Differential Requirement for Type I and Type II Transforming Growth Factor β Receptor Kinase Activity in Ligand-mediated Receptor Endocytosis*

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Transforming growth factor β (TGFβ) superfamily polypeptides regulate cell growth and differentiation by binding to single pass serine/threonine kinases referred to as TGFβ type I and type II receptors. Signal propagation is dependent upon heteromeric (type I-type II) complex formation and transphosphorylation of the type I receptor by the type II receptor. While many of the phosphorylation events necessary for receptor signaling have recently been characterized, the role of TGFβ receptor kinase activity in modulating receptor endocytosis has not been addressed. To that end, we have used chimeric receptors consisting of the extracellular domain of the granulocyte/macrophage colony-stimulating factor α and β receptors spliced to the TGFβ type I and type II transmembrane and cytoplasmic domains to address the specific role of type I and/or type II receptor kinase activity in TGFβ receptor internalization, down-regulation, and signaling. To inactivate chimeric receptor kinase activity, point mutations in the ATP binding site were made at amino acids 232 and 277 in the type I and type II receptor, respectively. Either of these mutations abolished plasminogen activator inhibitor 1 protein expression stimulated by granulocyte/macrophage colony-stimulating factor activation of chimeric heteromeric type I-type II TGFβ receptors. They did not, however, modulate TGFβ signaling stimulated through the endogenous TGFβ receptor. Although TGFβ receptor signaling was dependent upon the kinase activity of both chimeric receptors, the initial endocytic response was distinctly regulated by type I and type II receptor kinase activity. For instance, while heteromeric receptor complexes containing a kinase-inactive type I receptor were endocytosed similarly to wild type complexes, the kinase activity of the type II TGFβ receptor was necessary for optimal internalization and receptor down-regulation. Furthermore, these responses were shown to occur independently of type II receptor auto-phosphorylation but require a type II receptor capable of transphosphorylation.

The transforming growth factor β (TGFβ)1 superfamily of proteins regulate a number of diverse biologic processes (1–3). While the cellular response can be as distinct as growth stimulation or growth inhibition, it appears as though a similar receptor system is utilized for both pathways. Understanding how the receptors are regulated for one family of proteins will ultimately extend the knowledge for the entire superfamily. The model most commonly accepted for receptor activation requires oligomerization of a type I and type II TGFβ receptor (4–7). This occurs through ligand binding to a type II receptor and recruitment of a type I receptor into a dimeric and/or tetrameric complex (7–11). The serine/threonine kinase activity of the type I receptor is then activated by specific type II receptor phosphorylations in the juxtamembrane region of the type I receptor (12–16). This cascade of receptor interactions and phosphorylations ultimately results in the propagation of the TGFβ signal to downstream effectors in which the Smad family of proteins has a fundamental role (17–24).

Although a great deal of information has been generated documenting potential receptor interactions required for TGFβ signaling, the endocytic fate of the receptor-ligand complex is essentially unexplored. Typically, once growth factor receptors bind ligand, they are endocytosed through structures referred to as clathrin-coated pits (25–27). The endocytic process usually requires the intrinsic enzymatic (kinase) activity of the receptor and is mediated via defined elements routinely found in the cytoplasmic domain of the receptor (28–34). Although no canonical sequences have been identified, a structure representing a tight turn conformation has been proposed as a common determinant for an internalization signal (34). While much of our current understanding surrounding growth factor receptor endocytosis derives from studies performed on the epidermal growth factor and insulin receptor tyrosine kinases, relatively little has been done investigating these processes in the TGFβ receptor superfamily. Since the signaling mechanism, intrinsic receptor kinase activity, and biology of the two receptor systems differ, it is unknown whether the paradigms developed for the receptor tyrosine kinases will be operative in the TGFβ serine/threonine receptor family.

TGFβ receptors have been previously reported to undergo down-regulation after ligand binding in some cell types but not in others (6, 35–37). While this might simply represent cell type differences, it is now possible to evaluate these earlier studies in the context that both heteromeric and homomeric TGFβ receptor interactions have been documented on the cell surface (38, 39). For instance, ligand binding to homomeric type II receptor oligomers might result in a distinct endocytic response...
from that observed following activation of signaling competent type I-type II receptor heteromers. In that regard, our recent studies have shown that while type I-type I, type II-type II, or type I-type II TGFβ receptor oligomerization in mesenchymal AKR-2B cells results in the internalization of bound ligand, only signaling-competent type I-type II TGFβ receptor heteromers are down-regulated (40). Although that study demonstrated distinct endocytic responses of heteromeric and homomeric TGFβ receptors, it did not address the potential regulatory role for receptor serine/threonine kinase activity, nor did it determine whether the endocytic and signaling responses were independently regulated.

In the present paper, we have employed chimeric receptors consisting of the granulocyte/macrophage colony-stimulating factor (GM-CSF) α and β receptor ligand binding domain fused to the transmembrane and cytoplasmic domain of kinase-inactive type I and type II TGFβ receptors to examine the role of TGFβ receptor kinase activity in receptor trafficking and signaling. Consistent with previous reports in epithelial cells (7, 8), we find an obligate requirement for both type I and type II TGFβ receptor kinase activity in mediating heteromeric receptor signaling in mesenchymal cells. However, in contrast to that observed for receptor signaling, a differential requirement for receptor kinase activity in modulating the endocytic response of the receptor complex was observed. For instance, while internalization and down-regulation occurred independently of the type I receptor kinase, type II receptor-transphosphorylating activity was needed for optimal endocytosis. Thus, in activating downstream effector molecules, type I receptor phosphorylation (by the type II TGFβ receptor) is similarly required to promote internalization and down-regulation of the TGFβ receptor complex.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human GM-CSF was a generous gift from DNAX Research Institute (Palo Alto, CA) and recombinant human TGFβ1 and TGFβ2 purchased from Austral Biologicals (San Ramon, CA) or R & D Systems (Minneapolis, MN). The pCMV-TβRII HA K to R plasmid was generously provided by J. Wrana (Toronto, Ontario).

Construction of Mutant Receptors—The β I K232R mutation was generated using the Transformer™ site-directed mutagenesis kit version 2 (CLONTECH, Palo Alto, CA). The mutagenic primer was 5′-GAAAGTGTGTTAGATATTCTGTCAGTA-3′, and the selection primer was 5′-TGACTGGTGAGcTCACAACAGT-3′, where the mutagenic site is indicated by lower case. The reaction and the rest of the mutagenesis reaction was verified by automated DNA sequencing. The β I receptor was eluted into the pH expression plasmid through the SalI site (4). To generate the β II K277R mutation, an HpaI/XcmI cassette from pCMV-TβRII HA K to R plasmid was first placed in pCR™III (Invitrogen) and then ligated back into pH in following HpaI and BamHI digestion.

The β II P525L mutation was generated using the QuickChange™ (Stratagene, La Jolla CA) mutagenesis kit. Mutagenic primers were 5′-GGGACACGACCGACGCGCCTG and 5′-GACGGGCTCTAGGACACGGCACACCCTG-3′. Mutant constructs in pCR™III were verified by automatic DNA sequencing and ligated into pH in following XbaI and BamHI digestion.

Cell Culture—AKR-2B cells were maintained in 5% fetal bovine serum (FBS) (Summit, Ft. Collins, CO) supplemented with Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.). Following selection of stable chimeric receptor-expressing clones, cells were cultured in 5% FBS/DMEM containing 100 μg/ml bioactive Geneticin (Life Technologies) and 50 μg/ml bioactive hygromycin (Sigma).

Isolation of Clones—Parental AKR-2B cells were plated at 1 × 105 cells/well in a six-well dish (22 cm2) 24 h prior to transfection. Cells were rinsed with serum-free DMEM and then incubated for 6 h in 2 ml of DMEM with transfection solution consisting of 2–4 μg of expression plasmid DNA and 2 μg/TransIT™ LT2 (PanVera Corp., Madison, WI) in a final volume of 100 μl with Opti-MEM (Life Technologies). Cells recovered for 16 h in 5% FBS/DMEM and then were placed in selective medium (5% FBS/DMEM with 400 μg/ml Geneticin and 150 μg/ml hygromycin B) for 24 h before splitting 1:40 by surface area. 2–3 weeks later, well separated colonies were isolated and expanded.

Fluorescence-activated Cell Sorting (FACS) —Cells were detached in DMEM containing 40 mM EDTA, 20 mM HEPES, pH 7.2. After washing with 5% FBS/DMEM and antibody buffer (PBS supplemented with 2% FBS, 0.02% NaN3, pH 7.4), approximately 5 × 107 cells were incubated with primary monoclonal antibody at 5 μg/ml (anti-human GM-CSF α receptor, Santa Cruz Biotechnology catalog no. SC 458; anti-human GM-CSF β receptor, Santa Cruz catalog no. 457; or control mouse ascities, Sigma catalog no. M-8273) for 1 h at 4 °C with rocking. Cells were washed twice with antibody buffer and incubated with a 1:50 dilution of secondary fluorescein isothiocyanate-conjugated antibody (Sigma catalog no. F-2012). Following a 45-min incubation at 4 °C, the cells were washed; fixed in 500 μl of PBS containing 1% paraformaldehyde, pH 7.4, and filtered through a 40-μm nylon filter prior to flow analysis using a Beckton Dickinson FACS Vantage with PCLYSIS version 1.1 software.

Plasminogen Activator Inhibitor-1 Production—Cells were plated in six-well tissue culture dishes at 2 × 105 cells/well 24 h before treatment. The serum containing medium was removed, and the cultures were placed in 1.0 ml of serum-free DMEM lacking methionine but supplemented with the indicated growth factors. Following a 2-h treatment at 37 °C, wells were pulsed with 2 μl with 50 μCi/ml [35S]Met/Cys Premix (Amersham Pharmacia Biotech) and processed by washing once with PBS; three times with 10 mM Tris, 0.5% deoxycholate, 50 μM phenylmethylsulfonyl fluoride (Sigma), pH 8.0; twice with 2 mM Tris, pH 8.0; and once with PBS (41). Matrix proteins were eluted from the wells by the addition of 100 μl of the 2 × Laemmli buffer containing 10% β-mercaptoethanol, separated by 8% SDS-polyacrylamide gel electrophoresis, and processed for fluorography.

Transient Transfections—Cells were plated in six-well dishes (9.6 cm2) at 1.5 × 105 per well 24 h prior to transfection. Three μg of 3TP-Lux, 0.5 μg of pCMV-β-galactosidase, and 7 μl of TransIT LT2 (Mirus Corp, Madison WI) were combined with Opti-MEM (Life Technologies) to a final volume of 100 μl, and transfection was performed as described (4). Cultures were then stimulated in 5% FBS/DMEM for 24 h in the presence or absence of TGFβ or GM-CSF, and luciferase activity was determined following normalization for transfection efficiency with β-galactosidase.

Internalization Assays—Cells were plated in six-well dishes (9.6 cm2) well at 1.5 × 105 cells/well in 5% FBS/DMEM. Following 24 h at 37 °C, internalization assays were initiated by incubation at 4 °C for 2–4 h in binding buffer (0.3 ml DMEM containing 200 mM HEPES, pH 7.4, 25 mg/ml bovine serum albumin) supplemented with 100 pM 125I-GM-CSF (119 μCi/g; NEN Life Science Products). Once equilibrium had been reached, the plates were washed twice with binding buffer containing 75% horse serum and then placed at 37 °C in 5% FBS/DMEM for various times to promote receptor endocytosis. To calculate the percentage of internalization (i.e. specific cpm in cell/specific surface cpm), the cultures were returned to 4 °C, and the remaining surface-bound ligand was removed by acid washing (PBS, pH 3.0) and the internalized ligand was determined by cell lysis in 0.2 mM NaOH, 40 μg/ml salmon sperm DNA. All time points contained parallel plates with 25-fold excess cold GM-CSF to document specificity of binding.

Potassium depletion of cells was performed essentially as described by Larkin et al. (42) and Sorkin et al. (43). Following an initial 5-min hypotonic shock with DMEM/H2O (1:1) at 37 °C, cultures were incubated for 10 min in buffer A (50 mM HEPES, 100 mM NaCl, pH 7.4) and 30 mM sucrose buffer B (50 mM HEPES, 100 mM NaCl, 1.5% bovine serum albumin, pH 7.4) at 37 °C. Cells were then placed at 4 °C (in buffer B) for 15 min and replaced with buffer B containing 100 pM 125I-GM-CSF. Control cells were treated similarly but did not undergo initial hypotonic shock, and subsequent incubations in buffers A and B contained 10 mM KCl.

Down-regulation Assays—Similar conditions as described above for internalization assays were used to determine receptor down-regulation using the difference being that the cultures were first incubated at 37 °C with 5% FBS/DMEM containing unlabeled GM-CSF (520 pM) for 45 min. The plates were then washed two times with cold 75% horse serum, 25% binding buffer, and specifically bound 125I-GM-CSF was determined. Control studies had shown that acid washing removed 90–95% of receptor-bound ligand without affecting subsequent binding (data not shown).
RESULTS

Chimeric Receptor Expression—Once the chimeric receptor cDNAs were mutated and their sequences were confirmed, they were stably transfected into mouse fibroblast AKR-2B cells in α and β pairs to generate high affinity ligand-dependent heteromeric complex formation. The designation αβII, for example, represents clones expressing chimeric receptors consisting of the ligand binding domain of the GM-CSF α receptor fused to the transmembrane and cytoplasmic domain of the type I TGFβ receptor and the ligand binding domain of the GM-CSF β receptor fused to the transmembrane and cytoplasmic domain of the type II TGFβ receptor (4). The 600 series of clones contain a wild type chimeric type I TGFβ receptor co-expressed with a kinase-inactive chimeric type II TGFβ receptor, and the 700 series contain a wild type chimeric type II TGFβ receptor co-expressed with a kinase-inactive chimeric type I TGFβ receptor. Individual clones were isolated by ring subcloning and initially screened for membrane expression of the chimeric receptors by FACS (Fig. 1). Our previous work has shown that the parental AKR-2B cell line does not show specific staining (Control). FACS analysis is described under “Experimental Procedures.” The shift in fluorescence (filled histogram) relative to the control (open histogram) indicates specific membrane binding of monomeric anti-GM-CSF receptor antibody.

Lack of GM-CSF-induced Signaling in Clones Expressing Kinase-dead Chimeric Receptors—Once expression and ligand binding of the chimeric receptors was confirmed, we wished to determine whether the chimeric kinase-dead receptors responded in a similar fashion to that reported for kinase-inactive endogenous TGFβ receptors (2, 44). This was addressed by examining the ability of the chimeric and endogenous TGFβ receptors to stimulate expression of the extracellular matrix-associated protein plasminogen activator inhibitor-1 (PAI-1). As shown in Fig. 2, clone A105 (expresses wild type chimeric receptors) stimulates PAI-1 production when treated with either GM-CSF or TGFβ (activates chimeric or endogenous TGFβ receptors, respectively). However, cells expressing type I or type II kinase-dead chimeric TGFβ receptors do not induce PAI-1 protein when treated with GM-CSF at either 10 or 100 ng/ml. This does not represent a general signaling defect in the TGFβ pathway(s), since the addition of TGFβ to activate the endogenous TGFβ receptors results in PAI-1 expression similar to that observed in the parental cell line (Fig. 2). In that regard, identical ligand-dependent results are seen when the cultures are examined for their ability to form colonies in soft agar (data not shown).

Transfection of kinase-dead TGFβ receptors has been previously shown to function as dominant/negative mediators of TGFβ action (45–50). This inhibitory activity is presumably a reflection of the requirement for TGFβ receptor oligomerization in signal propagation (4–7). Moreover, the cytoplasmic domains of the type I and type II TGFβ receptors have been shown to form both receptor heteromers and homomers in overexpressing COS cells and yeast two-hybrid screens (38, 51). Fig. 2, however, shows that signaling through endogenous TGFβ receptors occurs independently of the expression of kinase-inactive (signaling-competent) chimeric TGFβ receptors. To address this potential paradox, the studies shown in Fig. 3 were performed. Representative chimeric kinase-inactive type I and type II TGFβ-expressing clones (A706 and A608, respectively) were stimulated with TGFβ, GM-CSF, or TGFβ plus GM-CSF, and the effect on PAI-1 protein secretion was determined. As expected, treatment with GM-CSF at either 10 or 100 ng/ml was unable to activate the kinase-dead chimeric receptors, while the addition of TGFβ stimulated PAI-1 protein expression through endogenous TGFβ receptor signaling. In order to determine whether PAI-1 expression was regulated by
mined. The results show independent TGFβ receptor kinase-inactive type I or type II TGFβ receptor kinase activity but requires a functional type II receptor kinase for optimal internalization. Thus, in either the absence or presence of GM-CSF, kinase-dead chimeric receptors are unable to form a functional association (i.e. act as a dominant/negative) with endogenous TGFβ receptors.

Internalization of Kinase-dead Chimeric Receptors—Previous work from our laboratory has shown that both homomeric and heteromeric TGFβ receptor combinations internalize ligand at similar rates in a clathrin-dependent manner (40). However, the receptor elements or activities regulating this response have not been identified or characterized. Since the kinase activity of the chimeric type I and type II TGFβ receptor is required for signaling (Figs. 2 and 3) and internalization of tyrosine kinase receptor family members is dependent upon receptor kinase activity (31, 52, 53), we wished to determine the role(s) of TGFβ receptor serine/threonine kinase activity in ligand-mediated internalization (Figs. 4, 5, and 8) and down-regulation (Figs. 6 and 9). To control for clonal bias, the kinetics of ligand internalization for two (A700s) or three (A600s) individual clones over 1 h 37 °C incubation is shown (Fig. 4). Although each of the clonal families containing a kinase-dead TGFβ receptor is signaling-incompetent (Figs. 2 and 3), differential effects on ligand internalization are observed. While clones expressing a kinase-inactive type I receptor (in the context of a wild type type II receptor; the A700 family) internalize labeled GM-CSF similar to the wild type A105 clone, expression of a kinase-inactive type II receptor (in the context of a wild type type I receptor; the A600 family), diminishes both the rate and extent of ligand internalization (Fig. 4).

The finding that internalization occurs independently of type I TGFβ receptor kinase activity but requires a functional type II receptor kinase for optimal internalization could reflect a unique endocytic mechanism for each kinase-inactive receptor complex. Since we have previously shown that wild type chimeric TGFβ receptors are internalized through a clathrin-dependent mechanism (40), we next determined whether the internalization of kinase-inactive type I or type II TGFβ receptors occurred through a similar mechanism. As shown in Fig. 5, when clathrin-dependent internalization is inhibited by cytosolic K+ depletion (42, 43), both type I and type II kinase-impaired chimeric TGFβ receptor heteromers are unable to internalize bound ligand. Thus, although the A600 clones show a diminished ability to internalize ligand, this is not a consequence of diverting to an alternative clathrin-independent pathway but reflects an intrinsic role(s) of the type II receptor kinase.

Down-regulation of Cell Surface Binding following Ligand Binding—Fig. 4 shows that the kinase activity of only the type II receptor is required for optimal internalization, yet both the type I and type II receptor kinases are required for signaling (Figs. 2 and 3). These findings indicate that TGFβ receptor endocytosis is not simply a reflection of receptor signaling but is a process controlled by distinct regulatory mechanisms. To address this question further, we next determined whether type I and/or type II TGFβ receptor kinase activity modulated the levels of ligand binding following a preincubation of cells with GM-CSF (i.e. down-regulation). As shown in Fig. 6, receptor down-regulation over 4-h ligand stimulation was determined for multiple clones (identical to the experiment performed in Fig. 4) of wild type (kinase active) chimeric TGFβ receptor heteromers as well as chimeric heteromers consisting of a kinase-dead type I (A700s) or type II (A600s) TGFβ receptor. Similar to what we observed for ligand internalization (Fig. 4), clones expressing a kinase-dead type I chimeric TGFβ receptor (A700s) also down-regulated surface binding as completely as the unmutated wild type heteromers, while clones expressing an inactive kinase in the type II TGFβ receptor (A600s) were impaired in their ability to down-regulate cell surface receptors (Fig. 6). These results support the hypothesis that inactivation of the type II TGFβ receptor has a dominant effect on both internalization and receptor down-regulation. Moreover, the data show that the endocytic response to heteromeric TGFβ receptor complex formation is regulated, at least in part, by the kinase activity of the type II TGFβ receptor.

Type II Receptor Transphosphorylation, but Not Autophosphorylation, Modulates Heteromeric Receptor Endocytosis—The type II TGFβ receptor has both auto- and transphospho-
rhythmic activity (8, 18). Moreover, the only known substrate for the type II receptor kinase is the type I TGFβ receptor. Since the K277R mutation in the ATP binding site would abolish both activities, we wished to determine whether either function could account for the decreased endocytic activity seen in the 600 series clones. To that end, Carcamo et al. (12) have described a type II receptor mutation that has autophosphorylating activity in vitro and in vivo but fails to transphosphorylate an associated type I receptor. When the identical (proline to leucine at amino acid 525) mutation was made in the chimeric type II TGFβ receptor, we also found the heteromeric receptor complex unable to stimulate expression of PAI-1 protein following ligand binding (Fig. 7A). A similar result is observed when luciferase activity is measured from the TGFβ-responsive 3TP-Lux reporter plasmid (Fig. 7B). Although the addition of TGFβ increased luciferase expression 30–40-fold through endogenous TGFβ receptors, stimulation of the mutant P525L chimeric receptor (in the same cell clone) did not increase luciferase activity. Since mutation at amino acid 525 in the type II receptor had a similar effect on chimeric receptor signaling as that reported for the endogenous TGFβ receptor, we next examined whether ligand internalization or receptor down-regulation were also modified. As shown in Figs. 8 and 9, internalization (Fig. 8) and down-regulation (Fig. 9) in the P525L clones were diminished to a similar extent as that observed for the kinase-inactive A600 cultures (compare Figs. 4 and 8 and Figs. 6 and 9). Thus, although TGFβ receptor signaling and endocytosis are distinctly regulated activities (Figs. 2–4 and 6), they are both highly dependent upon the transphosphorylating activity of the type II receptor kinase.

FIG. 5. Clathrin-dependent internalization of kinase-dead receptors. A, internalization of labeled GM-CSF was performed on clone A615 (αIIb K277R) in the absence (–KCl) or presence (+KCl) of potassium as described under “Experimental Procedures.” At the indicated times, surface-bound and internalized ligand was determined. B, similar studies as described in A were performed on clone A708 (αIIb K232R). Results are the average ± S.E. of two independent experiments each performed in duplicate.

FIG. 6. Receptor down-regulation is regulated by the kinase activity of the type II TGFβ receptor. Clones from the A600 (A608, A615, A618; αIIb K277R) (●), A700 (A706, A708; αIIb K232R) (■), or wild type heteromer (A105, αIIbI, A110 αIIbI) (○) transfection groups were treated with 10 ng/ml (520 pM) GM-CSF for the indicated times, and receptor down-regulation was performed. Following acid treatment to remove bound ligand, specific surface binding of radiolabeled GM-CSF (100 pM) was determined after a 2-h incubation at 4 °C in the presence or absence of 25-fold excess unlabeled GM-CSF. Percentage of control binding represents the percentage of zero time-specific binding observed following GM-CSF treatment for the indicated time. Each curve represents the average ± S.E. of two (wild type heteromers and A700s) or three (A600s) independent clones each of which were assayed twice in duplicate (i.e. each data point represents 8 or 12 independent analyses). Analysis of variance and Student’s t test for the 2- and 4-h points show significant differences between the A600s and the wild type heteromers (p values of 0.02 and 0.03 at 2 and 4 h, respectively).

DISCUSSION

Regulated control of the endocytic response constitutes one of the earliest cellular mechanisms for responding to environmental stimuli. Class I receptors, such as the low density lipoprotein receptor are constitutively endocytosed, while class II receptors, such as the epidermal growth factor and insulin receptor undergo ligand-dependent endocytosis. While it is well established that receptor tyrosine kinase activity is required for optimal internalization of full-length receptors (31, 52, 53), this obligate requirement can be partially overcome in truncated receptors (30, 54). These findings suggest that kinase activation removes an inhibitory signal or exposes a motif, which then allows internalization and down-regulation. While this complex relation of kinase activation and receptor endocytosis in tyrosine kinase receptors has been extensively investigated, the relationship between TGFβ receptor activation and endocytosis has not been similarly examined. There are two likely reasons for this: first, quantitative 125I-TGFβ binding studies are compromised by a high degree of nonspecific binding; second, the natural occurrence of both heteromeric and homomeric TGFβ receptor interactions makes any analysis problematic. In that regard, the chimeric system is ideally suited to address both of these concerns (4). For instance, our recent studies have shown that heteromeric and homomeric TGFβ receptor complexes in mesenchymal AKR-2B cells have distinct endocytic fates (40). While these results suggest a requirement for receptor cross-talk, the regulatory role(s) of the type I and/or type II TGFβ receptor kinase in the endocytic process has not been addressed. Since each of these receptor kinases have such distinct roles in TGFβ receptor activation, it is likely that novel paradigms will need to be developed defining the mechanisms whereby phosphorylation regulates TGFβ receptor endocytosis and trafficking.
type II receptors harboring the P525L mutation. A, clones 13 and 22 expressing a wild type chimeric α1 receptor and a chimeric β2 receptor with a mutation of proline 525 to leucine were treated for 4 h in methionine-free DMEM alone (Con) or supplemented with 10 ng/ml GM-CSF (GM) or TGFβ2 (TGF β). The extracellular matrix-associated PAI-1 protein was analyzed as described under “Experimental Procedures.” B, wild type heteromeric clone A105 or P525L clones 13 and 22 were transiently transfected with the 3TP-Lux reporter as described previously (4). Following recovery, arrested cells were stimulated for 24 h in 5% FBS/DMEM alone (clear bar), 5% FBS/DMEM supplemented with 10 ng/ml GM-CSF (gray bar), or 10 ng/ml TGFβ2 (black bar). Luciferase activity was then determined on normalized samples. The data are depicted as the fold increase in luciferase activity relative to the mean of the control-treated 525 clones and represent the mean ± S.D. of two separate experiments for each clone done in duplicate.

To initially address the question of whether TGFβ receptor kinase activity is linked to internalization and down-regulation, cell lines were generated stably expressing chimeric receptors containing mutations in the putative ATP binding site for both the type I and type II receptors (55, 56). Lysine to arginine mutations at position 232 in the type I receptor and 277 in the type II receptor were engineered into the chimeric receptors and transfected into parental AKR-2B fibroblasts in the heteromeric combinations α1IP II K277R and α1IP II K232R. Once stable clones expressing the receptor combinations were isolated (Fig. 1), each clone’s ability to signal TGFβ-dependent activity was assessed. As shown in Figs. 2 and 3, each of the clones expressing the kinase-inactive receptors did not induce secretion of PAI-1 protein in response to GM-CSF treatment. This was not due to a general lesion in the TGFβ signaling pathway, since activation of the endogenous TGFβ receptors resulted in PAI-1 protein expression. A similar response for all the clones was seen when the ability to form colonies in soft agar was measured (data not shown).

It has been previously shown that kinase-inactive TGFβ receptors can function as dominant/negatives to inactivate TGFβ signaling both in vitro and in vivo (45–50). In addition, both heteromeric and homomeric complexes of the type I and type II TGFβ receptor cytoplasmic domains have been observed in yeast two-hybrid screens and overexpressing COS cells (38, 51). Since we did not observe functional association between the kinase-inactive chimeric receptors and the endogenous TGFβ receptors (i.e. inactivation of TGFβ-dependent signaling) (Figs. 2 and 3), this suggested that either the level of chimeric receptor expression was not great enough for inhibition or the chimeric and endogenous TGFβ receptors associated into separate signaling complexes. In support of the latter possibility, ligand binding to the endogenous TGFβ receptors does not result in the heterologous down-regulation of the chimeric receptors (data not shown). Moreover, when both receptor families are activated by simultaneous treatment with GM-CSF and TGFβ (Fig. 3), the oligomerization state of one family does not affect the signaling activity of the other complex. Although these studies do not directly document the specific receptor interactions formed, they are consistent with the hypothesis, recently proposed by Luo and Lodish (5), that the TGFβ receptor associations formed in vivo are linked through interactions between their extracellular (not cytoplasmic) domains. Since the chimeric and endogenous receptors only share transmembrane and cytoplasmic domains, there would be no association (i.e. no dominant/negative effect) regardless of whether the receptor families were activated by ligand.

Mutation of the intrinsic kinase activity of the tyrosine kinase family of receptors abolishes their ability to internalize radiolabeled ligand (30, 31). When similar studies were per-
formed on the chimeric receptors containing a kinase-inactive type I or type II TGFβ receptor, differential effects on ligand internalization were observed (Fig. 4). While internalization was unaffected by the absence of a functional type I receptor kinase (A700 clones), cultures expressing a kinase-inactive type II TGFβ receptor (A600 clones) showed a diminished rate and extent of internalization. Although the underlying mechanism(s) regulating this response is currently unknown, it has been postulated that phosphorylation by receptor tyrosine kinases induces conformational changes necessary for revealing internalization motifs (30, 34); perhaps similarly acting elements are exposed in the type I TGFβ receptor following activation by the type II receptor kinase.

Ligand internalization is routinely followed by a decrease in cell surface receptor binding referred to as down-regulation. As shown in Fig. 6, treatment of heterologous wild type chimeric TGFβ receptors with GM-CSF results in a 60–80% down-regulation of surface binding by 2–4 h. Consistent with that observed for the internalization studies in Fig. 4, inactivation of the type I TGFβ receptor kinase (A700s, aβK232R) did not affect receptor down-regulation. Since type I receptor kinase activity is required for cellular signaling (Figs. 2 and 3 and data not shown), yet no effect is observed on heterologic TGFβ receptor internalization and down-regulation in the absence of a functional type I receptor kinase (Figs. 4 and 6), this supports the hypothesis that receptor endocytosis is not dependent upon, or the result of, TGFβ receptor signaling. However, in contrast to that observed in the A700 clones, mutation of the type II TGFβ receptor kinase (A600s, aβK277R) decreased both internalization and receptor down-regulation by approximately 50% (Figs. 4 and 6). While these data show a primary regulatory role for the type II TGFβ receptor kinase, it is of interest that the A600 clones have residual endocytotic activity. This suggests involvement of other receptor elements, substrates, and/or receptor interactions in addition to kinase activity in regulating TGFβ receptor endocytosis.

The signaling activity of the type II TGFβ receptor has been recently shown to be both positively and negatively regulated by various phosphorylations (18, 57, 58). In addition to these autophosphorylations, the type II receptor associates with, transphosphorylates, and activates the type I receptor. No other kinase has been shown to phosphorylate the type I receptor in vivo, nor have other substrates been reported for the type II receptor. Since Figs. 4 and 6 demonstrated that the endocytic response of the TGFβ receptor complex was dependent upon the type II receptor kinase, we next wished to determine whether this was a reflection of either the auto- or transphosphorylating activity of the type II receptor. To address this question, a proline to leucine mutation at amino acid 525 was made in the chimeric type II receptor, which had been previously shown to abolish type II receptor transphosphorylating activity but have no effect on autophosphorylation (12). When the endocytic response of these clones was examined, ligand internalization and receptor down-regulation was affected similarly to that seen in cultures containing a kinase-inactive type II receptor (compare Figs. 4 and 6 with Figs. 8 and 9, respectively). The data are consistent with a model whereby phosphorylation of the type I receptor (or some other substrate) by the type II TGFβ receptor is necessary for efficient receptor down-regulation and trafficking. Direct test of this model will include 1) identifying the particular site(s) in the type I receptor phosphorylated by the type II receptor necessary for appropriate receptor cross-talk and/or 2) determining whether a substrate (in addition to the type I TGFβ receptor) for the type II receptor kinase is required for endocytosis similar to that proposed for the epidermal growth factor receptor (31).
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