Regulation of TGF-β receptor hetero-oligomerization and signaling by endoglin

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ABSTRACT **Complex formation among transforming growth factor-β (TGF-β) receptors and its modulation by coreceptors represent an important level of regulation for TGF-β signaling. Oligomerization of ALK5 and the type II TGF-β receptor (TβRII) has been thoroughly investigated, both in vitro and in intact cells. However, such studies, especially in live cells, are missing for the endothelial cell coreceptor endoglin and for the ALK1 type I receptor, which enables endothelial cells to respond to TGF-β by activation of both Smad2/3 and Smad1/5/8. Here we combined immunoglobulin G–mediated immobilization of one cell-surface receptor with lateral mobility studies of a coexpressed receptor by fluorescence recovery after photobleaching (FRAP) to demonstrate that endoglin forms stable homodimers that function as a scaffold for binding TβRII, ALK5, and ALK1. ALK1 and ALK5 bind to endoglin with differential dependence on TβRII, which plays a major role in recruiting ALK5 to the complex. Signaling data indicate a role for the quaternary receptor complex in regulating the balance between TGF-β signaling to Smad1/5/8 and to Smad2/3.**

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INTRODUCTION

Ligands from the transforming growth factor-β (TGF-β) superfamily regulate endothelial cell migration and angiogenesis (Goumans *et al.*, 2009; Pinidiyaarachchi *et al.*, 2009; Lee *et al.*, 2012). They signal via a network of receptors, which in endothelial cells includes the endothelial cell coreceptor endoglin, the type II TGF-β receptor (TβRII), and the type I receptors ALK5 (TβRI) and ALK1 (expressed predominantly in endothelial cells; Massague, 1998; Goumans *et al.*, 2003a; Bernabeu *et al.*, 2009). Both ALK1 and endoglin, as well as ALK5 and TβRII, have essential roles in endothelial cell biology and angiogenesis, as demonstrated by germline mutations in endoglin and ALK1 resulting in the human vascular disease hereditary hemorrhagic telangiectasia (McAllister *et al.*, 1994; Johnson *et al.*, 1996; Bourdeau *et al.*, 1999). In addition, mice lacking ALK1 (Oh *et al.*, 2000; Urness *et al.*, 2000), endoglin (Bourdeau *et al.*, 1999; Li *et al.*, 1999; Arthur *et al.*, 2000), ALK5 (Larsson *et al.*, 2001), or TβRII (Oshima *et al.*, 1996) all exhibit embryonic lethal phenotypes due to vascular defects. Specifically, endoglin is critically important in developmental settings in the cardiovascular system, as endoglin-null mouse embryos show defects in vessel and heart development, resulting in a lethal phenotype (Li *et al.*, 1999; Arthur *et al.*, 2000). Moreover, in the adult vasculature, hereditary hemorrhagic telangiectasia was shown to occur after endoglin haploinsufficiency (McAllister *et al.*, 1994; Pece *et al.*, 1997).

The minimal TGF-β signaling complex, capable of signaling via the canonical Smad pathway and several non-Smad pathways, consists of a TβRII dimer, the dimeric TGF-β ligand, and a pair of type I receptors (Shi and Massague, 2003; Derynck and Miyazono, 2008; Ehrlich *et al.*, 2011). TGF-β binding triggers phosphorylation and activation of the type I receptor, which induces Smad signaling by phosphorylating R-Smads, followed by their hetero-oligomerization with Smad4 and translocation to the nucleus, where they regulate transcription (Shi and Massague, 2003; Feng and Derynck, 2005;

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Abbreviations used: BSA, bovine serum albumin; DαR, donkey anti-rabbit; FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; GIPC, GAIP-interacting protein C-terminal; HBSS, Hank's balanced salt solution; pSmad, phospho-Smad; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor; TGF-β, transforming growth factor-β; SαR, sheep anti-rabbit; WT, wild type.

Schmierer and Hill, 2007; Heldin *et al.*, 2009). TGF-β stimulus in endothelial cells activates Smad2/3 and Smad1/5/8 pathways via ALK5 and ALK1, respectively (Chen and Massague, 1999; Goumans *et al.*, 2003a; Shi and Massague, 2003; Moustakas and Heldin, 2009). In endothelial cells, ALK1 and ALK5 form a mutual complex with TβRII (Goumans *et al.*, 2003a), and it was proposed that ALK1/ALK5 cross-talk leads to a role for ALK5 in TGF-β–induced ALK1 signaling and for ALK1 in inhibiting ALK5 signaling (Goumans *et al.*, 2003b). ALK1 can also be activated by BMP-9 or BMP-10 to signal via Smad1/5/8 (Lebrin *et al.*, 2004).

TGF-β signaling can be modulated by structurally diverse coreceptors. The most abundant TGF-β superfamily coreceptor in endothelial cells is endoglin (Goumans *et al.*, 2003a; Gatza *et al.*, 2010). Endoglin is a 95-kDa glycoprotein that binds TGF-β1 and -β3 in conjunction with TβRII (Barbara *et al.*, 1999) and can also bind directly BMP-9 or -10 (David *et al.*, 2007, 2009). Endoglin has been reported to increase TGF-β1 binding to TβRII (Letamendia *et al.*, 1998; Barbara *et al.*, 1999), with controversial effects on TGF-β signaling (Lastres *et al.*, 1996; Letamendia *et al.*, 1998; Barbara *et al.*, 1999; Guerrero-Esteo *et al.*, 2002). Endoglin-mediated regulation of ALK1 signaling to Smad1/5/8 is contentious; loss-of-function studies with either small interfering RNA (siRNA)–mediated knockdown or in endoglin-null endothelial cells suggest either positive or negative regulation (Lebrin *et al.*, 2004; Pece-Barbara *et al.*, 2005). BMP-9 was also reported to promote Akt activation and endothelial cell tube stability via endoglin/GAIP-interacting protein C-terminal (GIPC)–mediated scaffolding to PI3K/Akt (Lee *et al.*, 2012). Whereas endoglin is regulated by phosphorylation of its short cytoplasmic domain by TβRII, ALK5, and ALK1 (Guerrero-Esteo *et al.*, 2002; Koleva *et al.*, 2006; Ray *et al.*, 2010), the role of the interactions between endoglin and the signaling TGF-β receptors in the regulation of TGF-β responses remains to be defined.

Multiple experimental approaches have been used to study the mode, extent, and function of TGF-β receptor oligomerization (reviewed in Ehrlich *et al.*, 2011). Crystallographic studies have shown that the extracellular domains of TβRII, ALK5, and dimeric ligand (TGF-β1 or -β3) form ternary complexes in which each receptor appears as a dimer (Groppe *et al.*, 2008; Radaev *et al.*, 2010). However, such studies yield only a static view of the endpoint of complex formation and do not necessarily reflect interaction between the full-length receptors situated in their native milieu, the plasma membrane. To address these issues, we developed the method of patch/FRAP (fluorescence recovery after photobleaching; Henis *et al.*, 1990) and applied it to study homomeric and heteromeric interactions among type II and type I TGF-β superfamily receptors (Rechtman *et al.*, 2009; Marom *et al.*, 2011). The emerging picture is that the cell surface population of these receptors comprises a heterogeneous and dynamic mixture of monomeric, homodimeric, and heteromeric complexes modulated by ligand binding (Ehrlich *et al.*, 2011). However, the interactions of ALK1 with itself, with TβRII, and/or with endoglin have not been investigated quantitatively in the cell membrane. Moreover, such data are lacking for the interactions of endoglin with itself, with TβRII, and with ALK5. Here we study these questions in live cells using epitope-tagged TGF-β receptor and endoglin constructs.

RESULTS

Endoglin forms stable homomeric complexes at the cell surface

The TGF-β coreceptor endoglin regulates TGF-β signaling and interacts with scaffolding proteins such as GIPC and β-arrestin2 (Lee and Blobe, 2007; Lee *et al.*, 2008). However, the molecular composition and dynamics of endoglin complexes with the full-length signaling TGF-β receptors situated at the cell plasma membrane have not been characterized. As the first step, we explored the homo-oligomeric interactions of endoglin and their potential dependence on GIPC and β-arrestin2 binding. The extent and dynamics of homomeric or heteromeric receptor complexes can be studied by the patch/FRAP method (Henis *et al.*, 1990), which we recently used to investigate TGF-β and bone morphogenetic protein receptor complexes (Rechtman *et al.*, 2009; Marom *et al.*, 2011). In patch/FRAP, one receptor is patched and laterally immobilized by cross-linking at the surface of live cells with a double layer of bivalent immunoglobulin Gs (IgGs); the effect on the lateral diffusion of a coexpressed receptor bearing a different extracellular epitope tag, labeled by monovalent fluorescent Fab′ fragments, is then measured by FRAP (see *Materials and Methods*). Depending on the FRAP timescale relative to the association-dissociation kinetics between the immobilized and the Fab′-labeled (non–cross-linked) receptors, one may observe a reduction in the mobile fraction (R_f) or in the lateral diffusion coefficient (D) of the latter. Reduction in R_f is obtained when the complex lifetimes are long relative to the characteristic FRAP recovery time, since bleached Fab′-labeled receptor molecules would not undergo measurable dissociation from the cross-linked patches during the FRAP measurement. Conversely, a short complex lifetime would lead to multiple association-dissociation cycles during the FRAP recovery phase, resulting in a slower diffusion rate (Henis *et al.*, 1990; Rechtman *et al.*, 2009).

To study endoglin homo-oligomerization, we coexpressed hemagglutinin (HA)- or myc-tagged endoglin at the surface of COS7 cells and conducted patch/FRAP studies on the tagged receptors. IgG-mediated cross-linking and immobilization of HA-endoglin significantly reduced (41%) the R_f of myc-endoglin, with no effect on D (Figure 1). Such an effect characterizes stable interactions between the differently tagged endoglin pairs (Henis *et al.*, 1990; Rechtman *et al.*, 2009), suggesting the formation of homomeric endoglin complexes that are stable on the time scale of the FRAP measurements (minutes). These complexes are detected already in the absence of exogenous ligand and are not affected by incubation with TGF-β1 or BMP-9. Of note, a statistical correction has to be applied to obtain the percentage of endoglin in homodimers (Ehrlich *et al.*, 2011; Marom *et al.*, 2011), since the probabilities of homodimer formation are 1:2:1 for dimers containing myc/myc, myc/HA plus HA/myc, and HA/HA. Because only myc-endoglin in mixed complexes with HA-endoglin would undergo immobilization and myc/myc endoglin complexes are labeled at twice the intensity of myc/HA endoglin complexes, the percentage reduction in R_f (ΔR_f) in patch/FRAP studies on homomeric complexes should be multiplied by 2 to derive the percentage of homodimerization (82%). Because endoglin is endogenously expressed in endothelial cells, we repeated the foregoing patch/FRAP studies in bEnd.3 endothelial cells transfected with myc-endoglin and HA-endoglin. These cells were chosen because they respond to TGF-β in both the Smad2/3 and Smad1/5/8 pathways (Figure 1G). The results of the patch/FRAP studies in these cells (Figure 1, E and F) were very similar to those obtained in COS7 cells, except that the initial R_f and D values of endoglin were somewhat lower in the bEnd.3 cells, reflecting the different cellular context. As in the COS7 cells, the reduction in the R_f value of myc-endoglin upon cross-linking HA-endoglin was high (47%), suggesting a high level in homodimers (47 × 2 = 94%), with no change in D, as expected for stable interactions. In line with the studies on COS7 cells, there were no significant effects of either TGF-β1 or BMP-9 on the patch/FRAP results.

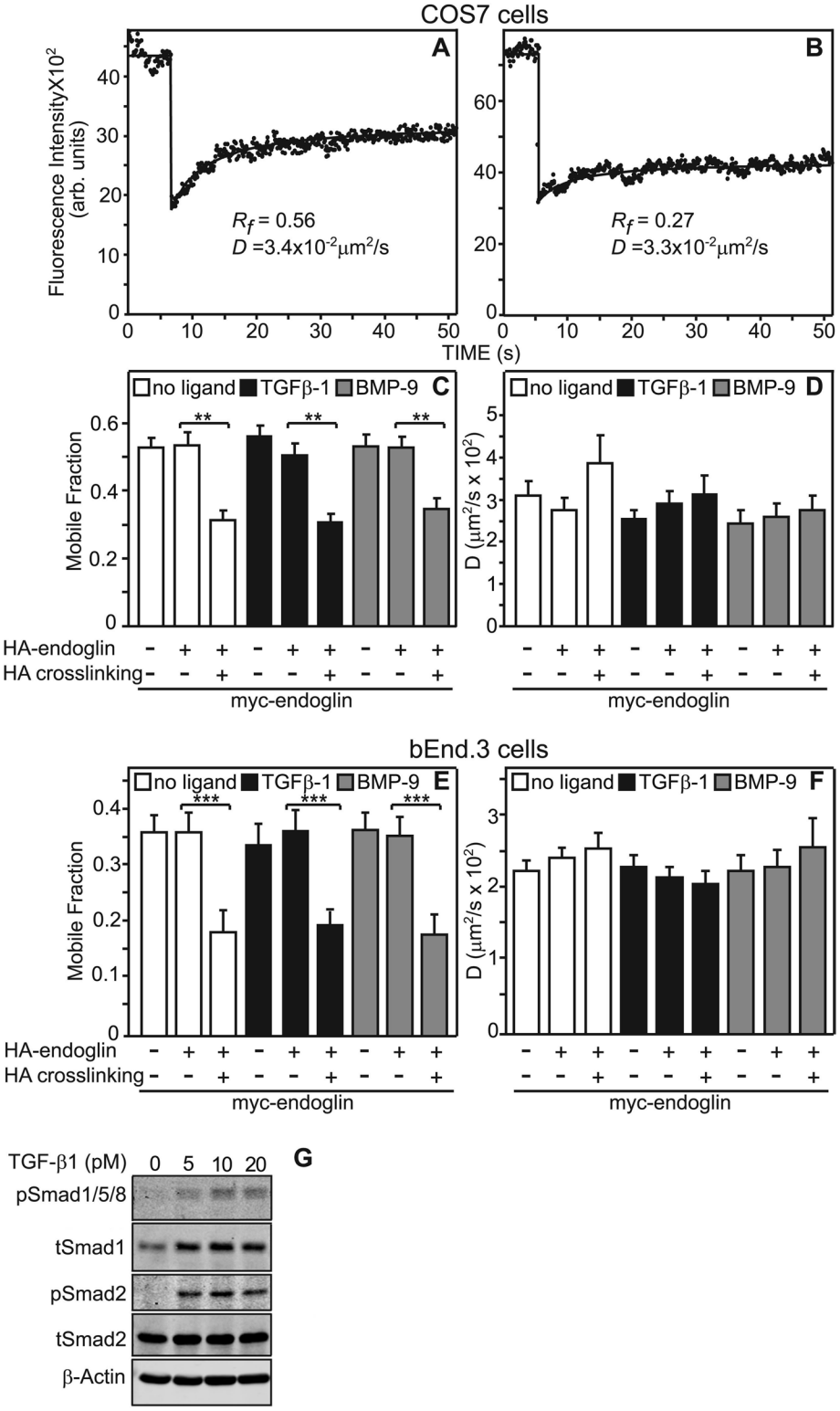

FIGURE 1: Patch/FRAP studies demonstrate stable endoglin homomeric complexes. COS7 or bEnd.3 cells were cotransfected with pairs of expression vectors encoding myc-endoglin and HA-endoglin. In control experiments with singly expressed myc-endoglin, HA-endoglin was replaced by empty vector. At 24 h posttransfection, live cells were subjected to the IgG-mediated patching/cross-linking protocol (*Materials and Methods*), resulting in HA-endoglin patched and labeled by Alexa 488 IgG GαR, whereas myc-endoglin is labeled by exclusively by monovalent Fab′ (with Fab′ Alexa 546–GαM as a secondary antibody). In control experiments without HA-endoglin cross-linking, the IgG labeling of HA-endoglin 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, B) Representative FRAP curves in COS7 cells of the lateral diffusion of myc-endoglin in a cell coexpressing HA-endoglin without (A) or with (B) IgG cross-linking of HA-endoglin. IgG–cross-linked HA-endoglin was laterally immobile (not shown). (C, D) Average R_f and D values derived from multiple patch/FRAP measurements in COS7 cells. (E, F) Average R_f and D values in bEnd.3 cells. Bars are mean ± SEM of 70 measurements in each case. Asterisks indicate significant differences between the R_f values of the pair indicated by brackets (**$p < 10^{-6}$; ***$p < 10^{-7}$; 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 TGF-β1 or BMP-9. (G) TGF-β1 stimulates the Smad1/5/8 and the Smad2/3 pathways in bEnd.3 cells. bEnd.3 cells were serum starved (6 h), stimulated (30 min) with the indicated TGF-β1 concentrations, and analyzed (see *Materials and Methods*) by immunoblotting for pSmad1/5/8, pSmad2, tSmad1, tSmad2, and β-actin. Data are of a representative experiment ($n = 3$).

To explore whether interactions involving the cytoplasmic domain of endoglin, such as with GIPC or β-arrestin2, are involved in the observed endoglin homo-oligomerization, we coexpressed wild-type (WT) myc-endoglin-WT with HA-endoglin-WT or with HA-endoglin mutants lacking interaction motifs with either GIPC (endoglin-Del) or β-arrestin2 (endoglin-T650A), cross-linked myc-endoglin-WT, and measured the effects on the lateral diffusion of the HA-endoglin mutants (Figure 2). The R_f and D values measured for the two HA-endoglin mutants without cross-linking were indistinguishable from that of HA-endoglin-WT (or myc-endoglin-WT; Figure 1), indicating that interactions of endoglin with GIPC or β-arrestin2 have a negligible effect on its lateral mobility. Of importance, the ΔR_f values of each HA-endoglin mutant upon cross-linking myc-endoglin were similar to the ΔR_f measured for HA-endoglin-WT, demonstrating that the homomeric interactions of endoglin do not depend on either GIPC or β-arrestin2 binding. The results in Figures 1 and 2 are in line with the reported disulfide-bond homo-dimerization of endoglin via its extracellular domain (Gougos and Letarte, 1988). However, it may well be that the endoglin subunits in the dimer interact with each other also without such an S–S bond, since reduction of the cells with 2 mM

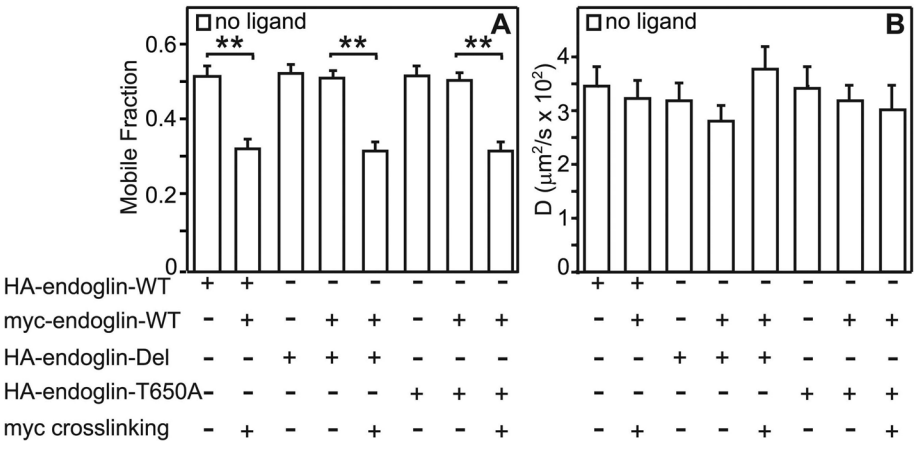

FIGURE 2: Endoglin homodimerization does not depend on interactions with GIPC or β-arrestin2. Patch/FRAP studies were carried out on COS7 cells expressing myc-endoglin-WT (or empty vector) together with HA-tagged endoglin-WT, endoglin-Del, or endoglin-T650A. The cells were labeled for the patch/FRAP experiments by the IgG-mediated patching/cross-linking protocol, leading to cross-linking/immobilization of myc-tagged endoglin (see *Materials and Methods*). The lateral mobility of Fab′-labeled HA-endoglin proteins was measured by FRAP at 15°C in the presence or absence of cross-linked myc-endoglin-WT. (A) R_f values; (B) 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). No significant differences were observed between HA-endoglin-WT and the mutants (HA-endoglin-Del or HA-endoglin-T650A).

dithiothreitol for 5–15 min at 37°C (as described in Gilboa *et al.*, 1998) did not affect the homomeric interactions in the cell membrane.

TβRII augments the association of ALK5 with endoglin

Next we used patch/FRAP to investigate heterocomplex formation between endoglin and TβRII. The studies were conducted on cells expressing HA-endoglin and myc-TβRII in the presence or absence of ligand (TGF-β1 or BMP-9), immobilizing (or not) HA-endoglin by IgG cross-linking, and measuring the lateral diffusion of Fab′-labeled myc-TβRII. In COS7 cells, cross-linking of HA-endoglin resulted in a 35% reduction in R_f (ΔR_f) of myc-TβRII, whereas D was unaffected (Figure 3, A–D). Similar results were obtained in the presence or absence of ligands. Analogous experiments on bEnd.3 cells (Figure 3, G and H) yielded comparable results, with a slightly higher ΔR_f (42%). Note that heterocomplex formation is directly proportional to the ΔR_f value and does not require the statistical correction needed for homo-dimerization (Ehrlich *et al.*, 2011). We conclude that a significant portion (0.3–0.4) of TβRII is in stable complexes with endoglin at the cell surface, independent of ligand stimuli. Moreover, analogous measurements on the interactions of TβRII with endoglin cytoplasmic-domain mutants (endoglin-Del or endoglin-T650A), yielded identical results to those obtained with endoglin-WT, suggesting that TβRII-endoglin interactions are also independent of GIPC or β-arrestin2 binding (Figure 3, E and F).

ALK5 was reported to phosphorylate endoglin (Goumans *et al.*, 2003a; Ray *et al.*, 2010), suggesting that the two proteins should interact. To explore such interactions, we conducted patch/FRAP studies analogous to those described in Figure 3, except that myc-ALK5 replaced myc-TβRII. A small yet significant portion of ALK5 exhibited stable association with cross-linked endoglin (ΔR_f = 15–17%, with no effect on D; Figure 4), indicating a weaker association with endoglin relative to TβRII. These interactions were unaffected by ligand (TGF-β1 or BMP-9; only the first is shown since both had no effect) or by mutations that disrupt endoglin interactions with GIPC or β-arrestin2 (Figure 4, C and D). The independence of the weak endoglin–ALK5 interactions from ligand supports the notion that these interactions do not require TβRII (which, unlike ALK5 and endoglin, is not exogenously expressed in these experiments).

Because TβRII and ALK5 form heteromeric complexes without endoglin (Gilboa *et al.*, 1998; Rechtman *et al.*, 2009; Ehrlich *et al.*, 2011), and strong interactions were found between endoglin and TβRII (Figure 3), we assessed the effects of TβRII expression on endoglin–ALK5 interactions. To this end, we coexpressed HA-endoglin and myc-ALK5 together with an excess of TβRII (replaced by empty vector in some experiments). Under these conditions, we cross-linked/immobilized HA-endoglin and measured the lateral diffusion of ALK5. The presence of TβRII confers a much more pronounced reduction in R_f of myc-ALK5 upon cross-linking of HA-endoglin without affecting D (Figure 5, A and B). Ligand (TGF-β1 or BMP-9) had no effect. The ΔR_f value of myc-ALK5 in the presence of TβRII increases to 45–50%. These values are even higher than the ΔR_f of TβRII upon cross-linking endoglin (Figure 3).

To explore further the interactions within this ternary complex, we ran a variation of the foregoing experiment to assess the effect of expression of untagged endoglin on HA-TβRII/myc-ALK5 interactions, measured by cross-linking HA-TβRII and conducting FRAP studies on myc-ALK5. Here TβRII/ALK5 formed stable complexes (reduction in R_f with no effect on D) to a mild degree in the absence of ligand (Figure 5, C and D), increasing approximately twofold upon TGF-β1 addition (Figure 5C; no endoglin). These results are in full accord with our earlier observations, which show that TGF-β1 enhances complex formation between TβRI and TβRII (Rechtman *et al.*, 2009). Of note, cross-linking HA-TβRII in the presence of endoglin already yields maximal reduction in R_f of myc-ALK5 (~50%), which does not increase further upon incubation with TGF-β1 (Figure 5C; + endoglin). We conclude that endoglin forms mutual complexes with both TβRII and ALK5, thus bringing the last two into close proximity.

ALK1 forms stable complexes with itself, with TβRII, and with endoglin

In spite of its involvement in TGF-β signaling in endothelial cells, neither the homomeric interactions of ALK1 nor its hetero-oligomerization with TβRII and/or endoglin have been investigated for the full-length receptors at the plasma membrane. To study the homomeric interactions of ALK1, we conducted patch/FRAP studies on cells coexpressing HA-ALK1 and myc-ALK1. Cross-linking/immobilization of HA-ALK1 induced a reduction by ~30% in R_f of non–cross-linked, coexpressed myc-ALK1, with no effect on the D value (Figure 6). This is indicative of stable homo-oligomerization, which in the case of dimers would reflect ~60% of the receptors existing in homo-dimeric complexes. Because ALK1 can bind BMP-9, we also tested the effects of BMP-9 on ALK1 homo-oligomerization; we found no significant effects (Figure 6).

Next we investigated complex formation between ALK1 and TβRII. To this end, we conducted patch/FRAP studies on cells coexpressing HA-TβRII and myc-ALK1. In the absence of endoglin,

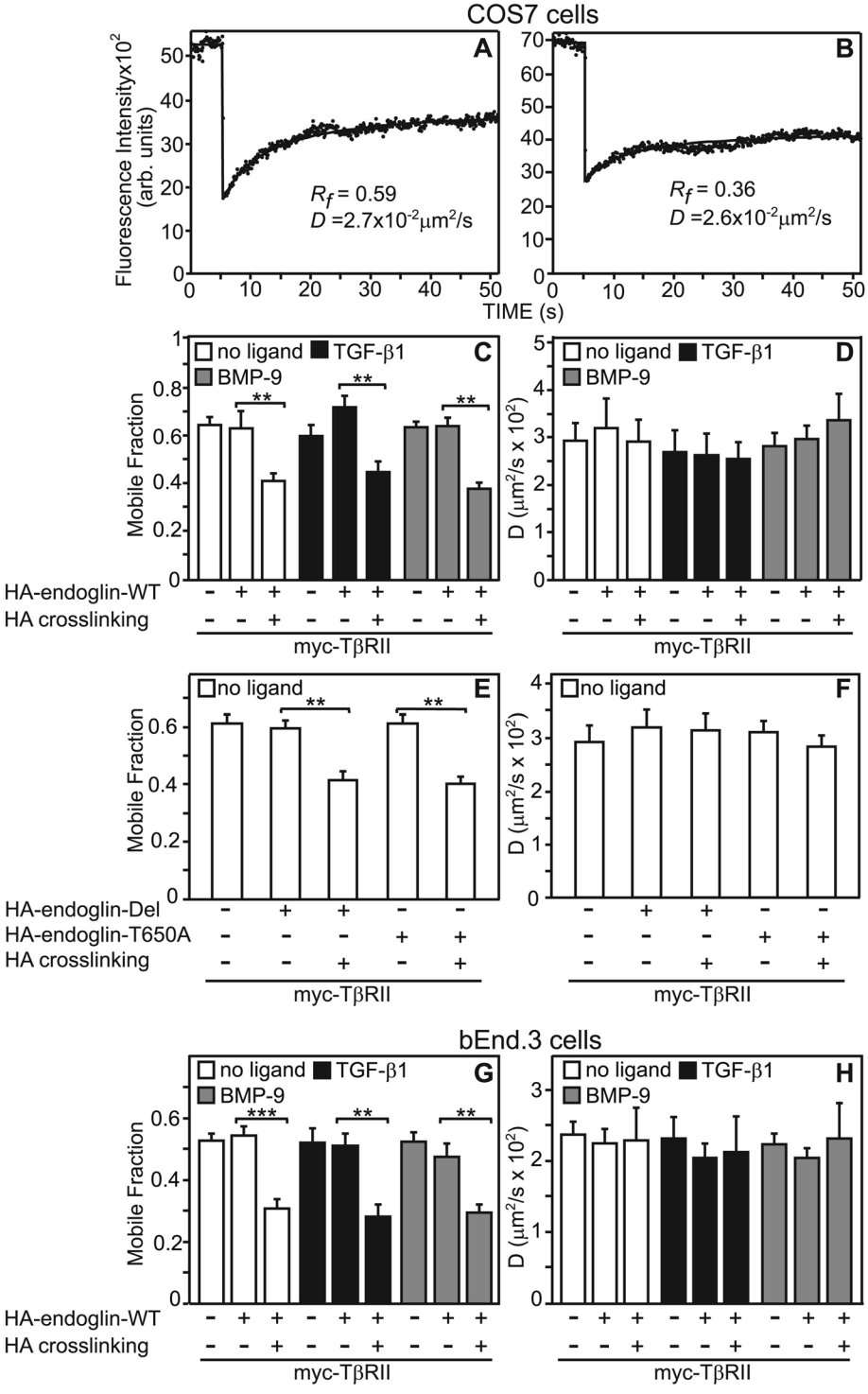

FIGURE 3: Stable TβRII/endoglin heteromeric complexes form independently of ligand and of endoglin interaction with GIPC and β-arrestin2. COS7 or bEnd.3 cells were cotransfected with pairs of expression vectors encoding myc-TβRII and HA-endoglin-WT, HA-endoglin-Del, or HA-endoglin-T650A. In control experiments with singly expressed myc-TβRII, HA-endoglin-WT or the mutants endoglin-Del and endoglin-T650A were replaced for empty vector. Labeling and patch/FRAP measurements (measuring the lateral diffusion of myc-TβRII) were carried out 24 h after transfection as described in Figure 1. (A, B) Representative FRAP curves of the lateral diffusion of myc-TβRII in a COS7 cell coexpressing HA-endoglin-WT without (A) or with (B) cross-linking of the HA-tagged proteins. (C, E, G) Average R_f and (D, F, H) average D values derived from multiple patch/FRAP measurements in COS7 cells (A–F) or bEnd.3 cells (G, H). Bars are mean ± SEM of 45–60 measurements. IgG cross-linking of HA-endoglin markedly reduced R_f of myc-TβRII, independent of TGF-β1, BMP-9 (whose addition yielded indistinguishable results from those obtained in the presence of TGF-β1), GIPC, or β-arrestin2 binding motifs (**$p < 10^{-5}$; ***$p < 10^{-6}$; Student's t test). No significant differences were found in the D values as a result of IgG-mediated cross-linking of HA-endoglin or after addition of TGF-β1 (or BMP-9) under similar experimental conditions.

cross-linking of HA-TβRII reduced the R_f of myc-ALK1 by ~30% (Figure 7, –endoglin). Because D was not affected, this suggests that 30% of ALK1 forms stable complexes with TβRII. Similar results were obtained in the presence of TGF-β1 or BMP-9. Subsequently, we probed for potential effects of untagged endoglin expression on HA-TβRII/myc-ALK1 interactions. Here, too, cross-linking of HA-TβRII resulted in ~30% reduction in R_f of myc-ALK1 (Figure 7A; +endoglin). Taken together, these findings suggest stable interactions between TβRII and ALK1, which are not significantly affected by either ligand or endoglin. However, they do not exclude the possibility that ALK1 may interact with endoglin independent of TβRII. To answer this question, we coexpressed myc-endoglin and HA-ALK1 and conducted patch/FRAP studies to measure the effects of cross-linking myc-endoglin on the lateral diffusion of HA-ALK1. Expression of endoglin (without cross-linking) had no effect on R_f or D of HA-ALK1; however, R_f of HA-ALK1 (but not its D value) was markedly reduced (~30%) upon myc-endoglin cross-linking (Figure 8, A and B), suggesting a significant level of stable heterocomplex formation. Similar results were obtained after addition of TGF-β1 (Figure 8, A and B) or BMP-9. Of note, coexpression of TβRII (untagged) on the background of myc-endoglin and HA-ALK1 did not affect the interactions between the latter. Moreover, endoglin–ALK1 interactions were insensitive to mutations that interfere with endoglin binding to the scaffold proteins GIPC or β-arrestin2 (Figure 8, C and D). We conclude that a significant portion of ALK1 can bind to endoglin independent of TβRII.

Endoglin differentially augments TGF-β–mediated activation of Smad1/5/8

Because endoglin appears to form complexes with TβRII, ALK5, and ALK1, we examined its ability to modulate TGF-β signaling via ALK5 (phopho-Smad2 [pSmad2] formation) and/or ALK1 (pSmad1/5/8 formation). To this end, we used endoglin-null murine embryonic endothelial cells derived from endoglin$^{-/-}$ mice (MEEC$^{-/-}$) and their

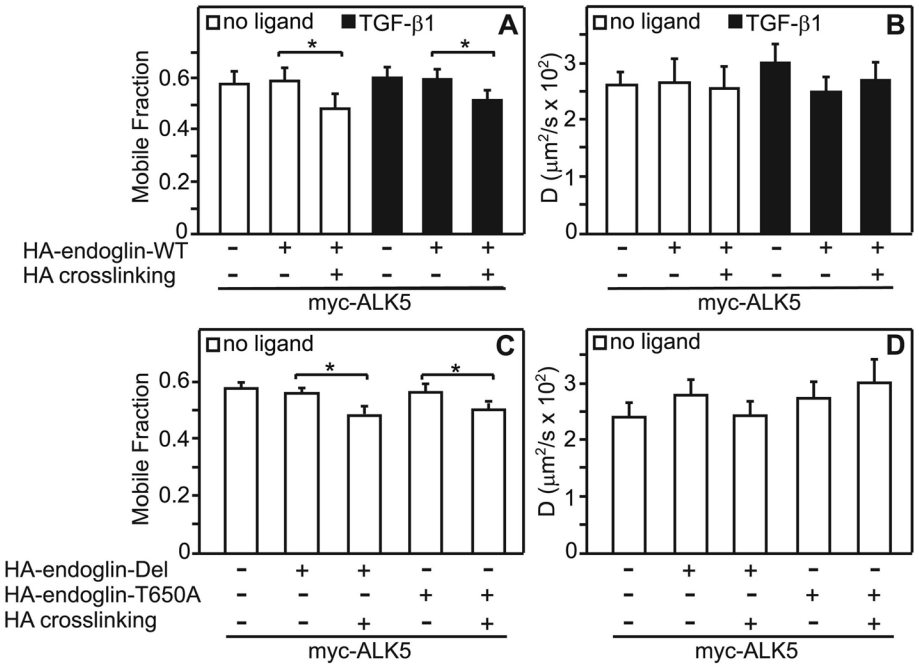

FIGURE 4: Patch/FRAP studies demonstrate weak but stable endoglin-ALK5 interactions. Experimental conditions were as in Figure 3, except that myc-TβRII was replaced by myc-ALK5. (A, C) Average R_f and (B, D) average D values. Bars are mean ± SEM of 45–60 measurements. Asterisks indicate significant differences between the R_f values of the pair indicated by brackets (*$p < 0.05$; Student's t test). Cross-linking of coexpressed HA-endoglin-WT, HA-endoglin-Del or HA-endoglin-T650A had a mild yet significant effect on the R_f values of myc-ALK5. Addition of TGF-β1 or endoglin C-terminal mutants that lose binding to GIPC or β-arrestin2 had no effect. BMP-9 addition yielded results similar to those obtained in the presence of TGF-β1.

wild-type control (MEEC$^{+/+}$). The cells were stimulated by increasing concentrations of TGF-β1, and the resulting phosphorylation of Smad2 and Smad1/5/8 was measured by immunoblotting (Figure 9). A low but measurable level of pSmad1/5/8 was observed in both cell lines already before addition of TGF-β1. Stimulation by TGF-β1 increased the pSmad1/5/8 levels in both cell types but was significantly higher in MEEC$^{+/+}$ cells at TGF-β1 concentrations ≥10 pM. A markedly different scenario was observed for Smad2 phosphorylation, for which no significant differences between MEEC$^{+/+}$ and MEEC$^{-/-}$ cells were detected without or with TGF-β1 stimulation (Figure 9, A and C). In light of earlier findings that demonstrated that TGF-β–mediated phosphorylation of Smad1/5/8 proceeds via ALK1 in human umbilical vein endothelial cells and mouse embryonic endothelial cells (Oh *et al.*, 2000; Goumans *et al.*, 2002), we conclude that endoglin expression alters the balance between the activation of ALK1-dependent (Smad1/5/8) and ALK5-dependent (Smad2/3) TGF-β signaling.

DISCUSSION

TGF-β signaling is regulated by complex formation among numerous receptors and coreceptors. Although oligomerization of TβRII and ALK5 and its role in Smad2/3 signaling have been extensively studied (reviewed in Ehrlich *et al.*, 2011), such information is lacking for ALK1–TβRII interactions, as well as for the coreceptor endoglin and its interactions with ALK1, ALK5, and TβRII in the intact cell membrane. Here we present biophysical studies on the extent and dynamics of complex formation among these receptors. Our main findings are as follows: 1) endoglin forms stable homomeric complexes, as well as heteromeric complexes with ALK1 and TβRII; 2) ALK5 displays limited binding to endoglin, which is significantly enhanced by TβRII; and 3) endoglin tilts the balance of activation between pSmad1/5/8 and pSmad2/3 toward the former. These findings, together with our previous results on TβRII–ALK5 interaction (Wells *et al.*, 1999; Rechtman *et al.*, 2009; Ehrlich *et al.*, 2011), support a model (Figure 10) in which endoglin, assisted by recruitment of ALK5 via binding to TβRII, functions as a scaffold that is sequentially phosphorylated by ALK5 and ALK1 after their activation by TβRII and ligand. This enables optimal activation of the Smad1/5/8 pathway.

Our patch/FRAP results demonstrated that homomeric endoglin complexes do not appreciably dissociate on the time scale of FRAP measurements (minutes), indicating stable interactions. The percentage reduction in the R_f (ΔR_f) value was 41%, which for homomeric interactions predicts a homodimerization level of 41 × 2 = 82%. This high dimerization level and its stable nature are in accord with the reported extracellular disulfide bond linkage between two endoglin monomers (Gougos and Letarte, 1988). Consistent with this model, endoglin homomeric interactions were independent of GIPC or β-arrestin2 binding (Figure 2). These results suggest that the interactions of endoglin with GIPC or β-arrestin2 do not link it to large immobile structures (e.g., the cytoskeleton), since endoglin mutants that lack binding to these scaffold proteins display the same D and R_f values as endoglin-WT.

Because endoglin does not bind TGF-β ligands on its own (Cheifetz *et al.*, 1992; Barbara *et al.*, 1999), its ability to modulate TGF-β signaling most likely reflects interactions with type II and/or type I TGF-β receptors. Activation of Smad2/3 by TGF-β depends on TβRII and ALK5; we therefore measured their ability to form heteromeric complexes with endoglin. Endoglin–TβRII interactions were ligand independent, stable on the FRAP time scale (patch/FRAP-mediated reduction in R_f of myc-TβRII upon cross-linking of coexpressed HA-endoglin; Figure 3), and involved approximately one-third of the cell-surface TβRII population (ΔR_f of 35%). Endoglin–ALK5 interactions were different: the ΔR_f value of myc-ALK5 was 15–17%, indicating that a much lower fraction approximately one-sixth) of ALK5 is immobilized along with cross-linked endoglin (Figure 4). Here, too, the interactions were stable over the FRAP time scale and ligand independent. Of note, the interactions of endoglin with either TβRII or ALK5 did not require GIPC or β-arrestin2 binding, indicating that they do not depend on recruitment to immobile scaffolds (e.g., the cytoskeleton). In view of the well-documented interactions between TβRII and ALK5 (Gilboa *et al.*, 1998; Rechtman *et al.*, 2009; reviewed in Ehrlich *et al.*, 2011), we assessed whether coexpression with TβRII contributes to endoglin–ALK5 interactions (compare Figures 4 and 5). Expression of TβRII dramatically enhanced ALK5–endoglin association (increasing ΔR_f of myc-ALK5 after HA-endoglin cross-linking from 17 to >40%; Figures 4A and 5A). This enhancement occurred already in the absence of ligand. Accordingly, coexpression of endoglin enhanced TβRII–ALK5 interactions (approximately twofold increase in ΔR_f of myc-ALK5 upon cross-linking of HA-TβRII) without ligand (Figure 5C). The

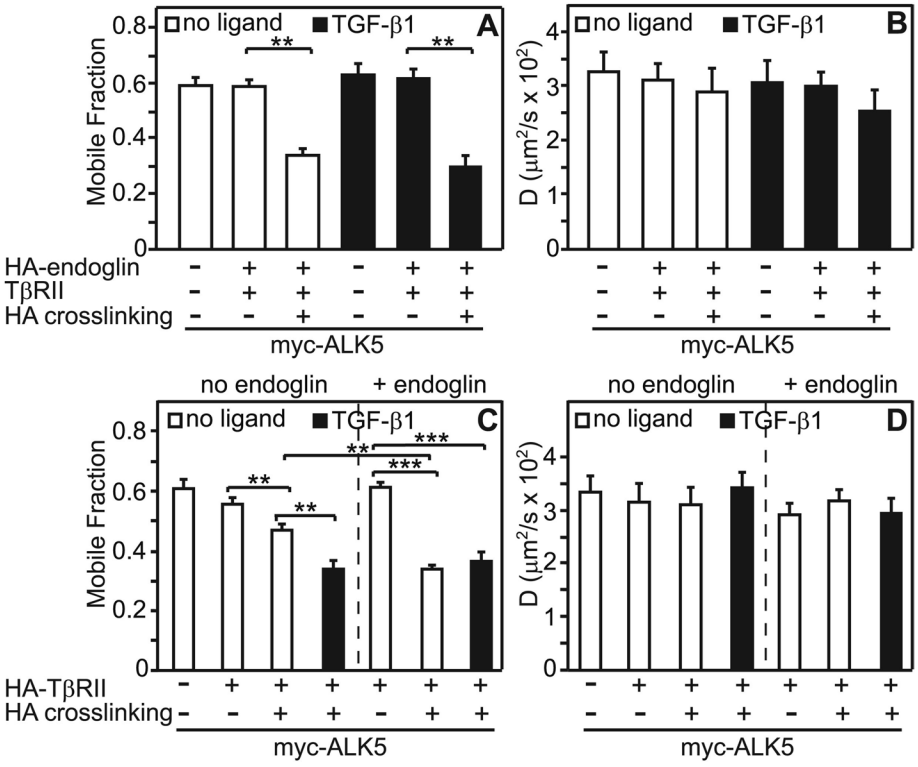

FIGURE 5: Patch/FRAP studies show ternary complex formation between endoglin, TβRII, and ALK5. (A, B) Effects of TβRII expression on endoglin/ALK5 interactions. COS7 cells were cotransfected with myc-ALK5 together with HA-endoglin and excess untagged TβRII (both replaced by pcDNA3 vector as control). Myc-ALK5 diffusion was measured by FRAP with or without cross-linking of HA-endoglin, as described in Figure 3. TGF-β1 was added where indicated; BMP-9 addition yielded similar results and is not shown. (A) R_f values and (B) D values of 50–75 measurements in each condition. Significant differences (**$p < 10^{-22}$) between the indicated pairs were observed only for R_f values of myc-ALK5 upon cross-linking of HA-endoglin either in the presence or absence of TGF-β1 (or BMP-9). (C, D) effects of endoglin expression on TβRII/ALK5 interactions. COS7 cells were cotransfected with myc-ALK5 together with HA-TβRII or a combination of HA-TβRII and excess untagged endoglin. Empty pcDNA3 vector served as cotransfection control. Myc-ALK5 diffusion was measured by FRAP with or without crosslinking of HA-TβRII and ± TGF-β1, as described in Figure 3. (C) R_f values and (D) D values of 50–75 measurements in each case. Endoglin expression was sufficient to induce highly significant differences (**$p < 10^{-4}$, ***$p < 10^{-15}$; Student's t test) in the R_f values of myc-ALK5 when HA-TβRII was cross-linked. This effect was as high as the TGF-β1–induced association between ALK5 and TβRII in the absence of endoglin.

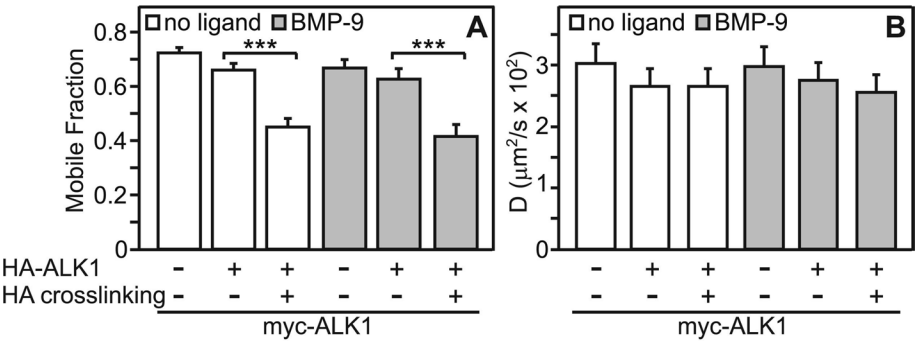

FIGURE 6: Patch/FRAP studies demonstrate stable ALK1 homomeric complexes. COS7 cells were cotransfected with pairs of expression vectors encoding myc-ALK5 and HA-ALK1, replacing HA-ALK1 by empty vector in control experiments. Labeling, IgG cross-linking (XL) of HA-ALK1, and patch/FRAP studies (measuring the lateral diffusion of myc-ALK5) were as in Figure 1. (A) Average R_f and (B) average D values derived from multiple patch/FRAP measurements. Bars are mean ± SEM of 50–100 measurements. Asterisks indicate significant differences between the R_f values of the pair indicated by brackets (***$p < 10^{-10}$; Student's t test). The R_f value of myc-ALK1 is markedly reduced after cross-linking of HA-ALK1, whereas the D value is unaffected. This indicates the formation of stable ALK1 homomeric complexes. As shown, addition of BMP-9 had no significant effects.

endoglin-mediated enhancement of TβRII–ALK5 interactions was indistinguishable from that mediated by TGF-β1 without endoglin (Figure 5C), indicating that triple expression of endoglin/TβRII/ALK5 leads to a significant fraction of mutual and stable complexes containing all three receptors. These findings support our model (Figure 10) in which endoglin binding to TβRII and TβRII–ALK5 interactions enhance the proximity of endoglin and ALK5, a prerequisite for efficient phosphorylation of endoglin by ligand-activated ALK5 and ALK1 (Ray *et al.*, 2010). Moreover, TβRII–ALK5 interactions appear to be different without endoglin and in the triple complex with endoglin, since although association with endoglin enhances TβRII-ALK5 interactions similarly to TGF-β1, it does not suffice to activate the Smad2/3 pathway (Figure 9).

Endothelial cells express both endoglin and ALK1, which mediate Smad1/5/8 signaling after TGF-β stimulation (Goumans *et al.*, 2002; Lebrin *et al.*, 2004). It is therefore possible that endoglin modulates TGF-β signaling via ALK1 by altering TβRII–ALK1 association. The present studies represent the first measurements of ALK1 homomeric (Figure 6) and heteromeric (Figures 7 and 8) complex formation at the surface of intact cells. ALK1 homomeric interactions resembled ALK5, showing a high level of homodimerization (~60%) already without ligand (Figure 6). This dimerization was insensitive to TGF-β1 (unpublished data), in line with the inability of ALK1 alone to bind this ligand. Of interest, in contrast to the TGF-β1–mediated increase in TβRII–ALK5 interaction (Figure 5C; Rechtman *et al.*, 2009), TGF-β1 did not increase the already high (~30%) TβRII–ALK1 association (Figure 7A). However, even in endoglin-null cells (MEEC$^{-/-}$), TGF-β1 stimulated signaling to Smad1/5/8 via ALK1 (Figure 9), indicating that ligand binding likely modifies the TβRII–ALK1 complex to an active state. Similar to ligand, coexpression with endoglin did not affect the stability or percentage of interacting TβRII and ALK1 molecules. Here, too, endoglin expression enhanced signaling to Smad1/5/8, both without and with ligand (Figure 9), supporting the notion that endoglin can modulate TGF-β signaling via Smad1/5/8 (Ray *et al.*, 2010). Of note, endoglin exhibited strong and stable association

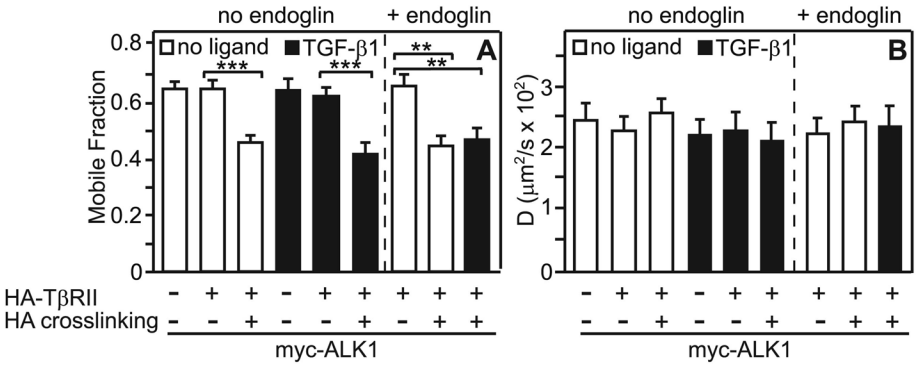

FIGURE 7: ALK1 forms stable heterocomplexes with TβRII. Experimental conditions were as in Figure 3, except that myc-ALK1 replaced myc-TβRII, and HA-TβRII was used instead of HA-endoglin. In some experiments, untagged endoglin was coexpressed (marked as "+endoglin"). The lateral diffusion of myc-ALK1 was measured under the indicated experimental conditions. (A) Average R_f and (B) average D values. Bars are mean ± SEM of 30–70 measurements. Asterisks indicate significant differences between the R_f values of the pair indicated by brackets (**$p < 10^{-5}$, ***$p < 10^{-10}$; Student's t test). The studies show a clear reduction in R_f of myc-ALK1 after cross-linking of HA-TβRII, and this reduction is unaffected by endoglin. Similar results were obtained in the presence of TGF-β1 or BMP-9 (since both had no effect, the latter is not shown).

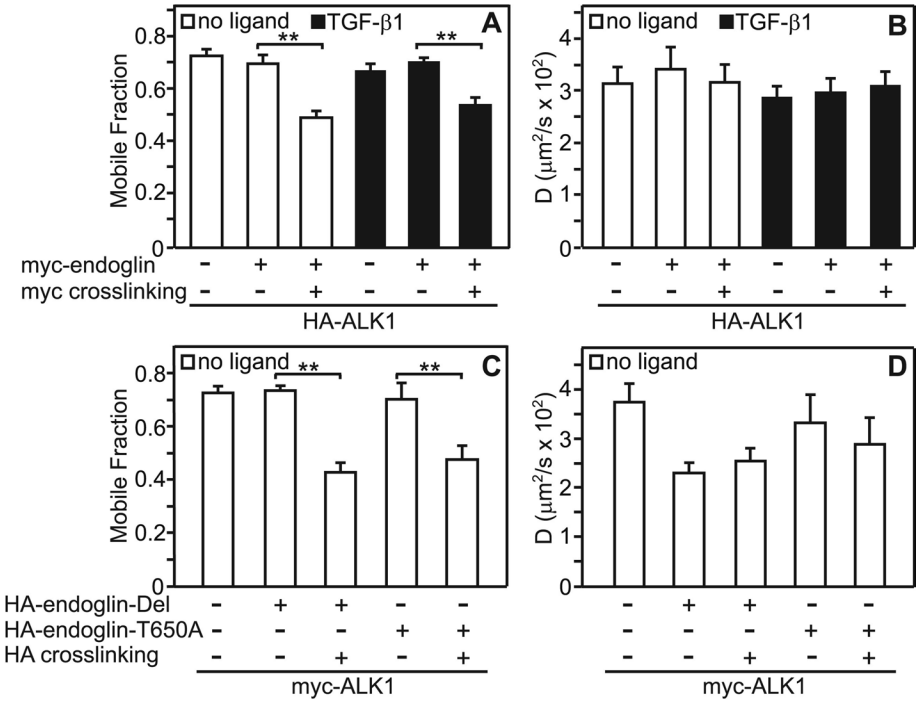

FIGURE 8: ALK1 strongly interacts with endoglin to form stable heteromeric complexes. COS7 cells were cotransfected with pairs of expression vectors encoding HA-ALK1 and myc-endoglin, myc-ALK1 and HA-endoglin-Del, or myc-ALK1 and HA-endoglin T650A. In control experiments, the vector encoding endoglin or its mutants was replaced by empty vector. IgG-mediated patching/cross-linking of myc-endoglin (A, B) was as in Figure 2; patching/cross-linking of HA-endoglin was as in Figures 1 and 3. The lateral diffusion of HA-ALK1 (A, B) or myc-ALK1 (C, D) was measured without or with myc-endoglin or HA-endoglin cross-linking. (A, C) Average R_f and (B, D) average D values. Bars are mean ± SEM of 45–65 measurements. Asterisks indicate significant differences between R_f values of the pair indicated by brackets (**$p < 10^{-6}$; Student's t test). Cross-linking of coexpressed myc-endoglin-WT, HA-endoglin-Del, or HA-endoglin-T650A elicited similar reduction in R_f of HA-ALK1 or myc-ALK1, suggesting that the strong ALK1/endoglin association does not rely on endoglin binding to GIPC or β-arrestin2. TGF-β1 or BMP-9 addition did not alter ALK1-endoglin association; only the first is shown, as both ligands had no effect.

with ALK1 already without TβRII transfection; this association was ligand independent and persisted with endoglin mutants lacking binding to GIPC or β-arrestin2 (Figure 8). Thus, unlike ALK5, ALK1–endoglin association is independent of TβRII. In contrast to the lack of dependence of TGF-β1–mediated Smad 2/3 activation on endoglin, Smad1/5/8 activation was markedly augmented by endoglin (Figure 9). These results cannot be explained by ligand-mediated modulation of the interactions between endoglin, TβRII, and ALK1, as all of these interactions were insensitive to TGF-β1. Therefore a molecular mechanism capable of supporting the potentiation of Smad1/5/8 signaling (both basal and ligand induced) by endoglin expression (Figure 9) is required. We propose that TβRII and ALK1 bind to endoglin stably and directly. On the other hand, ALK5 binding to endoglin is augmented by TβRII residing in the same complex (Figure 10). Thus endoglin and TβRII act together to recruit ALK5 to endoglin (Figure 10). Ligand binding allows 1) activation of the endoglin-bound ALK5 by TβRII and the ensuing phosphorylation of endoglin by ALK5 (Ray *et al.*, 2010) and 2) phosphorylation of endoglin by ligand-activated ALK1; 3) these lead to enhanced Smad1/5/8 signaling, without affecting signaling via Smad2/3, tilting the balance of TGF-β–mediated signaling toward Smad1/5/8 (Figure 10).

Although the effects of the Smad2/3 and Smad1/5/8 pathways on endothelial cell function and angiogenesis are controversial, multiple studies suggest that the balance of signaling between these pathways regulates the functional response to TGF-β ligands. The present studies, which support a central role for endoglin in differentially regulating signaling through the Smad2/3 and Smad1/5/8 pathways, including preferentially promoting ALK1 signaling to Smad1/5/8 (Figure 9), are consistent with the central role of endoglin and ALK1 in both physiological and pathophysiological angiogenesis. Indeed, both endoglin and ALK1 are being targeted in antiangiogenic cancer therapy. Whether these agents function by disrupting the complexes characterized here remains to determined.

MATERIALS AND METHODS

Reagents

Recombinant TGF-β1 was obtained from PeproTech (Rocky Hill, NJ), and BMP-9 from R&D Systems (Minneapolis, MN). Fatty acid–free bovine serum albumin (BSA; fraction V) was from Sigma-Aldrich (St. Louis, MO). Media and Hank's balanced salts solution

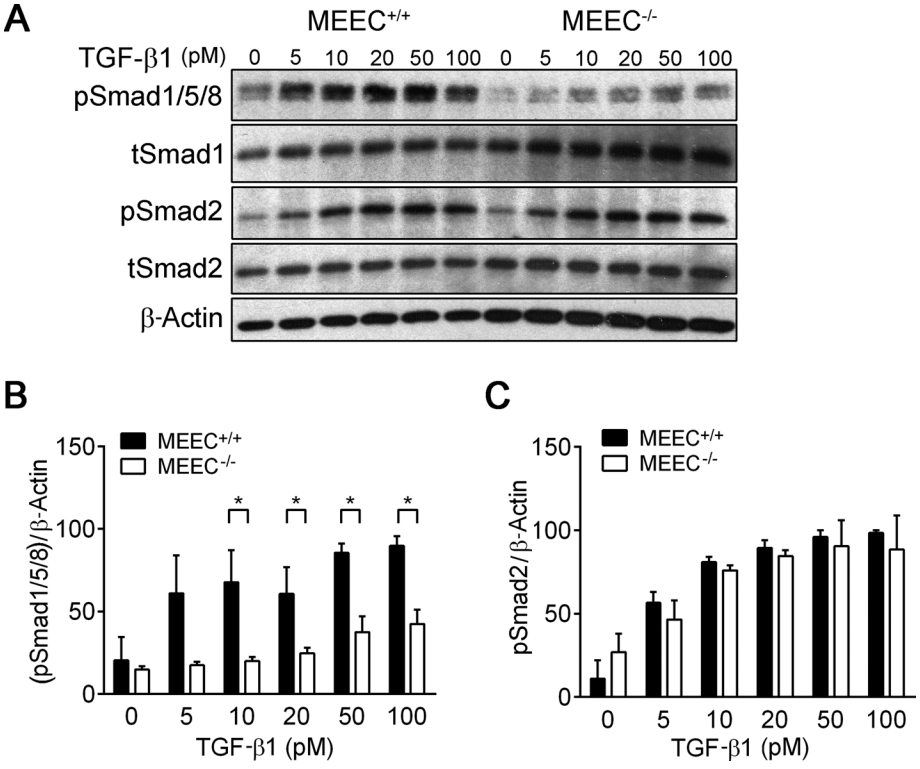

FIGURE 9: Endoglin expression enhances phosphorylation of Smad1/5/8 but not of Smad2/3. (A) Biochemical analysis of Smad activation in response to TGF-β1 was performed in mouse embryonic endothelial cells derived from endoglin wild-type (+/+) or endoglin null (−/−) mice. MEEC$^{+/+}$ and MEEC$^{-/-}$ cells were serum starved (6 h) and stimulated (30 min) with TGF-β1 at the indicated concentrations. Lysates were resolved by SDS–PAGE and subjected to Western blot analysis with antibodies to pSmad1/5/8, pSmad2, tSmad1, tSmad2, and β-actin. Data are representative of at least three independent experiments. (B) Densitometric analysis of band intensities of pSmad1/5/8 relative to β-actin; similar results were obtained for calibration relative to tSmad1. (C) Densitometric analysis of band intensities of pSmad2 relative to β-actin; similar results were obtained relative to tSmad2. Bars represent normalized mean ± SEM of three independent experiments. In B and C, results were calibrated relative to the value in MEEC$^{+/+}$ cells stimulated with 100 pM TGF-β1, taken as 100%. Asterisks indicate significant differences between the values obtained in MEEC$^{+/+}$ and MEEC$^{-/-}$ cells stimulated with the same TGF-β1 concentration (*$p < 0.05$).

(HBSS) were from Biological Industries (Beit Haemek, Israel) or Invitrogen (Carlsbad, CA). Rabbit anti-Smad2, anti–phospho-Smad2 (pSmad2), anti-Smad1, and anti-pSmad1/5/8 (antibody 9511, recognizing the C-terminal S*XS* motif, where S* stands for phospho-Ser) antibodies were from Cell Signaling Technologies (Danvers, MA). Mouse anti–β-actin was from MP Biomedicals (Solon, OH). Anti–myc tag 9E10 mouse ascites (Evan *et al.*, 1985), anti-influenza hemagglutinin epitope tag (anti-HA) 12CA5 mouse ascites, and HA.11 rabbit antiserum to the HA tag were purchased from Covance Research Products (Denver, PA). IgG and monovalent Fab′ fragments were prepared as described (Henis *et al.*, 1994). Chicken anti-myc affinity-purified IgY was from EMD Millipore-Chemicon (Temecula, CA). Peroxidase-conjugated donkey anti-rabbit (DαR) and sheep anti-rabbit (SαR) antibodies used for immunoblotting were from Amersham/GE Life Sciences (Pittsburgh, PA). Goat γ-globulin, Cy3-conjugated AffiniPure GαM F(ab′)$_2$, and fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey IgG against chicken IgY (DαC) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 488–GαR IgG and Alexa Fluor 546–GαM F(ab′)$_2$ were from Invitrogen-Molecular Probes (Eugene, OR); fluorescent F(ab′)$_2$ was converted to Fab′ as described (Gilboa *et al.*, 1998).

Plasmids

Expression vectors encoding human TβRI (in pcDNA3) or TβRII (in pcDNA1) with extracellular myc or HA epitope tags, as well as untagged TβRII, were described earlier (Henis *et al.*, 1994; Gilboa *et al.*, 1998; Ehrlich *et al.*, 2001; Shapira *et al.*, 2012). HA-tagged WT endoglin (endoglin-L), HA-endoglin-Del (lacking the last three C-terminal amino acids, resulting in loss of binding to GIPC), and HA-endoglin-T650A (a point mutation that abrogates endoglin binding to β-arrestin2) in pDisplay were described (Lee and Blobe, 2007; Lee *et al.*, 2008). HA- or myc-tagged ALK1 in pcDNA3.1 were delineated previously (Lee *et al.*, 2009; Tian *et al.*, 2012). Myc-tagged endoglin was generated by PCR incorporation of myc sequence into untagged pcDNA3.1 endoglin.

Cell culture and transfections

COS7 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in DMEM with 10% fetal calf serum (FCS; Biological Industries) as described (Gilboa *et al.*, 2000; Shapira *et al.*, 2012). Murine endothelial polyoma bEnd.3 cells (ATCC) were grown in the same medium under similar conditions. Murine embryonic endothelial cells (MEECs) from endoglin wild-type (MEEC$^{+/+}$) and endoglin-null (MEEC$^{-/-}$) (Pece-Barbara *et al.*, 2005; a gift from E. Dejana, University of Milan, Milan, Italy) were grown in MCDB-131 medium (Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml heparin (Sigma-Aldrich), and 50 μg/ml endothelial cell growth supplement (Sigma-Aldrich). The MEEC cell lines were cultured in flasks coated with 0.05% gelatin.

For patch/FRAP experiments, COS7 or bEnd.3 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. For endoglin, which expresses at higher levels than the TGF-β receptors, 300 ng of plasmid DNA was used per transfection, and the DNA amounts of TβRII, ALK5, or ALK1 vectors were adjusted to yield similar cell-surface expression levels, determined by quantitative immunofluorescence as described earlier (Marom *et al.*, 2011). The total DNA level was complemented by empty vector to 2 μg.

IgG-mediated patching/cross-linking

At 24 h posttransfection, COS7 or bEnd.3 cells transfected with various combinations of expression vectors for endoglin and TGF-β receptors were serum starved (30 min, 37°C), washed with cold HBSS supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) 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

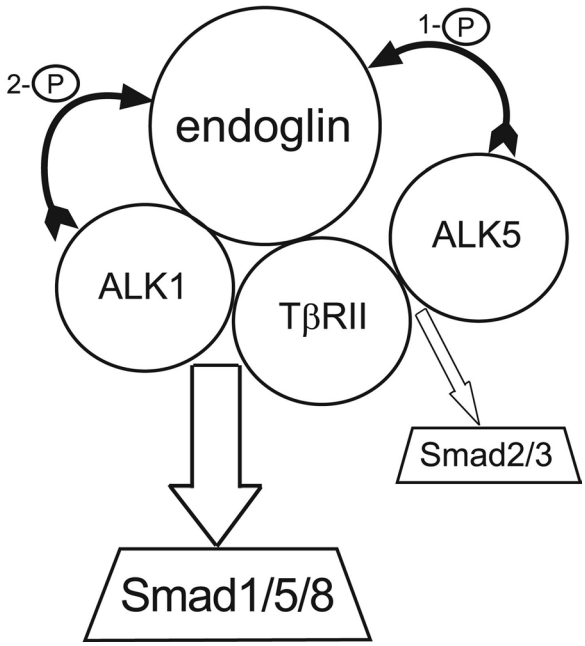

FIGURE 10: Model of endoglin regulation of Smad signaling via TβRII, ALK5, and ALK1 interactions. All receptors are drawn as monomers for simplicity. TβRII and ALK1 can bind to endoglin stably and directly, whereas ALK5 binding to endoglin is augmented by TβRII residing in the same complex. TGF-β1 binding to the complex (not drawn, for simplicity) enables activation of the endoglin-bound ALK5 by TβRII and the phosphorylation of endoglin first by ALK5 (designated 1-P) and subsequently by ligand-activated ALK1 (designated 2-P since this is a second phosphorylation event, which was shown to occur only after the first one; Ray *et al.*, 2010). In this complex, the signaling through ALK5/Smad2/3 is not affected by endoglin, whereas signaling via ALK1/Smad1/5/8 is enhanced.

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′. Alternatively, for immobilizing the myc-tagged receptor and measuring the lateral diffusion of Fab′-labeled HA-tagged receptor, the following labeling protocol was used: 1) monovalent mouse Fab′ anti-HA (40 μg/ml) together with chicken IgY anti-myc (20 μg/ml) and 2) Cy3-Fab′ GαM (40 μg/ml) together with FITC-IgG DαC (20 μg/ml). This protocol results in the myc-tagged receptor being immobilized and the HA-tagged receptor labeled by monovalent Fab′. In experiments with TGF-β1 or BMP-9, the ligand (250 pM TGF-β1 or 5 ng/ml BMP-9) was added after starvation along with the normal goat γ-globulin and maintained at this concentration during the following labeling steps and FRAP measurements.

FRAP and patch/FRAP

Cells coexpressing epitope-tagged receptors labeled fluorescently by anti-tag Fab′ fragments as described were subjected to FRAP and patch/FRAP studies as described previously (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 (Plan Apochromat 63×/1.4 numerical aperture 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 or endoglin (described in the foregoing) preceded the measurement (Henis *et al.*, 1990; Rechtman *et al.*, 2009). This enabled us to determine 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).

Smad phosphorylation assay and Western blotting

MEEC$^{+/+}$ (WT) or MEEC$^{-/-}$ (endoglin-null) cells were serum starved for 6 h, followed by a 30-min incubation with increasing doses of TGF-β1 as indicated in the legend to Figure 9. The cells were then lysed and subjected to electrophoresis on 10% SDS–PAGE, followed by immunoblotting as described (Ray *et al.*, 2010). The blots were probed by rabbit anti-pSmad1/5/8 (1:500), rabbit anti-total Smad1 (tSmad; 1:1000), rabbit anti-pSmad2 (1:1000), rabbit anti-tSmad2 (1:1000), or mouse anti-β-actin (1:10000), followed by peroxidase DαR or SαM IgG (1:5000). The bands were visualized by ECL (Amersham) and quantified by densitometry (ImageJ; National Institutes of Health, Bethesda, MD).

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