ALK1 regulates the internalization of endoglin and the type III TGF-β receptor

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ABSTRACT Complex formation and endocytosis of transforming growth factor-β (TGF-β) receptors play important roles in signaling. However, their interdependence remained unexplored. Here, we demonstrate that ALK1, a TGF-β type I receptor prevalent in endothelial cells, forms stable complexes at the cell surface with endoglin and with type III TGF-β receptors (TβRIII). We show that ALK1 undergoes clathrin-mediated endocytosis (CME) faster than ALK5, type II TGF-β receptor (TβRII), endoglin, or TβRII. These complexes regulate the endocytosis of the TGF-β receptors, with a major effect mediated by ALK1. Thus, ALK1 enhances the endocytosis of TβRIII and endoglin, while ALK5 and TβRII mildly enhance endoglin, but not TβRIII, internalization. Conversely, the slowly endocytosed endoglin has no effect on the endocytosis of either ALK1, ALK5, or TβRII, while TβRII has a differential effect, slowing the internalization of ALK5 and TβRII, but not ALK1. Such effects may be relevant to signaling, as BMP9-mediated Smad1/5/8 phosphorylation is inhibited by CME blockade in endothelial cells. We propose a model that links TGF-β receptor oligomerization and endocytosis, based on which endocytosis signals are exposedFUNCTIONAL IN SPECIFIC RECEPTOR COMPLEXES. This has broad implications for signaling, implying that complex formation among various receptors regulates their surface levels and signaling intensities.

INTRODUCTION The transforming growth factor-β (TGF-β) superfamily ligands regulate diverse physiologic and pathologic cellular processes, which in endothelial cells (ECs) include migration and angiogenesis, and were implicated in the development of diseases such as hereditary hemorrhagic telangiectasia (HHT) (McAllister et al., 1994; Johnson et al., 1996; Bourdeau et al., 1999; Lebrin et al., 2005; Goumans et al., 2009; McDonald et al., 2015; Roman and Hinck, 2017). They exert their effects through ligand-receptor interactions, mediated via type I and type II dual-specificity (Ser/Thr and Tyr) kinase receptors (Shi and Massague, 2003; Derynck and Miyazono, 2008; Ehrlich et al., 2012; Heldin and Moustakas, 2016). In ECs, typical type I receptors are ALK5 (which acts with the TGF-β–responsive type II TGF-β receptor, TβRII) and ALK1 (which can interact with several type II receptors, including TβRII, and BMP9/10-responsive type II receptors for activin or bone morphogenetic proteins (BMPs)) (Oh et al., 2000; Seki et al., 2003; Lebrin et al., 2004, 2005;
Roman and Hinck, 2017). In general, ligand binding to type II and type I receptors triggers phosphorylation and activation of the type I receptor, which phosphorylates specific R-Smads, followed by their association with Smad4 and accumulation in the nucleus, where they regulate transcription (Shi and Massague, 2003; Feng and Derynick, 2005; Schmierer and Hill, 2007; Heldin et al., 2009; Budi et al., 2017). TGF-β stimuli in ECs can activate Smad2/3 and Smad1/5/8 via ALK5 and ALK1, respectively (Chen and Massague, 1999; Goumans et al., 2003; Shi and Massague, 2003; Lebrin et al., 2005; Moustakas and Heldin, 2009), and ALK1 activation by BMP9 or BMP10 signal via Smad1/5/8 (Lebrin et al., 2004). ALK1 activity was shown to be essential for vascular development, as ALK1 knockout mice exhibit embryonic lethality at day 10.5 due to defective angiogenesis (Oh et al., 2000; Srinivasan et al., 2003), and ALK1 is often mutated in HHT (Johnson et al., 1996; Lebrin et al., 2005). ALK1 heterozygous mice phenocopy this multisystemic vascular dysplasia syndrome (Oh et al., 2000; Urness et al., 2000). In spite of the biological significance of ALK1 signaling, and the controversy on the potential roles for TGF-β superfamily receptor trafficking in regulating signal output (Ehrlich et al., 2001; Penheiter et al., 2002; Di Guglielmo et al., 2003; Mitchell et al., 2004; Shapira et al., 2012; Amsalem et al., 2016; Ehrlich, 2016), the internalization of ALK1 and its potential modulation by interactions with other TGF-β receptors remain to be explored.

TGF-β superfamily signaling can be modulated by several coreceptors. The most abundant are the type III TGF-β receptor (TβRIII) and endoglin (Jonker and Arthur, 2002; Goumans et al., 2003; Lebrin et al., 2005; Bernabeu et al., 2009; Mahmoud et al., 2009; Gatza et al., 2010). While endoglin is known to be coexpressed in ECs along with ALK1, this was unclear for TβRIII, which was reported to be found in cardiac ECs and in some microvascular but not macrovascular bovine ECs (Morello et al., 1995; Brown et al., 1999), and which we now find to be expressed in multiple EC cell types (Figure 1). TβRIII is a proteoglycan that binds several ligands, including TGF-βs, inhibin, and some BMPs, and presents them to the signaling receptors (Lopez Casillas et al., 1994; Kirkbride et al., 2008). It

![FIGURE 1: Expression of TβRIII, endoglin, and ALK1 in human and murine cell lines. (A, B) TβRIII is expressed in human and murine ECs. The various cell lines were grown in six-well plates and subjected to experiments to determine TβRIII mRNA (A) or protein (B) expression. Authentication of all immortalized human cell lines was carried out by short tandem repeat analysis at the Duke University DNA Analysis Sequencing Facility. (A) RT-PCR of TβRIII in human and murine cells. RNA isolation was followed by conversion to cDNA and RT-PCR (see Materials and Methods). GAPDH cDNA levels are shown as control. A representative experiment (n = 3) is shown. TβRIII mRNA expression is demonstrated for human ECs of arterial (HAEC; cat. #CC-2535; Lonza, Basel, Switzerland), venous (HUVEC and ECRF , gifts from C. Kontos, Duke University, Durham, NC, and R. Fontijn, Academic Medical Centre, Amsterdam, the Netherlands, respectively), and microvascular (HMEC-1 [cat. #CRL-3243; ATCC] and HMEC-d [cat. #CC-2543; Lonza] origin, as well as for murine ECs of arterial (MAEC; a gift from C. Kontos), microvascular (MsMVEC-d, obtained from D. Kirsch, Duke University, Durham, NC), and embryonal (MEEC) origin. Non-ECs (NMuMG [cat. #CRL-1636 ATCC] and NIH3T3 [cat. #CRL-1658; ATCC]) were included for comparison. (B) Cell surface TβRIII was detected by [125I]TGF-β binding/cross-linking followed by immunoprecipitation as described under Materials and Methods. Non-ECs (HEK293T [cat. #CRL-3216; ATCC], Panc-1 [cat. #CRL-1469; ATCC], and MCF7 [obtained from the Michigan Cancer Foundation]) were included for comparison. Data are representative of three experiments. (C–E) RT-qPCR quantification of TβRIII (C), ALK1 (D), and endoglin (E) in human ECs (HAEC, HUVEC, ECRF, and HMEC-1). Data were normalized to the cDNA levels of GAPDH, taking the value of the mRNA transcript measured in HAEC cells as 1 (see Materials and Methods). The results are the mean ± SEM of three independent experiments, each conducted in triplicate.](image-url)
can also modulate signaling by forming mutual complexes with the signaling receptors (Henis et al., 1994; Lopez Casillas et al., 1994; Eickelberg et al., 2002; reviewed in Gatza et al., 2010). In this context, we have recently shown (Tazat et al., 2015) that ALK5 and TβRII bind to TβRII simultaneously but not as a complex, competing with ALK5-TβRII signaling complex formation and thus inhibiting TGF-β-mediated Smad signaling in MDA-MB-231 cells. TβRII was shown to have a role in developmental angiogenesis in a zebrafish model (Kamai et al., 2015). In mammals, it is important for vasculogenesis, as TβRII knockout mice are embryonic lethal at day 14.5 due to defective vasculogenesis (Compton et al., 2007). Endoglin, the most abundant TGF-β superfamily coreceptor in ECs, regulates differentiation and angiogenesis (Li et al., 1999; Arthur et al., 2000), and mutations in endoglin cause HHT1 (McAllister et al., 1994; Lebrin et al., 2005). At the cell surface, endoglin can bind TGF-β1/3 and BMP9/10 (and other BMPs to a lesser extent) and can interact with signaling TGF-β superfamily receptors (Bernabeu et al., 2009; Alt et al., 2012). In this context, employing quantitative studies that measure directly the receptors’ interactions at the cell surface, we have demonstrated that endoglin functions as a scaffold for binding TβRII, ALK5, and ALK1, thus regulating the balance between TGF-β signaling to Smad1/5/8 and to Smad2/3 (Pomeraniec et al., 2015).

The cell surface levels of TβRII and endoglin were reported to be affected by diverse cellular processes and experimental manipulations. Thus, alteration of the β-arrestin2 expression level was shown to affect the cell surface localization of both TβRII (Chen et al., 2003; Finger et al., 2008; McLean and Di Guglielmo, 2010) and endoglin (Lee and Blobe, 2007), suggesting regulation of their intracellular distribution by endocytosis. The endocytic pathways involved remained controversial (Finger et al., 2008; McLean and Di Guglielmo, 2010), largely due to measurement of receptor down-regulation rather than internalization per se. Importantly, the kinetics of the endocytosis of these receptors and their modulation by interactions with the signaling TGF-β receptors remained unexplored. In the current paper, we employed biophysical studies on epitope-tagged TGF-β receptors to demonstrate that ALK1 forms stable complexes at the cell surface with TβRII, as we reported formerly for ALK1-endoglin interactions (Pomeraniec et al., 2015). Using quantitative point-confocal microscopy for direct endocytosis measurements, we found that ALK1 undergoes fast clathrin-mediated endocytosis (CME), significantly faster than that of ALK5 or TβRII, and much faster than TβRII and endoglin. Of note, we show that the interactions between ALK1 and TβRII or endoglin enhance the endocytosis rates of the latter two receptors, while TβRII (but not endoglin) inhibits the internalization of ALK5 and TβRII. These phenomena correlate with the blockade of BMP9-mediated signaling to Smad1/5/8 by inhibition of CME but not by nystatin in murine embryonic endothelial cells (MEECs). We propose a model that links TGF-β receptors complex formation with their endocytosis, based on which endocytosis signals are exposed (available for binding to the endocytosis machinery) in the specific receptor complex, with potential implications for signaling regulation.

**RESULTS**

**ALK1 forms stable heteromeric complexes with TβRII**

ALK1 and endoglin are characteristically expressed in ECs (Jonker and Arthur, 2002; Seki et al., 2003; Lebrin et al., 2004, 2005; Bernabeu et al., 2009; Mahmoud et al., 2009; Roman and Hinck, 2017). They were shown to interact with each other (Bernabeu et al., 2009; Alt et al., 2012), and ALK1 at the surface of live cells was shown to form stable complexes with itself and with endoglin (Pomeraniec et al., 2015). TβRII was also reported to be expressed in some ECs (Morello et al., 1995; Brown et al., 1999). As shown in Figure 1, A and B, we find TβRII expression in multiple types of human and murine ECs, as well as in epithelial cell lines. Comparison between the mRNA expression levels of TβRII, endoglin, and ALK1 in several human EC lines by real-time quantitative reverse-transcriptase PCR (RT-qPCR) showed that they express the mRNA transcripts for all three proteins, but at different levels (Figure 1, C–E). In view of the interactions between ALK1 and endoglin, it was important to explore whether ALK1 and TβRII interact. To this end, we initially co-expressed extracellularly tagged HA-ALK1 (wild type [WT], constitutively active Q201D, or kinase-dead K221R) together with myc-TβRII. Immunoprecipitation of myc-TβRII resulted in coprecipitation of all the HA-ALK1 variants (Figure 2, A and B), suggesting that the two receptors interact irrespective of ALK1 kinase activity. Of note, the high-molecular-weight smear of the heavily glycosylated TβRII is more difficult to detect at low expression levels, and the failure to detect it in the immunoprecipitated TβRII bands may suggest that the two receptors interact already at the endoplasmic reticulum (ER), yielding coimmunoprecipitation, which includes a significant contribution from the ER population. This interpretation is in line with the enrichment in the lower band of HA-ALK1 coprecipitated with TβRII, which most likely represents its ER form. Nevertheless, the two receptors interact also at the cell surface, as indicated by the experiments shown in Figure 2, C–F.

To measure the interactions between TβRII and ALK1 at the plasma membrane in live cells, we employed the patch/FRAP (fluorescence recovery after photobleaching) method, which measures interactions between receptors situated at the cell surface (Henis et al., 1990; Rechtman et al., 2009; Pomeraniec et al., 2015; Tazat et al., 2015). In this method, cross-linking of a tagged receptor by a double layer of immunoglobulins G (IgGs) results in its patching and lateral immobilization. The effects of this immobilization on the lateral diffusion of a coexpressed, differently tagged receptor labeled exclusively by monovalent Fab’ fragments are then measured by FRAP (see Materials and Methods). The nature of the effect depends and reports on the extent and mode of mutual complex formation between the receptors. Complex lifetimes longer than the characteristic FRAP times (interactions that are stable at this time range) are reflected by a reduction in the mobile fraction (fR), because bleached Fab’-labeled receptors associated with cross-linked, immobilized receptors do not appreciably dissociate from the immobile patches during the FRAP measurement. On the other hand, transient complexes (short complex lifetimes) would lead to multiple association–dissociation cycles during the FRAP measurement, resulting in a reduced apparent diffusion rate (D), without affecting fR (Henis et al., 1990; Eisenberg et al., 2006; Rechtman et al., 2009).

In the studies depicted in Figure 2, C–F, myc-TβRII was laterally mobile when labeled by monovalent Fab’ fragments (Figure 2C) and became immobile when cross-linked by IgGs (Figure 2D). Singly expressed HA-ALK1 exhibited lateral mobility similar to other TGF-β superfamily receptors (Yao et al., 2002; Rechtman et al., 2009; Marom et al., 2011; Pomeraniec et al., 2015; Tazat et al., 2015), which is typical of transmembrane proteins (Figure 2, E and F). The lateral diffusion parameters of ALK1 were not affected by TGF-β1 or TGF-β2, while BMP9 induced some reduction in the Rf value, suggesting that BMP9 may induce stable interactions of a fraction of the ALK1 population with other endogenous protein scaffolds/structures. For patch/FRAP studies on TβRII-ALK1 interactions, we co-expressed HA-ALK1 and myc-TβRII and investigated the effects of cross-linking myc-TβRII without and with ligand on HA-ALK1 diffusion. Immobilization of myc-TβRII induced an ~30% reduction in the Rf of Fab’-labeled HA-ALK1, with no change in D (Figure 2, E and F).
FIGURE 2: Coimmunoprecipitation and patch/FRAP experiments demonstrate that ALK1 and TβRIII form mutual heteromeric complexes. (A) Representative immunoblots of coimmunoprecipitation of HA-ALK1 with myc-TβRIII. COS7 cells were cotransfected with vectors encoding myc-TβRIII and a HA-ALK1 variant (WT, constitutively active Q201D, or kinase dead K221R). Cell lysates were assayed for input of myc-TβRIII (probing the Western blot with murine αmyc) and of HA-ALK1 (using rabbit αHA). The lysates were immunoprecipitated with murine αmyc antibody and assayed for pull down of myc-TβRIII (with murine αmyc) and for HA-ALK1 with rabbit αHA antibody (see Materials and Methods). A representative blot (n = 4) is shown. The band marked with n.s. for HA-ALK1 is nonspecific. (B) Quantification of the coimmunoprecipitation of HA-ALK1 variants with myc-TβRIII. The bands were quantified (see Materials and Methods), and the level of coimmunoprecipitation was determined by dividing the intensity of the band of the coprecipitated HA-ALK1 variant by that of the input of the same HA-ALK1 protein. The value obtained for the coprecipitation of HA-ALK1-WT was defined as 1. There were no significant differences between the coimmunoprecipitation level of the various HA-ALK1 variants (P > 0.6; Student’s two-tailed t test, n = 4). (C) A representative FRAP curve of the lateral diffusion of myc-TβRIII labeled exclusively by Fab’ fragments. (D) Representative FRAP curve of myc-TβRIII immobilized by IgG cross-linking. (E, F) Patch/FRAP studies were carried out on COS7 cells cotransfected with vectors encoding...
This effect was not altered by ligand (TGF-β1, TGF-β2, or BMP9). These results indicate that a significant fraction of ALK1 at the cell surface is constitutively and stably associated with TßRII. The identification of TßRIII expression in ECs and the demonstration that it forms stable complexes with ALK1 raises the possibility that TßRIII may regulate signaling via ALK1. Because in ECs ALK1 is known to transduce signals initiated by BMP9, leading to Smad phosphorylation and gene transcription changes, we investigated whether TßRIII regulated the ALK1 response to BMP9 in MEECs. To this end, we employed CRISPR to silence TßRIII in MEECs. TßRIII knockout was validated by [125]TGF-β1 binding and cross-linking (Figure 3A). Loss of TßRIII resulted in a twofold decrease in BMP9-induced pSmad1/5/8 signal (Figure 3, B and C). The attenuation of the response to BMP9 was also apparent in farther downstream signaling, as measured by the reduction in the ability of BMP9 to induce Id1, one of the master regulator genes whose transcription is activated by this pathway (Figure 3D). The reduction in Id1 was modest, in line with the twofold reduction in BMP9-mediated pSmad1/5/8 formation, most likely because the original expression level of TßRIII in the ECs is not very high. Similar to the effect of TßRIII loss on BMP9 signaling to Smad1/5/8 in MEECs, the mild but distinct ability of TGF-β1 to induce pSmad1/5/8 formation was also decreased (Figure 3, E and F). On the other hand, the loss of TßRIII elevated TGF-β1 signaling (mediated via ALK5) to the Smad2/3 pathway (Figure 3, G and H), in line with former reports (Lambert et al., 2011; Tazat et al., 2015). Taken together, these data suggest that TßRIII facilitates ALK1-mediated signaling and downstream functions in ECs, while inhibiting ALKS-mediated Smad3 responses.

The short cytoplasmic tail of TßRIII has been demonstrated to interact with β-arrestin2 and GARP-interacting protein C-terminal (GIPC) (Bloba et al., 2001; Chen et al., 2003). To examine whether the TßRIII cytoplasmic domain or TßRIII glycosylation is required for its interactions with ALK1, we coexpressed HA-TßRIII (WT, the Del mutant lacking the three C-terminal amino acids required to bind GIPC, the Tß814A point mutant defective in binding β-arrestin2, the ΔGAM mutant lacking the two glycosaminoglycan attachment sites, or the ΔCyto mutant lacking most of the cytoplasmic domain) together with myc-ALK1. To determine quantitatively the association between these HA-TßRIII mutants and myc-ALK1 situated at the plasma membrane, we conducted patch/FRAP studies on cells coexpressing myc-ALK1 and each specific HA-TßRIII variant (Figure 4, A and B). Immobilization of any of the HA-TßRIII mutants reduced the $R_t$ of myc-ALK1 similar to immobilization of WT HA-TßRIII (compare Figures 4A and 2E), with no effect on D of ALK1. We conclude that the stable interactions of ALK1 with TßRIII at the cell surface do not depend on motifs in the TßRIII cytoplasmic domain or on its glycosylation sites. These results are in accord with our studies on endoglin-ALK1 interactions (Pomeraniec et al., 2015), which were also independent of motifs located in the short cytoplasmic tail of endoglin.

### ALK1 undergoes fast endocytosis while the internalization of TßRIII and endoglin is slow

Given the detection of interactions of ALK1 with both TßRIII (Figure 2) and endoglin (Pomeraniec et al., 2015) at the cell surface, and the role of endocytosis in regulating surface receptor levels, we next measured the internalization kinetics of ALK1, TßRIII, and endoglin. To this end, we expressed one of these receptors (carrying an extra-cellular epitope tag), fluorescence labeled the cell surface population of this receptor at 4°C, and followed its endocytosis over time at 37°C by the point-confocal endocytosis assay (Ehrlich et al., 2001; see Materials and Methods). The time-dependent internalization of myc-ALK1 was observed as an alteration in its staining pattern from homogeneous to vesicular (Figure 5, A–C). ALK1 endocytosis was quantified by the reduction in the fluorescence intensity at the plasma membrane, measuring the receptor population remaining at the cell surface (Figure 5F). Interestingly, the half-time ($t_h$) of ALK1 internalization (2.5 min) was markedly shorter than the $t_h$ values for ALK5 (∼13 min; Shapira et al., 2012), TßRIII (∼15 min; Ehrlich et al., 2001), or the type II BMPR receptor (15–20 min; Amsalem et al., 2016). This fast endocytosis is mediated mainly via clathrin-coated pits, as indicated by its inhibition with PitStop 2, which is a specific CME inhibitor (von Kleist et al., 2011), but not with nystatin, an inhibitor of cholesterol-dependent endocytic pathways (Schnitzer et al., 1994; Di Guglielmo et al., 2003) (Figure 5G).

In contrast to the fast endocytosis of ALK1, TßRIII (Figure 6) and endoglin (Figure 7) undergo internalization at much slower rates. Measurements of HA-TßRIII endocytosis by the point-confocal method (Figure 6F) yielded a $t_h$ value of 20 min. This value was not affected by ligand (TGF-β1 or BMP9), indicating ligand-independent constitutive endocytosis, in line with earlier reports (Finger et al., 2008; McLean and Di Guglielmo, 2010). TßRIII internalization appears to be mediated mainly via CME, because it was strongly inhibited by PitStop 2 but not by nystatin (Figure 6, D–F). In this context, it should be noted that the TßRIII endocytic pathway is controversial (Finger et al., 2008; McLean and Di Guglielmo, 2010), largely due to measurement of receptor down-regulation rather than internalization per se. The current results using the point-confocal method measure directly and with high sensitivity the internalization of the receptors from the cell surface and suggest a major role of CME in TßRIII endocytosis. In accord with these findings, the cytoplasmic domain of TßRIII is necessary for its endocytosis, as shown by the failure of the HA-TßRIII-ΔCyto mutant to undergo...
FIGURE 3: TβRIII facilitates Smad1/5/8 phosphorylation induced by BMP9 or TGF-β1 and inhibits TGF-β1-mediated pSmad2 formation. (A) CRISPR silencing of TβRIII in MEECs. CRISPR knockout of TβRIII employed stable transduction with lentivirus, using nontargeting guide sequences for control (NTC; see Materials and Methods). Cell surface TβRIII was detected by [125I]TGF-β1 binding/cross-linking followed by immunoprecipitation. A representative experiment (n = 3) is shown. (B–H) Effects of TβRIII knockout in MEECs on signaling to distinct Smad pathways. MEECs transduced with CRISPR-mediated TβRIII knockout (crTβRIII cells) or with NTC lentivirus (control; NTC cells) were compared. After overnight serum starvation, cells were treated with BMP9 or TGF-β1 as indicated, lysed, and analyzed by immunoblotting for total (t) and phospho (p) Smad1/5/8 or Smad2, Id1, and β-actin. The bands were quantified by the Odyssey system (see Materials and Methods). (B) Representative blot showing that TβRIII knockout reduces BMP9-induced pSmad1/5/8 formation. Stimulation was with increasing doses of BMP9 (15 min). (C) Quantification of the reduction in BMP9-induced pSmad1/5/8 formation following TβRIII knockout. Stimulation was with 1 ng/ml BMP9.
ALK1 interacts with TβRIII and endoglin

The signaling TGF-β receptors, and especially ALK1, enhance endoglin endocytosis

The interactions between endoglin and the signaling TGF-β receptors (TβRII, ALK5, and ALK1) result in the formation of mutual complexes at the cell surface (Pomeraniec et al., 2015), raising the intriguing possibility that they may regulate the endocytosis of the receptors and thus affect their cell surface levels and signaling. Because the endocytosis rate of endoglin is much slower than those of ALK1, ALK5, or TβRII, complex formation between endoglin and these receptors could either increase the rate of endoglin internalization or inhibit the endocytosis rates of the signaling receptors residing in the mutual complexes. To explore these possibilities, we coexpressed HA-endoglin with myc-tagged ALK1, ALK5, or TβRII, labeled the cell surface receptors by fluorescent antibody fragments, and measured HA-endoglin endocytosis in cells coexpressing one of the myc-tagged receptors (Figure 8). Coexpression with any of the signaling TGF-β receptors enhanced the internalization rate of endoglin, with no additional effect by ligand (TGF-β1 or BMP9; Figure 8). The degree of enhancement was correlated with the internalization rate of the coexpressed receptor, with ALK1 (characterized by the fastest endocytosis rate) leading to a twofold faster internalization of HA-endoglin, while ALK5 and TβRII induced a weaker but significant enhancement. The dependence of the effect on the endocytosis rate of the receptor coexpressed with HA-endoglin was validated by coexpression with endocytosis-defective myc-TβRIII-HA (Ehrlich et al., 2001), which had the opposite effect and slowed the internalization of endoglin to a half-time of ~70 min (Figure 8). These findings suggest that in the endoglin-TGF-β receptor complexes, the internalization signals of the latter are dominant. In line with this notion, the reciprocal experiment testing the effects of HA-endoglin on the internalization rate of myc-tagged ALK1, ALK5, or TβRII showed no significant effect of endoglin on the endocytosis of any of the latter receptors, in either the absence or presence of ligands (TGF-β1 or BMP9; Figure 9).

(15 min). Results are shown as the pSmad1/5/8 ratio to β-actin, defining the value obtained for BMP9-treated NTC cells as 1. Each bar is the mean ± SEM value of four independent experiments (**, P < 0.003; Student’s two-tailed t test). (D) Loss of TβRIII reduces Id1 induction in response to BMP9. A representative blot (n = 3) is shown. Cells (NTC control or TβRIII-silenced) were serum starved overnight and treated with 1 ng/ml BMP9 for 4 h, and the level of Id1 induction was measured by Western blotting. Each Id1 band was calibrated relative to β-actin. The value obtained for BMP9-stimulated NTC cells was defined as 1. (E) Representative blot depicting an increase in TGF-β1–stimulated pSmad1/5/8 formation upon loss of TβRIII. Stimulation (30 min) was with 100 pM TGF-β1. (F) Quantification of TGF-β1–induced pSmad1/5/8 formation. The cells were stimulated with 100 pM TGF-β1 for 15 or 30 min. Results depict the mean ± SEM of three independent experiments, with the ratio of pSmad1/5/8 to β-actin in NTC cells taken as 1 (*, P < 0.05; Student’s t test). (G) Representative blot depicting an increase in TGF-β1–stimulated pSmad2 formation upon loss of TβRIII. Cells were stimulated by 100 pM TGF-β1 for 15 or 30 min. (H) Quantification of TGF-β1–mediated pSmad2 formation. The ratio of pSmad2 to β-actin in TGF-β1–stimulated control TβRIII cells was taken as 1. Bars, mean ± SEM of three or four independent experiments (*, P < 0.05; Student’s t test).
ALK1 but not ALK5 or TβRII enhances the endocytosis of TβRIII

Because ALK1, ALK5, and TβRII form complexes with TβRIII (Tazat et al., 2015, and Figures 2 and 4), we next explored the effects of these receptors (myc-tagged) on HA-TβRIII endocytosis. As shown in Figure 10, the pattern of these effects was different from that exerted on the endocytosis of endoglin. While coexpression with ALK1 significantly increased the internalization rate of TβRIII, ALK5 and TβRII had no significant effects on TβRIII endocytosis, in either the absence or presence of ligands (Figure 10). These results imply that in the mutual complexes with TβRIII, the endocytosis signal of ALK1 prevails, but this is not the case for the complexes of TβRIII with ALK5 or TβRII. The results of mirror experiments testing the effects of HA-TβRIII coexpression on the internalization rates of myc-tagged ALK1, ALK5, or TβRII are in complete agreement with this suggestion (Figure 11). Thus, if the ALK1 endocytosis signal in the TβRII-ALK1 complex is dominant, it is expected that endoglin would not affect ALK1 endocytosis, as is indeed the case (with or without ligand). On the other hand, the endocytosis rates of ALK5 and TβRII, which do not enhance TβRII internalization, are inhibited by coexpressed TβRIII (Figure 11), suggesting that the endocytosis signal of the latter receptor (which undergoes slow endocytosis) is dominant in the complexes with ALK5 or TβRII.

Given the differential regulation of endocytosis of the various receptors involved in BMP9 signaling in ECs, we tested whether disruption of either CME or caveolar endocytosis in MEECs (which express all the receptors studied here) affects Smad1/5/8 activation by BMP9. As shown in Figure 12, the CME inhibitor PitStop 2 significantly inhibited pSmad1/5/8 formation, while the caveolar inhibitor nystatin had no effect. These findings suggest that CME is required for BMP9 signaling to Smad1/5/8 in these cells and are in line with CME being the major pathway for endocytosis of ALK1 (the signal transducing receptor in this setting) and of most of the other TGF-β family receptors studied here.

DISCUSSION

Interactions between multiple TGF-β signaling receptors and coreceptors are at the core of TGF-β superfamily signaling (Seki et al., 2003; Shi and Massague, 2003; Lebrin et al., 2004; Derynck and Miyazono, 2008; Ehrlich et al., 2012; Heldin and Moustakas, 2016). ECs coexpress distinct TGF-β receptors (ALK1, ALK5, TβRII), as well as the coreceptors endoglin (Jonker and Arthur, 2002; Lebrin et al., 2005) and TβRIII (Morello et al., 1995; Brown et al., 1999; see Figure 1). The signaling output of TGF-β receptors is determined by the composition and cellular localization of heteromeric receptor complexes (Ehrlich et al., 2012; Ehrlich, 2016). Typically, the cellular localization of transmembrane receptors is regulated by molecular determinants (e.g., endocytic motifs), which mediate their inclusion

incubation at 37°C or 4°C (time zero) in media containing inhibitors where indicated. Endocytosis of myc-ALK1 was quantified by the point-confocal method. Results are mean ± SEM; the number of measurements at 10 min is depicted on each bar. For each treatment, the fluorescence intensity of the same sample at time zero (n = 167, 159, and 134 for untreated, PitStop 2-treated, or nystatin-treated cells, respectively) was taken as 100%. The percentage of the fluorescence intensity remaining at the cell surface after 10 min at 37°C was subtracted to obtain the % internalization. Treatment with PitStop 2, which inhibits CME, significantly reduced the ALK1 endocytosis (***, P < 10⁻¹⁰; Student’s two-tailed t test), while nystatin had no significant effect.
ALK1 interacts with TβRIII and endoglin

FIGURE 6: TβRIII undergoes slow endocytosis via clathrin-coated pits, which depends on its short cytoplasmic tail. COS7 cells were transfected with HA-TβRIII, HA-TβRIII-ΔCyto, HA-TβRIII-Del, or HA-TβRIII-T841A. After 24 h, they were either left untreated or subjected to an internalization-inhibiting treatment (PitStop 2 or nystatin). In experiments with ligands, TGF-β1 (250 pM) or BMP9 (5 ng/ml) was added after starvation along with the NGG at the start of the fluorescence labeling procedure (see Materials and Methods) and maintained during the following labeling and endocytosis steps. The surface receptors on live cells were then labeled at 4°C (time zero) with monoclonal mouse αHA followed by Alexa 546-GocM Fab’, incubated for defined intervals at 37°C, returned to 4°C, and fixed (Materials and Methods). (A–E) Typical images of HA-TβRIII internalization. Bar, 20 μm. The incubation time at 37°C is designated for each panel. Panels D and E depict cells treated to inhibit CME (PitStop 2) or caveolar endocytosis (nystatin), respectively. (F) Quantitative measurements of HA-TβRIII endocytosis. The fluorescence intensity remaining at the cell surface was measured by the point-confocal method (Materials and Methods) as described for FIGURE 5. Results are mean ± SEM; the number of measurements (each conducted on a different cell) is depicted in a table within the panel. For each sample, the intensity at time zero was taken as 100%. Because incubation with either TGF-β1 or BMP9 had no effect on HA-TβRIII internalization, only the results of one ligand (BMP9) are shown. Asterisks indicate significant differences at a given time point between drug-treated and untreated cells (***, \( P < 10^{-12} \); Student’s two-tailed \( t \) test). (G) The short cytoplasmic tail of TβRIII is required for its endocytosis. Experiments were conducted as in panel F, comparing the internalization rates of WT HA-TβRIII (shown in this panel as well for reference) with those of the designated mutants. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Asterisks indicate significant differences at a given time point between a mutant and the WT HA-TβRIII (***, \( P < 10^{-12} \); Student’s \( t \) test). The HA-TβRIII-ΔCyto mutant, which misses most of the cytoplasmic domain, exhibited defective endocytosis, while deletion of only the last three amino acids (HA-TβRIII-Del) or point mutation to interfere with binding to β-arrestin2 (HA-TβRIII-T841A) had no significant effects.
The coexpression and mutual complex formation among the multiple TGF-β receptors and coreceptors motivated us to explore whether these interactions can modulate their endocytosis. To this end, we studied the endocytosis of the different receptors under similar conditions and in the same cells, using the same method (point-confocal internalization measurements) employed earlier to measure ALK5 and TβRII endocytosis (Ehrlich et al., 2001; Shapira et al., 2012). We find that ALK1 undergoes constitutive internalization mainly via CME, in line with our earlier studies on the endocytosis of TβRII, ALK5, and the long form of BMP-RII (Ehrlich et al., 2001; Shapira et al., 2012; Amsalem et al., 2016). In this context, it should be noted that while the endocytosis pathways of the type I and II TGF-β receptors have been controversial due to contrasting data and methods, the current near-consensus is that CME is their main internalization pathway (Ehrlich et al., 2001; Hayes et al., 2002; Penheiter et al., 2002; Yao et al., 2002; Di Guglielmo et al., 2003; Mitchell et al., 2004; Chen, 2009), possibly complemented by a contribution from caveolar-like endocytosis (Ehrlich et al., 2001; Hayes et al., 2002; Penheiter et al., 2002; Yao et al., 2002; Di Guglielmo et al., 2003; Mitchell et al., 2004; Chen, 2009). However, ALK1 endocytosis is significantly faster (approximately fivefold) than that of ALK5 or TβRII (Figure 5) and is therefore potentially important for modulating the endocytosis of interacting receptors and for signaling. Indeed, blockade of CME by PitStop 2 inhibited pSmad1/5/8 formation following BMP9 stimulation in MEECs (Figure 12), in line with a role for endocytosis in the modulation of this signaling pathway. On the other hand, under the same conditions, the coreceptors TβRIII and endoglin undergo significantly slower endocytosis. For TβRIII, the internalization was found to proceed mainly via CME (Figure 6F), in line with some studies but not with others (Finger et al., 2008; McLean and Di Guglielmo, 2010). The current studies show no dependence of TβRIII endocytosis on its TβRIII knockout MEECs (Figure 7), which binds β-arrestin2. This differs from our prior report, which measured internalized iodinated TGF-β1 after 2 h (Chen et al., 2003). This apparent discrepancy may stem from methodological differences, as the current studies focus on direct measurement of the internalization of the T841 mutant. Moreover, we now find that the slow internalization of endoglin is insensitive to mutations that interfere with its ability to bind β-arrestin2 or GIPC (Figure 7G). This finding is seemingly at odds with our earlier reports on the effects of these mutations on the endocytosis of endoglin proceeds via both clathrin-coated pits and a nystatin-sensitive pathway. Experiments were conducted exactly as in Figure 6, except that the receptors whose endocytosis was followed were HA-endoglin (WT) or its HA-tagged mutants HA-endoglin-Del and HA-endoglin-T650A. (A–E) Typical images of HA-endoglin endocytosis. Bar, 20 μm. The incubation time at 37°C is designated for each panel. Panels D and E depict cells treated to inhibit CME (PitStop 2) or caveolar endocytosis (nystatin), respectively. (F) Quantitative measurements of HA-endoglin endocytosis, measured by the point-confocal method as described for Figures 5 and 6. Results are mean ± SEM; the number of measurements (each conducted on a different cell) is depicted in a table within the panel. No significant differences were observed in the internalization of the endoglin mutants as compared with WT endoglin. 4). Similar features (stable complex formation with no dependence on ligand, cytoplasmic domain of the coreceptor or mutations in this domain) were reported for the interactions of endoglin with ALK1 and for the interactions of both endoglin and TβRII with TβRII or ALK5 (Pomeraniec et al., 2015; Tazat et al., 2015). Of note, the TβRII-ALK1 interactions reported here modulate ALK1 signaling, as demonstrated by the reduced BMP9 or TGF-β1 signaling to Smad1/5/8 upon silencing of TβRII (Figure 3, B–E). These effects are distinct from those exerted via ALK5, as shown by the increased pSmad2 formation in response to TGF-β1 in TβRII knockout MEECs (Figure 3, G and H).
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% Fluorescence remaining at the cell surface

Time at 37°C, h

0 1 2

E

HA-endoglin

HA-endoglin+myc-TßRII

HA-endoglin+myc-ALK5

HA-endoglin+myc-ALK1

HA-endoglin+myc-ALK1+TßGF1

HA-endoglin+myc-TßRII-3A

n= 155 155 155 111 102

n= 155 155 111 100

n= 156 201 191 150

n= 145 70 202 138 37

n= 105 72 90 89 2

n= 131 148 91 64

% Fluorescence remaining at the cell surface

Time at 37°C, min

0 5 10

G

myc-ALK5

myc-ALK5+HA-endoglin

myc-TßRII

myc-TßRII+HA-endoglin

myc-ALK1

myc-ALK1+HA-endoglin

myc-ALK1+HA-endoglin+BMP9

n= 98 93 100 103 90

n= 100 100 108 91

n= 114 114 79 107

n= 387 122 209 357 136

n= 156 94 158 158 99

n= 87 87 88 88 87

FIGURE 8: Endoglin endocytosis is enhanced strongly by ALK1 and mildly by ALK5 or TßRII. COS7 cells were transfected with HA-endoglin alone or together with another myc-tagged receptor (myc-ALK1, myc-ALK5, myc-TßRII, or its endocytosis-defective myc-TßRII-3A mutant). The HA-tagged receptors were labeled with rabbit monoclonal HA.11 αHA (20 μg/ml, 45 min, 4°C) followed by Alexa 546-Green Fab′ (40 μg/ml, 45 min, 4°C); coexpression of the myc-tagged receptors was validated by labeling in parallel with murine αmyc followed by Alexa 488-GoM Fab′. Endocytosis studies were conducted as in Figure 6. (A–D) Typical images of HA-endoglin internalization. Bar, 20 μm. The incubation time at 37°C (0 or 30 min) is indicated to the left of the panels. (A, B) Cells expressing HA-endoglin alone; (C, D) HA-endoglin coexpressed with myc-ALK1 or myc-ALK5, respectively. (E) Quantitative measurements of HA-endoglin endocytosis, measured by the point-confocal method (see Figure 6). Coexpression with ALK1 enhanced markedly the internalization rate of HA-endoglin, while ALK5 or TßRII induced a mild effect in the same direction, suggesting that the stronger endocytosis signal of the receptors complexed with endoglin is exposed in the complex and prevails. This notion is supported by the reverse effect of the endocytosis-defective TßRII-3A mutant. The effect of ligands (250 pM TGF-ß1 or 5 ng/ml BMP9) was insignificant for HA-endoglin endocytosis in combination with any of the receptors; thus, for simplicity, only the effect of TGF-ß1 on the internalization of HA-endoglin in the presence of myc-ALK1 is shown. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Asterisks indicate significant differences between HA-endoglin endocytosis in the presence of the depicted coexpressed receptor and the value measured for singly expressed HA-endoglin at the same time point (**, P< 0.01; ***, P< 10.12; Student’s t test).

complex formation between receptors that undergo fast versus slow endocytosis can modulate their internalization. Depending on the special conformation acquired by the cytoplasmic domains in the complex, the cytoplasmic endocytosis signals of one receptor or both may be sequestered from interacting with the endocytic machinery. If the endocytosis signals of both receptors remain functional in the complex, the fast internalization rate of the fast-endocytosed receptor is expected to prevail. A similar scenario of functional prevalence of the strong endocytosis signal is expected when this signal is exposed while the weaker counterpart (of the slow-endocytosed receptor) is sequestered (e.g., as in

FIGURE 9: Endoglin expression does not affect the endocytosis of the signaling TGF-ß receptors. COS7 cells were transfected with myc-tagged ALK1, ALK5, or TßRII alone or together with HA-endoglin. The myc-tagged receptors were labeled with murine monoclonal αmyc (20 μg/ml, 45 min, 4°C) followed by Alexa 546-Green Fab′ (40 μg/ml, 45 min, 4°C); coexpression of HA-endoglin was validated by labeling in parallel with HA.11 rabbit αHA followed by Alexa 488-GoM Fab′. The effect of HA-endoglin coexpression on the internalization of the myc-tagged receptors was measured by the point-confocal method, as described in Figure 6. (A–F) Representative images of myc-ALK1 (top row) or myc-ALK5 (bottom row) internalization; HA-endoglin was coexpressed in panels C and F. Bar, 20 μm. The incubation time at 37°C (time 0 or 10 min) is indicated above the top panels. (G) Quantitative point-confocal measurements of myc-ALK1, myc-ALK5, or myc-TßRII endocytosis. No significant effect of HA-endoglin on the endocytosis of any of the other receptors was observed. Addition of ligand (250 pM TGF-ß1 or 5 ng/ml BMP9) had no effect on the endocytosis of either myc-ALK5, myc-TßRII, or myc-ALK1 coexpressed with HA-endoglin; for simplicity, only the effect of one ligand (BMP9) on the internalization of myc-ALK1 is shown. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Endoglin had no significant effects on the internalization of ALK1, TßRII, or ALK5.

cell surface levels of the endoglin mutants (Lee and Blob, 2007; Lee et al., 2008). However, the earlier reports measured the cell surface steady state levels of endoglin, while the current studies specifically assessed endoglin internalization.
the complexes of ALK1-endoglin or ALK1-TβRII; Figure 13). Conversely, in cases in which the strong signal is sequestered and the weak signal is the sole determinant of the internalization rate, slower internalization of the receptor complex is expected. In accord with this model, the internalization rates of TβRII or endoglin complexed with ALK1 were significantly enhanced (Figures 8 and 10), while the endocytosis rate of ALK1 was unaffected by coexpression of TβRII or ALK5, but not that of ALK1. COS7 cells were transfected with myc-tagged TβRII, ALK5, or ALK1 alone or together with HA-TβRIII. The cell surface receptors were labeled exactly as in Figure 9, and the internalization of the myc-tagged receptors was measured by the point-confocal method as in Figure 9. Addition of ligand (250 pM TGF-β1 or 5 ng/ml BMP9) had no effect on the endocytosis of any of the myc-tagged receptors coexpressed with HA-TβRIII; for simplicity, only the results of myc-ALK1 internalization in the presence of HA-TβRIII with one ligand (BMP9) are shown. (A–F) Typical images of myc-ALK1 (top row) and myc-ALK5 (bottom row) internalization; HA-TβRIII was coexpressed in panels C and F. Bar, 20 μm. The incubation time at 37°C (time 0 or 10 min) is indicated above the top panels. (G) Quantitative point-confocal endocytosis measurements of myc-ALK1, myc-ALK5, or myc-TβRII endocytosis. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Asterisks indicate significant differences between the internalization of a given myc-tagged receptor without (black symbols) and with HA-TβRIII. The cell surface receptors were labeled exactly as in Figure 9, and the internalization of the myc-tagged ALK1, ALK5, or TβRII together with myc-ALK1 was measured in the presence of BMP9. Addition of ligand (250 pM TGF-β1 or 5 ng/ml BMP9) had no effect on the endocytosis of any of the myc-tagged receptors coexpressed with HA-TβRIII; for simplicity, only the results of myc-ALK1 internalization in the presence of HA-TβRIII with one ligand (BMP9) are shown. (A–F) Typical images of myc-ALK1 (top row) and myc-ALK5 (bottom row) internalization; HA-TβRIII was coexpressed in panels C and F. Bar, 20 μm. The incubation time at 37°C (time 0 or 10 min) is indicated above the top panels. (G) Quantitative point-confocal measurements of myc-ALK1, myc-ALK5, or myc-TβRII endocytosis. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Asterisks indicate significant differences between the internalization of a given myc-tagged receptor without (black symbols) and with HA-TβRIII. The cell surface receptors were labeled exactly as in Figure 9, and the internalization of the myc-tagged receptors was measured by the point-confocal method as in Figure 9. Addition of ligand (250 pM TGF-β1 or 5 ng/ml BMP9) had no effect on the endocytosis of any of the myc-tagged receptors coexpressed with HA-TβRIII; for simplicity, only the results of myc-ALK1 internalization in the presence of HA-TβRIII with one ligand (BMP9) are shown. (A–F) Typical images of myc-ALK1 (top row) and myc-ALK5 (bottom row) internalization; HA-TβRIII was coexpressed in panels C and F. Bar, 20 μm. The incubation time at 37°C (time 0 or 10 min) is indicated above the top panels. (G) Quantitative point-confocal measurements of myc-ALK1, myc-ALK5, or myc-TβRII endocytosis. Results are mean ± SEM; the number of measurements is depicted in a table within the panel. Asterisks indicate significant differences between the internalization of a given myc-tagged receptor without (black symbols) and with (green symbols) coexpressed HA-TβRIII at the same time point (*, P < 0.01; **, P < 10⁻²;***, P < 10⁻¹²; Student’s t test).
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Reagents

Recombinant human TGF-β1 (cat. #100-21C) was from PeproTech (Rocky Hill, NJ). Recombinant human BMP9 (cat. #3209-BP-010) and goat IgG against the extracellular domain of TβRIII (cat. #AF-242-PB) were from R&D Systems (Minneapolis, MN). Protein G-Sepharose (cat. #P3296) and mouse anti-β-actin (cat. #A2066) were from Sigma-Aldrich (St. Louis, MO), and disuccinimidyl suberate (DSS; cat. #21655) was from Thermo Scientific Pierce (Grand Island, NY). Fatty acid free bovine serum albumin (BSA) (fraction V; cat. #10-775-835-001) and 12CA5 murine monoclonal anti-influenza hemagglutinin tag (αHA) IgG (cat. #11-66-606-001) were obtained from Roche Diagnostics (Manheim, Germany). Murine monoclonal anti-β-catenin (αCTn; cat. #626802) 9E10 IgG (Evan et al., 1985) and HA-11 rabbit polyclonal IgG to the HA tag (rabbit αHA; cat. #923502) were from Biologend (San Diego, CA). Monovalent Fab’ fragments were prepared from the 9E10 and 12CA5 IgG as described (Henis et al., 1994). Chicken anti-β-catenin tag affinity-purified IgG (cat. #AB3252) was from Merck Millipore (Burlington, MA). Alexa Fluor (Alexa) 488-GST IgG (cat. #R37116), Alexa 488-GST Fab’ (cat. #A-11070), and Alexa 546-goat anti-mouse (GxM) F(ab’)2 (cat. #A-11018) were from Invitrogen–Molecular Probes (Eugene, OR). Normal goat γ-globulin (cat. #005-000-002), Cy3 conjugated AffiniPure GxM F(ab’)2 (cat. #115-166-146), and Alexa 488-conjugated AffiniPure donkey IgG against chicken IgY (DxC; cat. #703-545-155) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescent F(ab’)2 was converted to Fab’ as described (Gilboa et al., 1998). Rabbit antibodies to phospho (p) Smad1/5/8 (cat. #9511), total (t) Smad1 (cat. #9743), and phospho (p) Smad2 (cat. #19338), as well as murine antibody to total (t) Smad 2 (cat. #3103) and infrared-tagged secondary donkey IgG against rabbit (DxR 800; cat. #5151) or mouse (DxM 680; cat. #5470) were from Cell Signaling Technology (Danvers, MA). Rabbit antibodies to Id1 (C-20) (cat. #sc-488) were from Santa Cruz Biotechnology (Santa Cruz, CA). [125I]TGF-β1 (cat. #NEX267010UC) was from PerkinElmer (Waltham, MA). PitStop 2 (cat. # ab120687) was obtained from Abcam (Cambridge, United Kingdom), and nystatin suspension (cat. # 03-030) was from Biological Industries Israel.

Plasmids

Expression vectors encoding WT human TβRII (in pcDNA3) or TβRII (in pcDNA1) with extracellular myc or HA epitope tags, as well as HA-TβRII or myc-TβRII in pcDNA3, were described by us earlier (Henis et al., 1994; Gilboa et al., 1998; Ehrlich et al., 2001; Chetrit et al., 2001; Tazat et al., 2015). HA- or myc-tagged ALK1, HA-ALK1-Q201D (constitutively active point mutant), and HA-ALK1-K221R (kinase dead mutant) in pcDNA3 (Henis et al., 1998; Chen et al., 2009; Kirkbride et al., 2015), HA- or myc-tagged ALK1, HA-ALK1-Q201D (constitutively active point mutant), and HA-ALK1-K221R (kinase dead mutant) in pcDNA3.1 (Nakao et al., 1997; Lee and Blobe, 2007; Tian et al., 2012) were a gift from D. A. Marchuk (Duke University, Durham, NC). HA-TβRIII–ΔCyto (truncated after IYSD, lacking most of the cytoplasmic domain), HA-TβRIII–ΔEho (lacking the last three C-terminal amino acids comprising a class I PDZ binding domain, resulting in loss of binding to GIPC), HA-TβRIII–ΔGAG (lacking two of the glycosaminoglycan attachment sites) in pcDNA3.1 were described (Blobe et al., 2001; Chen et al., 2003; Kirkbride et al., 2008). HA-tagged 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), as well as untagged endoglin and myc-tagged endoglin in pcDNA3.1 (Pomeraniec et al., 2015).

MATERIALS AND METHODS

Reagents

CME blockage inhibits BMP9 signaling in MEECs. (A) Immunoblot analysis of BMP9-induced Smad1/5/8 activation. MEECs were serum starved overnight and treated (or not) with an endocytosis inhibitor (25 μg/ml nystatin or 30 μM PitStop 2, 15 min, 37°C). Vehicle (0.5% dimethyl sulfoxide) or the inactive negative control of PitStop 2 (PitStop NC) was employed as a control. Cells were stimulated (or not) with BMP9 (1 ng/ml, 30 min), lysed, and analyzed by immunoblotting for pSmad1/5/8, total Smad1, and β-actin. The blot depicts a typical experiment (n = 5). (B) Quantification of pSmad1/5/8 formation. The graph depicts the mean ± SEM of the pSmad1/5/8 over β-actin ratio of five independent experiments. The value obtained for BMP9-stimulated untreated cells was defined as 1. Asterisks indicate a significant difference between the pairs indicated by brackets (**, P < 0.01; Student’s two-tailed t test).

Taken together, these studies provide further insight into another mechanism for regulating TGF-β superfamily signaling and downstream biology, which may also be relevant to signaling by receptors from other families that form specific complexes. Of note, the different expression levels of distinct receptors in different cells and organs have potential functional implications, because the effects of complex formation among the different interacting receptors on their endocytosis and signaling are expected to depend on their surface expression levels. As the function and expression of the TGF-β superfamily receptors studied here are altered in many human diseases, including HHT and cancer, how these alterations affect the trafficking and signaling of other TGF-β superfamily receptors to contribute to the pathophysiology of these diseases remains an active area of exploration.
**Cell culture and transfection**

COS7 cells (cat. #RCL-1651) from the American Type Culture Collection (ATCC; Manassas, VA) were grown in DMEM with 10% fetal calf serum (Biological Industries Israel, Beit Haemek, Israel) as described (Gilboa et al., 1998; Shapira et al., 2012). MEECs from endoglin WT (MEEC⁺/⁺) mice (Pece-Barbara et al., 2005; a gift from E. Dejana, University of Milan, Italy) were grown on 0.02% gelatin-coated plates in MCDB-131 medium (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μg/ml heparin (Sigma-Aldrich), and 50 μg/ml EC growth supplement (Sigma-Aldrich). All cells were routinely analyzed by reverse transcriptase-PCR (RT-PCR) for mycoplasma contamination and found to be clean.

For patch/FRAP experiments and endocytosis studies, COS7 cells were grown on glass coverslips in six-well plates and transfected by TransIT-LT1 transfection reagent (Mirus Bio LLC, 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 plasmid DNA was used per transfection, while the DNA amounts of the vectors encoding the various TGF-β receptors were adjusted to yield similar cell surface expression levels (around 0.5 μg), determined by quantitative immunofluorescence as described by us earlier (Marom et al., 2011). The total DNA level was complemented by empty vector to 2 μg. (250 pM TGF-β1 or 5 ng/ml BMP9) were 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 above were subjected to FRAP and patch/FRAP studies as described by us earlier (Rechtman et al., 2009; Pomeraniec et al., 2015; Tazat et al., 2015). FRAP studies were conducted at 15°C, replacing samples after 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 Microlmaging, Jena, Germany) to a Gaussian spot of 0.77 ± 0.03 μm (Planapochromat 63x/1.4 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 R1 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 above) preceded the measurement (Henis et al., 1990; IgG-mediated patching/cross-linking.

At 24 h posttransfection, COS7 cells transfected with various combinations of expression vectors for endoglin and TGF-β receptors were serum starved (30 min, 37°C), washed with cold Hank's balanced salt solution (HBSS; Biological Industries Israel) supplemented with 20 mM 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 incubations) with 1) monovalent mouse Fab’ αmyc (40 μg/ml) together with HA.11 rabbit αHA IgG (20 μg/ml) and 2) Alexa 546-Fab’ GoM (40 μg/ml) together with Alexa 488-IgG GoR (20 μg/ml). This protocol (protocol 1) 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, is labeled exclusively by monovalent Fab’. Alternatively, for immobili- zing the myc-tagged receptor and measuring the lateral diffusion of a coexpressed Fab′-labeled HA-tagged receptor, the labeling protocol used (protocol 2) was 1) monovalent mouse Fab’ αHA (40 μg/ml) together with chicken IgY αmyc (20 μg/ml) and 2) Cy3-Fab’ GoM (40 μg/ml) together with Alexa 488-IgG GoR (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 BMP9, the ligands...
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Rechtman et al., 2009; Pomeraniec et al., 2015). This enables determination of the effects of immobilizing one receptor type on the lateral diffusion of the coexpressed receptor (labeled exclusively with monovalent Fab'), allowing identification of complex formation between the receptors and distinction between transient and stable interactions (Henis et al., 1990; Rechtman et al., 2009; Tazat et al., 2015).

Internalization measurements

COS7 cells grown on glass coverslips in six-well plates were transfected as described for the IgG cross-linking and FRAP studies. After serum starvation (30 min, 37°C), they were blocked with NFG (200 μg/ml, 30 min, 4°C) and labeled with mouse monoclonal αmyc or αHA IgG (20 μg/ml, 45 min, 4°C) followed by Alexa 546-GluM Fab' (40 μg/ml, 45 min, 4°C), all in HBSS/HEPES/BSA. In experiments in which the effects of another, differently tagged receptor (e.g., HA-tagged) on the internalization of the measured receptor (e.g., myc-tagged) were measured, coexpression of the HA-tagged receptor was validated by labeling in parallel with rabbit monoclonal HA-11 followed by Alexa 488-GluR Fab'. In experiments with TGF-β1 (250 pM) or BMP9 (5 ng/ml), the ligands were added after starvation along with the NFG and maintained during the following labeling and endocytosis steps. The internalization of the tagged receptors was quantified by the point-confocal method employing the FRAP setup under nonbleaching illumination conditions (Ehrlich et al., 2001; Shapira et al., 2012). Labeled cells were either fixed immediately with 4% paraformaldehyde or warmed to 37°C for the indicated periods to allow endocytosis; they were then transferred back to 4°C, fixed, and mounted for immunofluorescence as above. Endocytosis was quantified by measuring the reduction in the fluorescence intensity levels at the plasma membrane, focusing the laser beam through the 63x objective at defined spots (1.86 μm²) in the focal plane of the plasma membrane away from vesicular staining, passing the fluorescence through a pinhole in the image plane to make it a true confocal measurement (Ehrlich et al., 2001).

Treatments affecting internalization

Endocytosis assays involving the use of inhibitors were initiated by a 15-min preincubation (37°C) with the inhibitory drug. The cells were kept under the inhibitory condition throughout the labeling and internalization measurement. Nystatin treatment to inhibit caveolar endocytosis (Schnitzer et al., 1994; Di Guglielmo et al., 2003; Mitchell et al., 2004) employed 25 μg/ml drug. Treatment with the clathrin inhibitor PitStop 2 was at 30 μM (von Kleist et al., 2011).

CRISPR to silence TβRIII expression in ECs

CRISPR knockout of MEECs was achieved by stable transduction with lentivirus from puromycin-expressing pLentiCRISPRV2 constructs (Addgene Plasmid 52961; Addgene, Watertown, MA) subcloned with guide RNAs targeting murine TβRIII, or nontargeting control (NTC) guide sequences from the GeCKoV2 database (Heckl et al., 2014; Shalam et al., 2014). Targeting sequences used were MscRNCTC crNTC, MGLiBa_66406, GCGAGGTATCGGCCTGGCCGG; MscrTβRIII-1, MGLiBa_53624, CTTCAACCCAAAGGCCGCCGA; MscrTβRIII-3, MGLiBa_53626, AACCTTCGCGATACAGACCA. Viral particles were made by cotransfecting the subcloned CRISPR vectors bearing Cas9 and the guide sequences with virus packaging plasmids pPA2X and pMD2.G in 293FT cells. Media was changed after 24 h, and the viral supernatants were collected at 48 and 72 h posttransfection, filtered through 0.45 micron membranes, and stored at −80°C or used immediately. Infection of target cells was achieved by incubation with 8 μg/ml polybrene for 24–48 h. Cells were allowed to recover in regular growth media for at least 24 h before selection in puromycin and analysis for gene expression or other experimental procedures.

Western blotting and coimmunoprecipitation

MEECs plated in six-well dishes were serumstarved overnight in MCDB-131 media, followed by incubation with ligands (BMP9 or TGF-β1) for the indicated times and doses. They were then lysed in 2x sample buffer and subjected to electrophoresis on 10% SDS–PAGE followed by immunoblotting. The blots were probed by rabbit anti-pSmad1/5/8 (1:500), rabbit anti-Smad1 (1:1000), rabbit anti-pSmad2 (1:1000), mouse anti-Smad2 (1:1000), or mouse anti–β-actin (1:10,000), followed by Cell Signaling DxrR 800 or DxM 680 (1:5000) infrared-tagged secondary antibodies. The bands were visualized by the Odyssey Classic infrared imaging system and quantified by Li-COR Image Studio software (both from Li-COR Biotechnology, Lincoln, NE).

For coimmunoprecipitation, COS7 cells coexpressing HA- or myc-tagged TβRIII and ALK1 were washed with phosphate-buffered saline and lysed on ice 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 (0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 5 mM dithiothreitol). Lysates were centrifuged (20 min, 4°C), and the supernatants were incubated overnight with protein G-Sepharose beads and antibody as indicated. Beads were washed three times with HNTG buffer (20 mM HEPES, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 10% glycerol) and boiled in 1x sample buffer before Western blot analysis.

Iodinated ligand binding and cross-linking

Cells grown in six-well plates were incubated with 100 pM (125I) TGF-β1 in the presence of fatty acid–free BSA and protease inhibitors (3 h, 4°C). The ligand was then cross-linked to the receptors using 0.5 mg/ml DSS and quenched with 20 mM glycine. Cells were lysed with RIPA buffer supplemented with protease inhibitors. Ligand–receptor complexes were immunoprecipitated overnight at 4°C using goat IgG directed against the extracellular domain of TβRIII. The resulting complexes were separated by SDS–PAGE (7.5% polyacrylamide), and dried gels were subjected to autoradiography with Typhoon 9200 Variable mode Imager (Molecular Dynamics, Pittsburgh, PA). Images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

RT-PCR and RT-qPCR

Total RNA was isolated from cells grown in six-well plates using the Qiagen RNAeasy kit (cat. #74104; Qiagen, Hilden, Germany), and cDNA synthesis was performed using the iScript cDNA Synthesis Kit (cat. #170-8891; Bio-Rad, Hercules, CA). The expression of TβRIII in several human and murine cell lines was determined by RT-PCR with GAPDH as loading control, using the iQ SYBR Green SuperMix (cat. #170-8892; Bio-Rad) and the following primers: 1) murine TβRIII—5′-GGTGTGAACCTGCACGATCA-3′ (forward) and 5′-GGTTAGGATTGAACCTCCCCTTG-3′ (reverse); 2) human TβRIII—5′-CGTT-CACCCGACCTGAAAT-3′ (forward) and 5′-CGTCAAGAGGCGAC-CAC-3′ (reverse); 3) mouse GAPDH—5′-GTCTCAGATCATCCAC-CTG-3′ (forward) and 5′-AGTGGATGTTCATACTTCGTTGTG-3′ (reverse); and 4) human GAPDH—5′-GAGTCACGGATTTTCATGTG-3′ (forward) and 5′-TTATTGATGGGATG-TACCGTCA-3′ (reverse).
To quantify the transcript mRNA levels of TβRII, endoglin, and ALK1 in several human cell lines, we employed RT-qPCR. mRNA level and cDNA synthesis were carried out as above. Using the iQ SYBR Green SuperMix in a BioRad iCycler, relative mRNA levels were determined by the comparative threshold method (Livak and Schmittgen, 2001), normalizing the data to GAPDH. The RT-qPCR data (run in triplicate in each experiment) were analyzed using the BioRad CFX Connect real-time system software. For human TβRII and human GAPDH, the primers used were those described above for RT-PCR. For human endoglin, the primers were 5′-AGTGAAGGCTCTGAGGATTTG-3′ (forward) and 5′-GCCATA-TCCCGACCAACTG-3′ (reverse). For human ALK1, the primers were 5′-GCCCACCAACCTCTCTCGG-3′ (forward) and 5′-ACA-CATCCACCAAGGCACCA-3′ (reverse).

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