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Distinct Endocytic Responses of Heteromeric and Homomeric Transforming Growth Factor β Receptors

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Transforming growth factor β (TGFβ) family ligands initiate a cascade of events capable of modulating cellular growth and differentiation. The receptors responsible for transducing these cellular signals are referred to as the type I and type II TGFβ receptors. Ligand binding to the type II receptor results in the transphosphorylation and activation of the type I receptor. This heteromeric complex then propagates the signal(s) to downstream effectors. There is presently little data concerning the fate of TGFβ receptors after ligand binding, with conflicting reports indicating no change or decreasing cell surface receptor numbers. To address the fate of ligand-activated receptors, we have used our previously characterized chimeric receptors consisting of the ligand binding domain from the granulocyte/macrophage colony-stimulating factor α or β receptor fused to the transmembrane and cytoplasmic domain of the type I or type II TGFβ receptor. This system not only provides the necessary sensitivity and specificity to address these types of questions but also permits the differentiation of endocytic responses to either homomeric or heteromeric intracellular TGFβ receptor oligomerization. Data are presented that show, within minutes of ligand binding, chimeric TGFβ receptors are internalized. However, although all the chimeric receptor combinations show similar internalization rates, receptor down-regulation occurs only after activation of heteromeric TGFβ receptors. These results indicate that effective receptor down-regulation requires cross-talk between the type I and type II TGFβ receptors and that TGFβ receptor heteromers and homomers show distinct trafficking behavior.

INTRODUCTION

The transforming growth factor β (TGFβ) superfamily of polypeptides are involved in many processes regulating cellular growth and development (Massagué *et al.*, 1990). These proteins bind to a novel superfamily of single-pass transmembrane serine/threonine kinase receptors (Massagué *et al.*, 1992; Kingsley 1994; ten Dijke *et al.*, 1994). Nearly all mammalian cells express three TGFβ receptor species referred to as the type I, type II, and type III receptor (Massagué *et al.*, 1990). Recently, the receptor interactions necessary for TGFβ signaling have been defined (Wrana *et al.*, 1994; Anders and Leof, 1996; Luo and Lodish, 1996). The type II receptor is a constitutively active kinase capable of binding free ligand and recruiting the type I receptor into an oligoheteromeric (di- and/or tetramers) complex (Wrana *et al.*, 1992, 1994; Yamashita *et al.*, 1994; Anders and Leof, 1996; Luo and Lodish, 1996; Weis-Garcia and Massagué, 1996). Phosphorylation of the type I receptor by the type II initiates a signaling cascade(s) to downstream effectors of which the Smad family of proteins may play a fundamental role (Lagna *et al.*, 1996; Lechleider *et al.*, 1996; Macias-Silva *et al.*, 1996; Massagué, 1996; Yingling *et al.*, 1996; Nakao *et al.*, 1997).

The fate of the TGFβ receptor/ligand complex after receptor transphosphorylation is unclear. Earlier studies were inconclusive and suggested that TGFβ receptors either did not undergo significant down-regulation (Massagué, 1985; Wakefield *et al.*, 1987) or showed an approximate 50% decrease in TGFβ surface binding

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by 2 h (Frolik *et al.*, 1984). At first glance it is difficult to understand why such important basic information would be lacking in a growth factor/receptor system with such a fundamental role in growth and development. The reasons for this are probably twofold. First, quantitative ^{125}I-labeled TGFβ binding assays are extremely difficult due to the high amount of nonspecific binding usually observed and although cross-linking studies are capable of identifying which TGFβ binding species are present, they are inadequate for quantitation. Secondly, the natural occurrence of both TGFβ receptor heteromers and homomers would make any analysis problematic (Chen and Derynck, 1994; Henis *et al.*, 1994). For instance, because type II receptors have been shown to homodimerize and heterodimerize in the presence and absence of ligand (Henis *et al.*, 1994), there may be distinct endocytic responses for each of the receptor combinations.

In the present study, we wished to determine 1) whether signaling-competent heteromeric type I/type II TGFβ receptors were internalized and down-regulated; 2) the associated mechanism by which any endocytic event(s) was regulated; and 3) whether a similar response was observed for signaling-incompetent homomeric type I/type I or type II/type II TGFβ receptors. To address these questions, we have used a cellular system capable of specifically and independently examining heteromeric or homomeric TGFβ receptor interactions. Stable cell lines have been previously characterized expressing chimeric TGFβ receptors that have the extracellular ligand binding 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 TGFβ receptor (Anders and Leof, 1996). Because high-affinity ligand binding requires dimerization of the GM-CSF α and β subunits (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991; Shanafelt *et al.*, 1991), the endocytic response of defined heteromeric or homomeric TGFβ receptor interactions can be examined (Anders and Leof, 1996; Muramatsu *et al.*, 1997).

The data presented in this manuscript show that internalization per se is not sufficient for cellular signaling because both heteromeric and homomeric TGFβ receptor combinations are internalized. However, although both resulting receptor complexes are internalized, only signaling-competent heteromeric type I/type II TGFβ receptor complexes undergo ligand-mediated receptor down-regulation.

MATERIALS AND METHODS

Materials

Recombinant human GM-CSF was a generous gift from DNAX Research Institute (Palo Alto, CA), and ^{125}I-labeled recombinant human GM-CSF and epidermal growth factor (EGF) were purchased from DuPont/NEN (Boston, MA). To document activation of endogenous TGFβ receptors, recombinant human TGFβ1 or TGFβ2 was purchased from Austral Biologicals (San Ramon, CA) or R & D Systems (Minneapolis, MN).

Cell Culture

Parental AKR-2B cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS; Summit, Ft. Collins, CO). Chimeric receptor expressing clones were grown in 5% FBS/DMEM containing 100 μg/ml bioactive Geneticin (Life Technologies) and 50 μg/ml Hygromycin B (Sigma, St. Louis, MO). The designation αIβII (i.e., clone A105) represents a ligand-activated heteromeric receptor interaction consisting of the extracellular ligand binding domain of the GM-CSF α receptor fused to the transmembrane and cytoplasmic domain of the type I TGFβ receptor (i.e., αI), and the extracellular ligand binding domain of the GM-CSF β receptor fused to the transmembrane and cytoplasmic domain of the type II TGFβ receptor (i.e., βII). A similar designation is used for homomeric TGFβ receptor interactions seen with clones A120 (i.e., αIβI) and A122 (i.e., αIIβII). For instance, αIβI would indicate ligand binding to the GM-CSF α and β receptor extracellular domains resulting in homomerization of type I TGFβ receptor transmembrane and cytoplasmic domains and αIIβII would represent homomeric interactions of the type II TGFβ receptor (Anders and Leof, 1996).

Internalization Assays

Cultures were plated at 1×10^5 cells/well in six-well dishes (9.6 cm^2/well) for 24 h prior to the experiment in 5% FBS/DMEM. Ligand binding was performed at 4°C in binding buffer [DMEM containing 200 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 7.4, 25 mg/ml bovine serum albumin (BSA)] supplemented with 100 pM ^{125}I-labeled GM-CSF (119 μCi/μg) in the presence or absence of a 25-fold molar excess of unlabeled GM-CSF to document specific binding (routinely 70–90% of total binding). Once equilibrium had been reached (2–4 h), the plates were washed and placed at 37°C for the indicated times to promote receptor endocytosis. Percent internalization (i.e., specific cpm in cell/specific surface cpm) was determined after acid washing (PBS, pH 3.0; two 3-min washes) to remove surface bound ligand and cell lysis in 0.2 M NaOH and 40 μg/ml salmon sperm DNA. EGF internalization was measured as described for GM-CSF except the binding buffer was 20 mM HEPES, 0.1% BSA, and DMEM, pH 7.4, and the acid stripping solution was 50 mM acetic acid, 135 mM NaCl, and 2.5 mM KCl. ^{125}I-labeled EGF binding was measured at 150 pM and specific binding was determined by incubation with a 200-fold excess of unlabeled EGF.

Down-Regulation Assays

To determine the effect of ligand on receptor down-regulation, cultures were incubated at 37°C with unlabeled GM-CSF (520 pM or 10 ng/ml) for the indicated times. Surface-bound ligand was removed by acid washing (PBS, pH 3.0) and remaining cell surface receptor binding was determined by incubation at 4°C for 2–4 h with 100 pM ^{125}I-labeled GM-CSF. The plates were washed twice with ice-cold binding buffer, containing 75% horse serum (Life Technologies) and specifically bound ^{125}I-labeled GM-CSF was determined. Control studies have shown that acid washing removes 90–95% receptor-bound ligand without affecting subsequent ligand binding (our unpublished results). For recovery from down-regulation, cells were incubated for 4 h with unlabeled GM-CSF (520 pM or 10 ng/ml) at 37°C and acid washed with PBS, pH 3.0. Cells were then returned to 37°C in the appropriate test medium for the indicated times and specific ^{125}I-labeled GM-CSF binding was determined after 2–4 h at 4°C as described above.

Cytosolic Acidification

Cells were plated at 1×10^5 cells/well in six-well dishes containing 5% FBS/DMEM and grown for 24 h at 37°C. The medium was replaced with 1.0 ml of buffer 1 (30 mM NH_4Cl, 200 mM HEPES, DMEM, pH 7.2). After a 30-min incubation at 37°C, the cultures were rinsed and incubated in buffer 2 (140 mM KCl, 20 mM HEPES, 2.0 mM $CaCl_2$, 1 mM $MgCl_2$, 1.0 mM amiloride, pH 7.2) at 37°C for an additional 90 min. Cells were then placed at 4°C for 15 min prior to ^{125}I-labeled GM-CSF (100 pM) binding (buffer 2 containing 2.5% BSA) for 2–4 h at 4°C. Ligand internalization was determined as described above. Control cells were treated identically except that NH_4Cl was omitted from the first 30-min incubation and amiloride was not present in subsequent incubations.

Potassium Depletion

Potassium depletion of cells was performed essentially as described by Larkin *et al.* (1983) and Sorkin *et al.* (1995). Cultures initially received a 5-min hypotonic shock with DMEM/H_2O (1:1) at 37°C followed by a 10-min incubation at 37°C in buffer A (50 mM HEPES, 100 mM NaCl, pH 7.4) and a 30-min in buffer B (50 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, 2.5% BSA, pH 7.4). Cells were then placed at 4°C (in buffer B) for 15 min and replaced with buffer B containing 100 pM ^{125}I-labeled GM-CSF. Binding and internalization were measured as described above. Control cells were treated as above but did not undergo initial hypotonic shock, and subsequent incubations in buffers A and B were performed in the presence of 10 mM KCl.

Determination of Bulk Flow Uptake

To ascertain that potassium depletion did not disrupt bulk-phase endocytosis, we used the method described by Cupers *et al.* (1994), with minor modifications. Potassium depletion was as previously described with horseradish peroxidase (HRP; 2 mg/ml) substituted for radiolabeled GM-CSF. HRP was dissolved in buffer B with and without potassium and incubated with the cells on ice for 15 min prior to warming for various times. Plates were warmed to 37°C and at the indicated times internalization was stopped by placing the plates on ice. Cells were then washed as described by Cupers *et al.* (1994), lysed with 0.4 ml of 0.05% Triton X-100 in 10 mM Tris(hydroxymethyl)aminomethane, pH 7.4, and scraped from the wells. Endocytosis of HRP was measured by the colorometric assay using *o*-dianisidine (Sigma) as a substrate (Marsh *et al.*, 1987). Cell lysates (25 μl) were mixed with 0.342 mM *o*-dianisidine in 0.5 M KH_2PO_4, pH 5.0, 0.3% Triton X-100, and 0.006% H_2O_2, and HRP activity measured at 490 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). Standard curves were used to determine the mass of HRP internalized and expressed relative to total BCA protein (Pierce Chemicals, Rockford, IL).

RESULTS

Internalization of Chimeric Receptor Heteromeric and Homomeric Combinations

The fate of TGFβ receptors after ligand-induced association is unclear. To address these general questions relating TGFβ receptor internalization and activation, we have devised a chimeric receptor system capable of distinguishing the contribution of TGFβ receptor homomers, either type I/type I or type II/type II, from heteromeric type I/type II TGFβ receptors (Anders and Leof, 1996; Muramatsu *et al.*, 1997). To study the fate of ligand-bound receptors, internalization assays were performed on the two heteromer clones (A105 and A110) and a representative type I (A120) or type II (A122) receptor homomer expressing clone. Equilibrium ^{125}I-labeled GM-CSF binding was performed at 4°C on each of the clones. The cultures were shifted to 37°C, and at the indicated times, the internalized and surface-bound ligand was determined as described in MATERIALS AND METHODS. As shown in Figure 1, similar rates (and amount) of internalization were observed for all the receptor combinations although there was some variation between individual clones (i.e., A110 showed an higher ratio of internalized to surface-bound ligand at 120 or 240 min than any of the other clones).

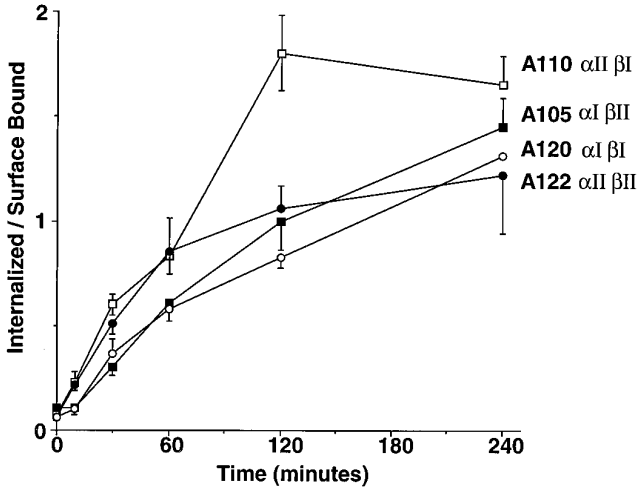

Figure 1. Internalization of chimeric receptors. Individual clones were cultured as described in MATERIALS AND METHODS and incubated with 100 pM radiolabeled GM-CSF for 2 h at 4°C. Unbound ligand was removed, and cells were placed at 37°C for the indicated times. Surface-bound ligand was stripped by washes with PBS, pH 3.0, and radioactivity was measured. The remaining cell pellet was solublized and measured as internalized ligand. Each curve for each clone represents the mean ± SEM of two (A105), three (A122), or four (A120 or A110) independent experiments assayed in duplicate. The specific endocytotic rate constant (k_e) was determined for each of the clones over the first four data points (A105, 0.01/min; A110, 0.02/min; A120, 0.02/min; A122, 0.01/min). Analysis of variance shows no statistical difference between the rates ($p > 0.60$). The initial and maximal percent internalization, respectively, for clone A105 was 10.6% and 53.0%, for clone A110 was 7.5% and 51.9%, for clone A120 was 6.3% and 42.9%, and for clone A122 was 5.3% and 43.0%.

The endocytotic rate constant (k_e) for each clone representing the slope of the line when the ratio of internalized ligand to surface-bound ligand is plotted as a function of time is shown in Figure 1 (Wiley and Cunningham, 1982). Although no significant difference was observed for any of the clones, the calculated rates for the chimeric receptors were 5- to 10-fold slower than that reported for the EGF receptor (Wiley and Cunningham, 1982; Waters *et al.*, 1990; Rajagopalan *et al.*, 1991; Chang *et al.*, 1993; Sorkin *et al.*, 1996) and about 3- to 4-fold slower than the growth hor-

mone receptor or chimeric EGF/Erb B receptors (Baulida *et al.*, 1996; Harding *et al.*, 1996). Although the significance of these relatively slow receptor kinetics is presently unknown, it may provide a possible explanation to why classic signaling pathways have not been consistently documented as downstream mediators of TGFβ receptor activation.

Clathrin-mediated Internalization of Heteromeric and Homomeric GM-CSF/TGFβ Receptors

Growth factor receptors are frequently internalized through structures referred to as clathrin-coated pits (Schmid, 1992). Because signaling-incompetent homomeric receptors were internalized to a similar extent as signaling-competent heteromeric receptors (Figure 1), we wished to first determine the mechanism of internalization and second to determine whether it differed between these receptor groups. To examine this question, clathrin-dependent internalization was prevented by K^+ depletion (prevents clathrin lattice formation; Larkin *et al.*, 1983; Sorkin *et al.*, 1995) or cytoplasmic acidification (prevents the pinching off of clathrin buds; Fire *et al.*, 1995). As shown in Figure 2, A and B, ^{125}I-labeled GM-CSF internalization was inhibited 80–90% by either K^+ depletion or cytoplasmic acidification of the heteromeric receptor expressing A105 clone. Moreover, there was no effect of amiloride alone (i.e., in the absense of NH_4Cl and subsequently no acidification) on ligand internalization (our unpublished results). The Figure 2B inset represents a positive control performed in parallel showing comparable clathrin dependence to EGF internalization (Sorkin and Water, 1993). To determine whether homomeric type I/type I or type II/type II receptors internalized ligand in an analogous manner, the experiments shown in Figure 2, C and D, were performed. Similar to that observed for heteromeric receptor internalization (Figure 2, A and B), homomeric receptors were unable to internalize radiolabeled ligand in the absence of K^+, yet internalization occurred once K^+ was restored to the cultures.

The preceding data support the hypothesis that the primary mechanism for chimeric TGFβ receptor internalization is clathrin-dependent. However, although the pharmacologic approaches in Figure 2 are commonly used to indicate clathrin dependence, they are not specific. To determine whether our treatments were resulting in a generalized disruption of the endocytic machinery, we examined the effect of K^+ depletion on the clathrin-independent uptake of the fluid-phase marker HRP (Cupers *et al.*, 1994; Damke *et al.*, 1994). As shown in Figure 3, similar rates and amounts of HRP were internalized over the first 10 min in the presence or absence of potassium. In agreement with the results of Cupers *et al.* (1994), HRP accumulation decreased approximately 30.3% in treated cells after a 10-min internalization, presumably a reflection of an intermediate compartment regurgitating its content. Because the intracellular uptake of the fluid-phase tracer HRP was not impaired by K^+ depletion, the results of Figure 2 are most consistent with a clathrin-dependent process being the primary mechanism through which chimeric TGFβ receptors are internalized. Thus, although homomeric receptor combinations are unable to signal (Anders and Leof, 1996; Luo and Lodish, 1996; Muramatsu *et al.*, 1997), they undergo clathrin (and ligand)-dependent internalization with kinetics similar to signaling-competent heteromeric TGFβ receptors.

Differential Down-Regulation of Chimeric Heteromeric and Homomeric Receptors

The finding that homomeric TGFβ receptor interactions were internalized similarly to heteromeric TGFβ receptors (Figures 1 and 2) indicated that internalization, per se, was in itself not sufficient to transmit a biological response. Although internalization is usually followed by a decrease in ability to bind ligand due to receptor down-regulation, we observed differing results when down-regulation assays were performed on the heteromeric and homomeric receptor expressing clones (Figure 4). Cells were treated with 10 ng/ml unlabeled GM-CSF for 4 h at 37°C and acid stripped to remove any remaining receptor-bound ligand, and ^{125}I-labeled GM-CSF surface binding was measured at 4°C. As shown in Figure 4, heteromeric receptor expressing clones A105 and A110 showed only 20–30% binding, relative to time zero, after a 2- to 4-h treatment with 10 ng/ml GM-CSF (70–80% receptor down-regulation). In contrast, homomeric clones A120 and A122 showed no decrease in surface binding throughout the 4-h GM-CSF treatment. In fact, clone A120 showed an increase in surface binding by 4 h, the significance of which is presently unknown. These results (Figures 1 and 2, and see Figure 4) suggest that heteromeric and homomeric TGFβ receptors undergo distinct trafficking behavior after ligand-induced oligomerization.

Dose and Temperature Dependence of Ligand-induced Down-Regulation

Receptor down-regulation occurs through an endocytic mechanism dependent upon the dose of the ligand and the temperature of incubation. To determine whether down-regulation of the chimeric TGFβ receptor showed a similar dependence, down-regulation assays were performed with various GM-CSF concentrations and differing temperatures. Figure 5A shows that receptor down-regulation of heteromeric clone A105 occurs in a dose-dependent manner with a half-maximal response seen at 0.2–0.4 ng/ml (10.4–20.8 pM) GM-CSF. This concentration of GM-CSF corre-

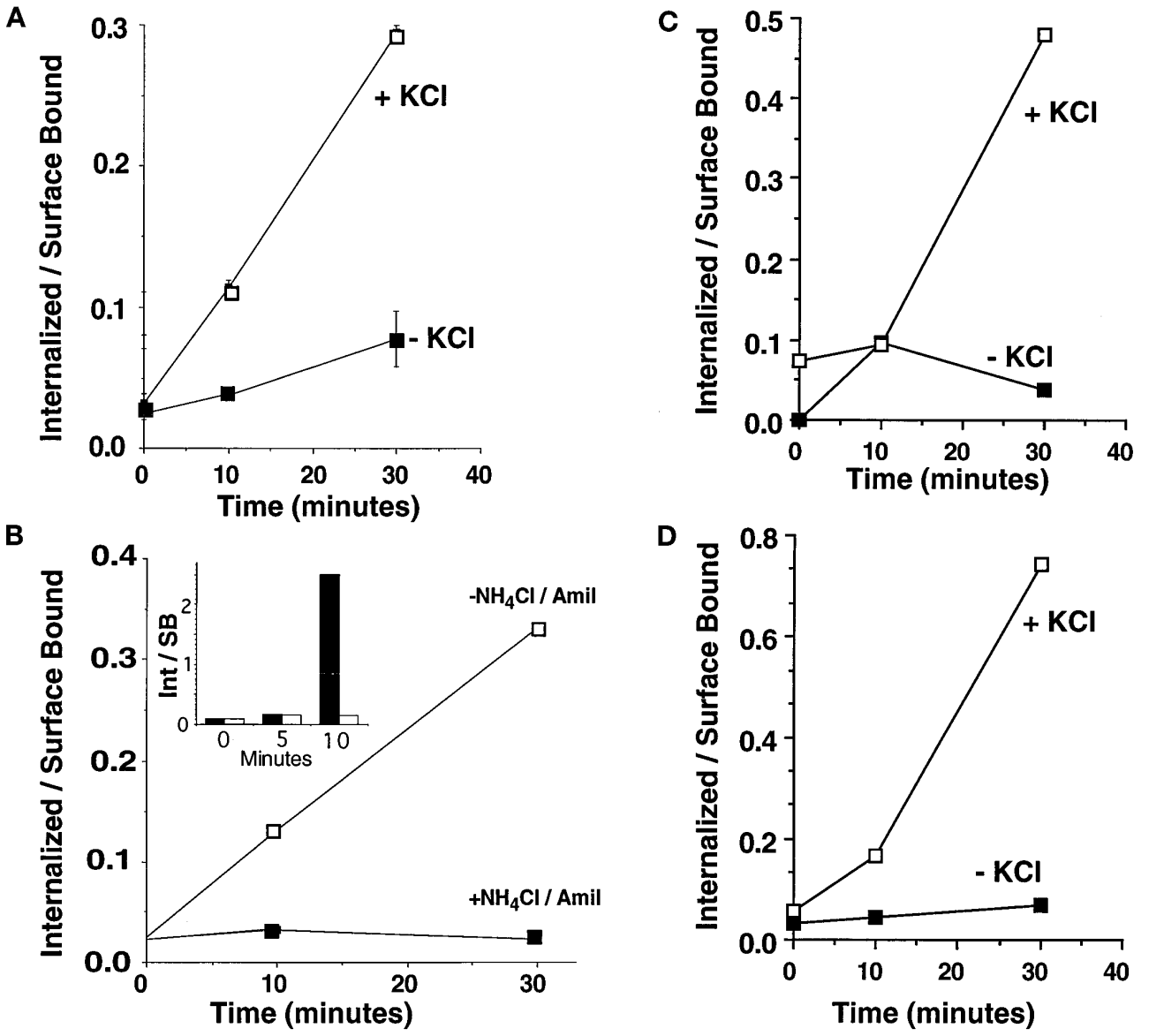

Figure 2. Clathrin-dependent internalization. (A) Heteromeric receptor expressing clone A105 was K^+ depleted (■) or placed in the identical buffers supplemented with 10 mM KCl (□) as described in MATERIALS AND METHODS. Internalization of ^{125}I-labeled GM-CSF was determined at the indicated times. (B) Clone A105 was treated with (■) or without (□) NH_4Cl and amiloride at 37°C as described in MATERIALS AND METHODS. Cells then underwent 100 pM radiolabeled GM-CSF binding with or without amiloride for 2 h at 4°C and internalization proceeded as described in Figure 1. (Inset) AKR-2B cells were treated with (open bars) or without (solid bars) NH_4Cl and amiloride. After treatment, 150 pM EGF was bound at 4°C for 2 h. Internalization was initiated by removing unbound ligand and warming to 37°C for 0, 5, or 10 min. (C and D) Homomeric clones A120 (type I/type I; C) and A122 (type II/type II; D) were K^+ depleted (■) or supplemented with 10 mM KCl (□) as discussed in A and MATERIALS AND METHODS. Cells bound 100 pM radiolabeled GM-CSF in appropriate buffer B for 2 h at 4°C and internalization was determined at the times indicated. The results represent the mean ± SEM of two independent experiments done in duplicate.

lates well with the apparent chimeric receptor K_d of 7.8 ± 13.4 pM (our unpublished results) for the A105 clone and is similar to the GM-CSF dose dependence shown previously for induction of plasminogen activator inhibitor 1 protein (Anders and Leof, 1996).

Earlier studies have shown that endocytosis through clathrin-coated pits is highly dependent upon the temperature of incubation (Pearse and Robinson, 1990; Schmid, 1992). Although Figure 2 indicated a clathrin requirement for chimeric receptor internalization,

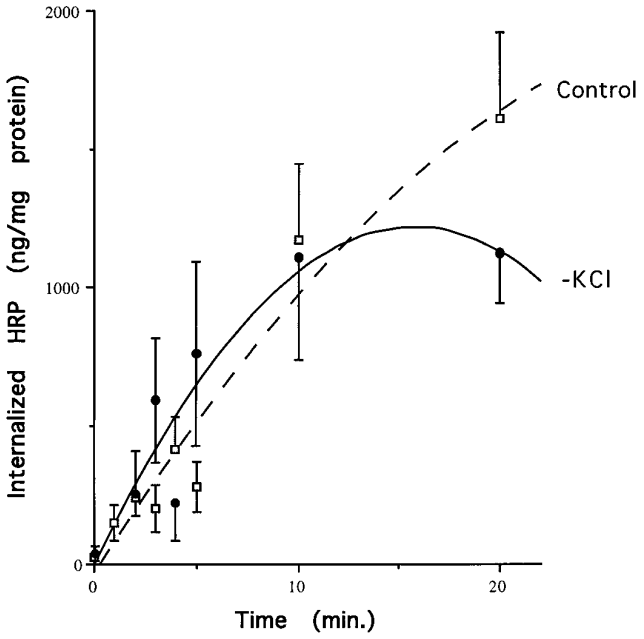

Figure 3. Effect of potassium depletion on HRP accumulation. Control (□) or potassium-depleted (●) A105 cells were incubated on ice for 15 min with 2 mg/ml HRP. The cultures were then placed at 37°C and at the indicated times internalized HRP was determined and normalized to total cellular protein. The data represent the mean ± SEM of three separate experiments done in triplicate and the R^2 value was 0.95 and 0.87 for control and potassium-depleted cultures, respectively.

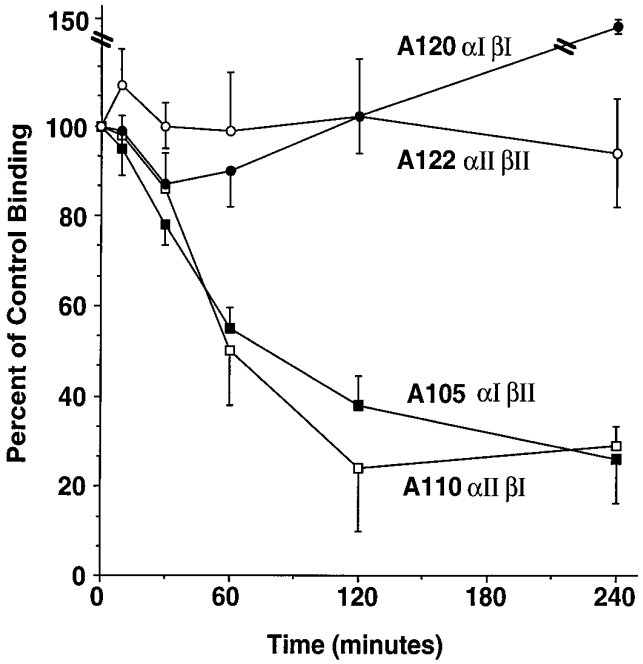

Figure 4. Down-regulation of chimeric receptor surface binding. Individual clones A120αIβI (●), A122αIIβII (○), A105αIβII (■), and A110αIIβI (□) were treated with 10 ng/ml (520 pM) GM-CSF for the indicated times. Cells were acid stripped, and specific surface binding of 100 pM radiolabeled GM-CSF was determined after a 2-h incubation at 4°C with or without a 25-fold excess of unlabeled GM-CSF. Percentage of control binding is calculated as the percent of zero time (no prior GM-CSF treatment) specific binding. Each curve represents the mean ± SEM of three independent experiments assayed in duplicate.

down-regulation assays were performed with GM-CSF treatment at 4°C, 25°C, or 37°C. In agreement with the results of Figure 4, 37°C GM-CSF treatment resulted in a 70% decrease in cell surface receptor binding by 2 h (Figure 5B). However, treatment with GM-CSF at 4°C or 25°C caused no appreciable down-regulation of the chimeric receptors. These results (Figures 2, 4, and 5) are consistent with the hypothesis that the endocytosis of heteromeric signaling competent TGFβ receptors is dependent upon 1) functional clathrin lattice formation, 2) the dose of ligand, and 3) the temperature of incubation.

Recovery from Down-Regulation

Receptor down-regulation is usually considered one mechanism by which the cellular response to bioactive molecules is dampened. It follows, however, that the decrease in cell surface receptor expression must be replenished to respond to subsequent signals. Because the clones (A105 and A110) expressing heteromeric combinations of chimeric receptors underwent a ligand-dependent decrease in surface receptor binding, we wished to determine the cellular requirement(s) for returning receptor binding to the prestimulatory level. To address that question, cells were treated with GM-CSF to down-regulate the receptors and allowed to recover in normal growth medium (5% FBS), growth medium supplemented with actinomycin D, or growth medium containing cycloheximide (Figure 6). At the indicated recovery times, cell surface binding was measured with radiolabeled GM-CSF. As shown in Figure 6, receptor binding attained the initial control level 6–8 h after addition of growth medium. Although the recovery in binding was dependent upon new protein synthesis, as cycloheximide treatment blocked recovery, control binding levels were attained in the presence of the mRNA synthesis inhibitor actinomycin D.

DISCUSSION

Understanding the events after TGFβ receptor activation have been limited by poor TGFβ binding assays and the finding that homomeric and heteromeric TGFβ receptor interactions occur on the cell surface (Henis *et al.*, 1994). To circumvent these problems, we have generated a chimeric receptor system that qualitatively and quantitatively recapitulates both short-

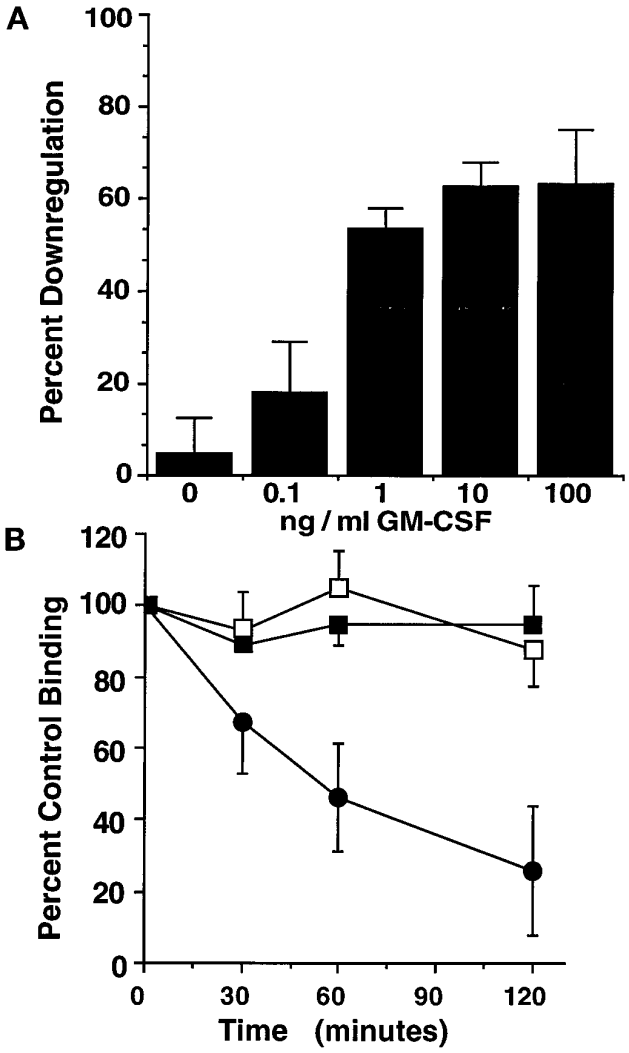

Figure 5. Dose and temperature requirements for heteromeric receptor down-regulation. (A) Chimeric receptor down-regulation in clone A105 was induced by treatment with the indicated amounts of GM-CSF at 37°C for 2 h. After incubation, cells were acid stripped of ligand and remaining receptor surface binding was determined as described in Figure 4. The data represent the mean ± SEM of two independent experiments performed in duplicate. (B) Cells were treated with 10 ng/ml GM-CSF for the indicated times at either 4°C (■), 25°C (□), or 37°C (●). Acid stripping and receptor surface binding were performed after each time point. Data are reported as the percent of binding compared with zero time for each dose or temperature. The data represent the mean ± SEM of three independent experiments performed in duplicate.

term gene expression and long-term biological responses dependent upon TGFβ signaling events (Anders and Leof, 1996; Muramatsu *et al.*, 1997). Isolation of clones expressing homomeric type I/type I or type II/type II and heteromeric type I/type II TGFβ receptor combinations has allowed us to initiate studies examining the endocytic response of distinct receptor complexes.

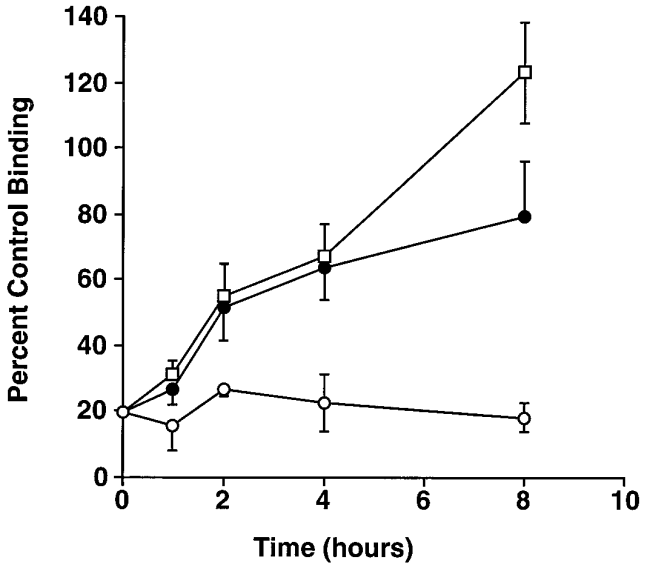

Figure 6. Recovery from down-regulation of surface binding. Heteromeric clone A105 was treated with 10 ng/ml GM-CSF for 4 h at 37°C. Cells were acid stripped and returned to 37°C in DMEM supplemented with 5% FBS (□), 5% FBS plus 1.0 μg/ml actinomycin D (●), or 5% FBS plus 20.0 μg/ml cycloheximide (○). At the indicated times, specific ^{125}I-labeled GM-CSF binding (100 pM) was determined after a 2-h incubation at 4°C. Percent binding of control is the ratio of specific binding observed after each treatment time to specific binding found in cells initially down-regulated. The data represent the mean ± SEM of three or four independent experiments done in duplicate. Analysis of variance between the 8-h FBS alone and 8-h FBS plus actinomycin D points showed no statistical difference ($p = 0.13$).

It was first determined that heteromeric and homomeric receptor/ligand-induced complexes were internalized (Figure 1). Although there is some variation in the degree of internalization, the overall rate of internalization is statistically similar between all the clones tested (Figure 1 and our unpublished results). It is of interest that the chimeric TGFβ receptors internalize ligand at a slower rate from that observed in tyrosine kinase receptors, such as the EGF receptor. For instance, the endocytotic rate constant (k_e), defined as the probability of an occupied receptor being internalized in 1 min at 37°C, is 5- to 10-fold slower for the chimeric TGFβ receptors than the EGF receptor. Although one can never disprove the possibility that these relatively slow kinetics are a reflection of the chimeric receptor system, the endocytic constants reported from other chimeric systems are similar to those seen in the native receptor (Rajagopalan *et al.*, 1995; Tseng *et al.*, 1995). Moreover, the elements regulating receptor endocytosis are commonly found in the cytoplasmic domains of most receptors (Trowbridge *et al.*, 1993) and GM-CSF receptors have been reported to not internalize in mouse fibroblasts (Watanabe *et al.*, 1993). Because our previous findings

showed that the chimeric receptors quantitatively and qualitatively reconstituted TGFβ receptor signaling, this further suggests that the observed internalization rates reflect the endogenous receptor system. One possibility might be that because the endogenous (and the chimeric) TGFβ receptor requires the formation of an heteromeric complex between differing receptor types (i.e., a dimeric and/or tetrameric complex), analogies to one-component systems (i.e., EGF receptor) may be inappropriate.

Although our results and data from other laboratories document internalization of ligand bound to chimeric and native TGFβ receptors (Massagué and Like, 1985; O'Grady *et al.*, 1991; Rakowicz-Szulczynska *et al.*, 1994; Dickson *et al.*, 1995; Muramatsu *et al.*, 1997), a recent publication by Koli and Arteaga (1997) reports minimal internalization of ^{125}I-labeled TGFβ at 37°C. It is unclear why these investigators obtained such different results. One explanation might be that because their internalization assays were not done after steady-state binding, the dissociation rate at 37°C would mask the apparent slow (relative to the EGF receptor) rate of internalization. Furthermore, their data appeared to show increasing ligand internalization at the end of the assay.

Although receptor-mediated pinocytosis has most commonly been reported to occur through clathrin-coated vesicles, recent reports have suggested other mechanisms such as caveolae, macropinocytosis, or noncoated vesicles for various growth factor receptors (Lamaze and Schmid, 1995). Because there was essentially no information concerning the mechanism(s) through which TGFβ receptors were internalized, we wished to determine whether the chimeric receptors were endocytosed through a clathrin-dependent or -independent process. Moreover, because this system has the distinct advantage of separately examining the endocytic response of TGFβ receptor homomers and heteromers, we could also determine whether the comparable endocytic rate seen for both receptor families reflected similar or distinct mechanisms. As shown in Figure 2, the internalization of both heteromeric and homomeric receptor complexes was inhibited by K^+ depletion or cytosolic acidification. Although both treatments are known to disrupt clathrin-dependent processes, they are not entirely specific. To document that the inhibitory effect seen in Figure 2 was not reflecting a generalized disruption of the endocytic machinery, we also determined whether the clathrin-independent uptake of the fluid-phase tracer HRP was affected by K^+ depletion (Figure 3). In agreement with previous studies (Cupers *et al.*, 1994), we found no effect of K^+ depletion on the initial rate of HRP internalization. Whether the effect seen at later times reflects regurgitation from an early endosomal compartment as shown by Cupers *et al.* (1994) is unknown. An additional mechanism by which receptors are internalized is through the process of macropinocytosis (West *et al.*, 1989; Hewlett *et al.*, 1994; Lamaze and Schmid, 1995). However, macropinocytosis is inhibited by amiloride in the absence of cytosolic acidification (West *et al.*, 1989), and we found no effect of amiloride alone (i.e., no NH_4Cl and subsequently no cytosolic acidification) on chimeric receptor internalization (our unpublished results). Thus, although morphologic studies such as electron microscopic colocalization will be needed to definitively prove a clathrin-dependent mechanism, the data strongly support that conclusion. Whether internalization also requires the activity of a GTPase such as dynamin for the pinching off of the endocytic bud (Urrutia *et al.*, 1997) or the association with proteins composing the AP-2 complex (Pearse and Robinson, 1990; Nesterov *et al.*, 1995; Sorkin *et al.*, 1995, 1996) is currently under investigation.

Internalization of bound ligand is usually followed by a decrease in cell surface receptors referred to as down-regulation. There have been conflicting reports whether TGFβ receptors undergo ligand-mediated down-regulation. Initial studies suggested that a large intracellular pool of recycling receptors replenished cell surface binding after internalization (Massagué, 1985; Massagué and Kelly, 1986; Sathre *et al.*, 1991). However, these data were based upon the results of ligand binding to both endogenous heteromeric and homomeric TGFβ receptors and/or the earlier consideration that the type III receptor (beta glycan) was the primary signaling receptor for TGFβ. More recent studies (O'Grady *et al.*, 1991; Zhao and Buick, 1995; Muramatsu *et al.*, 1997) and the results of the present investigation show that type I and type II TGFβ receptors are down-regulated and that distinct endocytic effects are observed for TGFβ receptor heteromers and homomers. For instance, although heteromeric type I/type II TGFβ receptor interactions result in both internalization and receptor down-regulation, homomeric type I/type I or type II/type II TGFβ receptors are internalized but not down-regulated (Figures 1 and 4). One possible mechanism to account for this difference might be that the ability to down-regulate is directly tied to the signaling capability of the receptor combination. Because homomeric receptor interactions are signaling incompetent (Anders and Leof, 1996; Luo and Lodish, 1996; Muramatsu *et al.*, 1997), only signaling-competent heteromeric receptors would be down-regulated. However, this possibility is unlikely because signaling-incompetent heteromeric receptors containing a kinase dead type I TGFβ receptor are down-regulated to the same extent and with similar kinetics as functional heteromers (our unpublished results). A similar result was reported by Opresko and Wiley (1990) showing that kinase-negative EGF receptors would internalize ligand but not down-regulate when expressed in *Xeno-*

pus oocytes. Another explanation might be that homomeric and heteromeric receptor/ligand complexes are processed through distinct endocytic pathways. This would be consistent with ligand binding assays in which homomeric receptors were internalized but not down-regulated. Moreover, it might also account for the initial confusion in the endocytic fate of TGFβ receptors because earlier binding studies would have generated results based upon the overall endocytic response of both heteromeric and homomeric TGFβ receptor interactions. For instance, because type II TGFβ receptor homomers occur in the presence or absence of ligand (Henis *et al.*, 1994), the down-regulation of endogenous heteromeric TGFβ receptors might be masked by the binding of ligand to homomeric type II receptors still present on the cell surface. In addition, the previous determination that TGFβ receptors are recycled (Frolik *et al.*, 1984; Massagué and Kelly, 1986; Sathre *et al.*, 1991) could also be similarly accounted for by our finding that TGFβ receptor homomers are internalized but not down-regulated. Because these earlier TGFβ binding studies would not differentiate the response of heteromeric from homomeric TGFβ receptor interactions, the "recycled" receptor component might reflect ligand binding to type II TGFβ homomers that have trafficked back to the plasma membrane after internalization. We are not hampered by these considerations because the chimeric receptor system is capable of distinguishing the cellular response of defined heteromeric and homomeric receptor populations.

Additional studies examined both the temperature requirement for down-regulation and the synthetic requirements for replenishing ligand binding after down-regulation (Figures 5B and 6). The lack of receptor down-regulation at 25°C was somewhat surprising and differs from that routinely observed in other growth factor receptors, such as EGF or insulin. However, similar findings (i.e., down-regulation at 37°C but not at room temperature or 15°C) have been reported for both the human chorionic gonadotropin receptor (Rebois and Fishman, 1984) and the T cell receptor β-chain (Makida *et al.*, 1996). Whether this response is unique to the chimeric receptors or reflects an unusual property of TGFβ receptors is presently unknown and under investigation. Nonetheless, it suggests a step other than clathrin-coated pit assembly and budding being rate limiting for this class of receptors.

The present data suggest several new ideas regarding TGFβ receptor interactions including 1) proposing internalization rates for heteromeric and homomeric TGFβ receptor complexes, 2) providing evidence that the mechanism of receptor endocytosis is clathrin-dependent, 3) demonstrating that only heteromeric type I/type II TGFβ receptor combinations down-regulate in a dose- and temperature-dependent manner, and 4) showing that the subsequent recovery from down-regulation is dependent upon new protein synthesis but can occur in the presence of actinomycin D, indicating that a large intracellular pool of receptors may not be present. Although our results using a defined chimeric receptor system agree and/or extend many earlier reports analyzing endogenous TGFβ receptors, it is readily apparent that additional studies will be required to appropriately characterize this extremely complex receptor system.

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