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Transforming Growth Factor β Receptor Signaling and Endocytosis Are Linked through a COOH Terminal Activation Motif in the Type I Receptor

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Transforming growth factor β (TGF-β) coordinates a number of biological events important in normal and pathophysiological growth. In this study, deletion and substitution mutations were used to identify receptor motifs modulating TGF-β receptor activity. Initial experiments indicated that a COOH-terminal sequence between amino acids 482–491 in the kinase domain of the type I receptor was required for ligand-induced receptor signaling and down-regulation. These 10 amino acids are highly conserved in mammalian, *Xenopus*, and *Drosophila* type I receptors. Although mutation or deletion of the region (referred to as the NANDOR BOX, for nonactivating non–down-regulating) abolishes TGF-β–dependent mitogenesis, transcriptional activity, type I receptor phosphorylation, and down-regulation in mesenchymal cultures, adjacent mutations also within the kinase domain are without effect. Moreover, a kinase-defective type I receptor can functionally complement a mutant BOX expressing type I receptor, documenting that when the BOX mutant is activated, it has kinase activity. These results indicate that the sequence between 482 and 491 in the type I receptor provides a critical function regulating activation of the TGF-β receptor complex.

INTRODUCTION

Normal cellular proliferation is a complex process that requires the coordinated integration of both stimulatory and inhibitory growth factors. Transforming growth factor β (TGF-β) is unique in this regard in that it is capable of both stimulating and inhibiting cell growth, depending on the cellular context (Roberts *et al.*, 1985; Moses *et al.*, 1990). The pivotal role that TGF-β plays in modulating a number of biological activities makes it critical to identify the mechanisms through which TGF-β actions are regulated. To more systematically address these questions, the three TGF-β receptor (TGF-βR) species seen in most cell types have been characterized (Wang *et al.*, 1991; Lin *et al.*, 1992; Franzén *et al.*, 1993). Although the type III receptor (also referred to as betaglycan) has been shown to present TGF-β to the signaling receptors (i.e., type I and type II receptors) and enhance cell responsiveness to TGF-β, its short cytoplasmic tail and absence of known signaling motifs suggested a limited role in the direct regulation of TGF-β signal transduction (Lopez *et al.*, 1993). This activity seems to be mediated primarily by the type I and II TGF-β receptors. Although both receptors are capable of ligand binding, TGF-β initially binds to cell surface type II receptors. Once ligand binds to a type II receptor, this results in type I receptor recruitment, transphosphorylation (by the type II receptor), and activation of a heteromeric TGF-βR complex (Wrana *et al.*, 1992, 1994).

TGF-βR signaling is regulated by both positive and negative acting sites in the type I and type II receptors (Wieser *et al.*, 1995; Heldin *et al.*, 1997; Luo and Lodish, 1997; Hoodless and Wrana, 1998). The type I TGF-βR contains a highly conserved juxtamembrane region of 30 amino acids referred to as the GS domain. Interest in these sites arose from studies that showed the region to be phosphorylated on serine and threonine residues when complexed with a type II TGF-βR (Wrana *et al.*, 1994; Wieser *et al.*, 1995). Although single amino acid changes within the GS domain were subsequently shown to have no detectable effect on cellular signaling, multiple GS domain mutations resulted in a dose-dependent loss in receptor signaling capacity. Although a number of additional sites have been documented that regulate TGF-βR signaling (Cárcamo *et al.*, 1995; Wieser *et al.*, 1995; Saitoh *et al.*, 1996; Doré *et al.*, 1998), it is presently unclear as to the manner in which they function.

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Plasma membrane receptors constitute the initial "sorting organelle" controlling the cellular response to environmental stimuli. Distinct sequence elements have been identified within the cytoplasmic (primarily) domains of various membrane receptors controlling the endocytic process as well as association with coated pit proteins (Itin *et al.*, 1995; Marks *et al.*, 1996; Mellman, 1996; Mukherjee *et al.*, 1997; Floyd and De Camilli, 1998). Although defined sequence elements control, at least in part, receptor internalization, the process of endocytosis occurs through the coordinate interplay of a number of plasma membrane proteins (Ohno *et al.*, 1996; Cao *et al.*, 1998; Kao *et al.*, 1998; Sweitzer and Hinshaw, 1998; Nesterov *et al.*, 1999; Ramjaun *et al.*, 1999). In that regard, we have recently initiated studies designed to identify and characterize endocytic regulation of heteromeric and homomeric TGF-β receptors in mesenchymal and epithelial cells. Although cell type differences have been observed (Doré *et al.*, 1998, 2001), regulatory control in fibroblasts is mediated through the transphosphorylating activity of the type II receptor (Anders *et al.*, 1998) and the formation of a heteromeric TGF-βR complex (Anders *et al.*, 1997; Doré *et al.*, 1998). Because TGF-βR signaling is also dependent on heteromeric complex formation (Wrana *et al.*, 1992; Anders and Leof, 1996; Luo and Lodish, 1996; Muramatsu *et al.*, 1997), we wished to extend these analyses and further define the relationship between TGF-βR endocytosis and signaling.

In the present report we have used our chimeric TGF-βR system (Anders and Leof, 1996) to determine whether there are additional sequence motifs in the transmembrane and/or cytoplasmic domain of the type I TGF-βR controlling activation of the TGF-βR complex. This system uses the ligand binding domain of the GM-CSF α or β receptor fused to the transmembrane and cytoplasmic domain of the type I or type II TGF-βR. Because high-affinity GM-CSF binding requires the presence of both the α and β subunits, defined heteromeric (i.e., type I/type II) and homomeric (i.e., type I/type I or type II/type II) TGF-βR cytoplasmic interactions can be examined independently. The data demonstrate that a highly conserved COOH-terminal sequence between residues 482 and 491 (referred to as the NANDOR BOX, for nonactivating non–down-regulating) controls down-regulation of TGF-β receptors in mesenchymal cultures. In addition to modulating TGF-βR endocytic activity, both Smad4-dependent and -independent TGF-β-mediated signaling stimulated through endogenous or chimeric TGF-β receptors was examined in fibroblasts and epithelial cells and shown to similarly require an intact BOX motif. Moreover, further studies determined the following: 1) the BOX was necessary for type I receptor phosphorylation and therefore, kinase activity; 2) the signaling activity of a BOX mutation could be complemented by a kinase-defective receptor, demonstrating that the BOX mutant is a functional kinase; and 3) epithelial cultures, in contrast to mesenchymal cells, do not require an intact BOX for effective down-regulation. Thus, the BOX further defines a differential cell type requirement for phosphorylation in regulating TGF-βR down-regulation (Doré *et al.*, 2001), as well as a novel activation domain within the type I receptor required for TGF-β signal transduction.

MATERIALS AND METHODS

Materials

Recombinant human GM-CSF was generously provided by the DNAX Research Institute (Palo Alto, CA), and recombinant human TGF-β was purchased from R&D Systems (Minneapolis, MN) or Austral Biologicals (San Ramon, CA). Cell culture media, horse serum, and geneticin (G418 sulfate) were purchased from Life Technologies (Gibco-BRL, Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from Summit (Fort Collins, CO), and hygromycin B was purchased from Boehringer Mannheim (Indianapolis, IN). Unless specifically noted, all other reagents were from Sigma (St. Louis, MO).

Cell Culture

AKR-2B fibroblasts expressing the chimeric TGF-β receptors were maintained in DMEM supplemented with 5% (vol/vol) FBS and 100 μg/ml geneticin and 50 μg/ml hygromycin B as described (Anders and Leof, 1996). Mv1Lu and R1B epithelial cells were grown in DMEM containing 10% (vol/vol) FBS. The receptors were placed in either the pNa or pPa expression vector (Anders and Leof, 1996). Cos7 cells were grown in DMEM containing 10% (vol/vol) FBS and transiently transfected with the indicated wild-type or mutated endogenous TGF-β receptors.

Plasminogen Activator Inhibitor-1 Production and Smad2 Phosphorylation

Plasminogen activator inhibitor-1 (PAI-1) protein expression was determined essentially as described (Anders and Leof, 1996). Briefly, ligand-treated cultures were pulsed with [^{35}S]-methionine and processed by washing (on ice) once with 1× PBS, three times with 10 mM Tris, pH 8.0, 0.5% deoxycholate, 50 μg/ml PMSF, twice with 2.0 mM Tris, pH 8.0, and once with 1× PBS. The remaining matrix proteins were eluted from the plate by addition of 100 μl of 2× Laemmli buffer containing 10% β-mercaptoethanol. The samples were separated by 8% SDS-PAGE followed by fluorography. To detect endogenous Smad2 phosphorylation, cells were plated on 100-mm culture dishes at 2–2.5 $\times$ 10^7 cells per dish. The following day, cultures were serum-starved for 24 h in serum-free DMEM containing 0.1% FBS and stimulated with the indicated growth factors for 30–45 min. After induction, the cells were washed twice with PBS and lysed on ice in 50 mM Tris, pH 7.4, 1% NP40, 0.25% DOC, 50 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail (Boehringer Mannheim). The cell debris was removed, and equivalent supernatant protein was separated on an 8% SDS-PAGE. Total and phospho-Smad2 were detected with antibodies 06–654 and 06–829, respectively, from Upstate Biotechnology.

GM-CSF Binding and Down-regulation

Receptor binding assays were used to determine plasma membrane expression of chimeric receptors as described previously (Anders *et al.*, 1997; Doré *et al.*, 1998). For down-regulation assays, cells were incubated at 37°C with 10 ng/ml cold GM-CSF for the times indicated. Wells were then washed twice at 4°C with acid PBS (pH 3.0), and the remaining surface binding was determined by incubating for 2 h at 4°C with 100 pM ^{125}I-GM-CSF alone or in the presence of 25-fold molar excess of cold GM-CSF before cell lysis with 0.2 M NaOH, 40 μg/ml sheared salmon sperm DNA (Anders *et al.*, 1997; Doré *et al.*, 1998).

TGF-β Binding and Cross-linking

^{125}I-TGF-β1 binding was performed on Mv1Lu, R1B, and transfected clones. Cultures were plated at 3 $\times$ 10^5 cells per well in six-well dish plates 24 h before use in 10% FBS/DMEM. The me-

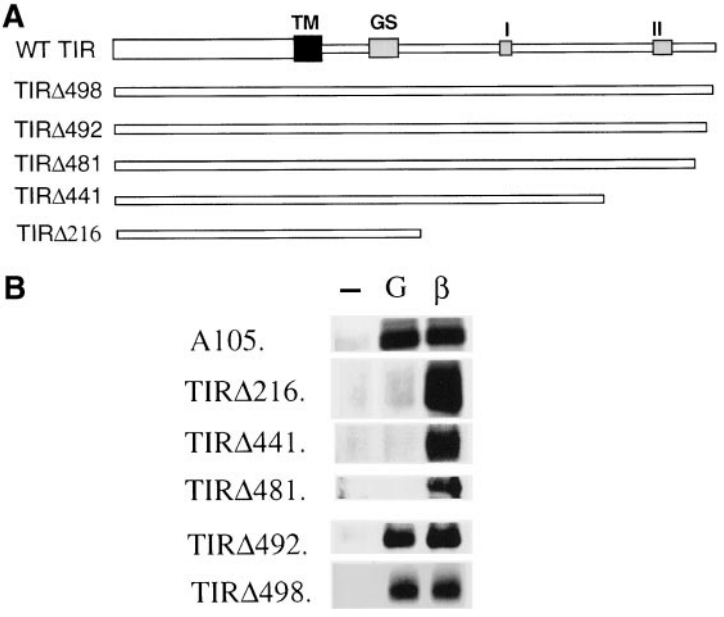

Figure 1. Effect of type I receptor truncations on ligand-induced signaling. (A) The full-length (503 amino acids) type I TGF-βR (WT TIR) and the location of the transmembrane (TM) and GS domains as well as kinase inserts I and II are shown at the top. Below are depictions of five chimeric type I receptor truncations. The designation TIRΔ481 (for instance) represents a chimeric type I TGF-βR that expresses the alanine at 481 as the final COOH-terminal residue, whereas TIRΔ492 expresses an additional 11 amino acids (the leucine at 492 is the final COOH-terminal residue). (B) Parental A105 cells or clones expressing a full-length type II chimeric TGF-βR and a type I receptor truncated at amino acid 216, 441, 481, 492, or 498 were stimulated with 5% FBS/DMEM alone (−) or supplemented with 10 ng/ml GM-CSF (GM) or TGF-β2 (β) as described (Anders and Leof, 1996). Endogenous PAI-1 protein expression was analyzed in the indicated clones after 4 h stimulation.

dium was removed, replaced with 2 ml of binding buffer (BB: DMEM, 2.5% BSA, 0.2 M HEPES, pH 7.4), and rocked for 30 min at room temperature. After incubation for 15 min at 4°C, the medium was removed and washed two times with cold BB, and 250 μl of 200 ng/ml cold TGF-β1 was added to nonspecific binding wells and 500 μl of 1 ng/ml ^{125}I-TGF-β1 (Amersham Pharmacia, Piscataway, NJ) was added to test wells. The plates were rocked at 4°C for 15 min, and 250 μl of 2 ng/ml ^{125}I-TGF-β1 was added to the nonspecific wells. After a 2 h incubation (with rocking) at 4°C, the cells were washed three times with BB and lysed in 0.5 ml TGF-β lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100) for 30 min at room temperature.

Cross-linking of TGF-β membrane receptors was performed after ligand binding. Cultures were washed once with BB and once with PBS at 4°C before addition of 1 ml of 2 mM BS^3 (Pierce, Rockford, IL) in PBS. The plates were rocked at 4°C for 1 h, and the medium was replaced with 1 ml of 1% ethanolamine, pH 7.4, for 15 min at 4°C (no rocking). Cells were lysed in 100 μl 2× Laemmli sample buffer and sonicated, and the supernatant was run on 8% SDS-PAGE.

Site-directed Mutagenesis of Chimeric cDNA

Truncated chimeric αI receptors were prepared by introducing two in-frame tandem stop coding sequences (TAA and TAG) with the use of the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). The BOX-ANA mutant (amino acids 482–492) was generated with primers 5′-GCC AAT GGA GCA GCT GCC GCA GCA AAT GCC GCA GCA GCC GCA GCA GCA GCC CAA CTC AGT CAA CAG GAA GGC-3′ as sense, and 5′-GCC TTC CTG TTG ACT GAG TTG GGC TGC TGC TGC GGC TGC TGC GGC ATT TGC TGC GGC AGC TGC TCC ATT GGC-3′ as antisense. After a 3-min predenaturation step, the 50-μl samples were cycled at 95°C for 1 min, annealed at 42°C for 1 min, and extended at 68°C for 14 min through 18 cycles. A final 20 min 68°C finishing was performed, and the samples were held at 4°C. To generate the conservative overlapping 4 × 2 point mutations, the desired nucleotide changes were flanked by 18 5′ and 3′ perfect nucleotide matches. The mutagenized constructs were generated in pGEM-3Z, verified by automated DNA sequencing, and then subcloned into the eukaryotic expression vector pNa at the *Sal*I and *Hin*dIII sites.

Type I Receptor Phosphorylation

Cos7 cells (1.5 × 10^6/p100) were transfected with the indicated constructs with the use of Fugene6 (Boehringer Mannheim), and the receptors were expressed for 36 h (total DNA 16.5 μg). For in vivo labeling, the media was replaced with phosphate-free medium for 2 h and then replaced with fresh phosphate-free medium (4 ml) containing 0.5 mCi/ml ^{32}P-P$_i$ for an additional 2 h, all at 37°C. Cultures were left untreated or stimulated with ligand and then lysed on ice in 700 μl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM Triton X-100, 50 mM NaF, 10 mM Na-pyrophosphate, 1 mM $NaVO_3$, 25 mM imidazole, and protease inhibitor cocktail). The TGF-βR complex was purified from β-galactosidase–normalized samples after overnight incubation at 4°C with His-Bind resin (Novagen, Madison, WI) to capture the type I/type II receptor complex, washed three times with lysis buffer containing 50 mM imidazole, and eluted in lysis buffer plus 350 mM imidazole for 4–6 h at 4°C. The hemagglutinin (HA)-tagged type I receptor was specifically immunoprecipitated from the complex by 4°C overnight incubation with anti-HA mouse monoclonal 12CA5 antibody and analyzed on 9% SDS-PAGE.

RESULTS

Type I TGF-βR Truncations Define a New Receptor Domain Controlling Receptor Signaling and Endocytosis

Activation of the type I TGF-βR is required for ligand-stimulated signaling. This occurs primarily through phosphorylation of juxtamembrane residues located within the GS domain (Wieser *et al.*, 1995). Because the cellular response to ligand is often controlled through the cooperative interaction of various receptor elements, we wished to identify other type I TGF-βR motifs necessary for ligand action. To address this question, we expressed truncated chimeric type I TGF-β receptors in the context of a full-length chimeric type II TGF-βR. Initial type I receptor truncations were made by inserting two tandem stop codons after amino

acids 216, 441, 481, 492, or 498 (Figure 1A). These sites were chosen for their ability to examine the role of the entire kinase domain (Δ216), kinase inserts 1 and 2 (Δ441 and Δ481), or the cytoplasmic tail (Δ492 and Δ498) in receptor signaling (Kingsley, 1994). As shown in Figure 1B, although AKR-2B clones expressing the Δ216, Δ441, or Δ481 chimeric type I receptor truncations were unable to stimulate endogenous PAI-1 protein expression, addition of 11 cytoplasmic tail amino acids (Δ492) restored PAI-1 expression to wild-type levels. Although the Δ492 type I receptor truncation stimulated endogenous PAI-1 protein to a similar extent as the wild-type receptor, the inability of the Δ216, Δ441, or Δ481 truncations to propagate a signal after GM-CSF binding does not simply reflect a general signaling anergy or absence of an intact TGF-β signaling pathway(s) in these cultures, because each of the clones was responsive to TGF-β activation of endogenous TGF-β receptors (Figure 1B). Identical results were observed if transient luciferase activity, growth in soft agar, morphologic transformation, and fibronectin protein expression were examined.

A common question in receptor biology is the relationship between the endocytic and signaling systems. Although initial reports suggested that receptor down-regulation was a response to modulate excess receptor activity (Wells *et al.*, 1990), more recent findings indicate that this may not be so straightforward (Kranenburg *et al.*, 1999; Leof, 2000). Moreover, we determined previously that although down-regulation could occur in the absence of receptor signaling, optimal down-regulation required the kinase activity of the type II TGF-βR (not the type I receptor) as well as the formation of a heteromeric type I/type II receptor complex (Anders *et al.*, 1997, 1998). Because these results documented the potential for multiple regulatory mechanisms being operative in the TGF-βR system, we wished to determine whether the type I receptor sequence identified in Figure 1B to control TGF-βR signaling also affected down-regulation. As shown in Figure 2, when the truncated type I receptors were coexpressed with a wild-type chimeric type II receptor, positive- and negative-acting effects on down-regulation were observed. For instance, although deletion of residues 442–503 (TIRΔ441) prevented down-regulation, subsequent truncation to amino acid 216 (TIRΔ216) resulted in a receptor complex that down-regulates similarly to wild-type receptors. Although the ability of the TIRΔ216 construct to down-regulate likely reflects an action of the GS domain, it is presently unknown whether a distinct element(s) exists between residues 217 and 441 negatively regulating endocytosis, because clones expressing a type I receptor truncated after amino acid 160 are unable to down-regulate. Moreover, the ability of cells expressing the TIRΔ216 truncation to down-regulate but not signal (Figures 1B and 2) 1) documents that the absence of the type I receptor kinase domain (amino acids 207–498) does not negatively impact on receptor down-regulation and 2) provides further support for independent regulation of these activities (Anders *et al.*, 1998).

Because the C-terminal deletion at amino acid 441 suggested the presence of an activity in a region that had not been previously shown to provide a critical receptor function, we further investigated the remaining 62 amino acids. Although addition of 40 amino acids to include kinase insert II (T1RΔ481) had only a modest effect on the endocytic

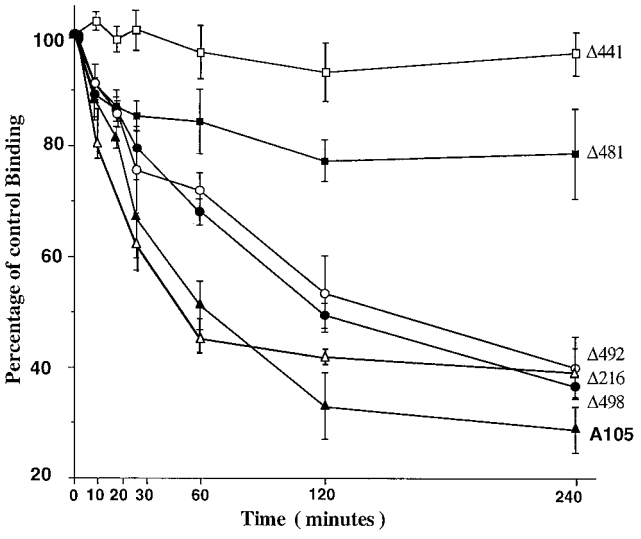

Figure 2. Type I receptor amino acids 482–492 are critical for down-regulation. Down-regulation assays were performed on clones expressing a full-length chimeric type II TGF-βR and either a full-length chimeric type I receptor (▲; A105) or type I receptors truncated after amino acid 216 (△; Δ216), 441 (□; Δ441), 481 (■; Δ481), 492 (○; Δ492), or 498 (●; Δ498) as described in MATERIALS AND METHODS. Percentage of control binding is calculated as the percentage of zero time (no prior GM-CSF treatment) specific binding. The data represent the mean ± SE of three independent clones assayed three times for the truncated receptors and five times for the A105 cells, all in duplicate.

response (Figure 2), inclusion of 11 additional amino acids (T1RΔ492) restored receptor down-regulation to a similar extent as that observed in A105 cells, which express a full-length type I and type II chimeric TGF-β receptor.

Although the T1RΔ492 construct promoted an approximately 60% decrease in chimeric TGF-βR membrane binding, the rate of down-regulation was slower than that observed for the wild-type receptor complex (i.e., compare T1RΔ492 with A105). Various explanations could be proposed to account for this, including 1) a requirement for additional receptor sequence and 2) clonal variation (i.e., the T1RΔ492 data represent the mean response of three independent clones, whereas the A105 line was originally chosen for its ability to respond to chimeric receptor activation). To address these questions, additional clones expressing a type I receptor truncated at amino 498 (T1RΔ498) were examined. These clones show no significant difference in either the rate or extent of down-regulation relative to the T1RΔ492 lines (Figure 2). As such, the results of Figures 1 and 2 indicate that the sequence from amino acids 482–492 in the type I TGF-βR provides a critical function for regulating both the signaling and endocytic activities of the TGF-βR complex in fibroblastic AKR-2B cells.

Requirement for the BOX Region in Chimeric and Endogenous TGF-βR Activity

Analysis of the amino acid sequence between residues 482 and 492 in the type I TGF-βR indicated a positively charged region with little homology to known signaling or endocytic

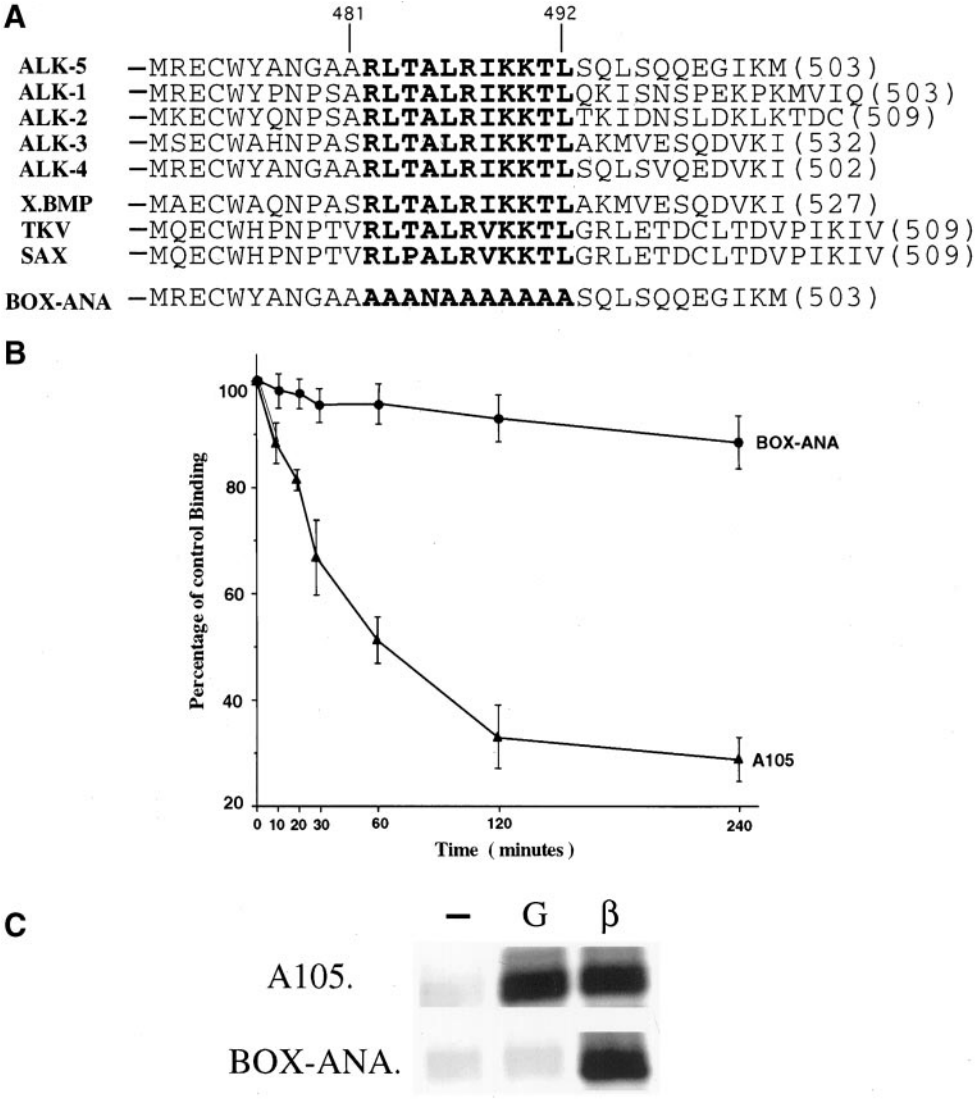

Figure 3. Mutagenesis of an 11 amino acid domain in the type I receptor abolishes chimeric TGF-βR down-regulation and PAI-1 expression. (A) The amino acid sequence of the COOH-terminal 37–41 residues in the activin-like kinases ALK-1 to ALK-5 as well as the *Xenopus* BMP (X.BMP) and *Drosophila* thickveins (TKV) and saxophone (SAX) type I receptors is depicted. The conserved region from amino acid 482–492 is in bold. ALK-5 is the type I TGF-βR; ALK-1 is also referred to as TSR-1 or R3; ALK-2 binds activin and is also called ActR-1, Tsk7L, SkrR1, or R1; ALK-3 binds activin and BMP and has been referred to as Brk-1, BMPR-IA, or mTFRII; and ALK-4 can also bind activin or BMP and is called ActR-IB, R2, or SKR-2 (Kingsley, 1994; Miyazono *et al.*, 1994; Massagué, 1996). (B) Down-regulation assays were performed on the parental A105 cells (▲) and clones expressing a chimeric type I receptor containing the depicted 11 amino acid changes in residues 482–492 (●; BOX-ANA) shown in A. The BOX-ANA results represent the mean ± SE of three clones analyzed three separate times in duplicate. Because this study was run in parallel with the truncations shown in Figure 2, the control A105 curve is the same. (C) Cells expressing the wild-type chimeric type I receptor (A105) or a chimeric type I receptor with the 11 amino acid substitution (BOX-ANA), both in the context of a wild-type type II chimeric receptor, were left untreated (−) or treated with 10 ng/ml GM-CSF (G) or TGF-β2 (β) for 4 h at 37°C to activate the chimeric or endogenous TGF-β receptors, respectively. Expression of endogenous PAI-1 was determined as described in MATERIALS AND METHODS.

elements (Figure 3A); however, this region shows 100% identity to other type I activin-like kinases (ALKs) and shares 10/11 and 9/11 residues with the *Drosophila* thickveins and saxophone receptors, respectively (Figure 3A). Although the previous truncation data indicated a fundamental role for amino acids 482–492 (referred to as the NANDOR BOX) in TGF-βR signaling and down-regulation (Figures 1 and 2), we wished to determine whether mutagenesis of those 11 residues (in the context of a full-length type II receptor) would similarly prevent receptor down-regulation and signaling. As shown in Figure 3B, ligand addition to the BOX region mutant (BOX-ANA) resulted in no significant decrease in membrane binding (>90% initial binding) after 4 h of GM-CSF stimulation. This is contrasted by the approximately 30% remaining binding observed in A105 cells expressing wild-type chimeric receptors over the same time period. Similarly, when induction of endogenous PAI-1 protein was examined, cells expressing the BOX-ANA mutant were unable to stimulate PAI-1 expression through the chimeric mutant receptor (Figure 3C). They were able, however, to induce PAI-1 when the endogenous TGF-β receptors were activated (Figure 3C).

Figures 1–3 suggest an important signaling and endocytic role for amino acids 482–492 in the type I receptor; however, the results reflect chimeric and not endogenous TGF-βR activity. Although the chimeric receptor system has been shown to recapitulate all tested TGF-β–dependent responses (Anders and Leof, 1996; Anders *et al.*, 1997, 1998; Doré *et al.*, 1998, 2001), we next determined whether the BOX region controlled signaling of endogenous TGF-β receptors. The wild-type type I TGF-βR or the endogenous receptor harboring the BOX-ANA mutations in residues 482–492 was

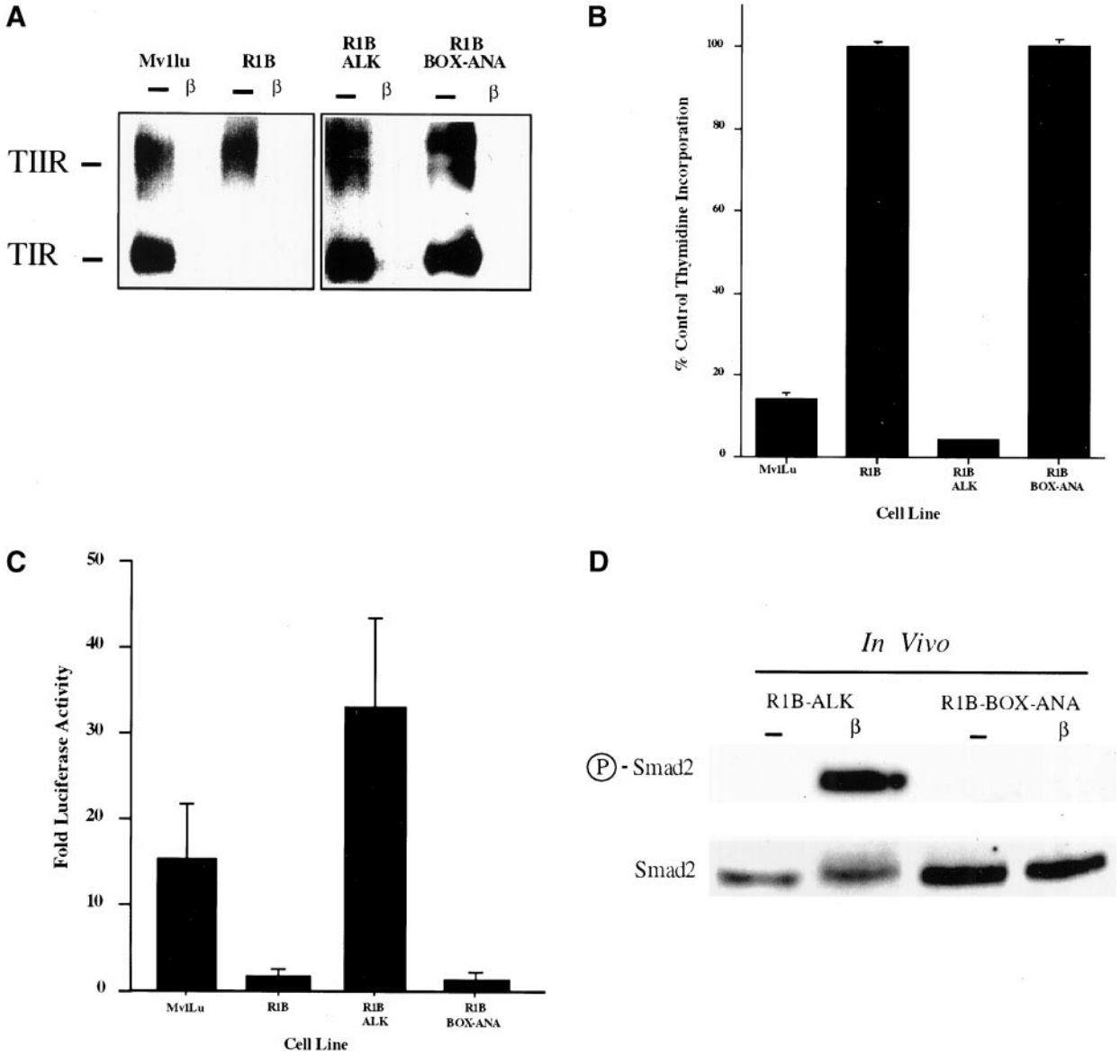

Figure 4. Endogenous TGF-βR signaling is dependent on the type I receptor box. (A) Cross-linking of ^{125}I-TGF-β1 was performed as described in MATERIALS AND METHODS on parental Mv1Lu or R1B cells in the absence (−) or presence (β) of a 200× excess of cold TGF-β2. The last four lanes represent similar cross-linking studies on R1B clones stably expressing a wild-type type I TGF-βR (R1B ALK; clone 9) or a type I TGF-βR containing the BOX mutations shown in Figure 3A (R1B BOX-ANA; clone 144). (B) The effect of TGF-β2 on ^{3}H-thymidine incorporation was determined on the epithelial cell cultures described in A. Percentage control reflects the incorporation observed in the presence of TGF-β2 divided by the incorporation in the absence of TGF-β2 for each cell type. The data represent the mean ± SE of four experiments on the parental Mv1Lu and R1B cells and four experiments on five R1B ALK (clones 6, 9, 27, 40, and 44) and four R1B BOX-ANA (clones 11, 46, 50, and 144) clones, all done in duplicate. (C) The same cultures as discussed in A were transiently transfected with the 3TP-Lux reporter plasmid, and normalized luciferase activity was determined. The data represent the mean ± SE of three separate experiments, each done in duplicate. (D) R1B cells stably expressing the endogenous wild-type (R1B-ALK) or BOX-ANA mutant (R1B-BOX-ANA) type 1 receptor were serum-starved and stimulated for 30 min at 37°C with DMEM alone (−) or containing 2 ng/ml TGF-β2 (β). Equivalent cellular protein (100 μg) was separated by SDS-PAGE and Western blotted with antibodies specific for phospho-Smad2 (top lane), then stripped and reprobed for total Smad2 (bottom lane).

transfected into type I TGF-βR–negative R1B cells (Laiho *et al*., 1991), and clones were isolated. Membrane expression and ligand binding for the wild-type and mutant receptors was documented by ^{125}I-TGF-β1 cross-linking (Figure 4A). When the cultures were tested for their ability to respond to TGF-β inhibition of DNA synthesis, mutant BOX receptor expression (R1B BOX-ANA) did not restore the growth inhibitory response (Figure 4B). This is contrasted by R1B cells

expressing the wild-type TGF-βR (R1B ALK), which restored TGF-β growth inhibition to a similar extent as that seen in parental Mv1Lu cells. Because the BOX-ANA mutant was unable to provide the necessary signal(s) required for growth inhibition, we next determined whether earlier responses involved in TGF-β action, such as transcriptional activity and phosphorylation of Smad2, might occur in BOX-ANA–expressing R1B cells (Figure 4, C and D). As shown in Figure 4C, luciferase activity from the TGF-β–responsive 3TP-Lux reporter was observed in Mv1Lu cells and R1B cells stably expressing the wild-type type I TGF-βR; however, expression of the BOX-ANA mutant was unable to induce luciferase activity to any greater extent than that seen in the parental R1B cells. A similar response was observed when ligand-stimulated Smad2 phosphorylation was examined (Figure 4D). The data (Figures 1, 3, and 4) clearly show that the BOX motif regulates both endogenous and chimeric TGF-βR signaling by modulating an early event(s) in TGF-βR action.

To determine whether a functional motif within the BOX could be defined more specifically, overlapping four-by-two conservative point mutations were made between residues 476 and 499 (Figure 5). The 13 constructs fall within three general groups: group 1 mutations lie amino terminal to the BOX region (Figure 5A); group 2 mutants include at least two amino acids within the BOX (Figure 5B); and group 3 mutations are COOH terminal to the BOX (Figure 5C). No effect on chimeric receptor down-regulation was observed when mutations were made outside the BOX region (groups 1 and 3) (Figure 5, A and C); however, when two to four amino acids were mutated within the BOX (group 2), the resulting receptor complex was unable to down-regulate after ligand addition (Figure 5B). Although quantitative differences in the role(s) of particular amino acids within the BOX were observed, with residues 484–487 (Box 3 clones) providing the most critical function, the data support the hypothesis that amino acids 482–491 in the type I TGF-βR provide a functional motif required for receptor down-regulation. Moreover, when we looked at the signaling capabilities of these constructs, they followed a pattern identical to that observed with the down-regulation results (Figure 6). For instance, when the type I receptor cytoplasmic domain sequence is intact (A105), or mutated outside of the BOX region (i.e., clones −1, 0, 6/2, 7, 8, 9), the chimeric receptor is capable of stimulating PAI-1 protein and Smad2 phosphorylation similar to the endogenous TGF-βR (Figure 6, A and B). This is contrasted by mutations within the BOX (i.e., clones 1, 2, 3, 4, 5, 6/1, 6) that result in an inability to stimulate expression of PAI-1 (Figure 6A), Smad2 phosphorylation (Figure 6B), or fibronectin (Figure 6C). Because the Smad4-independent induction of fibronectin is similarly regulated as PAI-1, this suggests that the BOX region is controlling a fundamental function in TGF-βR signaling (Engel *et al.*, 1999; Hocevar *et al.*, 1999; Sirard *et al.*, 2000). Moreover, of the 13 independent mutations shown in Figures 5 and 6, only the Box 9 mutation falls outside of the kinase domain (Franzén *et al.*, 1993; Kingsley, 1994; ten Dijke *et al.*, 1994), yet Box clones −1, 0, 7, 6/2, and 8 (mutations all within the kinase domain) signal and down-regulate similar to wild-type receptors. Thus, the sequence encompassed by amino acids 482–491 provides a critical function in ligand-dependent TGF-βR activation.

The BOX Region Controls TGF-βR Activation

Signaling and endocytosis of the TGF-βR complex in mesenchymal cells is dependent on type I receptor recruitment and transphosphorylation by the type II receptor (Wrana *et al.*, 1994; Anders *et al.*, 1998). Because mutations in the BOX region prevent both signaling and down-regulation, we determined whether effects on type I receptor phosphorylation might be the mechanism through which the BOX modulated these activities. To address this question, Cos7 cells were transiently transfected with a wild-type type II TGF-βR and either a wild-type or BOX-ANA mutant type I receptor, and type I receptor phosphorylation was determined. Although phosphorylation of the wild-type type I TGF-βR occurred in a ligand-dependent manner, there was no detectable phosphorylation of the BOX-ANA mutant despite both receptors showing similar plasma membrane expression (Figures 4A and 7). Thus, by preventing TGF-β-stimulated type I receptor phosphorylation in vivo, residues 482–491 regulate activation of the TGF-βR complex.

A previous publication has defined two residues, Gly-261 and Gly-322, that provide a critical role in promoting type I receptor phosphorylation by the type II receptor (Weis-Garcia and Massagué, 1996). Although these amino acids were necessary for type I receptor activation (i.e., phosphorylation) and subsequent signaling, receptors mutated at these sites were shown to have a functional kinase through their ability to be complemented by cotransfection with a kinase-defective type I receptor mutant. As such, to document that the loss-of-function mutations in the BOX were not reflecting a misfolding of the kinase domain but rather a new activation motif, the ability of the Box 3 clone (Figures 5 and 6) to transcomplement a kinase-impaired mutant type I receptor was determined. As shown in Figure 8A, although the Box 3 mutation was unable to stimulate 3TP-luciferase activity when expressed alone (in the context of a wild-type type II receptor), cotransfection of a kinase-defective type I receptor resulted in a five- to sixfold increase in signaling. This is similar to that observed with the Gly-261 and Gly-322 activation mutants (Weis-Garcia and Massagué, 1996) (Figure 8A).

Although Figure 8A shows that the Box 3 mutant can function as a receptor kinase, it was of interest to document whether the endocytic machinery, in addition to the signaling machinery (Figure 8A), could respond appropriately to the Box 3 mutation. To address this question, epithelial cell clones were isolated expressing the type II TGF-βR and either the wild-type or Box 3 mutant type I receptor. Because epithelial cultures, in contrast to fibroblasts, do not require type I receptor phosphorylation for down-regulation (Doré *et al.*, 2001), this allows a direct determination of whether the Box 3 mutation simply generates a receptor structure that is unable to be recognized by the endocytic system. As shown in Figure 8B, epithelial cells expressing a Box 3 mutant type I receptor down-regulate to a similar extent as wild-type receptors. Thus, BOX mutations that modulate TGF-βR signaling and down-regulation reflect an absence of TGF-βR activation and not an overall defect in receptor recognition or function.

DISCUSSION

TGF-βR signaling is dependent on the formation of a heteromeric complex consisting of a type I and type II receptor(s). Because ligand-specific signaling is defined by the

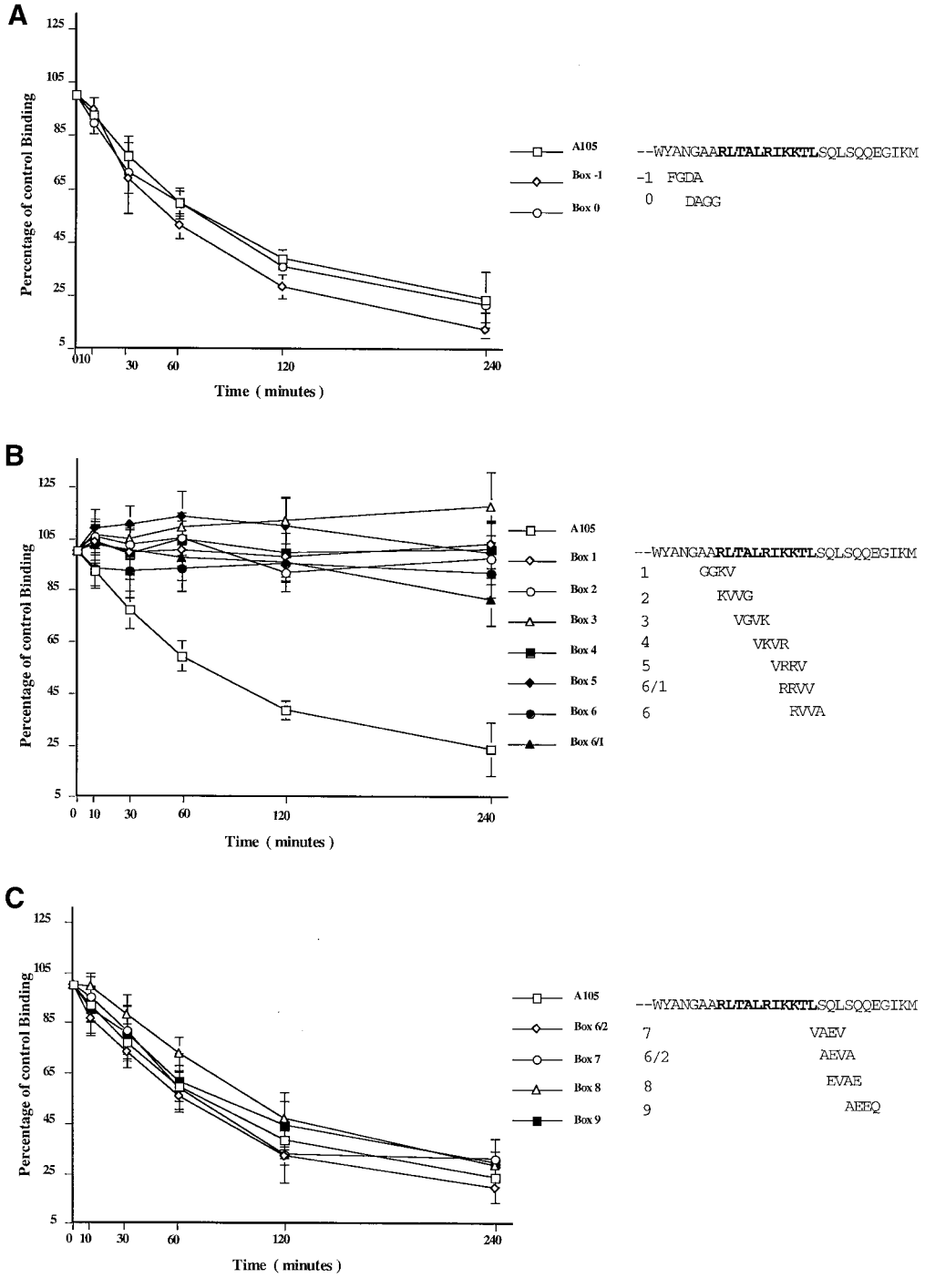

Figure 5. Endocytic effect of BOX region mutations. The right-hand side depicts the location of the 13 4 × 2 shift mutants. The conserved BOX region is in bold. (A) Mutagenesis NH2 terminal of the BOX does not affect down-regulation. Down-regulation assays were performed in the A105 cells (□) and clones Box-1 (◇) and Box 0 (○) as described (Doré *et al.*, 1998). The data represent the mean ± SE of three separate experiments for the A105 cells and three independent clones for each of the two Box region mutants done in duplicate. (B) Mutagenesis within the BOX prevents receptor down-regulation. The indicated seven Box clones are depicted, and down-regulation assays were performed as above for the A105 cells (□) and clones Box 1 (◇), Box 2 (○), Box 3 (△), Box 4 (■), Box 5 (◆), Box 6 (●), and Box 6/1 (▲). The data represent the mean ± SE of three separate experiments on three independent clones for each of the seven Box mutants done in duplicate. (C) Mutagenesis COOH terminal of the BOX does not affect down-regulation. The type I TGF-βR sequence and the point mutations COOH terminal to the BOX region are indicated. Down-regulation assays were performed in the A105 cells (□) and clones Box 6/2 (◇), Box 7 (○), Box 8 (△), and Box 9 (■). The data represent the mean ± SE of three separate experiments on three independent clones for each of the four Box mutants done in duplicate.

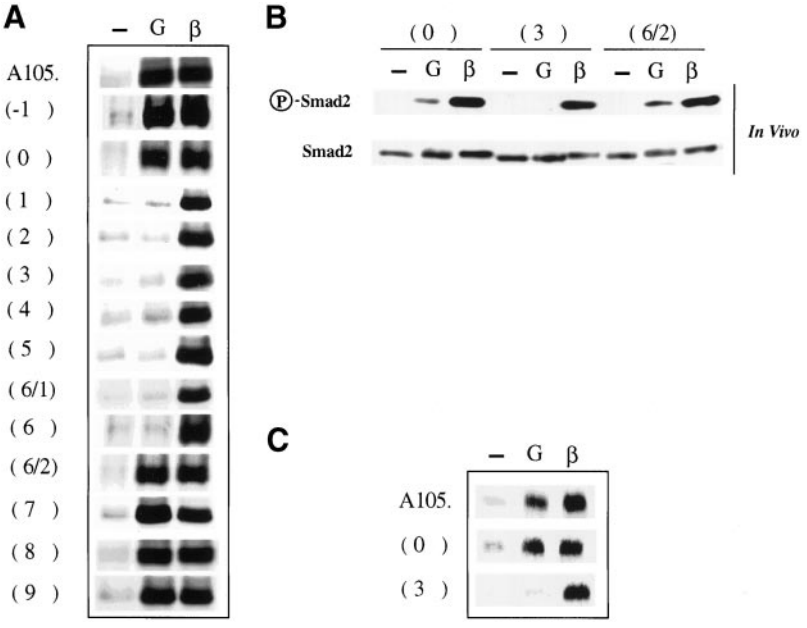

Figure 6. Signaling effect of BOX region mutations. To analyze the signaling capability of the mutated chimeric receptors, endogenous PAI-1 production (A), Smad2 phosphorylation (B), or fibronectin activation (C) was determined in control (−) and GM-CSF (G) or TGF-β2 (β) stimulated cultures. The clone numbers are identical with the labeling of Figure 5 and document that point mutations within the BOX are unable to stimulate TGF-βR induction of PAI-1, Smad2 phosphorylation, or fibronectin.

type I receptor (Feng and Derynck, 1997; Chen *et al.*, 1998; Massagué, 1998), we wished to determine whether this reflected defined protein binding sites and/or activation motifs distinct from the GS domain. As such, truncation mutants were made in the type I receptor after amino acids 216, 441, 481, 492, and 498 (Figure 1A). Although it was to be expected that receptor truncations at 216 and 441, which delete significant amounts of the kinase domain (encompassing amino acids 207–498), would be unable to signal, the differential signaling response from the Δ481 and Δ492 receptors indicated that residues 482–492 might have a significant role in TGF-βR function (Figure 1B).

The relationship between ligand-stimulated signaling and receptor endocytic activity is currently unclear (DiFiore and Gill, 1999; Ceresa and Schmid, 2000; Leof, 2000). Although much of our current understanding surrounding growth factor receptor endocytosis derives from studies performed on the epidermal growth factor and insulin receptor tyrosine kinases (Wiley *et al.*, 1991; Ware *et al.*, 1997; Ceresa *et al.*, 1998; Contreres *et al.*, 1998; Kil *et al.*, 1999), relatively little has been done to investigate these processes in the TGF-βR superfamily. Because the signaling mechanism, intrinsic receptor kinase activity, and biology of the two receptor systems differ dramatically, it is unknown whether paradigms developed for receptor tyrosine kinases will be operative in the TGF-β serine/threonine receptor family. To that end, we designed a chimeric receptor system that has allowed us to determine that 1) there are distinct differences in the endocytic fates of ligand-activated heteromeric and homomeric TGF-β receptors (Anders *et al.*, 1997), 2) the kinase activity of

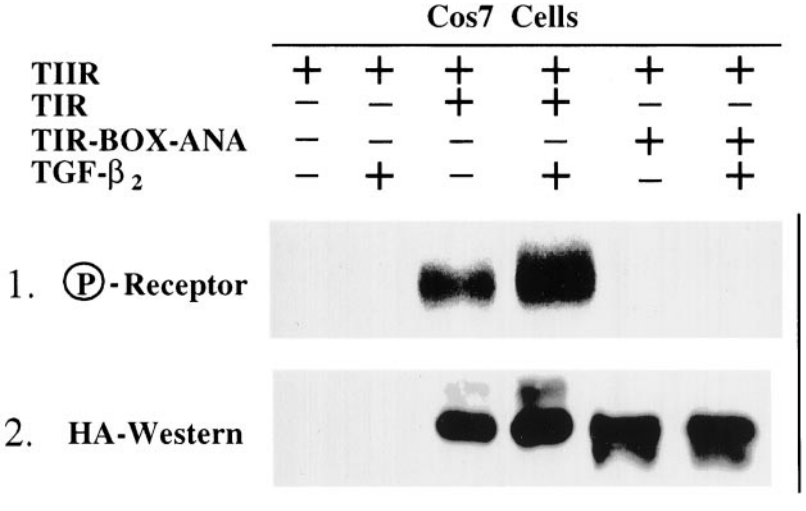

Figure 7. BOX region mutations prevent type I receptor phosphorylation in vivo. Lane 1, in vivo receptor phosphorylation was performed in Cos cells after transfection of the indicated (i.e., wild-type type II receptor, TIIR; wild-type type I receptor, TIR; and/or type I receptor with BOX-ANA mutation, TIR BOX-ANA) CMV promoter-driven native TGF-βR constructs and 0.5 μg CMV-β-galactosidase. The type II receptor was His tagged and both type I receptors contained an HA epitope. After incubation for 30 h at 37°C, the cultures were pulsed for 2 h with 0.5 mCi/ml [^{32}P] and treated as indicated with 10 ng/ml TGF-β2 for 15 min. The samples were normalized for β-galactosidase expression, and the TIR/TIIR complex was prepared by overnight incubation at 4°C with His-Bind resin. The receptor complex was eluted with 350 mM imidazole, immunoprecpitated with 12CA5 monoclonal anti-HA antibody, and analyzed by SDS-PAGE. Lane 2, parallel plates were treated identical to those in lane 1 except without orthophosphate labeling to document equivalent expression of the type I receptor constructs. The receptor was detected by Western blotting with 12CA5 conjugated to horseradish peroxidase.

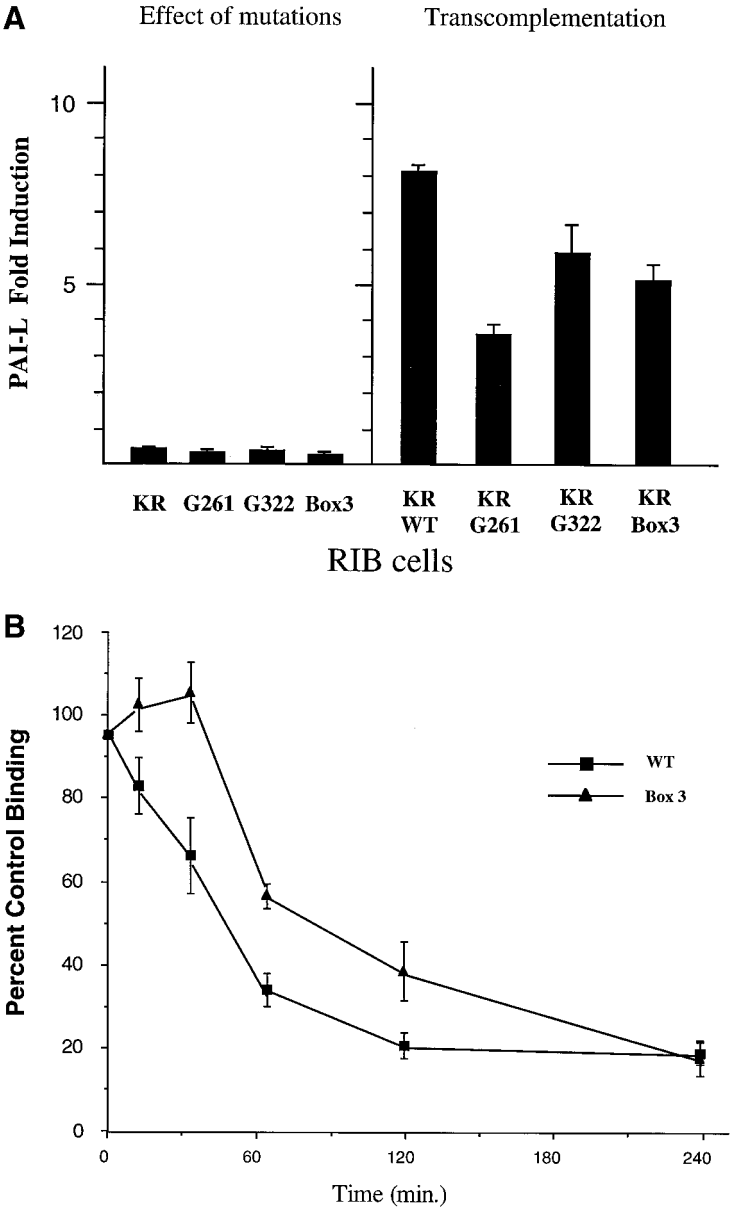

Figure 8. Box 3 mutations have a functional receptor kinase and down-regulate in epithelial cells. (A) R1B cells were transiently transfected with 3TP-Lux, a wild-type chimeric type II receptor and the indicated chimeric type I receptor(s) (WT, wild-type; KR, K232R mutation in ATP binding site; G261 and G322; G261E and G322D activation mutants, respectively) (Weis-Garcia and Massagué, 1996) (Box3, 284–287 VGVK mutant shown in Figure 5). After stimulation with 15 ng/ml GM-CSF for 24 h, the cultures were normalized for β-galactosidase expression, and the fold induction of luciferase expression (relative to no ligand) was determined. The data represent the mean ± SE of two separate experiments done in duplicate. (B) Mv1Lu clones expressing wild-type chimeric type II and either wild-type (MB202–4 and MB202–18) (Doré *et al.*, 2001) or Box 3 mutant (MBox3–1, MBox3–3, MBox3–8) type I TGF-βRs were isolated. Down-regulation assays were performed as described and represent the mean ± SE of one experiment for each of the three MBox clones and MB202–4, and two experiments for MB202–18, all done in duplicate.

the type II (but not the type I) TGF-βR is required in fibroblasts for optimal endocytosis (Anders *et al.*, 1998), and 3) mesenchymal and epithelial cultures respond to and process endocytosed TGF-β receptors in a distinct manner (Doré *et al.*, 1998, 2001). Because the kinase activity of the type I receptor was not required for effective endocytosis, yet only a heteromeric complex of type I and type II receptors down-regulate, we wished to determine whether a defined element(s) in the type I receptor provided this endocytic information and how this activity could be integrated into a more comprehensive model of receptor signaling.

When the truncated type I receptors were coexpressed with a wild-type chimeric type II receptor, a pattern of positive- and negative-acting effects on down-regulation were observed (Figure 2). For instance, although deletion of residues 442–503 (TIRΔ441) prevented down-regulation, subsequent truncation to amino acid 216 (TIRΔ216) resulted in a receptor complex that down-regulated similarly to wild-type receptors (Figure 2). Although it is presently unknown whether a distinct element exists between residues 217 and 441 negatively regulating down-regulation, the Δ216 construct clearly documents that down-regulation is not dependent on receptor signaling or an intact kinase domain (Figures 1 and 2).

Because our previous data supported a functional role for amino acids 482–492 in receptor signaling, endocytic studies were performed to further characterize this region. As shown in Figure 2, although the absence or presence of kinase insert II (TIRΔ441 and TIRΔ481, respectively) did not restore heteromeric receptor down-regulation, addition of

11 amino acids (TIRΔ492) generated a type I receptor capable of significant down-regulation (when complexed with a type II TGF-βR). Although the TIRΔ492 construct down-regulated to a similar extent as the wild-type chimeric receptors in the A105 cells, the rate of down-regulation was slower. To determine whether this reflected a requirement for additional receptor sequence, clones were generated expressing a wild-type type II chimeric TGF-βR and a chimeric type I TGF-βR truncated at amino acid 498 (TIRΔ498). Because these clones showed an identical response as the TIRΔ492 lines (Figure 2), and the TIRΔ498 receptor is only missing the five most COOH-terminal residues, the different kinetics of receptor down-regulation observed in the A105 cells is likely a reflection of clonal variation.

The sequence encompassing amino acids 482–492 for various type I receptors is depicted in Figure 3A. No canonical motifs or significant sequence conservation with protein kinases other than type I family members was found within this region; however, an extremely high degree of conservation was noted within the activin-like kinase family, including identity at 10/11 and 9/11 residues with the *Drosophila* thickveins and saxophone type I receptors (Figure 3A). As such, the BOX region seems to be a sequence uniquely restricted to regulating type I TGF-βR activity. In that regard, recent studies by O'Connor and colleagues have shown that the decreased type I receptor activity of saxophone (relative to thickveins) in *Drosophila* can be mapped to the nonconserved proline within the BOX (M. O'Connor, personal communication). Thus, the functional activity of the BOX seems to be evolutionarily conserved throughout the type I TGF-βR family.

The BOX region was shown to be necessary for receptor down-regulation, PAI-1 and fibronectin protein expression, transcriptional activation, Smad2 phosphorylation, and growth inhibitory responses from both chimeric and endogenous TGF-β receptors (Figures 1–6). Moreover, the finding that similar affects are observed on Smad4-dependent and -independent signaling, as well as receptor endocytic activity in mesenchymal cells, indicates that mutation of the BOX region interferes with an early event(s) in receptor activation. As such, we determined the role of the BOX region in TGF-βR complex formation and type I receptor phosphorylation. Although the native type I receptor BOX-ANA mutant was capable of forming a heteromeric complex with the type II TGF-βR to a similar extent as the wild-type type I receptor (Figures 4A and Figure 7, row 2), the associated type II receptor was unable to transphosphorylate and activate the mutant receptor in vivo (Figures 6D and 7, row 1).

Computer modeling with the use of Insight II and Sybyl 6.6 indicates that although the BOX region (amino acids 482–491) is significantly distal (33.7 Å, on average) to the regulatory juxtamembrane GS domain (amino acids 176–205), it is exposed on the same surface as Gly-261 and Gly-322, two residues required for activation of type I receptor subunits (Weis-Garcia and Massagué, 1996; Huse *et al.*, 1999). Because it was shown previously that Gly-261 and Gly-322 could be transcomplemented by inactive type I receptors containing a mutation in the ATP binding site (Weis-Garcia and Massagué, 1996), we determined whether the BOX motif would respond similarly. As expected for an activation domain, cotransfection of the Box 3 mutant with a kinase-impaired type I receptor restored TGF-βR signaling (Figure 8A). Because type I receptor kinase activity is required for TGF-β signaling, and the only receptor capable of providing this function harbors the Box 3 mutation, this shows that the BOX mutation does not directly impair the receptor kinase. Moreover, because the endocytic requirement for type I receptor phosphorylation was shown recently to differ between epithelial and fibroblast cells (Doré *et al.*, 2001), this provided an ideal opportunity to assess whether the observed effects on down-regulation were a specific reflection of an absence of type I receptor phosphorylation or caused by a general misfolding of the receptor. When the Box 3 mutant receptor was expressed in epithelial cells, the receptor complex down-regulated to a similar extent as wild-type (Figure 8B). Thus, not only does a Box 3 mutant receptor have a functional kinase, but the mutation is capable of being recognized by the endocytic machinery.

These results suggest that type I receptor activation involves the coordinated action of multiple regulatory domains. Furthermore, although the BOX is within the type I receptor kinase domain (amino acids 207–498), the absence of receptor activity cannot be explained simply by disrupting this region. For instance, 1) analogous mutations 5′ or 3′ to the BOX have no apparent effect on either receptor endocytosis or signaling (Figures 5 and 6); 2) the absence of type I receptor kinase activity, per se, has no effect on TGF-βR down-regulation in fibroblasts (Anders *et al.*, 1998); 3) modeling of energy-minimized Box 3 substitution mutants shows only a minor structural perturbation with a 0.63 Å overall shift in the backbone; 4) truncation after amino acid 216 (i.e., missing essentially the entire kinase domain) generates a type I receptor that down-regulates similar to wild-type (Figure 2); and 5) cotransfection with a kinase-impaired type I receptor generates an active signaling complex (Figure 8A). As such, the manner in which mutations within this motif block type I receptor activation is not apparent from the structure. These observations indicate that the sequence between amino acids 482 and 491 in the type I receptor provides a critical function regulating GS domain phosphorylation and subsequent activation of the TGF-β receptor complex.

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