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 TβRIII 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.**

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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, or ALK5) 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 TβRI, 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

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Abbreviations used: BSA, bovine serum albumin; FRAP, fluorescence recovery after photobleaching; GαM, goat anti-mouse; GαR, goat anti-rabbit; GIPC, Gα-interacting protein–interacting protein C-terminus; HBSS, Hank's balanced salt solution; TGF-β, transforming growth factor-β; TβRI, ALK5 type I TGF-β receptor; TβRII, type II TGF-β receptor; TβRIII, type III TGF-β receptor; WT, wild type.

(López-Casillas *et al.*, 1994; Elderbroom *et al.*, 2014), although in some cases, shedding-independent inhibition was also demonstrated (Eickelberg *et al.*, 2002). TβRIII was found to interact with the scaffolding proteins β-arrestin2 and Gα-interacting protein–interacting protein C-terminus (GIPC) through its short, conserved cytoplasmic domain. Binding to GIPC stabilized TβRIII at the cell surface and increased TGF-β signaling (Blobe *et al.*, 2001a), whereas interaction with β-arrestin2 mediated cointernalization of TβRIII and TβRII, down-regulation of these receptors, and a decrease in TGF-β signaling (Chen *et al.*, 2003; Finger *et al.*, 2008).

TβRIII expression is lost or reduced in most cancer cell line models (Segarini, 1990; Chen *et al.*, 1997; Sun and Chen, 1997) and human cancers of the breast, prostate, ovary, kidney, lung, and pancreas (Copland *et al.*, 2003; Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007), as well as in myelomas (Lambert *et al.*, 2011), in line with an inhibitory role for TβRIII in cancer progression. Accordingly, increasing or restoring TβRIII expression in such cancer cells was reported to decrease cell motility/invasion in vitro and reduce angiogenesis, invasion, and metastasis in vivo (Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007; Lee *et al.*, 2010; Lambert *et al.*, 2011). Reciprocally, decreasing TβRIII expression by short hairpin RNA increased migration and invasion of such cancer cells (Gordon *et al.*, 2008; Mythreye and Blobe, 2009). On the other hand, TβRIII was reported to have cancer-promoting effects in colon cancer cells, enhancing their migration, growth, and colony formation in soft agar (Gatza *et al.*, 2011).

Complex formation among TGF-β receptors has important roles in signaling. This was investigated mainly for type I and type II TGF-β receptors, both by crystallographic studies on their extracellular domains (Groppe *et al.*, 2008; Radaev *et al.*, 2010) and by fluorescence-based quantitative methods measuring interactions of the full-length receptors situated in the plasma membrane (reviewed in Ehrlich *et al.*, 2012). In previous studies, we combined immunoglobulin G (IgG)–mediated patching of epitope-tagged cell surface receptors with fluorescence recovery after photobleaching (FRAP) to investigate homomeric and heteromeric complex formation among type I and type II TGF-β receptors and among BMP receptors (Rechtman *et al.*, 2009; Marom *et al.*, 2011). Here we use patch/FRAP to study quantitatively the interactions of TβRIII with TβRII and TβRI at the surface of live cells, their dependence on association with the scaffold proteins GIPC and β-arrestin2, and their relation to Smad signaling. Our studies demonstrate that TβRIII homo-oligomerization depends on its cytoplasmic domain and on binding to GIPC scaffolds. Of interest, we find that TβRII and TβRI bind to TβRIII independently and in a ligand-independent manner, suggesting binding to nonoverlapping sites rather than as a TβRII/TβRI complex. Signaling studies in MDA-MB-231 cell lines that do or do not express wild-type (WT) or mutant TβRIII showed that TβRIII inhibits TGF-β–mediated Smad2/3 nuclear translocation and transcriptional activation. This inhibitory effect required the TβRIII cytoplasmic domain and could be exerted also by an ectodomain shedding–defective TβRIII mutant. We propose a model in which binding of TβRII and TβRI to TβRIII competes with formation of the TβRII/TβRI complex, inhibiting signaling to the Smad pathway.

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 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 (R_f) or the lateral diffusion coefficient (D) of the Fab′-labeled receptor, depending on the FRAP time scale relative to the dissociation/association 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 R_f (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 R_f 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 R_f 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 R_f in patch/FRAP studies should be multiplied by 2 to obtain the percentage of homodimeric receptors

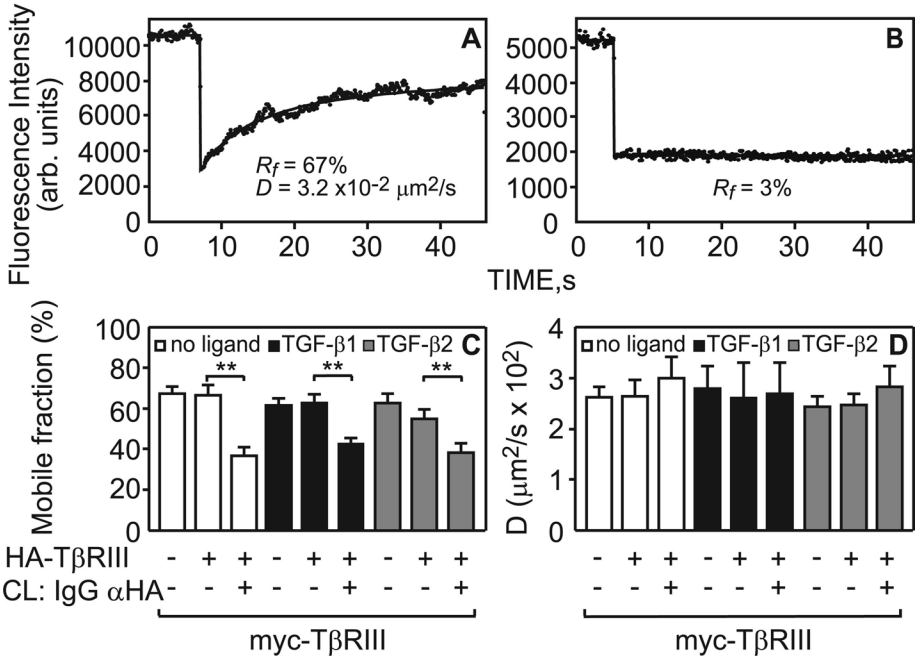

FIGURE 1: Patch/FRAP studies demonstrate stable TβRIII homomeric complexes. COS7 cells were cotransfected with pairs of expression vectors encoding myc- and HA-tagged TβRIII. In control experiments with singly expressed myc-TβRIII receptors, the HA-tagged construct was replaced by empty vector. After 44–48 h, live cells were subjected to the IgG-mediated patching/cross-linking (CL) protocol (*Materials and Methods*), resulting in HA-TβRIII patched and labeled by Alexa 488–GαR IgG (designated IgG αHA), whereas myc-TβRIII is labeled exclusively by monovalent Fab′ (with Alexa 546-GαM Fab′ as a secondary antibody). In control experiments without HA-TβRIII cross-linking, the IgG labeling of HA-TβRIII was replaced by exclusive Fab′ labeling (replacing the cross-linking IgGs by their respective Fab′ fragments). FRAP studies were conducted at 15°C to minimize internalization. Solid lines are the best fit of a nonlinear regression analysis to the lateral diffusion equation (Petersen *et al.*, 1986). (A) A representative FRAP curve of the lateral diffusion of myc-TβRIII in a cell coexpressing HA-TβRIII (no IgG cross-linking). (B) A representative FRAP curve of HA-TβRIII immobilized by IgG cross-linking. (C, D) Average R_f and D values derived from multiple patch/FRAP measurements. Bars are mean ± SEM of 30–50 measurements in each case. Asterisks indicate significant differences between the R_f values of the pair indicated by brackets (**$p < 3 \times 10^{-5}$; Student's t test). No significant differences were found between D values as a result of IgG- mediated cross-linking. Neither the D nor the R_f values were significantly affected by ligand.

(Ehrlich *et al.*, 2011). Therefore the 45% reduction in R_f of myc-TβRIII upon immobilization of HA-TβRIII 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 TβRIII 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-TβRIII cytoplasmic domain mutants with WT myc-TβRIII (Figure 2). In contrast to full-length TβRIII, cross-linking of a TβRIII mutant whose cytoplasmic domain was truncated right after the IYSD sequence (replacing the cytoplasmic domain with RR to retain a positive charge where the transmembrane domain ends; TβRIII-Cyto; Blobe *et al.*, 2001a) did not reduce the R_f of the coexpressed myc-TβRIII but instead reduced its D value (Figure 2, A and B). Similar results were obtained upon cross-linking of HA-TβRIII-Cyto-1, a TβRIII mutant truncated after the IYSHTGETAGRQ 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 TβRIII in the homomeric interactions. Of interest, analogous effects on myc-TβRIII-WT diffusion were observed upon cross-linking of HA-TβRIII-Del, a TβRIII 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-TβRIII-T841A, a TβRIII mutant that does not bind β-arrestin2 (Chen *et al.*, 2003), compromised but did not abolish the reduction in R_f of myc-TβRIII-WT (Figure 2, E and F). These findings suggest that TβRIII 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.

TβRIII forms stable heteromeric complexes with TβRI and TβRII

Previous studies demonstrated that TβRIII forms TGF-β1–induced complexes with TβRII independently of TβRI (López-Casillas *et al.*, 1993) and enhances ligand binding to TβRII. In addition, TβRII was shown to phosphorylate TβRIII, resulting in dissociation of TβRIII from the TβRII/TβRI signaling complex (Blobe *et al.*, 2001b). However, subsequent studies reported that some TβRIII might remain associated with TβRII as the complex internalizes in endocytic vesicles (Chen *et al.*, 2003). To explore the dynamics of the interactions between TβRIII and TβRII, we conducted patch/FRAP studies on cells expressing HA-TβRIII and myc-TβRII in the presence or absence of ligand (TGF-β1 or -β2), measuring the effects of patching HA-TβRIII on the lateral diffusion of the Fab′-labeled myc-TβRII (Figure 3). Unexpectedly, some reduction in R_f (17%) of TβRII occurred already upon coexpression with TβRIII (without cross-linking), suggesting that a subpopulation of TβRII interacts preferentially with slowly diffusing or immobile TβRIII molecules/clusters (Figure 3A). This demonstrates that TβRIII/TβRII complexes exist before ligand binding. The mild reduction in R_f was markedly increased upon TβRIII cross-linking (from 17 to 33%; no effect on D), indicating that a second population of TβRII interacts with an initially mobile subclass of TβRIII, which is immobilized after IgG cross-linking (Figure 3, A and B). Of note, coexpression of excess untagged TβRII canceled this reduction, indicating both specificity and saturability in the binding of TβRII to TβRIII. TGF-β1 or -β2 had no significant effect on these interactions (Figure 3, A and B). Note that for heterocomplexes (e.g., TβRII/TβRIII), no statistical correction is needed, and the percentage reduction in R_f of myc-TβRII upon cross-linking of HA-TβRIII is a direct measure of their hetero-oligomerization (Rechtman *et al.*, 2009; Ehrlich *et al.*, 2012).

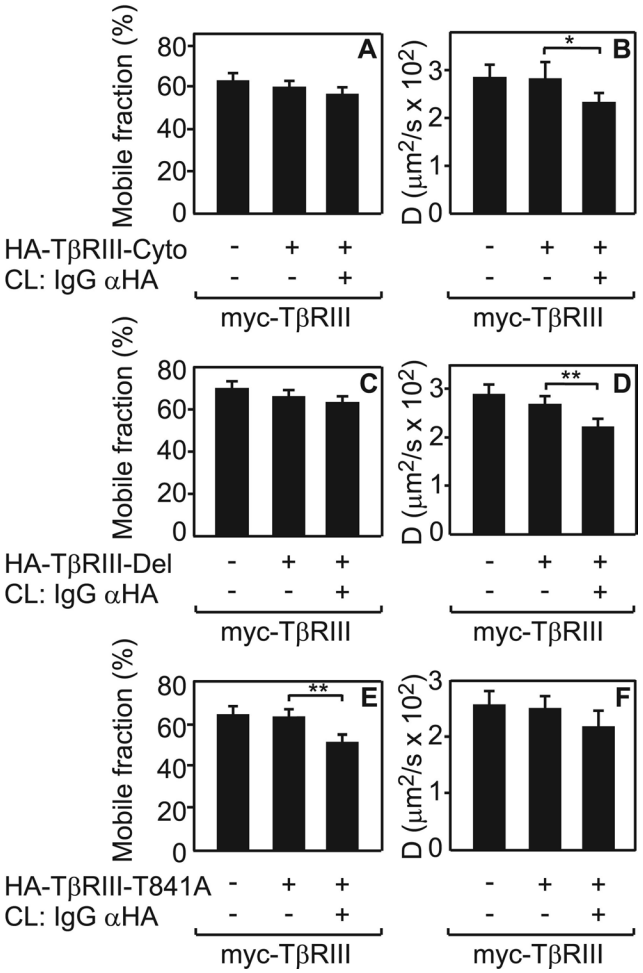

FIGURE 2: TβRIII homo-oligomerization depends on its cytoplasmic domain and GIPC binding. COS7 cells were cotransfected by myc-TβRIII-WT together with HA-TβRIII-Cyto, HA-TβRIII-Del, or HA-TβRIII-T841A (or empty vector). After 44–48 h, the cells were labeled for patch/FRAP experiments by the IgG-mediated patching/cross-linking protocol using rabbit IgG αHA and mouse Fab' αmyc, leading to immobilization of HA-tagged TβRIII (see *Materials and Methods*). The lateral mobility of Fab'-labeled myc-TβRIII-WT proteins was measured by FRAP at 15°C with or without cross-linking of the HA-TβRIII mutant. (A, C, E) Average R_f values. (B, D, F) Average D values. Bars are mean ± SEM of 30–70 measurements in each case. Asterisks indicate significant differences between the R_f values of the pairs indicated by brackets (*$p < 0.03$; **$p < 6 \times 10^{-4}$; Student's t test). IgG cross-linking of HA-TβRIII-Cyto and HA-TβRIII-Del had only minor effects on the D value of myc-TβRIII-WT, whereas cross-linking of HA-TβRIII-T841A was capable of reducing R_f of myc-TβRIII-WT, albeit somewhat more weakly than after cross-linking of HA-TβRIII-WT (Figure 1).

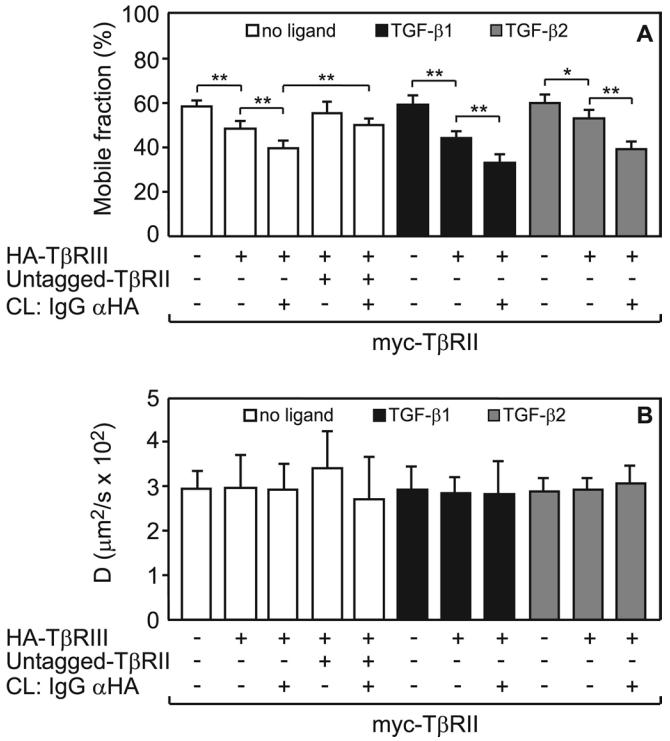

FIGURE 3: TβRIII forms stable heteromeric complexes with TβRII. Patch/FRAP studies were carried out on COS7 cells expressing myc-TβRII together with HA-TβRIII (or empty vector). In some cases, excess untagged TβRII was coexpressed along with myc-TβRII and HA-TβRIII. The cells were subjected to the IgG cross-linking protocol, leading to immobilization of HA-TβRIII (see *Materials and Methods* and Figure 2). The lateral mobility of the Fab'-labeled myc-TβRII proteins was measured by FRAP at 15°C with or without IgG cross-linking of HA-TβRIII. (A) Average R_f values. (B) Average D values. Bars are mean ± SEM of 30–70 measurements in each case. Asterisks indicate significant differences between the R_f values of the pairs indicated by brackets (*$p < 0.05$; **$p < 7 \times 10^{-3}$; Student's t test). No significant differences were found between D values as a result of IgG cross-linking of TβRIII or after addition of ligand (250 pM) under similar experimental conditions. A subpopulation of myc-TβRII was immobilized upon coexpression with TβRIII, and a further reduction in R_f occurred after IgG cross-linking of HA-TβRIII. Of note, coexpression with excess untagged TβRII canceled the latter effect, indicating both specificity and saturability in the binding of TβRII to TβRIII.

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

Analogous studies on TβRIII/TβRI interactions yielded a different pattern, suggesting weaker interactions (Figure 5), as R_f of myc-TβRI was not reduced merely by coexpression with HA-TβRIII, and the percentage reduction in its R_f upon cross-linking of coexpressed HA-TβRIII was lower (17%). This effect essentially disappeared upon inclusion of excess untagged TβRI in the cotransfection, demonstrating the specificity and saturability of these interactions. The heteromeric TβRIII/TβRI 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 TβRIII cytoplasmic domain mutants (Cyto, Del, and T841A) yielded results identical to those obtained with TβRIII-WT (Figure 6), suggesting that TβRI/TβRIII interactions are independent of GIPC or β-arrestin2 binding.

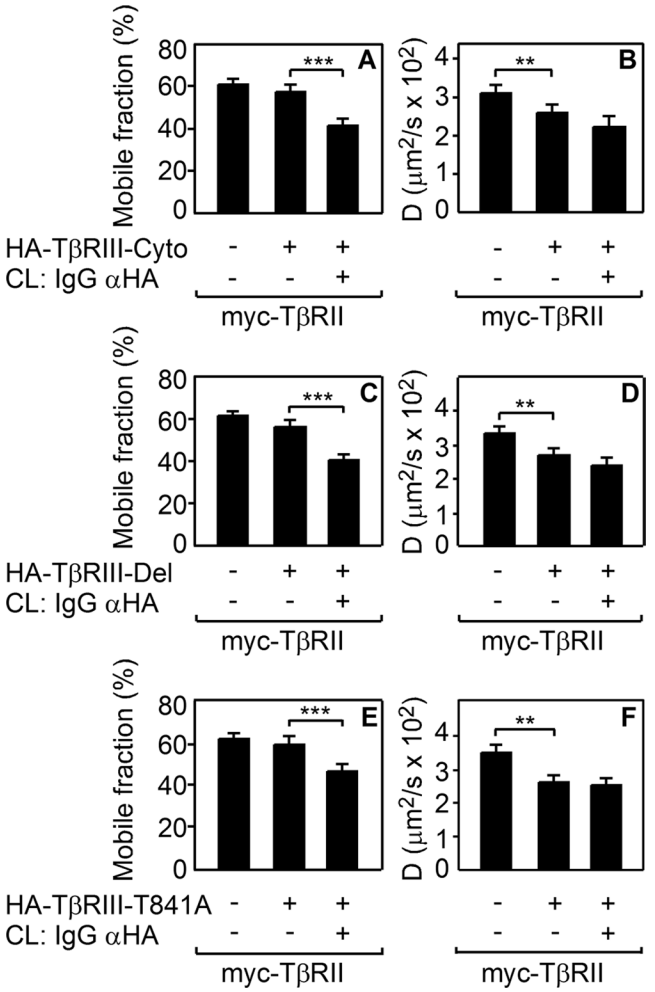

FIGURE 4: The immobile subpopulation of TβRII coexpressed with TβRIII depends on the TβRIII cytoplasmic domain. Experimental conditions were as in Figure 2, except that myc-TβRIII was replaced by myc-TβRII. (A, C, E) Average R_f values. (B, D, F) Average D values. Bars are mean ± SEM of 30–40 measurements in each case. Asterisks indicate significant differences between the R_f or D values of the pairs indicated by brackets (**$p < 0.005$; ***$p < 10^{-4}$; Student's t test). The reduction in R_f of myc-TβRII coexpressed with HA-TβRIII without cross-linking disappeared in the TβRIII cytoplasmic domain mutants. Instead, the D values of myc-TβRII were decreased upon coexpression with these TβRIII mutants.

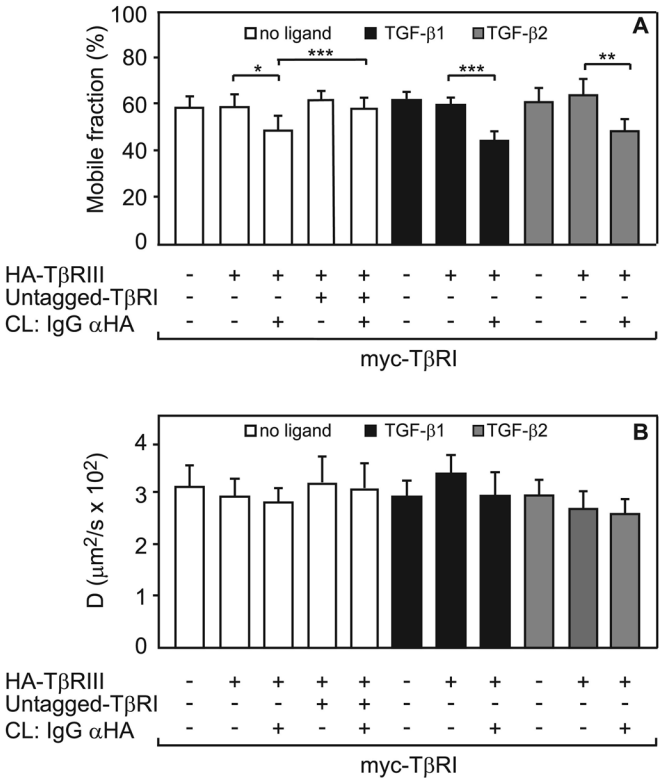

FIGURE 5: Patch/FRAP studies demonstrate mild stable TβRIII/TβRI interactions. Experimental conditions were as in Figure 3, except that myc-TβRII and untagged TβRII were replaced by myc-TβRI and untagged TβRI, respectively. (A) Average R_f values. (B) Average D values. Bars are mean ± SEM of 30–40 measurements in each case. Asterisks indicate significant differences between the R_f values of the pairs indicated by brackets (*$p < 0.03$; **$p < 0.002$; ***$p < 10^{-5}$; Student's t test). No significant differences were found between the D values as a result of IgG-mediated cross-linking. Neither the D nor the R_f values were significantly affected by ligand (250 pM).

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 TβRIII/TβRI interactions. The reduction in R_f of myc-TβRI after HA-TβRIII cross-linking was not affected by overexpression of untagged TβRII (Figure 7), as shown by the fact that it remained identical to that observed in the absence of untagged TβRII (compare with Figure 5). Moreover, in contrast to the reduced R_f of myc-TβRII upon coexpression with TβRIII (Figure 3A), mere coexpression of untagged TβRII did not confer reduction in R_f of myc-TβRI coexpressed with TβRIII (Figure 7A), suggesting that TβRI binding to TβRIII is not enhanced by TβRI/TβRII complex formation.

The lack of effect of TβRII on TβRIII/TβRI 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-TβRI, cross-linked (or not) HA-TβRII, and used FRAP to measure the lateral diffusion of myc-TβRI (Figure 8). In accord with our earlier studies on TβRI/TβRII interactions (Rechtman *et al.*, 2009), when myc-TβRI and HA-TβRII were coexpressed without TβRIII, the R_f of myc-TβRI 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 R_f (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-TβRI interactions (Figure 8A). These results suggest the formation of a triple complex containing TβRIII, TβRII, and TβRI and are in line with distinct binding domains on TβRIII 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 TβRIII-WT, its shedding-defective mutant TβRIII-ΔShed, and the

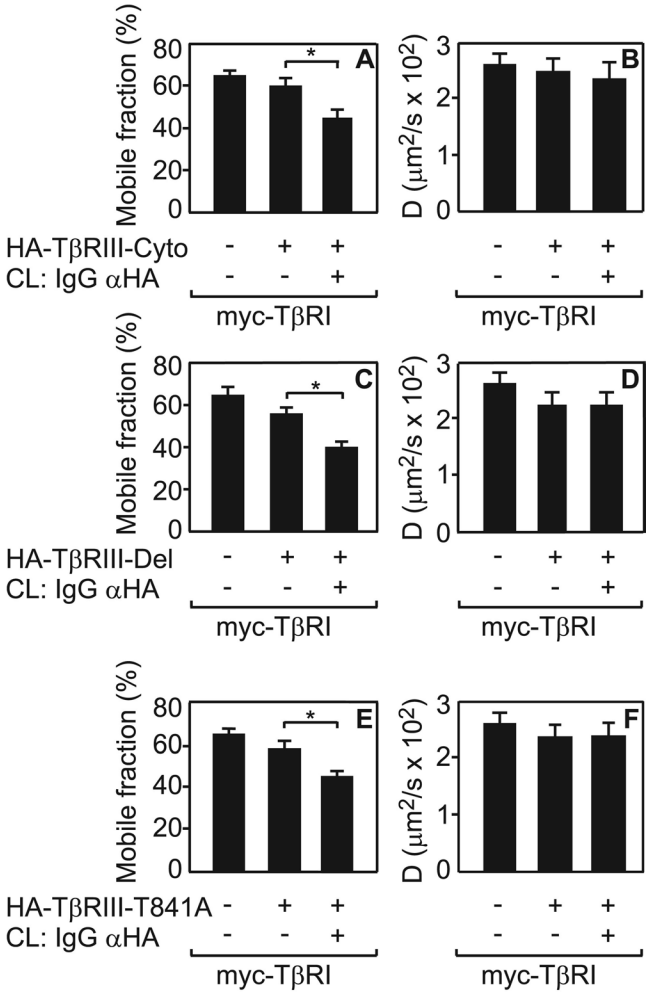

FIGURE 6: Stable TβRIII/TβRI heteromeric complexes form independently of the TβRIII cytoplasmic domain. Experimental conditions were as in Figure 2, except that myc-TβRIII was replaced by myc-TβRI. (A, C, E) R_f values. (B, D, F) *D* values. Bars are mean ± SEM of 30–50 measurements in each case. Asterisks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

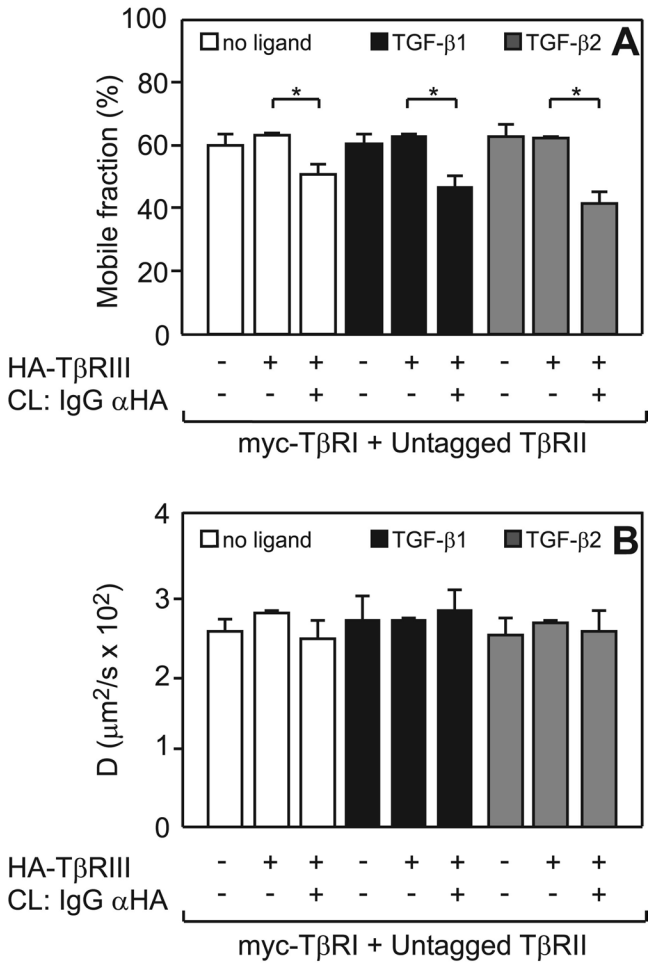

FIGURE 7: Expression of TβRII does not affect TβRI/TβRIII interactions. COS7 cells were cotransfected with myc-TβRI together with HA-TβRIII (or empty vector) and excess untagged TβRII. The diffusion of myc-TβRI was measured by FRAP with or without cross-linking of HA-TβRIII, as described in Figure 3. (A) Average R_f values. (B) Average *D* values. Bars are mean ± SEM of 30–50 measurements in each case. Asterisks indicate significant differences between the R_f values of the pairs indicated by brackets (*$p < 10^{-5}$; Student's *t* test). Cross-linking of HA-TβRIII reduced R_f of myc-TβRI without affecting the *D* values. Neither the *D* nor the R_f 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 R_f of myc-TβRI upon cross-linking HA-TβRIII remains exactly as in the absence of untagged TβRII (Figure 5). This suggests that TβRI binds to TβRIII independently and not through TβRII.

TβRIII-Cyto mutant (lacking most of the cytoplasmic domain) on TGF-β–mediated Smad signaling. To this end, we used MDA-MB-231 cell lines stably expressing one of these receptors (Mythreye and Blobe, 2009; Elderbroom *et al.*, 2014), with MDA-MB-231-Neo (stably transfected with empty vector) as a control. Except for MDA-MB-231-Neo, these cell lines expressed comparable levels of the respective TβRIII mutants at the cell surface, as determined by [^{125}I]TGF-β1 binding/cross-linking assays (Figure 9A), and only the TβRIII-ΔShed mutant failed to accumulate the soluble shed form in conditioned medium (Elderbroom *et al.*, 2014; Figure 9A). Figure 9, B and C, depicts Smad2/3 nuclear translocation in these cell lines in response to TGF-β1. Of interest, expression of TβRIII-WT markedly inhibited Smad2/3 nuclear translocation, with a weaker but significant inhibition by TβRIII-ΔShed expression. This implies that although shedding of soluble TβRIII can inhibit TGF-β signaling in these cells (Elderbroom *et al.*, 2014), TβRIII can also inhibit Smad2/3 signaling by an alternative, shedding-independent mechanism. 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.

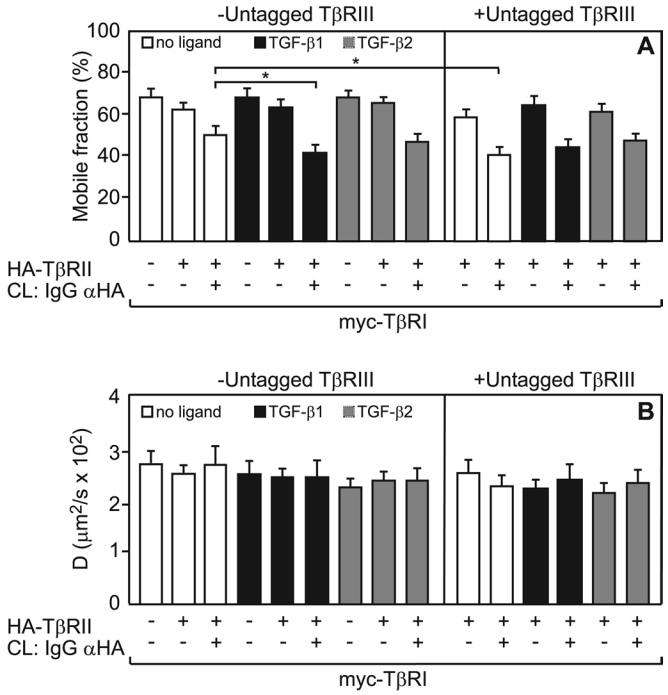

FIGURE 8: Patch/FRAP studies demonstrate that TβRII/TβRI interactions are enhanced by expression of untagged TβRIII. COS7 cells were cotransfected with myc-TβRI together with HA-TβRII and excess untagged TβRIII (both replaced by empty vector in control experiments). The diffusion of myc-TβRI was measured by FRAP with or without cross-linking of HA-TβRII, as described in Figure 3. (A) Average R_f values. (B) Average D values. Bars are mean ± SEM of 30–60 measurements in each case. Asterisks indicate significant differences between the R_f values of the pairs indicated by brackets (*$p < 10^{-3}$; Student's t test). IgG cross-linking of HA-TβRII significantly reduced R_f of myc-TβRI. This effect was augmented by TGF-β1 (250 pM). Expression of untagged TβRIII was sufficient to induce further reduction in the R_f values of myc-TβRI when HA-TβRII was cross-linked, reaching the level of enhancement mediated by ligand in the absence of TβRIII. In all cases, the D values were not significantly altered.

The stronger inhibition by TβRIII-ΔShed in this assay relative to the Smad2/3 nuclear translocation assay most likely reflects the fact that transcriptional activation is downstream of Smad nuclear translocation and measures cumulative response over several hours.

DISCUSSION

TβRIII is a TGF-β coreceptor involved in ligand presentation to TβRII, which regulates numerous TGF-β signaling pathways (López-Casillas *et al*., 1994; Eickelberg *et al*., 2002; reviewed in Gatza *et al*., 2010). These roles are expected to depend on complex formation between TβRIII, TβRII, and TβRI. Whereas complex formation between TβRI and TβRII has been extensively studied (Henis *et al*., 1994; Gilboa *et al*., 1998; Rechtman *et al*., 2009; Ehrlich *et al*., 2012), no such data were available for TβRIII complex formation with TβRI and TβRII, their potential dependence on TβRIII cytoplasmic domain interactions, and their effects on the signaling TβRI/TβRII complex. Here we investigated these issues using patch/FRAP and Smad signaling assays. We found that TβRIII homo-oligomerization is largely indirect and reflects its association with scaffold proteins, mainly GIPC-containing scaffolds. We show that TβRI/TβRII heterocomplex formation is enhanced by their simultaneous binding to distinct sites on TβRIII and that these receptors bind independently to TβRIII rather than as a complex. Of interest, TβRIII inhibited TGF-β Smad2/3 signaling in MDA-MB-231 cells, an effect that required the TβRIII cytoplasmic domain and persisted when TβRIII ectodomain shedding was abrogated. We propose that the independent binding of TβRI and TβRII to TβRIII competes with TβRI/TβRII signaling complex formation, thus inhibiting TGF-β–mediated Smad signaling (Figure 10).

Our earlier semiquantitative immunofluorescence copatching studies already indicated TβRIII homomeric complex formation (Henis *et al*., 1994). The present patch/FRAP experiments (Figure 1) demonstrate that HA-TβRIII/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 TβRIII oligomerization before ligand binding, leaving little margin for an incremental increase in homo-oligomerization. Analogous experiments with TβRIII mutants that lack binding to GIPC or β-arrestin2 (Figure 2) show that TβRIII homo-oligomerization depends on its binding to GIPC and, to a lesser degree, to β-arrestin2. Thus TβRIII homomeric complexes are indirect, reflecting mutual binding to GIPC (and/or β-arrestin2)–containing scaffolds.

To assess heterocomplex formation between TβRIII and the signaling TGF-β receptors, we investigated TβRIII interactions with TβRII and TβRI. Complex formation between TβRIII and TβRII (Figure 3) was characterized by two distinct TβRII populations. One subpopulation was immobilized (reduction in R_f) directly upon coexpression with TβRIII (without the need to immobilize TβRIII by IgG cross-linking), most likely reflecting binding to TβRIII clusters that form due to association with intracellular scaffolds. This view is reinforced by the demonstration (Figure 4) that TβRIII mutants with defective cytoplasmic interactions (Cyto, Del, T841A) lose the "direct immobilization" effect on the TβRII subpopulation, and the reduction in R_f 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 TβRIII cytoplasmic domain and GIPC mutants (Figure 2) and suggests that the oligomerization of TβRIII 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 R_f of TβRII after IgG cross-linking of TβRIII (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 distinctively 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 TβRIII cytoplasmic domain (Figures 5 and 6). The different characteristics of TβRI versus TβRII binding to TβRIII raise the possibility that TβRI and TβRII bind to nonoverlapping sites on TβRIII. This view is supported by the finding that TβRII overexpression had no effect under any condition on myc-TβRI/HA-TβRIII 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/TβRIII 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 TβRIII may serve here as a scaffold by itself, bridging indirectly between TβRI and TβRII that

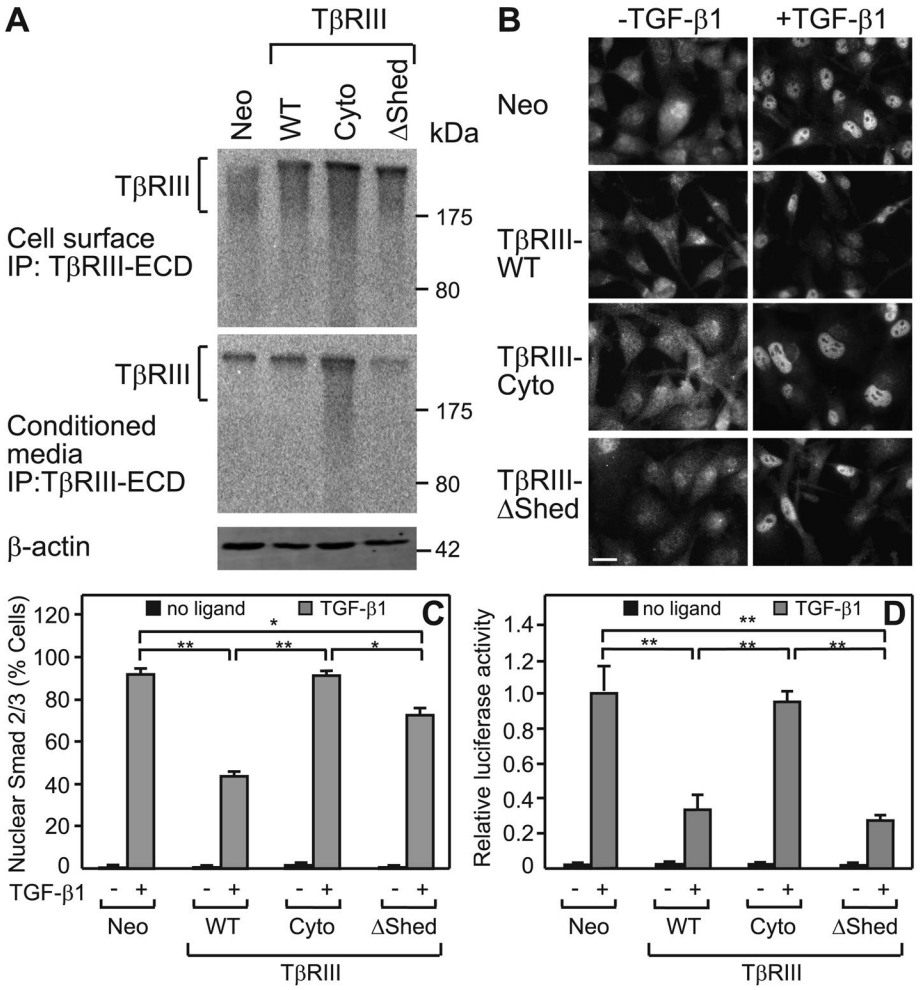

FIGURE 9: TβRIII expression inhibits Smad2/3 signaling, depending on its cytoplasmic domain. (A) Affinity labeling of TβRIII in stably expressing MDA-MB-231 cell lines. Cells were incubated with [^{125}I]TGF-β1 (100 pM), and bound ligand was cross-linked to the cell surface receptors. Cell lysates and 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 $(CAGA)_{12}$-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-β 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).

bind to TβRIII independent of each other (not as the TβRI/TβRII signaling complex; see Figure 10).

Formation of an alternative TβRI/TβRII/TβRIII complex that competes with the TβRI/TβRII 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, TβRIII-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 TβRIII-Cyto mutant, might have enhanced avidity for binding most of the TβRII molecules. The ability of TβRIII to bind independently TβRII and TβRI, competing with the signaling TβRI/TβRII 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 TβRI/TβRII signaling complex formation (Eickelberg *et al.*, 2002). Note that there are time-domain differences between the two mechanisms: inhibition due to interactions of TβRI and TβRII with TβRIII 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.

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

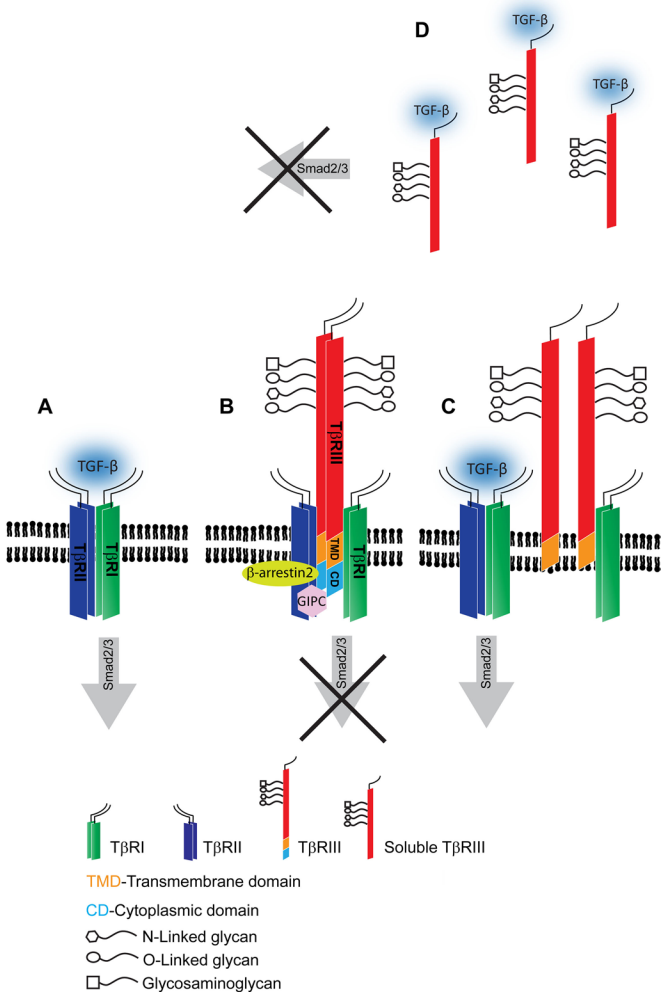

FIGURE 10: Model of TβRIII regulation of Smad2/3 signaling via interactions with TβRII and TβRI. (A) In the absence of TβRIII, ligand binding to TβRII enhances the formation of a heterotetrameric complex with TβRI, leading to activation of TβRI, which stimulates TGF-β–mediated Smad2/3 signaling. (B) When TβRIII is expressed, TβRII and TβRI bind independently to nonoverlapping sites on TβRIII. 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 TβRIII, 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 homomeric 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.

soluble TβRIII (Eickelberg *et al.*, 2002). The latter report concluded that the TβRIII inhibition of TGF-β signaling in renal epithelial LLC-PK(1) 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 TβRIII, TβRII, and TβRI identified here regulate other aspects of TGF-β signaling remains to be determined.

MATERIALS AND METHODS

Reagents

Recombinant TGF-β1 was obtained from PeproTech (Rocky Hill, NJ) and fatty acid–free bovine serum albumin (BSA; fraction V) from Sigma-Aldrich (St. Louis, MO). Media and cell culture reagents were from Biological Industries (Beit Haemek, Israel) or Invitrogen (Carlsbad, CA). Rabbit immunoglobulin G (IgG) against Smad3 (reactive with Smad3 and Smad2; sc-528) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). G418 was purchased from Calbiochem (La Jolla, CA). Dual-luciferase reporter (DLR) assay system was from Promega (Fitchburg, WI). Affinity-purified biotinylated goat anti-rabbit (GαR) IgG and Cy3-streptavidin were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-myc tag (αmyc) 9E10 mouse ascites (Evan *et al.*, 1985) and HA.11 rabbit antiserum to the HA tag (αHA) were from Covance Research Products (Denver, PA). IgG and monovalent Fab′ fragment αmyc were prepared from the 9E10 ascites as described (Henis *et al.*, 1994). Alexa Fluor 488–GαR IgG and Alexa Fluor 546–goat anti-mouse (GαM) $F(ab')_2$ were from Invitrogen-Molecular Probes (Eugene, OR); fluorescent $F(ab')_2$ was converted into Fab′ as described (Gilboa *et al.*, 1998). [^{125}I]TGFβ-1 was from PerkinElmer (Waltham, MA). Goat IgG against the extracellular domain of TβRIII was from R&D Systems (Minneapolis, MN). Mouse anti–β-actin was from Sigma-Aldrich.

Plasmids

Expression vectors encoding human TβRI (in pcDNA3) or TβRII (in pcDNA1) with extracellular myc or HA tags or HA-TβRIII, as well as untagged TβRII and TβRI, were described by us earlier (Henis *et al.*, 1994; Gilboa *et al.*, 1998; Ehrlich *et al.*, 2001; Chetrit *et al.*, 2009). Myc-tagged WT TβRIII and untagged TβRIII in pcDNA3 were transferred into pcDNA3 from the formerly described constructs in pcDNA1 (Henis *et al.*, 1994). The HA-TβRIII-Cyto or -Cyto1 (lacking most of the cytoplasmic domain), HA-TβRIII-Del (lacking the last three C-terminal amino acids comprising a class I PDZ binding domain, resulting in loss of binding to GIPC), and HA-TβRIII-T841A (a point mutation that abrogates TβRIII binding to β-arrestin2) in pcDNA3.1 were described (Blobe *et al.*, 2001a; Chen *et al.*, 2003). pRL-TK was from Promega. The TGF-β–responsive luciferase reporter construct $(CAGA)_{12}$-Luc in pGL3ti (Dennler *et al.*, 1998) was a gift from P. Knaus (Free University of Berlin, Berlin, Germany). The construct is considered to be highly specific for TGF-β–mediated Smad activation due to specific binding of Smad3 and Smad4 to the CAGA boxes in the promoter (Dennler *et al.*, 1998).

Cell culture and transfection

COS7 cells (American Type Culture Collection, Manassas, VA) were grown in DMEM with 10% fetal calf serum (FCS; Biological Industries) as described earlier (Gilboa *et al.*, 2000; Shapira *et al.*, 2012). Breast cancer stable cell lines of MDA-MB-231 were grown in MEME (Biological Industries), supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, L-glutamine, penicillin/streptomycin, and 250 μg/ml G418 (Mythreye and Blobe, 2009; Elderbroom *et al.*, 2014). All cells were incubated at 37°C with 5% CO_2.

For patch/FRAP experiments, COS7 cells were grown on glass coverslips in six-well plates and transfected by TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI) with different combinations of vectors encoding myc- and HA-tagged (or untagged) receptor constructs. The DNA amounts of the various vectors were adjusted to yield similar cell surface expression levels of the coexpressed differently tagged receptors as described by us earlier (Marom *et al.*, 2011).

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) monovalent mouse Fab' anti-myc (40 μg/ml) together with HA.11 rabbit IgG anti-HA (20 μg/ml) and 2) Alexa Fluor 546–Fab' GαM (40 μg/ml) together with Alexa Fluor 488–IgG GαR (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 ± 0.03 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_f 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-β1 (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-GαR 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-β1 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 [^{125}I]TGFβ-1 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/HCl, 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 TβRIII. 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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