Chimeric Granulocyte/Macrophage Colony-stimulating Factor/Transforming Growth Factor-β (TGF-β) Receptors Define a Model System for Investigating the Role of Homomeric and Heteromeric Receptors in TGF-β Signaling*

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Transforming growth factor-β (TGF-β) belongs to a family of ligands that regulate cell growth and differentiation. The most commonly observed receptors are referred to as the type I, type II, and type III (betaglycan) TGF-β receptors. Two receptor models have been presented to account for the various cellular responses to TGF-β. The first proposes that all TGF-β signaling results from the formation of a heteromeric type I/type II complex, while the second suggests that distinct type I or type II TGF-β receptor combinations mediate aspects of TGF-β signaling. We have addressed this general question relating to TGF-β signaling by constructing chimeric receptors consisting of the extracellular domain of the granulocyte/macrophage colony-stimulating factor (GM-CSF) α or β receptor fused to the transmembrane and cytoplasmic domain of the type I or type II TGF-β receptor. Since high affinity GM-CSF binding requires dimerization of the α and β ligand binding subunits, the response elicited by defined type I and/or type II TGF-β receptor cytoplasmic domain homomers or heteromers can be examined. We show in mesenchymal AKR-2B cells that while TGF-β-dependent transient luciferase activity, endogenous gene activity, and long-term biological responses are similarly induced by activating the chimeric heteromeric receptors with GM-CSF as the endogenous TGF-β receptor, chimeric homomeric type I/type I or type II/type II receptors are signaling-incompetent.

Transforming growth factor-β (TGF-β) is a 25-kDa polypeptide prototypic of a growth factor superfamily involved in events central to cell biology (1-3). These cytokines have important roles in regulating cell growth, pattern formation, and human disease. Although the downstream mediators activated after TGF-β receptor activation are not well characterized (1), the TGF-β receptors have been recently shown to constitute a new superfamily of single pass, transmembrane serine/threonine kinases (4-8). In general, the majority of mammalian cells express three TGF-β binding species referred to as the type I, type II, and type III (betaglycan) receptors (9-12). The type I and type II TGF-β receptors are transmembrane serine/threonine kinases with molecular masses of 53 and 75 kDa, respectively, when fully glycosylated (6, 8, 13). While the overwhelming majority of studies indicate that the cellular response to TGF-β is mediated through these receptors, there is controversy as to the particular receptor profile(s) responsible for individual activities. To that end, two competing but not mutually exclusive models have been proposed. The basis of the first model is that all TGF-β signaling requires heteromeric complex formation between the type I and type II TGF-β receptors. There is a great deal of genetic evidence using chemically derived cell mutants in support of such a model (14-19). For instance, Wrana et al. (18) have presented data indicating both a physical and functional association between the type I and type II TGF-β receptors. Moreover, recent publications have shown that the type II TGF-β receptor is a constitutively active kinase capable of binding ligand (19, 20). Once ligand is bound, this results in the recruitment and transphosphorylation of the type I receptor, which allows propagation of the signal to downstream effectors. A fundamental tenet to this model is that the type I TGF-β receptor is unable to bind ligand alone in the absence of the type II receptor (18, 19). Although a great deal of evidence has been generated in support of this model, there is similarly a large amount of data at odds with the heteromeric model. For instance, chemical cross-linking analysis of many nontransformed and transformed cell types has shown TGF-β binding to type I receptors in the absence of type II receptor binding (21-29). Additional data for which the heteromeric model cannot readily account are: (i) type I and type II receptor homomers have been documented in the absence or presence of ligand binding (18, 30, 31); (ii) the type I and type II receptor kinase domains have been shown to have identical substrate specificity (32), raising the question of whether the two receptors can functionally substitute for each other; (iii) effects on growth inhibition and gene expression have been uncoupled using dominant negative type II receptors and receptor mutants (28, 33); and (iv) other TGF-β family members such as OP-1 and BMP-4 are capable of binding type I receptors in the absence of type II receptor expression (34).

While a large amount of data have accumulated both in support of and in argument against the previous discussed TGF-β receptor models, most of the reports are heavily dependent on the use of chemically derived epithelial cell mutants resistant to TGF-β growth inhibition, cross-linking analysis (11, 12, 35), and/or transient transfections (15-17). Although these approaches have merit, the manner in which they have
been used does not preclude a number of alternative interpretations (hence the controversy in the literature). To that end, we addressed this question concerning the requirement for TGF-β receptor heteromers or homomers by generating stable transfectants expressing chimeric receptors consisting of the ligand binding domain of the granulocyte/macrophage colony stimulating factor (GM-CSF) α or β receptors (36, 37) fused to the transmembrane and cytoplasmic domains of the type I and type II TGF-β receptors. Since high affinity GM-CSF binding occurs through the formation of an αβ heterodimer (37, 38), we can generate cytoplasmic receptor heteromers (type I/type II) or homomers (type I/type I or type II/type II) with essentially total specificity. In addition to directly comparing the signaling capabilities of TGF-β receptor heteromers and homomers, this chimeric approach has a number of other advantages: (i) there is an absence of endogenous GM-CSF receptors which might complicate the analyses; (ii) it includes the ability to test TGF-β signaling in cell lines that have not been chemically mutated (i.e. avoids unknown mutagenic events); (iii) it is amenable to the generation of stable cell lines for examining endogenous cellular responses (i.e. is not solely dependent on transient assays); (iv) all assays are internally controlled in that one can directly compare the response seen with the chimeric receptor (i.e. activated by GM-CSF) to that of the endogenous TGF-β receptor (i.e. activated by TGF-β) in the same cell population; and (v) this approach can be used in essentially any cell system where one wishes to determine the TGF-β receptor complex mediating effects on cellular growth, differentiation, and/or cell signaling.

We have initially used this system in mesenchymal AKR-2B cells previously shown to be highly responsive to TGF-β receptor activation (39, 40). The data support the model initially proposed by Massagué and colleagues (18, 19) in that all TGF-β receptor signaling requires heteromeric complex formation. For instance, while chimeric receptor heteromers modulate transient, endogenous, and long term growth responses similar to activating the endogenous TGF-β receptor, no signaling activity is observed in chimeric receptor combinations consisting solely of type I/type I or type II/type II TGF-β receptor homomers.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human granulocyte/macrophage colony stimulating factor was a generous gift from DNAx Research Institute (Palo Alto, CA), and recombinant human TGF-β1 and TGF-β2 were purchased from Austral Biologicals (San Ramon, CA) or R&D Systems (Minneapolis, MN). No appreciable difference was observed for TGF-β1 or TGF-β2 ligands, and they are used interchangeably to document this response. The type I (ALK-5 or R4) TGF-β receptor was obtained from Dr. Kohei Miyazono (Tokyo Medical and Dental University, Tokyo, Japan), the type II TGF-β receptor cDNA was obtained from Dr. Harvey Lodish (Whitehead Institute for Biomedical Research, Cambridge, MA), the human GM-CSF α receptor cDNA was from Dr. Tony Troutt (Immune Research, Seattle, WA), and the canine GM-CSF β subunit was provided by Dr. Kenneth Kaushansky (University of Washington, Seattle, WA). Expression plasmids pNa and pHα were from Dr. Brad Nelson at the Fred Hutchinson Cancer Research Center (Seattle, WA), and the 3P-Lux reporter plasmid was from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY).

Cell Culture—AKR-2B cells were grown in 5% fetal bovine serum (FBS) (Summit, Ft. Collins, CO) supplemented Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies, Inc.). Following transfection with the chimeric receptor expression plasmids the transfected cells were maintained in 5% FBS/DMEM containing 100 μg/ml bioactive Geneticin (Life Technologies) and 50 μg/ml bioactive Hygromycin B (Sigma).

Equilibrium 125I-GM-CSF Binding—Cells were detached in RPMI 1640 medium (Life Technologies, Inc.), pH 7.2, containing 40 mM EDTA and 20 mM HEPEs. The cells were washed twice in binding buffer (20 mM HEPEs, 2 mg/ml bovine serum albumin, RPMI 1640 medium, pH 7.4), and approximately 5 × 10^6 cells were mixed at 4°C for 2 h with rocking in 200 μl of binding buffer, 100 pm 125I-labeled recombinant human GM-CSF (DuPont NEN) with or without 25–50-fold excess unlabeled GM-CSF. Bound was separated from free by centrifugation at 4°C for 15 min, × 10 for 2 min in chilled 25% binding buffer with 75% horse serum (41) (Life Technologies, Inc.). The pellets were counted in a MiniAxi 5000 series (Packard Instrument Co.) gamma counter, where specific binding routinely represented 70–90% of total binding.

Transfection and Selection of Stable Clones—Parental AKR-2B cells were plated at 5 × 10^5 cells/10 cm^2 culture dish 24 h prior to transfection. Four μg of plasmid DNA was mixed with 100 μl of 2.5 M CaCl_2 pH 7.4, and added dropwise to 500 μl of 2× HEBS (140 mM NaCl, 40 mM HEPEs, 11 mM dextrose, 10 mM KCl, 14 mM NaHPO_4, pH 7.2). The precipitate was then added to 10 ml of growth medium (5% FBS/DMEM) covering the cells and incubated for 6 h at 37°C. Following a 2-min 10% glycerol shock, the cultures were placed in fresh growth medium for 36 h prior to a 1:20–1:40 split into small medium (5% FBS/DMEM, 400 μg/ml Geneticin, 150 μg/ml Hygromycin B, bioactive concentrations). Well isolated clones (2 or 3/10-mm^2 plate) were subcloned with polyethylene 8-mm cylinders directly into a well of a 24-well dish (200 cm^2) and expanded.

Construction of Chimeric cDNAs—It was not possible to use known restriction sites to generate the four chimeric receptors. For that reason the chimeras were constructed using a modification of the overlap extension technique (42). Three polymerase chain reactions (PCRs) and four different oligonucleotides were required for each chimeric receptor. The following oligonucleotides were used, where the underlined portion denotes the indicated type I or type II TGF-β receptor, the lower case a unique restriction site plus four nucleotides, and the rest the appropriate GM-CSF α or β receptor. For α (essential ligand binding domain of the GM-CSF receptor and cytoplasmic domain of the type I TGF-β receptor): 1) gatagtcgacgtctttctttcttcttctgaccag; 2) gataaattctcatatgatctgtctgtgtctgatcag; 3) gataagacatagttagagttgctggcccagggccgctgctctcc; 4) gtaagaaatctatctatctatctatctatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatatactatat...
Chimeric TGF-β Receptor Signaling

Figure 1. Schematic of general strategy. A, PCR splicing reaction results in four chimeric cDNAs. The human GM-CSF receptor subunit (α or β) ligand binding domains (LB) were fused to the human TGF-β receptor (type I or type II) transmembrane (TM) and cytoplasmic (C) domain. These cDNAs were subcloned into the mammalian expression plasmids pNa and pHa. α1 and β2 refer to chimeric receptors expressing the ligand binding domain of the GM-CSF receptor α chain fused to the transmembrane and cytoplasmic domain of the TGF-β type I receptor and the ligand binding domain of the GM-CSF β chain fused to the transmembrane and cytoplasmic domain of the TGF-β type II receptor, respectively. Plasmids were then transfected into AKR-2B cells and clones stably expressing α and β pairs of chimeric receptors selected. This result in the isolation of two heteromeric families (α1β1 and α1β2) and two homomeric (α1β1 and α1β2) domain families. B, addition of ligand (GM-CSF) and high affinity binding results in the dimerization of the chimeric receptors. It is then determined whether GM-CSF induces similar biological responses as stimulation of parallel plates with TGF-β.

**Results**

General Strategy—As discussed in the introduction, the receptor complex responsible for TGF-β-dependent signaling is presently unclear. As a means to address the two receptor paradigms concerning TGF-β signaling, the chimeric receptors depicted in Fig. 1A were generated (described under “Experimental Procedures”). This system takes advantage of the plasticity inherent in many plasma membrane receptors (47, 48) and results in the fusion of the extracellular ligand binding domain of the GM-CSF α or β receptor with the transmembrane and cytoplasmic domain of the type I or type II TGF-β receptor. The two α receptor constructs were placed in the expression plasmid pNa and the two β receptors in pHa. Since high affinity GM-CSF binding only occurs through the generation of α/β heterodimers (37, 38), depending upon which TGF-β receptor domain is fused to the α or β GM-CSF subunit, essentially all the intracellular signaling interactions will consist of heteromeric (i.e. type I/type II) or homomeric (i.e. type I/type I or type II/type II) cytoplasmic TGF-β receptor combinations. The possible chimeric receptor combinations generated after addition of GM-CSF to four independent cellular clones are shown in Fig. 1B.

Chimeric Receptor Expression—After construction of the chimeric receptor cDNAs it was necessary to first confirm that the cDNAs encoded proteins of the expected mass and immunolog-
Although the results from Fig. 2 indicated high affinity GM-CSF binding through αβ heterodimers, to further confirm membrane expression of both GM-CSF receptor subunits the experiment shown in Fig. 3 was performed. Parental AKR-2B cells or each of the six indicated transfectants were stained with monoclonal antibodies specific to the human GM-CSF α or β receptor followed by fluorescein isothiocyanate-tagged secondary antibody or secondary antibody alone (control). Unfixed cells were stained with propidium iodide to exclude dead cells and/or any intracellular receptor staining. FACS analysis showed no specific binding of either the GM-CSF α or β antibody to the parental AKR-2B cells and roughly similar receptor numbers for five of the clones, with one clone (A122) showing slightly higher receptor numbers (Fig. 3, A and B).

Chimeric Receptor Heteromers Regulate TGF-β-dependent Transient Gene Expression—The data in Figs. 2 and 3 documented cell surface expression for each of the chimeric receptor groups. These results indicated that the selected clones were suitable candidates for examining the signaling capabilities of both TGF-β receptor heteromers and homomers. To initially address this question, we investigated whether any of the chimeric receptor expressing clones were able to stimulate luciferase activity from the 3TP-Lux reporter construct. This plasmid has been previously shown to respond to TGF-β receptor activation in a variety of cell types (18). The results from these studies are shown in Fig. 4. While GM-CSF stimulated luciferase expression 20–25-fold in both the chimeric intracellular TGF-β receptor heteromer expressing A105 and A110 clones (Fig. 4A), neither the type I/type I (A120 and A137) nor type II/type II homomers (A122 and A139) TGF-β receptor homomer expressing clones responded to GM-CSF over background levels (Fig. 4B). The inability of the αβ or αβ homomers to respond to GM-CSF is not simply a reflection of a general signaling defect in these four clones. For instance, the internal control of stimulating each homomeric clone with TGF-β (i.e. activating the endogenous TGF-β receptors) resulted in a 20–25-fold increase in luciferase activity similar to that observed in the parental AKR-2B cells and the A105 and A110 heteromers (Fig. 4, A and B).

Regulation of Endogenous PAI-1 Protein Expression by Chimeric Receptor Heteromers—As discussed earlier, one major advantage to this particular system is the ability to examine endogenous gene activity regulated through activation of the TGF-β receptor. Although a number of activities have been shown in AKR-2B cells to be controlled by TGF-β (50–53), we first determined whether the response seen in the transient PAI-1 reporter assay (Fig. 4) was similarly reflected in endogenous PAI-1 protein synthesis. As shown in Fig. 5, while all the tested cell types induced PAI-1 synthesis after TGF-β stimulation, only the heteromeric A105 and A110 chimeric receptor expressing clones showed increased endogenous PAI-1 protein with GM-CSF treatment. The inability of GM-CSF to induce PAI-1 protein in the chimeric receptor homomers is not just a reflection of differences in ligand binding, since concentrations of GM-CSF from 0.1 to 250 ng/ml were also without effect (data not shown).

Typically, receptor ligand interactions produce responses that vary with the ligand concentration. If the chimeric receptors were functioning similar to the endogenous TGF-β receptor, one would expect the response to GM-CSF to be dose-dependent. This was directly shown in Fig. 6, where the ability of various GM-CSF concentrations to induce endogenous PAI-1 protein synthesis in the heteromeric clone A105 (αββ) was compared with the cellular response to TGF-β stimulation. PAI-1 protein expression is shown in Fig. 6A and the quantitation of that data is reported in Fig. 6B. The results show that...
while GM-CSF and TGF-β both induced PAI-1 synthesis in a dose-dependent manner, the A105 cells were approximately 3–5-fold more responsive to GM-CSF and actually showed a decrease in PAI-1 expression at the highest GM-CSF concentration.

Chimeric Receptor Heteromers Modulate Cellular Growth Responses—The previous data (Figs. 4–6) show that GM-CSF stimulation of chimeric receptor heteromers recapitulates TGF-β receptor activation of PAI-1 expression in transient and endogenous assays. Although it was first necessary to document whether the chimeric receptors responded similarly as the normal TGF-β receptor complex in relatively short term gene expression studies, a more demanding test of this system would be to examine whether GM-CSF and TGF-β induced similar biological effects on the chimeric clones. To address this question, we investigated the ability of the chimeric receptors to modulate both the morphological transformation and growth in soft agar seen after addition of TGF-β to mesenchymal AKR-2B cells (54, 55). These results are shown in Figs. 7 and 8.

As shown in Fig. 7A and B, addition of TGF-β to quiescent cultures results in a dramatic change in the cellular morphology over 48 h. However, while GM-CSF treatment induces a similar phenotype in the heteromeric A105 and A110 clones (Fig. 7A), it has no effect on either the parental AKR-2B cells (Fig. 7A) or any of the homomeric clones (Fig. 7B).

TGF-β was originally identified by its ability to stimulate anchorage-dependent mesenchymal cells to grow in an anchorage-independent manner (56). The ability of anchorage-dependent cells to form colonies in soft agar is still one of the best in vitro correlates with tumorigenicity. Since there has been controversy over the role of TGF-β receptor complex regulation of gene expression and effects on growth (1, 19), it was next determined whether similar chimeric receptor interactions that controlled PAI-1 expression (Figs. 4–6) and morphological transformation (Fig. 7) would regulate colony growth in soft agar. In agreement with the previous data (Figs. 4–7), only the chimeric receptors expressing A105 and A110 cultures are capable of GM-CSF-dependent growth in soft agar. Neither the parental AKR-2B cells nor the chimeric type I or type II homomers show any GM-CSF response over control treatment (Fig. 8A and B). However, all tested cultures show soft agar growth after addition of TGF-β. This differential growth response to GM-CSF in soft agar is not reflected in an alteration in other growth characteristics. For instance, all of the chimeric receptor expressing clones show density-dependent growth arrest, a similar serum dependence as the parental AKR-2B cells for growth in monolayer, and a 12–14-h minimum G1 transit time prior to S phase commitment (data not shown). Thus, while similar TGF-β-dependent receptor activity is observed in AKR-2B cells after activation of different receptor complexes, these receptors appear to activate different intracellular signaling pathways in these cells. Although their extracellular ligand binding domains are only about 15% identical, the chimeric receptor expressing clones showed a similar TGF-β-dependent growth response in soft agar, indicating that the intracellular signaling pathways activated by these receptors are similar.

DISCUSSION

A large amount of data concerning TGF-β action have been generated after cloning of the primary TGF-β receptor species seen on most cell types. These receptor kinases have approximately 40% amino acid sequence identity in their cytoplasmic kinase domains. Although their extracellular ligand binding domains are only about 15% identical, the chimeric receptor expressing clones showed a similar TGF-β-dependent growth response in soft agar, indicating that the intracellular signaling pathways activated by these receptors are similar.
level, a 9-amino-acid cysteine box and conserved cysteine residues are found (5). A characteristic of the type I receptor is the presence of a series of tandem Ser/Gly residues referred to as a type I box or GS domain preceding the kinase domain. The GS domain has recently been shown to be a site for type II receptor phosphorylation (19, 57, 58).

As discussed previously, there are two general models to explain the manner by which TGF-β binding results in receptor activation (1, 19). There is a large amount of experimental evidence in support of both models (1, 12, 15, 17, 19, 33). Since these models contain paradigms that are mutually exclusive (i.e. ligand binding to type I receptor in absence of type II receptor and homomeric receptor signaling), the difficulty in resolving these questions using the experimental approaches currently used (see below) is emphasized. Considering the fundamental role played by TGF-β in cellular proliferation and
differentiation, an understanding of TGF-β signaling is of paramount importance for the development of appropriate intervention strategies.

We have addressed this general question relating to TGF-β signaling by generating chimeric receptors consisting of the extracellular ligand binding domain of the GM-CSF α or β receptor fused to the transmembrane and cytoplasmic domains of the type I or type II TGF-β receptors (Fig. 1). The absence of endogenous GM-CSF receptors and ability to isolate stable cell lines provides the means to determine whether specific cellular responses are dependent upon activation of type I/type I homomers, type II/type II homomers, type I/type II heteromers, and/or higher order complexes. In that regard, recent studies have suggested that the receptor complex may be a heterotetramer consisting of two molecules of both the type I and type II receptors (59–62). Although the present results do not directly address the oligomeric status of the chimeric receptor signaling complex, since both the type I2 and type II (30, 31) TGF-β receptors are capable of forming homo-oligomers, homo- or heterotetrameric receptor complexes or interactions with endogenous TGF-β receptors would be equally likely to form in each of the chimeric receptors expressing AKR-2B clones. To that end, preliminary data indicate that chimeric heteromers are signaling-competent when expressed in COS cells lacking endogenous TGF-β receptors and that activation of kinase dead type I or type II chimeric heteromers does not act in a dominant negative fashion (data not shown).

A number of stable AKR-2B cell lines capable of binding labeled GM-CSF were isolated after transfection with the chimeric receptors (Fig. 2). While no appreciable differences in ligand binding were observed dependent upon the transmembrane and cytoplasmic domains of the particular TGF-β receptors fused to the extracellular domain of the GM-CSF receptor, high affinity binding required expression of both the GM-CSF receptor α and β subunits (Fig. 2 and data not shown). To make subsequent analyses manageable, two clones from each group were chosen representing potential TGF-β receptor cytoplasmic interactions. Prior to performing functional studies, FACS analysis documented that each of the selected clones showed

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**Fig. 7.** Morphological transformation induced by GM-CSF or TGF-β. A, parental AKR-2B cells and clones A105 (αIβII) or A110 (αIIβI) were grown to confluence and serum-arrested as described under "Experimental Procedures." Fresh serum-free MCDB 402 medium was added alone or supplemented with 10 ng/ml GM-CSF or 10 ng/ml TGF-β2 for 48 h at 37 °C. Representative areas of the culture were photographed at 20× phase contrast. B, identical studies as described in A were performed on the chimeric receptor expressing clones A120 (αIβII), A137 (αIβII), A122 (αIIβII), and A139 (αIIβI).

**Fig. 8.** Anchorage-independent growth in soft agar stimulated by GM-CSF or TGF-β. A, parental AKR-2B cells and heteromeric clones A105 (αIβII) or A110 (αIIβI) were plated at 1 x 10^4 cells/ml in 0.4% agarose containing 10% FBS alone or supplemented with 10 ng/ml GM-CSF or 10 ng/ml TGF-β2 as described under "Experimental Procedures." Following 10 days' growth at 37 °C the plates were photographed at 10× bright field. B, identical studies as described in A were performed on the homomeric receptor expressing clones A120 (αIβI), A137 (αIβII), A122 (αIIβII), and A139 (αIIβI). The data are representative of three separate experiments, each performed in triplicate.

\(^2\) J. Wrana, personal communication.
appropriate membrane expression of the transfected receptors (Fig. 3). In agreement with the semiquantitative FACS analysis, preliminary saturation binding studies and Scatchard analysis indicate similar receptor affinities for all of the done, with one done (A122) showing higher receptor numbers (data not shown).

A major limitation of previous studies investigating the TGF-β receptor complex(es) mediating receptor signaling was an inability to conclusively document by cross-linking analysis that a small subpopulation of receptors (either heteromers or homomers) was not responsible for subsequent activities. In that regard, the system described in this paper avoids that potential caveat and is suited to determine whether TGF-β receptor homomers consisting of either the type I or type II TGF-β receptors have intrinsic signaling capabilities after ligand binding. Moreover, this analysis is not limited to a subset of transient responses but can be extended to endogenous cellular signaling as well as long term biological activities dependent upon TGF-β receptor activation. It was first determined whether the chimeric receptors were signaling-competent by examining transient luciferase activity regulated by TGF-β-dependent enhancer elements in the 3TP-Lux vector (Fig. 4). While addition of TGF-β to all the chimeric receptor-expressing lines resulted in a 20–25-fold increase in luciferase activity over basal levels, only the heteromeric A105 (αIIβ1) and A110 (αIIβ1δlll) lines similarly responded to GM-CSF stimulation. An identical response (i.e. activation of intracellular TGF-β receptor heteromers and an absence of signaling from intracellular TGF-β receptor homomers) was seen when endogenous PAI-1 protein synthesis was examined after ligand binding to the chimeric receptors (Fig. 5). Moreover, similar heteromer-dependent signaling is observed if endogenous fibronectin protein synthesis is assayed (data not shown). Since the chimeric heteromers are active regardless of the backbone by which the type I or type II TGF-β receptors are fused (i.e. αIIβ1 or αIIβ1δlll) and TGF-β can activate the endogenous TGF-β receptors in the homomeric clones, these results support and are consistent with the hypothesis that homeric TGF-β receptors are unable to propagate a signal after ligand-induced receptor dimerization. Alternatively, it is possible that previously observed differences between TGF-β receptors could be due to different proportions of the subunits in the active receptor complex not replicated with the GM-CSF/TGF-β chimeras. In that regard, whether a similar response would be seen in cell types previously reported to be responsive to ligand-activated type I receptor homomers is not known and is presently under investigation.

Although the results shown in Figs. 4–6 documented that the chimeric receptor system could reconstitute (at least some) TGF-β signaling activities, if this system was reflecting the cellular response to TGF-β receptor activation then it should also induce biological responses similar to the endogenous TGF-β receptor. This was directly shown in Figs. 7 and 8, where both the morphological transformation induced by TGF-β over 24–48 h as well as the colony formation in soft agar over 7–10 days were observed to be similarly regulated by GM-CSF activation of chimeric heteromers. Again, neither the type I nor type II chimeric receptor homomers showed any activity after GM-CSF binding.

The present findings add several new ideas to understanding TGF-β receptor action including (i) expanding the receptor signaling paradigm into a mesenchymal cell model equally important to TGF-β biology; (ii) examining long-term biological responses not directly addressable in transient systems; (iii) showing that TGF-β signaling can be reconstituted in a system completely independent of TGF-β ligand; (iv) defining a cellular system useful in cultures containing intact TGF-β signaling pathways thus avoiding potential complications of unknown mutations resulting from chemical mutagens; (v) documenting that the extracellular ligand binding domains of the type I or type II TGF-β receptors are not necessary for subsequent signaling; and (vi) describing a mechanism for studying TGF-β or other growth factor-dependent signaling activities in systems (in vitro or in vivo) not amenable to other methods and/or requiring receptor dimerization. Last, we believe that the present system provides compelling evidence that in ARK-2B cells both transient as well as endogenous cellular responses to TGF-β receptor activation require heteromer formation and that TGF-β receptor homomers are signaling-inactive.

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