TβRIII independently binds type I and type II TGF-β receptors to inhibit TGF-β signaling

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ABSTRACT Transforming growth factor-β (TGF-β) receptor oligomerization has important roles in signaling. Complex formation among type I and type II (TβRI and TβRII) TGF-β receptors is well characterized and is essential for signal transduction. However, studies on their interactions with the type III TGF-β coreceptor (TβRIII) in live cells and their effects on TGF-β signaling are lacking. Here we investigated the homomeric and heteromeric interactions of TβRIII with TβRI and TβRII in live cells by combining IgG-mediated patching/immobilization of a given TGF-β receptor with fluorescence recovery after photobleaching studies on the lateral diffusion of a coexpressed receptor. Our studies demonstrate that TβRIII homo-oligomerization is indirect and depends on its cytoplasmic domain interactions with scaffold proteins (mainly GIPC). We show that TβRII and TβRI bind independently to TβRIII, whereas TβRIII augments TβRI/TβRII association, suggesting that TβRI and TβRII bind to TβRIII simultaneously but not as a complex. TβRIII expression inhibited TGF-β–mediated Smad2/3 signaling in MDA-MB-231 cell lines, an effect that depended on the TβRIII cytoplasmic domain and did not require TβRII ectodomain shedding. We propose that independent binding of TβRI and TβRII to TβRIII competes with TβRI/TβRII signaling complex formation, thus inhibiting TGF-β–mediated Smad signaling.

INTRODUCTION

Transforming growth factor-β (TGF-β) ligands play critical roles in a variety of physiological and pathological processes (Massague, 1998, 2012; Elliott and Blobe, 2005; Clarke and Liu, 2008; Gordon and Blobe, 2008; Deheuninck and Luo, 2009; Heldin et al., 2009). They signal via the Ser/Thr kinase type I (TβRI) and II (TβRII) TGF-β receptors, assisted and/or regulated by distinct coreceptors, the best-characterized of which is the type III TGF-β receptor (TβRIII), or betaglycan; Lin et al., 1992; Franzen et al., 1993; Eickelberg et al., 2002; Shi and Massague, 2003; Bernabeu et al., 2009; Gatza et al., 2010). TGF-β signaling is initiated by ligand binding to TβRII, which recruits and phosphorylates TβRII, inducing signaling via the canonical Smad pathway and/or (depending on the cellular context) several non-Smad pathways (Shi and Massague, 2003; Moustakas and Heldin, 2009; Zhang, 2009; Ehrlich et al., 2012). In the Smad pathway, the activated TβRI phosphorylates R-Smads, followed by their hetero-oligomerization with Smad4. The resulting Smad complex accumulates in the nucleus, where it regulates gene transcription (Shi and Massague, 2003; Feng and Derynck, 2005; Schmierer and Hill, 2007).

TβRIII is the most abundant and well-characterized TGF-β coreceptor. It is a proteoglycan comprising 851 amino acids, which binds to several TGF-β–family ligands and presents them to the signaling receptors (López-Casillas et al., 1994). TβRIII also regulates TGF-β signaling to the p38 pathway (You et al., 2007), inhibits nuclear factor κB signaling (You et al., 2009), and activates Cdc42 to regulate cell proliferation and migration (Mythreye and Blobe, 2009). Moreover, TβRIII was shown to inhibit TGF-β superfamily signaling through ectodomain shedding–mediated generation of soluble TβRIII, which can bind and sequester TGF-β away from its receptors.
and were incapable of detecting transient complexes, which might dissociate during the patching or immunoprecipitation steps (Rechtman et al., 2009). Moreover, it was not known whether the oligomerization of TβRIII is direct or depends on association with scaffold proteins.

The mode of interaction (stable vs. transient) among cell-surface proteins can be determined by patch/FRAP (Henis et al., 1990; Eisenberg et al., 2006; Rechtman et al., 2009). In this method, one receptor is patched and immobilized by cross-linking with a double layer of IgGs, and the effect on the lateral diffusion of a coexpressed, extracellularly tagged receptor, labeled exclusively by monovalent Fab' fragments, is measured by FRAP (see Materials and Methods).

Complex formation between the receptors can reduce either the mobile fraction (Rf) or the lateral diffusion coefficient (D) of the Fab'-labeled receptor, depending on the FRAP time scale relative to the dissociationassociation rates of the complex. Complex lifetimes longer than the characteristic FRAP times (i.e., stable interactions) lead to a reduction in the mobile fraction without affecting the diffusion rate, since bleached Fab'-labeled receptors do not undergo appreciable dissociation from the immobile clusters during the FRAP measurements. Conversely, short complex lifetimes (transient interactions) result in several association/dissociation cycles for each fluorescent-labeled molecule during the FRAP measurement, thus reducing the diffusion rate (lower D) without altering Rf (Henis et al., 1990; Eisenberg et al., 2006; Rechtman et al., 2009). We previously demonstrated that the mobility-restricting effects of the IgG cross-linking are specific and do not involve nonspecific steric trapping (Shvartsman et al., 2003).

We first used patch/FRAP to investigate the interaction mode of homomeric TβRIII complexes at the surface of COS7 cells, which do not express detectable levels of TβRIII before transfection. To this end, we coexpressed differently tagged hemagglutinin [HA]-TβRIII and myc-TβRIII and subjected the cells to patch/FRAP studies. Fab'-labeled (un-cross-linked) myc-TβRIII was laterally mobile in the plasma membrane of COS7 cells, whereas HA-TβRIII subjected to IgG-mediated patching became laterally immobile (Figure 1, A and B). The average results of patch/FRAP experiments testing the effects of immobilization of HA-TβRIII on the lateral diffusion of coexpressed myc-TβRIII are summarized in Figure 1, C and D. Immobilization of HA-TβRIII mediated a significant reduction (45%) in Rf of the coexpressed myc-TβRIII without affecting the D value (Figure 1D).

Such an effect characterizes stable interactions between the HA- and myc-tagged TβRIII pairs (Henis et al., 1990; Rechtman et al., 2009), suggesting the formation of homomeric TβRIII complexes that are stable on the time scale of the FRAP measurements (minutes). These complexes were unaffected by TGF-β1 or TGF-β2, in line with the high homo-oligomerization level of TβRIII reported earlier based on immunofluorescence copatching (Henis et al., 1994). A statistical correction is required to convert the percentage reduction in Rf to percentage homodimerization (Ehrlich et al., 2011; Marom et al., 2011), since the probabilities of homodimer formation are 1:2:1 for HA/HA+, (myc/HA + HA/myc), and myc/myc-containing dimers. On immobilization of cross-linked HA-TβRIII and FRAP measurement of the lateral diffusion of myc-TβRIII, only myc-TβRIII in mutual complexes with HA-TβRIII would undergo immobilization, whereas the mobility of myc/myc-containing homodimers would not be affected, and HA/HA homodimers do not contribute to the measurement. In addition, myc-TβRIII/myc-TβRIII complexes contain two myc tags and are therefore labeled at twice the intensity of myc/HA-containing homodimers. Thus, for homodimers, the percentage reduction in Rf in patch/FRAP studies should be multiplied by 2 to obtain the percentage of homodimeric receptors.

RESULTS

TβRIII homomeric complex formation depends on its cytoplasmic domain and on binding to GIPC

Using immunofluorescence copatching and immunoprecipitation, we previously demonstrated that TβRIII forms homomeric complexes at the cell surface already in the absence of ligand (Henis et al., 1994). However, these experiments were semiquantitative.
Therefore the 45% reduction in \( R_f \) of myc-TβRII upon immobilization of HA-TβRII suggests a very high level of homodimerization (45 × 2 = 90%). Naturally, if the oligomers are larger than homodimers, the statistical correction is smaller, becoming negligible for oligomeric structures containing many subunits of the same receptor, since in a large oligomer, the probability that at least one subunit will carry a different tag is high, increasing with the number of subunits in the oligomer.

Because TβRII was shown to interact with the scaffolding proteins β-arrestin2 (Chen et al., 2003; Finger et al., 2008) and GIPC (Blobe et al., 2001a) through its short cytoplasmic domain, it is possible that these interactions regulate its homo-oligomerization. To test this hypothesis, we used patch/FRAP to measure the interactions of several HA-TβRII cytoplasmic domain mutants with WT myc-TβRII (Figure 2). In contrast to full-length TβRII, cross-linking of a TβRII mutant whose cytoplasmic domain was truncated right after the Y5SD sequence (replacing the cytoplasmic domain with RR to retain a positive charge where the transmembrane domain ends; TβRII-Cyo; Blobe et al., 2001a) did not reduce the \( R_f \) of the coexpressed myc-TβRII but instead reduced its D value (Figure 2, A and B). Similar results were obtained upon cross-linking of HA-TβRII-

Cyto-1, a TβRII mutant truncated after the YSYHTGARQ cytoplasmic sequence (Blobe et al., 2001b). The shift from an effect on \( R_f \) to an effect on D is characteristic of a transfer from stable to transient interactions (Rechtman et al., 2009; Ehrlich et al., 2011), suggesting an important role for the cytoplasmic domain of TβRII in the homeric interactions. Of interest, analogous effects on myc-TβRII WT diffusion were observed upon cross-linking of HA-TβRII-Del, a TβRII mutant lacking the last three C-terminal amino acids comprising a class I PDZ binding domain that mediates binding to GIPC (Blobe et al., 2001a; Figure 2, C and D). Interactions with β-arrestin2 appeared to have a lower contribution, since cross-linking of HA-TβRII-T841A, a TβRII mutant that does not bind β-arrestin2 (Chen et al., 2003), compromised but did not abolish the reduction in \( R_f \) of myc-TβRII-WT (Figure 2, E and F). These findings suggest that TβRII homo-oligomerization is indirect and primarily mediated by binding of its cytoplasmic domain to intracellular scaffolds containing GIPC and, to a lesser extent, β-arrestin2.

### TβRII oligomerization with TβRI and TβRII

Previous studies demonstrated that TβRII forms TGF-β1-induced complexes with TβRI independently of TβRI (López-Casillas et al., 1993) and enhances ligand binding to TβRI. In addition, TβRII was shown to phosphorylate TβRII, resulting in dissociation of TβRII from the TβRI/TβRII signaling complex (Blobe et al., 2001b). However, subsequent studies reported that some TβRII might remain associated with TβRII as the complex internalizes in endocytic vesicles (Chen et al., 2003). To explore the dynamics of the interactions between TβRII and TβRII, we conducted patch/FRAP studies on cells expressing HA-TβRII and myc-TβRII in the presence or absence of ligand (TGF-β1 or -β2), measuring the effects of patching HA-TβRII on the lateral diffusion of the Fab’-labeled myc-TβRII (Figure 3). Unexpectedly, some reduction in \( R_f \) of TβRII occurred already upon coexpression with TβRII (without cross-linking), suggesting that a subpopulation of TβRII interacts preferentially with slowly diffusing or immobile TβRII molecules/clusters (Figure 3A). This demonstrates that TβRII/TβRII complexes exist before ligand binding. The mild reduction in \( R_f \) was markedly increased upon TβRII cross-linking (from 17 to 33%; no effect on D), indicating that a second population of TβRII interacts with an initially mobile subclass of TβRII, which is immobilized after IgG cross-linking (Figure 3, A and B). Of note, coexpression of excess untagged TβRI canceled this reduction, indicating both specificity and saturability in the binding of TβRII to TβRII. TGF-β1 or β2 had no significant effect on these interactions (Figure 3, A and B). Note that for heterocomplexes (e.g., TβRI/TβRII), no statistical correction is needed, and the percentage reduction in \( R_f \) of myc-TβRII upon cross-linking of HA-TβRII is a direct measure of their hetero-oligomerization (Rechtman et al., 2009; Ehrlich et al., 2012).
plasmic domain of TβRII in the cotransfection, demonstrating the specificity and saturability of these interactions. The heteromeric TβRII/TβRII complexes were slightly enhanced by TGF-β1 (to 25%) or -β2 (to 24%), but this enhancement was not statistically significant. Moreover, analogous measurements of the interactions of TβRI with TβRII cytoplasmic domain mutants (Cyto, Del, and T841A) yielded results identical to those obtained with TβRII WT (Figure 6), suggesting that TβRI/TβRII interactions are independent of GIPC or β-arrestin2 binding.

Next we investigated whether interactions mediated by the cytoplasmic domain of TβRII are involved in its hetero-oligomerization with TβRII. To that end, we conducted patch/FRAP measurements of myc-TβRII with HA-TβRII cytoplasmic domain mutants (Cyto, Del, and T841). All of the TβRII cytoplasmic domain mutations abolished the reduction in $R_i$ of TβRII upon coexpression with TβRII before cross-linking (Figure 4, A, C, and E). Instead, the $D$ values of TβRII were decreased upon coexpression with these TβRII mutants (Figure 4, B, D, and F). Together with the observation that the cytoplasmic mutations of TβRII interfere with its homo-oligomerization, which occurs via binding to intracellular scaffolds (Figure 2), these results suggest that the immobile subpopulation of TβRII that interacts preferentially with TβRI arises due to association of TβRII with GIPC and/or β-arrestin2-containing scaffolds.

Analogous studies on TβRII/TβRI interactions yielded a different pattern, suggesting weaker interactions (Figure 5), as $R_i$ of myc-TβRII was not reduced merely by coexpression with HA-TβRII, and the percentage reduction in its $R_i$ upon cross-linking of coexpressed HA-TβRII was lower (17%). This effect essentially disappeared upon inclusion of excess untagged TβRII in the cotransfection, demonstrating the specificity and saturability of these interactions. The heteromeric TβRII/TβRII complexes were slightly enhanced by TGF-β1 (to 25%) or -β2 (to 24%), this enhancement was not statistically significant. Moreover, analogous measurements of the interactions of TβRI with TβRII cytoplasmic domain mutants (Cyto, Del, and T841A) yielded results identical to those obtained with TβRII WT (Figure 6), suggesting that TβRI/TβRII interactions are independent of GIPC or β-arrestin2 binding.
TβRI and TβRII bind to TβRIII independently of each other
We previously used patch/FRAP to demonstrate that TβRI and TβRII form stable heteromeric complexes (Rechtman et al., 2009; Ehrlich et al., 2011). In the present work, we show that TβRIII interacts with TβRII and TβRI (Figures 3 and 5). Therefore it was of interest to explore the effects of TβRII expression on TβRII/TβRII interactions. The reduction in Rf of myc-TβRII after HA-TβRIII cross-linking was not affected by overexpression of untagged TβRIII (Figure 7), as shown by the fact that it remained identical to that observed in the absence of untagged TβRIII (compare with Figure 5). Moreover, in contrast to the reduced Rf of myc-TβRII upon coexpression with TβRIII (Figure 3A), mere coexpression of untagged TβRII did not confer reduction in Rf of myc-TβRII coexpressed with TβRIII (Figure 7A), suggesting that TβRI binding to TβRIII is not enhanced by TβRI/TβRIII complex formation.

The lack of effect of TβRII on TβRIII/TβRII interactions indicates that TβRII and TβRI binding to TβRIII is mutually independent. If they bind to distinct sites, it is expected that they could bind to TβRIII simultaneously. To investigate this issue, we coexpressed an excess of untagged TβRIII with HA-TβRII and myc-TβRII, cross-linked (or not) HA-TβRII, and used FRAP to measure the lateral diffusion of myc-TβRII (Figure 8). In accord with our earlier studies on TβRI/TβRII interactions (Rechtman et al., 2009), when myc-TβRII and HA-TβRII were coexpressed without TβRIII, the Rf of myc-TβRII was significantly reduced (by 26%) upon cross-linking of HA-TβRII, whereas its D value was unaffected, demonstrating stable heterocomplex formation (Figure 8). These interactions were augmented by ligand, as ligand addition increased the reduction in Rf (to 35–38%; Figure 8A). Of importance, overexpression of untagged TβRIII had an augmenting effect similar to that induced by the ligand on HA-TβRII/myc-TβRII interactions (Figure 8A). These results suggest the formation of a triple complex containing TβRII, TβRII, and TβRI and are in line with distinct binding domains on TβRII for TβRII and TβRI.

TβRIII-mediated inhibition of Smad2/3 signaling depends on its cytoplasmic domain
TβRIII interactions with TβRI and TβRII can modulate TGF-β-induced signaling. Because TβRIII was shown to undergo ectodomain shedding that can inhibit TGF-β signaling by ligand sequestration (López-Casillas et al., 1994; Elderbroom et al., 2014), we studied the effects of TβRII-WT, its shedding-defective mutant TβRII-Dshed, and the
This notion is further supported by the observation that expression of TβRIII-Cyto, which is not shedding defective, does not inhibit TGF-β–induced Smad2/3 nuclear translocation (Figure 9, B and C). The latter finding suggests that the shedding-independent inhibition depends on the cytoplasmic domain of TβRIII. These conclusions are further supported by transcriptional activation assays conducted on the same cell lines (Figure 9D) using the TGF-β–responsive luciferase reporter construct CAGA-Luc (Dennler et al., 1998). These experiments, which measure the transcriptional response downstream the Smad2/3 signaling pathway, demonstrated marked inhibition of Smad2/3 transcriptional response after expression of TβRIII-WT and TβRIII-ΔShed, with no inhibition by TβRIII-Cyto.

**FIGURE 6:** Stable TβRIII/TβRI heteromeric complexes form independently of the TβRIII cytoplasmic domain. Experimental conditions were as in Figure 2, except that myc-TβRIII was replaced by myc-TβRI. (A, C, E) R₁ values. (B, D, F) D values. Bars are mean ± SEM of 30–50 measurements in each case. Asterisks indicate significant differences between the R₁ values of the pairs indicated by brackets (*p < 10⁻³; Student’s t test). Cross-linking of TβRIII cytoplasmic domain mutants reduced R₁ of myc-TβRI, whereas its D values were not affected, similar to the observations after IgG cross-linking of HA-TβRIII-WT.

**FIGURE 7:** Expression of TβRII does not affect TβRI/TβRIII interactions. COS7 cells were cotransfected with myc-TβRII together with HA-TβRIII (or empty vector) and excess untagged TβRII. The diffusion of myc-TβRI was measured by FRAP with or without cross-linking of HA-TβRIII, as described in Figure 3. (A) Average R₁ values. (B) Average D values. Bars are mean ± SEM of 30–50 measurements in each case. Asterisks indicate significant differences between the R₁ values of the pairs indicated by brackets (*p < 10⁻³; Student’s t test). Cross-linking of HA-TβRIII reduced R₁ of myc-TβRI without affecting the D values. Neither the D nor the R₁ values were significantly affected by ligand (250 pM). Thus coexpression of untagged TβRII together with HA-TβRIII/myc-TβRI has no effect on TβRI binding to TβRIII, as the reduction in R₁ of myc-TβRI upon cross-linking HA-TβRIII remains exactly as in the absence of untagged TβRII (Figure 5). This suggests that TβRII binds to TβRIII independently and not through TβRI.
Smad2/3 signaling in MDA-MB-231 cells, an effect that required the TβRII cytoplasmic domain and persisted when TβRII ectodomain shedding was abrogated. We propose that the independent binding of TβRI and TβRII to TβRII competes with TβRI/TβRII signaling complex formation, thus inhibiting TGF-β-mediated Smad signaling (Figure 10).

Our earlier semiquantitative immunofluorescence copatching studies already indicated TβRII homo-oligomeric complex formation (Henis et al., 1994). The present patch/FRAP experiments (Figure 1) demonstrate that HA-TβRII/myc-TβRIII complexes not only are formed, but are also stable on the FRAP time scale (minutes) and are independent of ligand binding. These findings validate the copatching results (Henis et al., 1994); the insensitivity to ligand binding is in line with the high level of TβRII oligomerization before ligand binding, leaving little margin for an incremental increase in homo-oligomerization. Analogous experiments with TβRII mutants that lack binding to GIPC or β-arrestin2 (Figure 2) show that TβRII homo-oligomerization depends on its binding to GIPC and, to a lesser degree, to β-arrestin2. Thus TβRII homo-oligomeric complexes are indirect, reflecting mutual binding to GIPC (and/or β-arrestin2)–containing scaffolds.

To assess heterocomplex formation between TβRII and the signaling TGF-β receptors, we investigated TβRII interactions with TβRI and TβRII. Complex formation between TβRII and TβRII (Figure 3) was characterized by two distinct TβRII populations. One subpopulation was immobilized (reduction in Rr) directly upon coexpression with TβRII (without the need to immobilize TβRIII by IgG cross-linking), most likely reflecting binding to TβRII clusters that form due to association with intracellular scaffolds. This view is reinforced by the demonstration (Figure 4) that TβRII mutants with defective cytoplasmic interactions (CytO, Del, T841A) lose the “direct immobilization” effect on the TβRII subpopulation, and the reduction in Rr of TβRII shifts to an effect on D, suggestive of weaker, transient interactions. A laterally immobile TβRIII subpopulation due to binding to scaffold proteins is in line with the parallel loss of TβRIII homo-oligomerization in TβRII cytoplasmic domain and GIPC mutants (Figure 2) and suggests that the oligomerization of TβRII via binding to the scaffold proteins may enhance its interactions with TβRII. Another TβRII subpopulation interacts with TβRIII molecules that are initially mobile, as shown by the further reduction in Rr of TβRII after IgG cross-linking of TβRII (Figure 3). Of note, all TβRII/TβRIII interactions, including the “directly immobilized” subpopulation, were insensitive to ligand binding, in line with the dependence of these complexes on the cytoplasmic domain of TβRIII.

Complex formation between TβRI and TβRIII was distinctly different from TβRII/TβRIII interactions. Thus no “directly immobilized” subpopulation of TβRI coexpressed with TβRIII was detected, and the interactions of TβRI with TβRIII were independent of the TβRII cytoplasmic domain (Figures 5 and 6). The different characteristics of TβRI versus TβRII binding to TβRII raise the possibility that TβRI and TβRIII bind to nonoverlapping sites on TβRII. This view is supported by the finding that TβRI overexpression had no effect under any condition on myc-TβRI/HA-TβRII complex formation (compare Figure 7A with Figure 5A). Because TβRI and TβRII form a ligand-dependent heteromeric complex (Rechtman et al., 2009; Figure 8), the insensitivity of TβRI/TβRII interactions to TβRII coexpression implies that TβRI and TβRII do not bind to TβRIII as a complex. Coexpression of untagged TβRIII mildly enhanced TβRI/TβRII interactions, similar to the effect of ligand (Figure 8). Nonetheless, the TβRIII-mediated enhancement of TβRI/TβRII association is independent of ligand binding. This implies that TβRII may serve here as a scaffold by itself, bridging indirectly between TβRI and TβRII...
bind to TβRIII independent of each other (not as the TβRII/TβRIII signaling complex; see Figure 10).

Formation of an alternative TβRI/βRII/TβRIII complex that competes with the TβRI/βRIII signaling complex may alter TGF-β-mediated signaling. The testing of this hypothesis is complicated by TβRIII ectodomain shedding, which by itself can inhibit TGF-β signaling (López-Casillas et al., 1994; Elderbroom et al., 2014). To circumvent this complication, we studied TGF-β-mediated Smad signaling in MDA-MB-231 cell lines stably expressing TβRIII-WT.

The characterization of TβRIII as a coreceptor that enhances TGF-β binding to TβRII and facilitates TGF-β-mediated biology has largely been based on studies performed in specific model systems, including L6 myoblasts (López-Casillas et al., 1993; Blobe et al., 2001a,b). Prior results demonstrating decreased TGF-β signaling by TβRIII have been attributed to shedding of soluble TβRIII (López-Casillas et al., 1994; Dong et al., 2007). However, there are reports of TβRIII decreasing TGF-β signaling in specific cell contexts (Ji et al., 1999), which in some cases was shown to be independent of

\[ TβRII ΔShed (incapable of shedding), and TβRIII-Cyto (Figure 9). The persistence of the inhibition of Smad signaling (Smad2/3 nuclear translocation and Smad-dependent transcriptional activation) by TβRIII in the TβRIII-ΔShed–expressing cells demonstrates that it does not require TβRIII shedding. This does not mean that there is no inhibition by TβRIII shedding, as the former results on such inhibition were derived under conditions optimized to detect shedding effects (e.g., incubation for 24 h with conditioned medium derived from the same cell lines; Elderbroom et al., 2014). Of note, the TβRIII-dependent inhibition measured here (Figure 9) disappeared in cells expressing TβRIII-Cyto. This most likely reflects scaffold-dependent interactions of the TβRIII cytoplasmic domain, which are responsible for both TβRIII homomeric interactions (Figure 2) and the directly immobilized subpopulation of TβRII upon coexpression with TβRIII (Figure 3). The latter subpopulation, which represents scaffold-associated clusters and disappears in the TβRII-Cyto mutant, might have enhanced avidity for binding most of the TβRII molecules. The ability of TβRII to bind independently TβRII and TβRIII, competing with the signaling TβRII/TβRIII complex (Figure 10), provides an alternative novel mechanism for the inhibition of TGF-β-mediated signaling. This model is consistent with a prior report demonstrating that TβRIII can inhibit TGF-β signaling in renal epithelial cells via interference with TβRII/TβRIII signaling complex formation (Eickelberg et al., 2002). Note that there are time-domain differences between the two mechanisms: inhibition due to interactions of TβRII and TβRIII with TβRII on the cell surface is immediate, whereas inhibition due to TβRIII ectodomain shedding requires accumulation of the shed ectodomain over longer periods. There may be an interplay between the two mechanisms, as the effectiveness of shedding-dependent inhibition would depend on the presence/absence or level of appropriate peptidases. Moreover, inhibition due to association with TβRIII at the cell surface is specific to the cells that express these receptors, whereas shedding from one cell type can induce inhibition in neighboring cells as well.

**FIGURE 9:** TβRIII expression inhibits Smad2/3 signaling, depending on its cytoplasmic domain. (A) Affinity labeling of TβRIII in stably expressing MDA-MB-231 cell lines. Cells were incubated with [125I]TGF-β1 (100 pM), and bound ligand was cross-linked to the cell surface receptors. Lysates and conditioned media were immunoprecipitated with antibody against the extracellular domain of TβRIII. β-Actin was used as a loading control. Representative data from three independent experiments. (B, C) MDA-MB-231 cell lines were serum starved for 16 h, followed by incubation with or without TGF-β1 (100 pM, 30 min, 37°C), fixed/permeabilized, and processed for immunofluorescent labeling of Smad2/3 (see Materials and Methods). (B) Typical images of Smad2/3 localization. Bar, 20 μm. (C) Quantification of Smad 2/3 localization. The percentages of cells with predominantly nuclear Smad2/3 localization (mean ± SEM) were determined by scoring 100 cells/sample in three independent experiments. (D) MDA-MB-231 cell lines were cotransfected with the TGF-β-responsive luciferase reporter plasmid (CAGA)₂⁻Luc together with pRL-TK. At 24 h posttransfection, cells were serum starved (16 h), stimulated (or not) with TGF-β1 (100 pM, 24 h, 37°C), lysed, and analyzed for luciferase activity by the DLR assay. The results were normalized for transfection efficiency using Renilla luminescence. Data are presented as relative activation, taking the Neo cell line stimulated with TGF-β1 as 1. Bars are mean ± SEM of four independent experiments, each measured in triplicate. Asterisks indicate significant differences between the pairs of cell lines indicated by the brackets after stimulation with TGF-β1 (*p < 0.003; **p < 10⁻⁴; Student’s t test).
FIGURE 10: Model of TβRIII regulation of Smad2/3 signaling via interactions with TβRII and TβRI. (A) In the absence of TβRIII, ligand binding to TβRII enhances the formation of a heterotetrameric complex with TβRI, leading to activation of TβRI, which stimulates TGF-β–mediated Smad2/3 signaling. (B) When TβRIII is expressed, TβRII and TβRI bind independently to nonoverlapping sites on TβRII. This competes with formation of the normal signaling TβRII/TβRI complex, resulting in inhibition of TGF-β–mediated Smad2/3 signaling. The binding of TβRII by TβRIII in the inhibitory complex depends on the cytoplasmic domain of TβRII, most likely involving interactions with the scaffolding proteins GIPC and β-arrestin2. For simplicity, bound ligand is not shown in this panel. (C) Deletion of the TβRIII cytoplasmic domain removes the GIPC and β-arrestin2 scaffolding domains, resulting in loss of TβRIII homomorphic clustering, leading to a parallel loss of avidity toward binding TβRII and ineffective competition with the formation of TβRII/TβRI signaling complexes. (D) Ectodomain shedding of TβRIII results in soluble TβRIII, which provides an alternative mechanism of inhibition by competing for TGF-β binding.

sustainable TβRIII (Eickelberg et al., 2002). The latter report concluded that the TβRIII inhibition of TGF-β signaling in renal epithelial LLC-PK1 cells involves interference with TβRI/TβRII association, reinforcing the model proposed in the present study. Thus the effects of TβRIII on TGF-β signaling and TGF-β–mediated biology, like many aspects of TGF-β signaling, are likely to be cell context dependent. Whether the stable or transient interactions among TβRII, TβRII, and TβRII identified here regulate other aspects of TGF-β signaling remains to be determined.
IgG-mediated patching/cross-linking

At 24 h posttransfection, COS7 cells transfected with various combinations of expression vectors for TGF-β receptors were serum starved (30 min, 37°C), washed with cold Hank’s balanced salt solution (HBSS) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.2) and 2% BSA (HBSS/HEPES/BSA), and blocked with normal goat γ-globulin (200 μg/ml, 30 min, 4°C). They were then labeled successively at 4°C (to avoid internalization and enable exclusive cell surface labeling) in HBSS/HEPES/BSA (45-min incubations) with 1) monoclonal mouse Fab′ anti-myc (40 μg/ml) together with HA.11 rabbit IgG anti-HA (20 μg/ml) and 2) Alexa Fluor 546–Fab′-GtR (40 μg/ml) together with Alexa Fluor 488–IgG GtR (20 μg/ml). This protocol results in the HA-tagged receptor cross-linked and immobilized by IgGs, whereas the myc-tagged receptor, whose lateral diffusion is then measured by FRAP (see later description), is labeled exclusively by monovalent Fab′.

FRAP and patch/FRAP

Coexpressed epitope-tagged receptors labeled fluorescently by anti-tag Fab fragments as described were subjected to FRAP and patch/FRAP studies as described by us earlier (Rechtman et al., 2009; Marom et al., 2011). The FRAP measurements were conducted at 15°C, replacing samples within 20 min to minimize internalization during the measurement. An argon-ion laser beam (Innova 70C; Coherent, Santa Clara, CA) was focused through a fluorescence microscope (Axioimager.D1; Carl Zeiss MicroImaging, Jena, Germany) to a Gaussian spot of 0.77 μm (PlanApochromat 63×/1.4 numerical aperture [NA] oil-immersion objective). After a brief measurement at monitoring intensity (528.7 nm, 1 μW), a 5-MW pulse (20 ms) bleached 60–75% of the fluorescence in the illuminated region, and fluorescence recovery was followed by the monitoring beam. Values of D and R were extracted from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process (Petersen et al., 1986). Patch/FRAP studies were performed similarly, except that IgG-mediated cross-linking/patching of an epitope-tagged TGF-β receptor (described in the preceding subsection) preceded the measurement (Henis et al., 1990; Rechtman et al., 2009). This enables determination of the effects of immobilizing one receptor type on the lateral diffusion of the coexpressed receptor (labeled exclusively with non-cross-linking Fab′), allowing identification of complex formation between them and distinction between transient and stable interactions (Henis et al., 1990; Rechtman et al., 2009).

Smad2/3 nuclear translocation assay

MDA-MB-231 cell lines were seeded in six-well plates. After 24 h, the cells were serum starved (for 16 h) and stimulated (or not) with 100 pM TGF-B1 (30 min). They were then fixed with 4% paraformaldehyde and permeabilized with Triton X-100 (0.2%, 5 min). After blocking with goat γ-globulin (200 μg/ml, 30 min, 22°C) in HBSS/HEPES/BSA, they were labeled successively by 1) rabbit IgG reactive with Smad2/3 (5 μg/ml), 2) biotin-GtR IgG (5 μg/ml), and 3) Cy3-streptavidin (1.2 μg/ml). Cells were mounted with fluorescence mounting medium (Golden Bridge International, Bothell, WA), and fluorescence digital images were captured by a charge-coupled device camera (CoolSNAP HQ-M; Photometrics, Tucson, AZ) mounted on an Axioimager D.1 microscope (Carl Zeiss Microimaging) with a 63×/1.4 NA objective.

Transcriptional activation assay

MDA-MB-231–derived cell lines were seeded in six-well plates. After 24 h, they were cotransfected with 0.5 μg of DNA of the luciferase reporter construct (CAGA)_12-Luc, and 0.1 μg of DNA of pRL-TK (Renilla luciferase). At 24 h posttransfection, the cells were serum starved (16 h), stimulated (or not) with 100 pM TGF-B1 for another 24 h, lysed, and analyzed by the DLR assay system. The results were normalized for transfection efficiency using the Renilla luminescence as described by us earlier (Shapira et al., 2012).

Binding and cross-linking

MDA-MB-231 cells (250,000/well) were seeded in six-well plates. The media were conditioned for 18–20 h and clarified by centrifugation. Both cells (cell surface labeling) and conditioned media were incubated with 100 pM [125I]TGF-B1 in the presence of fatty acid–deficient bovine serum albumin and protease inhibitors (3 h, 4°C). The ligand was then chemically cross-linked to the receptors using 0.5 mg/ml disuccinimidyl suberate (Thermo Scientific Pierce-Life Technologies, Grand Island, NY) and quenched with 20 mM glycine. Cells were lysed with RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 50 mM Tris/HC1, pH 7.4, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM sodium phosphate) supplemented with protease inhibitors. Ligand–receptor complexes were pulled down by immunoprecipitation overnight at 4°C using goat IgG directed against the extracellular domain of TβRII. The resulting complexes were separated by SDS–PAGE, and dried gels were exposed to an autoradiograph. Images were acquired with a phosphorimager and analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

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