receptor | Heteromeric (low binding) | Heteromeric (high binding) | A105 |
|----------|--------------------------|--------------------------|------|
| Affinity (pM) | 46.5 ± 19.9 | 38.6 ± 8.5 | 41.7 ± 14.9 | 41.8 ± 4.1 |
| Capacity (pM) | 3.32 ± 0.49 | 0.43 ± 0.09 | 1.93 ± 1.08 | 0.99 ± 0.20 |

Scatchard analysis performed on clones M202–18 (a Mv1Lu epithelial line expressing wild-type chimeric heteromeric receptors), S172A-8 (a representative of the S172A low binding Mv1Lu family), S172A-27 (a representative of the S172A high binding Mv1Lu family), and A105 (an AKR-2B mesenchymal line expressing wild-type chimeric heteromeric receptors). Values represent the mean ± S.E. of triplicate samples from three separate experiments. Analysis of the data utilized the Ligand program for a two-binding site receptor model (23). Only the affinity and capacity for the high affinity sites is presented since low affinity α alone binding is signaling incompetent (18, 24, 25).
also was unable to dissociate TGF-βR mediated transcriptional and growth inhibitory responses.

It is presently unknown why we should be unable to separate type I TGF-βR signaling pathways as previously reported (27) as well as what might account for the attenuation in signaling observed for the Ser172 mutation. Although it is possible that the difference seen between Saitoh et al. (27) and the present report is a reflection of limitations in both systems (i.e. other chemically-induced changes in the receptor mutant R4-2 line or a chimeric receptor, respectively), the attenuation seen with the S172A cells is similar to the receptor threshold effect of serine 165 required for cell overgrowth reported by Souchelnytskyi et al. (12).

The present data provide and extend a number of findings for understanding TGF-β action including: (i) documenting that only an heteromeric complex of a type I and type II TGF-βR can signal in Mv1Lu epithelial cells, (ii) reporting an endocytic rate constant for heteromeric type I/type II TGF-β receptors similar to that of tyrosine kinase-coupled receptors, (iii) determining that in epithelial cells homomeric type I-type I or type II-type II TGF-βR interactions do not result in significant internalization or down-regulation, (iv) demonstrating that chimeric type I TGF-βR-mediated transcriptional and growth inhibitory responses cannot be distinguished by juxtamembrane domain mutations, and (v) reporting that serine 172 in the type I TGF-βR is permissive for TGF-β signaling. The ability to investigate defined TGF-βR interactions in both epithelial and mesenchymal systems provides the opportunity to mechanistically address how these complexes might be regulated to generate such diverse cellular phenotypes.

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REFERENCES

1. Moses, H. L. & Serra, R. (1996) Curr. Opin. Genet. Dev. 6, 581–586
2. Massague, J. & Wei-Weis-Garcia, F. (1996) Cancer Surv. 27, 41–64
3. Hoodless, P. A. & Wrana, J. L. (1998) Curr. Top. Microbiol. Immunol. 228, 235–272
4. Lin, H. Y., Wang, X. F., Ng, E. E., Weinberg, R. A. & Lodish, H. F. (1992) Cell 68, 775–785
5. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schultz, P., Heldin, C.-H. & Miyazono, K. (1993) Cell 75, 681–692
6. Wrana, J. L., Attisano, L., Carcano, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F. & Massague, J. (1992) Cell 71, 1003–1014
7. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massague, J. (1994) Nature 370, 341–347
8. Henis, Y. I., Moustakas, A., Lin, H. Y. & Lodish, H. F. (1994) J. Cell Biol. 126, 129–154
9. Chen, R. H. & Derynck, R. (1994) J. Biol. Chem. 269, 22868–22874
10. Heldin, C.-H., Miyazono, K. & ten Dijke, P. (1997) Nature 390, 465–471
11. Wieser, R., Wrana, J. L. & Massague, J. (1995) EMBO J. 14, 2199–2208
12. Souchelnytskyi, S., ten Dijke, P., Miyazono, K. & Heldin, C.-H. (1996) EMBO J. 15, 6231–6240
13. Luo, K. & Lodish, H. F. (1997) EMBO J. 16, 1970–1981
14. Massague, J. (1985) Cancer Cells 3, 73–78
15. Wakefield, L. M., Smith, D. M., Masui, T., Harris, C. C. & Sporn, M. B. (1987) J. Cell Biol. 106, 965–975
16. Frolik, C. A., Wakefield, L. M., Smith, D. M. & Sporn, M. B. (1984) J. Biol. Chem. 259, 10959–11002
17. Anders, R. A., Arline, S. L., Doré, J. J. E. & Leof, E. B. (1997) Mol. Cell Biol. 8, 2133–2143
18. Anders, R. A. & Leof, E. B. (1996) J. Biol. Chem. 271, 21758–21766
19. Anders, R. A., Doré, J. J. E., Arline, S. L., Aaramo, N. & Leof, E. B. (1998) J. Biol. Chem. 273, 23118–23125
20. Luo, K. & Lodish, H. F. (1996) EMBO J. 15, 4485–4496
21. Muramatsu, M., Yan, J., Eto, K., Tomoda, T., Yamada, R. & Arai, K. (1997) Mol. Biol. Cell 8, 469–480
22. Shipley, G. D., Tucker, R. F. & Moses, H. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4147–4151
23. Rovati, G. E., Rodbard, D. & Munson, P. J. (1988) Anal. Biochem. 174, 636–649
24. Shanafelt, A., Miyajima, A., Kitamura, T. & Kastelan, R. A. (1991) EMBO J. 10, 4105–4112
25. Hayashida, K., Kitamura, T., Gorman, D. M., Arasi, K.-J., Yokota, T. & Miyajima, A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9656–9659
26. Chen, R. H., Ebner, R. & Derynck, R. (1993) Science 260, 1335–1338
27. Saitoh, M., Nishimoto, H., Amagasa, T., Miyazono, K., Takagi, M. & Ichijo, H. (1994) J. Biol. Chem. 269, 27170–27175
28. Wiley, H. S. & Cunningham, D. D. (1982) J. Biol. Chem. 257, 2422–2429
29. Sorkin, A., Mazzotti, M., Sorkina, T., Scotto, L. & Beggioni, L. (1996) J. Biol. Chem. 271, 13377–13384
30. Hardwick, P. A., Wang, X., Okada, S., Chen, W. Y., Wan, W. & Kopchick, J. J. (1996) J. Biol. Chem. 271, 6708–6712
31. Chang, C. P., Lazar, C. S., Walsh, B. J., Komuro, M., Collawn, J. F., Kuhn, L. A., Tainer, J. A., Trowbridge, I. S., Farquhar, M. G., Rosenfeld, M. G., Wiley, H. S. & Gill, G. N. (1993) J. Biol. Chem. 268, 1912–1920
32. Weis-Garcia, F. & Massague, J. (1996) EMBO J. 15, 276–289
33. Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O’Connor, M. M., McCourt, D. & Lodish, H. F. (1990) J. Biol. Chem. 265, 22215–22218
34. Kawabata, M., Chytil, A. & Moses, H. L. (1995) EMBO J. 14, 341–347
35. Bottinger, E. P., Jakubczak, J. L., Roberts, I. S. D., Mumy, M., Hemmati, P., Bagnall, K., Merlino, G. & Wakefield, L. M. (1997) EMBO J. 16, 2621–2633
36. Brand, T., MacLellan, W. R. & Schneider, M. D. (1993) J. Biol. Chem. 268, 11500–11503
37. Choi, M. E. & Ballermann, B. J. (1995) J. Biol. Chem. 270, 21144–21150
38. Filvaroff, E. H., Ehner, R. & Derynck, R. (1994) Development 120, 1085–1095
39. Wang, X. J., Greenhalgh, D. A., Bickenbach, J. R., Jiang, B., Lundman, D. S., Krieg, T., Derynck, R. & Roop, D. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2386–2391
40. Zhao, Y. & Young, S. L. (1996) J. Biol. Chem. 271, 2369–2372
41. Kingsley, D. M. (1994) Genes Dev. 8, 133–146
42. ten Dijke, P., Franzen, P., Yamashita, H., Ichijo, H., Heldin, C. H. & Miyazono, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 55–72