ALK5 phosphorylation of the endoglin cytoplasmic domain regulates Smad1/5/8 signaling and endothelial cell migration

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Endoglin, an endothelial cell-specific transforming growth factor-β (TGF-β) superfamily coreceptor, has an essential role in angiogenesis. Endoglin-null mice have an embryonic lethal phenotype due to defects in angiogenesis and mutations in endoglin result in the vascular disease hereditary hemorrhagic telangiectasia type I. Increased endoglin expression in the proliferating endothelium of tumors has been correlated with metastasis, tumor grade and decreased survival. Although endoglin is thought to regulate TGF-β superfamily signaling in endothelial cells through regulating the balance between two TGF-β-responsive pathways, the activin receptor-like kinase 5 (ALK5)/Smad1/5/8 pathway and the activin receptor-like kinase 1 (ALK1)/Smad1/5/8 pathway, the mechanism by which endoglin regulates angiogenesis has not been defined. Here, we investigate the role of the cytoplasmic domain of endoglin and its phosphorylation by ALK5 in regulating endoglin function in endothelial cells. We demonstrate that the cytoplasmic domain of endoglin is basally phosphorylated by ALK5, primarily on serines 646 and 649, in endothelial cells. Functionally, the loss of phosphorylation at serine 646 resulted in a loss of endoglin-mediated inhibition of Smad1/5/8 signaling in response to TGF-β and endothelial cell migration, whereas loss of phosphorylation at both serines 646 and 649 resulted in a loss of ALK5-mediated inhibition of Smad1/5/8 signaling in response to bone morphogenetic protein-9. Taken together, these results support endoglin phosphorylation by ALK5 as an important mechanism for regulating TGF-β superfamily signaling and migration in endothelial cells.

Introduction

The transforming growth factor-β (TGF-β) superfamily signaling pathways have important roles in regulating vascular biology. TGF-β has been reported to both inhibit and stimulate endothelial cell migration, proliferation and angiogenesis. This dichotomous effect of TGF-β on endothelial cells is thought to be mediated by the balance of signaling through two TGF-β-responsive signaling pathways (2–5). Similar to epithelial cells, endothelial cells express the type I transforming growth factor-β receptor (ALK5/TβRII) and the type II transforming growth factor-β receptor (TβRII). TβRII binds TGF-β and recruits and activates activin receptor-like kinase 5 (ALK5) by phosphorylation. The newly activated ALK5 then phosphorylates Smads 2/3, 15% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml heparin and 15 μg/ml endothelial cell growth supplement. MEECs were nucleofected using the Amaxa nucleofection system (Lonza, Germany). Briefly, 1 × 10^6 cells were nucleofected with 1–2 μg of DNA using solution L following the manufacturer’s protocol.

Materials and methods

Cells and nucleofection

COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. COS-7 cells were transiently transfected using FuGene 6 (Roche, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. Wild-type (WT) and knockout (KO) mouse embryonic endothelial cells (MEECs) were selectively immortalized from endoglin knockout (KO) mouse embryonic endothelial cells (MEECs) were selectively immortalized from endoglin kiss and endoglin kiss mice embryos and yolk sacs isolated at E9.0 with polyoma middle T antigen as described in Pecce-Barbara et al. (31). MEECs were maintained in MCD-131 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml heparin and 15 μg/ml endothelial cell growth supplement. MEECs were nucleofected using the Amaxa nucleofection system (Lonza, Germany). Briefly, 1 × 10^6 cells were nucleofected with 1–2 μg of DNA using solution L following the manufacturer’s protocol.

Plasmids and antibodies

pDisplay-Endoglin FL was created by polymerase chain reaction subcloning endoglin into the pDisplay vector. pDisplay-Endoglin-A3, -A10, -A21, -A34, -M1, -M2, -M3, -M4, -S646A, -T647A, -S649A and -T650A (Table I) were made using QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). Hemagglutinin (HA)-tagged constitutively active ALK1 (HA-caALK1) was obtained from Dr. Douglas Marchuk. HA-TβRII and HA-tagged constitutively active ALK5 (HA-caALK5) were obtained from Dr. Xiao-Fan Wang. GST-Endoglin cyto was created by polymerase chain reaction subcloning the cytoplasmic domain of endoglin into pGEX. The following antibodies were used for immunoprecipitation and western blotting: α-HA (Roche), α-GST, α-GAAP, α-Endoglin, α-β-arrestin2 (25) and α-SMAD1/5/8 (6–8). Mechanisms regulating the balance of signaling between these pathways have not been clearly defined.

Endothelial cells express endoglin, an endothelial cell-specific TGF-β superfamily coreceptor (9,10). Endoglin has been proposed to be a regulator of the balance between the ALK5/M1, -M2, -M3, -M4, -S646A, -T647A, -S649A and -T650A (Table I) were used for immunoprecipitation and western blotting: α-HA (Roche), α-GST, α-GAAP, α-Endoglin, α-β-arrestin2 (25) and α-SMAD1/5/8 (6–8). Mechanisms regulating the balance of signaling between these pathways have not been clearly defined.

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MEECs were nucleofected with empty vector, endoglin, S646A, S649A or noclipipitated with 2-ethanesulfonic acid for 30 min at room temperature. Cells were washed with 2-ethanesulfonic acid peroxidase. PAGE under reducing conditions and blotted with streptavidin–horseradish Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL)/649A as indicated. Cells were washed with PBS, incubated with 0.5 mg/ml MEECs were nucleofected with empty vector, endoglin, S646A, S649A or S646/Endoglin Biotinylation as indicated. Cells were washed with PBS, incubated with 0.5 mg/ml 2-ethanesulfonic acid. The reaction was stopped with the addition of 2-ethanesulfonic acid. The reaction was incubated for 30 min at 37°C. The reaction was stopped in the addition of 2-ethanesulfonic acid. The reaction was stopped with the addition of 2-ethanesulfonic acid sample buffer. The products were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to polyvinylidene difluoride and exposed to phosphorimager plates. Filters were mounted on slides and examined by microscope. Images of three random fields were taken and the cells in each image were counted. Cells numbers were normalized to the number of migrated WT-neo cells and then statistically analyzed using a two-tailed Student’s t-test.

### Results

**Basal phosphorylation of endoglin is due to ALK5**

Although endoglin phosphorylation by ALK5 has been reported, discrete sites of endoglin phosphorylation by ALK5 have not been determined (29). To determine the contribution of ALK5 in endoglin phosphorylation, we assessed whether ALK5 was responsible for the basal phosphorylation of endoglin observed in 32P-orthophosphate-labeled COS-7 cells and MEECs by assessing the effects of the ALK5 inhibitor, SB431542. SB431542 is an inhibitor of kinase activity of the TGF-β superfamily type I receptors: ALK5, which binds TGF-β, ALK4, which binds activin, and ALK7, which is thought to bind nodal. SB431542 inhibited the basal phosphorylation of exogenous-expressed endoglin in COS-7 cells and endogenous endoglin in MEECs in a dose-dependent manner (Figure 2A and B). Because SB431542 also inhibits ALK4 and ALK7, we examined endoglin phosphorylation in wild type mink lung cells (Mv1Lu) and mink lung cells that express ALK4 and ALK7 but do not express ALK5 (R1B). While endoglin was phosphorylated in WT mink lung (Mv1Lu) cells, endoglin was not phosphorylated in R1B cells (Figure 1C), further supporting ALK5 as the kinase that basally phosphorylates endoglin. In addition to interaction with TGF-β, endoglin has recently been reported to directly bind BMP-9, which has been reported to regulate endothelial cell biology (27,28). Accordingly, we investigated the effect of the inhibitor SB431542 on endoglin phosphorylation in TGF-β1- or BMP-9-stimulated endothelial cells. Surprisingly, SB431542 inhibited both TGF-β1- and BMP-9-induced phosphorylation of endoglin in endothelial cells (Figure 1D), supporting ALK5 as an important mediator of endoglin phosphorylation in endothelial cells.

**Ser646 and Ser649 are major sites of endoglin phosphorylation**

To define the sites of endoglin phosphorylation by ALK5, we began by generating four cytoplasmic domain truncation mutants of endoglin: A3, D10, D21 and D34 (Table I). WT and mutant endoglin constructs were 32P-orthophosphate labeled in the presence of either empty vector or HA-caALK5 (Figure 2A) in COS-7 cells. Again, we observed basal phosphorylation of endoglin due to endogenous ALK5 (Figure 2A–C). Coexpression of HA-caALK5 led to a modest induction of endoglin phosphorylation on both the fully (upper band) and the partially (lower band) processed forms of endoglin (Figure 2A). Compared with WT endoglin, endoglin phosphorylation was decreased in endoglinA10 and markedly diminished in endoglinD21 (Figure 2A). This suggests that phosphorylation of endoglin requires the last 21 amino acids of the cytoplasmic domain. To minimize structural changes that could disrupt a protein–protein interaction and to further define sites of phosphorylation, we made a series of mutations of the last nine serines/threonines of the cytoplasmic domain of endoglin: M1 (S643A/S646A/T647A), M2 (S649A/T650A), M3 (S653A/T654A) and M4 (S655A/S656A) (Table I). Each construct was orthophosphate labeled in the presence of empty vector or HA-caALK5 (Figure 2B). Phosphorylation of WT endoglin was modestly induced on the fully (upper band) processed form of endoglin in the presence of HA-caALK5 (Figure 2B). In comparison with WT endoglin, there was a decrease in M1 and to a lesser extent M2 phosphorylation in the presence of HA-caALK5, whereas there was no loss of phosphorylation for either M3 or M4 in the presence of HA-caALK5 (Figure 2B). As each of these constructs contained mutations of multiple potential phosphorylation sites to determine which discrete

<table>
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α-phospho-Smad1/5/8 and α-Smad1 (Cell Signaling Technologies, Danvers, MA).

Orthophosphorylation of COS-7 cells expressing endoglin or endoglin phosphorylation mutants and ALK1, ALK5 or TβRII were labeled with 32P-orthophosphate, lysed with radioimmunoprecipitation assay lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 25 mM β-glycerophosphate-Na, 10% glycerol, 25 mM NaF, 1 mM Na2VO3, 1 mM PMSF, 10 μg/ml leupeptin and 1 μg/ml pepstatin) followed by 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid. The immunocomplexes were incubated with 2 μl reaction buffer; 5 μl GST-endoglin cyto; 0.5 μl (0.25 mCi) gamma-32P-ATP and 12 μl 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid. The reaction was incubated for 30 min at 37°C. The reaction was stopped in the addition of 2-μl sample buffer. The products were resolved by 12% SDS-PAGE after reducing conditions and were transferred to polyvinylidene difluoride and exposed to phosphorimager plates. Smad phosphorylation

Equal numbers of endoglin +/+ or endoglin −/− mouse embryonic endothelial cells (WT MEECs or KO MEECs) nucleofected with the indicated constructs were seeded in six-well plates. Two days after nucleofection, cells were serum starved for 4 h. Cells were then stimulated with 100 pM TGF-β1 or 500 pM BMP-9 for 10 min. Cells were then washed with PBS and lysed with radioimmunoprecipitation assay lysis buffer. Lysates were analyzed on 10% SDS–PAGE under reducing conditions and blotted with α-Smad1/5/8 or α-phospho-Smad1 antibodies.

Biotinylation

Endoglin+/+ MEECs were nucleofected with empty vector and endoglin−/− MEECs were nucleofected with empty vector, endoglin, S646A, S649A or S646/649A as indicated. Cells were washed with PBS, incubated with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL)/2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid for 30 min at room temperature. Cells were washed with PBS and lysed with radioimmunoprecipitation assay lysis buffer. Endoglin was immunoprecipitated with α-endoglin (288). Lysates were analyzed on 10% SDS–PAGE under reducing conditions and blotted with streptavidin–horseradish peroxidase.

Fibronectin migration assay

Endoglin+/+ MEECs were nucleofected with empty vector and endoglin−/− MEECs were nucleofected with empty vector, endoglin, S646A, S649A or S646/649A as indicated. Fifty thousand cells in serum-free media were seeded onto the fibronectin-coated upper chamber of transwells. Complete MEEC media was placed in the lower chamber to act as a chemoattractant. Cells were then incubated at 37°C for 6 h. After incubation, cells left in the top chamber were scraped away using a cotton swab. The cells on the bottom of the filter were fixed and stained with the Three-Step Stain Set (VWR, West Chester, PA). Filters were mounted on slides and examined by microscope. Images of three random fields were taken and the cells in each image were counted. Cells numbers were normalized to the number of migrated WT-neo cells and then statistically analyzed using a two-tailed Student’s t-test.
Fig. 1. ALK5 basally phosphorylates endoglin. HA-endoglin expressed in COS-7 cells (A) and endogenous endoglin in WT MEECs (B) were orthophosphate labeled in the presence of increasing concentrations of the ALK5 inhibitor, SB431542. (C) HA-endoglin was expressed in WT mink lung cells (Mv1Lu) and mink lung cells that do not express ALK5 (R1B). (D) WT MEECs were 32P-orthophosphate labeled in the presence of 200 pM TGF-β in the presence or absence of the ALK5 inhibitor SB431542 or 500 pM BMP-9 in the presence or absence of the ALK5 inhibitor, SB431542. Endoglin phosphorylation was assessed by 32P-orthophosphate labeling of cells, immunoprecipitation of the HA-tagged endoglin and detection by phosphorimager (A, B, C, and D, top). Expression of endoglin was detected by western blot analysis (A, B, C, and D, bottom).

Fig. 2. Endoglin is phosphorylated by ALK5 on S646 and S649. Empty vector (EV), HA-endoglin, HA-D3 (Δ3), HA-Δ10 (Δ10), HA-Δ21 (Δ21) or HA-Δ34 (Δ34) were transiently coexpressed with HA-caALK5 in COS-7 cells (A). Empty vector (EV), HA-endoglin or mutants, HA-M1 (M1), HA-M2 (M2), HA-M3 (M3) or HA-M4 (M4) were transiently coexpressed with HA-caALK5 in COS-7 cells (B). Empty vector (EV), WT endoglin (End), HA-S646A (S646A), HA-T647A (T647A), HA-S649A (S649A) or HA-T650A (T650A) were transiently coexpressed with HA-caALK5 in COS-7 cells (C). Endoglin phosphorylation was assessed by 32P-orthophosphate labeling of COS-7 cells, immunoprecipitation of the HA-tagged receptors and detection by phosphorimager (top). Expression of HA-caALK5 was detected by autophosphorylation (middle) and expression of endoglin was detected by western blot analysis (bottom). Data are representative of three independent experiments.
site(s) in M1 and M2 contribute to the loss of phosphorylation observed, we made single point mutants: S646A, T647A, S649A and T650A (Table I). These constructs were orthophosphate labeled in the presence of empty vector or HA-caALK5 (Figure 2C). Phosphorylation of WT endoglin was modestly induced on both the fully (upper band) and the partially (lower band) processed forms of endoglin in the presence of HA-caALK5 (Figure 2C). Compared with WT endoglin, there was decreased basal phosphorylation on S646A, S649A and T650A and decreased HA-caALK5-stimulated phosphorylation of S646A and S649A (Figure 2C).

S646A and S649A were able to bind TGF-β1 in the presence of TβRII in COS-7 cells, as assessed by 125I-TGF-β binding and cross-linking, at least as well as WT endoglin (supplementary Figure 1 is available at Carcinogenesis Online). As endoglin can only efficiently bind TGF-β1 when in complex with TβRII, these results confirm that S646A and S649A can traffic to the cell surface, form complexes with TβRII and bind ligand. Taken together, these results support S646 and S649 as the major sites of basal endoglin phosphorylation, support ALK5 as the kinase responsible for this basal phosphorylation and establish that phosphorylation on these sites does not alter the trafficking of these receptors to the cell surface or their ability to form complexes with other TGF-β receptors.

To further investigate the loss of these phosphorylation sites, we made a double mutant, S646A/S649A (S6/9A). Endoglin and S6/9A were orthophosphate labeled in COS-7 cells in the presence of empty vector, HA-caALK1, HA-caALK5 or TβRII. Loss of both serines 646 and 649 led to a loss of basal phosphorylation of endoglin (Figure 3A, compare lanes 2 and 6). HA-caALK5-stimulated phosphorylation of endoglin (Figure 3A, compare lanes 4 and 8) as well as an impairment of ALK1 phosphorylation on both the fully (upper band) and the partially (lower band) processed forms (Figure 3A, compare lanes 3 and 7). However, there was no effect on TβRII phosphorylation (Figure 3A, compare lanes 5 and 9). Although S6/9A bound 125I-TGF-β at least as well as WT endoglin (supplementary Figure 1 is available at Carcinogenesis Online), it cannot be phosphorylated by HA-caALK5 or HA-caALK1. In orthophosphate-labeled endoglin—/— MEECs (KO MEECs) expressing WT endoglin, S646A, S649A or S6/9A, WT endoglin was robustly phosphorylated, whereas S649A was less phosphorylated and S646A and S6/9A exhibited no phosphorylation (Figure 3B). Taken together, these studies support S646 and S649 as the major sites of endoglin phosphorylation by ALK5 and perhaps by ALK1 and that loss of S646 and S649 might be necessary for phosphorylation of endoglin by other kinases.

Loss of ALK5 phosphorylation impairs the ability of ALK1 to phosphorylate endoglin

Since both ALK5 and ALK1 phosphorylation was impaired when serines 646 and 649 were mutated, we performed in vitro kinase assays with a GST-endoglin cytoplasmic domain fusion protein as the substrate and immunopurified HA-caALK5 or HA-caALK1 as the kinase. Although both HA-caALK5 and HA-caALK1 were active, only HA-caALK5 was able to directly phosphorylate the cytoplasmic domain of endoglin (Figure 4A). Since ALK1 phosphorylation was impaired when serines 646 and 649 were mutated and ALK1 did not phosphorylate endoglin in the in vitro kinase assay, we hypothesized that ALK1 phosphorylation of endoglin might require ALK5 phosphorylation. We further reasoned that if basal ALK5-mediated phosphorylation was required for subsequent ALK1 phosphorylation, the ALK5 inhibitor, SB431542, which does not inhibit caALK1 activity (33), would inhibit not only basal ALK5 phosphorylation but also caALK1-mediated endoglin phosphorylation. To assess this, we orthophosphate labeled

![Fig. 3. Loss of endoglin phosphorylation by ALK5 on serines 646 and 649 impairs ALK1 phosphorylation but not TβRII phosphorylation. (A) HA-endoglin or HA-endoglin with both serines 646 and 649 mutated to alanine (S6/9A) were transiently coexpressed with HA-caALK1, HA-caALK5 or HA-tagged TβRII (HA-TβRII) in COS-7 cells. (B) HA-endoglin or HA-endoglin with serine 646 mutated to alanine (S646A), serine 649 mutated to alanine (S649A) or both serines 646 and 649 mutated to alanine (S6/9A) were transiently expressed in endoglin—/— mouse embryonic endothelial cells (KO MEECs). Endoglin phosphorylation was assessed by 32P-orthophosphate labeling of cells, immunoprecipitation of the HA-tagged receptors and detection by phosphorimager (top). Expression of the kinases was detected by their autophosphorylation (top) and expression of endoglin was detected by western blot analysis (bottom). Data are representative of three independent experiments.](https://example.com/fig3)

![Fig. 4. ALK1 does not phosphorylate unmodified endoglin. (A) HA-tagged kinase-dead ALK1 (kdALK1) or HA-tagged constitutively active ALK1 (caALK1) and HA-tagged kinase dead ALK5 (kdALK5) or HA-tagged constitutively active ALK5 (caALK5) were immunoprecipitated from COS-7 cells transiently expressing these constructs. The immunocomplexes were incubated with a GST fusion protein of the cytoplasmic domain of endoglin (GST-endoglin cyto) and 32P-ATP, the GST-endoglin cyto was pulled down with glutathione beads and detected by phosphorimager (A, top). Expression of HA-endoglin, HA-caALK1 and HA-caALK5 was detected by western blot analysis (A, bottom). (B) HA-endoglin was expressed in COS-7 cells in the presence of empty vector or caALK1 and treated with the ALK5 inhibitor, SB431542. Endoglin phosphorylation was assessed by 32P-orthophosphate labeling of cells, immunoprecipitation of the HA-tagged endoglin and detection by phosphorimager (top). Expression of HA-caALK1 was detected by phosphorimager (middle). Expression of endoglin was detected by western blot analysis (bottom). Data are representative of three independent experiments.](https://example.com/fig4)
COS-7 cells transiently expressing endoglin with either empty vector or HA-caALK1 in the presence or absence of the ALK5-specific inhibitor, SB431542 (Figure 4B). In the presence of SB431542, basal ALK5 phosphorylation of endoglin was inhibited (Figure 4B, compare lanes 4 and 2), and there was no induction of endoglin phosphorylation with the addition of HA-caALK1 (Figure 4B, compare lanes 5 and 3). Taken together, these data suggest a model of sequential endoglin phosphorylation, first by ALK5 and then by ALK1.

Endoglin phosphorylation regulates TGF-β1 and BMP-9 signaling via the ALK1/Smad1/5/8 pathway

To determine whether endoglin phosphorylation affects TGF-β signaling, we focused on TGF-β-induced Smad1/5/8 phosphorylation in WT MEECs and KO MEECs as endoglin has been reported to have no effect on Smad2/3 phosphorylation in these cells (31). When WT MEECs were treated with TGF-β1, there was a robust induction of Smad1/5/8 phosphorylation (Figure 5A, lanes 1 and 2). In contrast, in the KO MEECs, there was little induction of Smad1/5/8 phosphorylation in response to TGF-β1 treatment (Figure 5A, lanes 3 and 4). Smad1/5/8 phosphorylation induction in response to TGF-β1 was restored in KO MEECs nucleofected with WT endoglin or with endoglin-S649A, establishing that the difference in Smad1/5/8 phosphorylation between WT MEECs and KO MEECs was due to endoglin and that phosphorylation at serine 649 is not essential for this endoglin function (Figure 5A, lanes 5, 6, 9 and 10). However, expression of S646A or S6/9A did not restore induction of Smad1/5/8 phosphorylation in response to TGF-β1 (Figure 5A, lanes 7, 8, 11 and 12). These results suggest that the phosphorylation of S646A of endoglin by ALK5 is required for promotion of ALK1/Smad1 signaling, whereas phosphorylation of S649 does not affect this signaling pathway.

Since BMP-9 has been reported to bind to both endoglin and ALK1 (34), we assessed BMP-9-induced phosphorylation of Smad1/5/8 in WT MEECs and KO MEECs (31). When WT MEECs were treated with BMP-9, there was a robust induction of Smad1/5/8 phosphorylation (Figure 5B, lanes 1 and 2). However, in KO MEECs, there was a marked decrease in phosphorylation in response to BMP-9 treatment (Figure 5B, lanes 3 and 4). Smad1/5/8 phosphorylation induction in response to BMP-9 was restored in KO MEECs nucleofected with WT endoglin, establishing that the difference in Smad1/5/8 phosphorylation between WT MEECs and KO MEECs was due to endoglin (Figure 5B, lanes 5, 6, 9 and 10). However, expression of S646A or S649A did not restore induction of Smad1/5/8 phosphorylation in response to BMP-9. In addition, induction of Smad1/5/8 phosphorylation was absent when S6/9A was expressed, suggesting that both serines 646 and 649 are essential for this endoglin function (Figure 5B, lanes 11 and 12).

![Fig. 5](image_url)

**Fig. 5.** Phosphorylation of endoglin on S646 and S649 is required for induction of Smad1/5/8 phosphorylation. WT MEECs and KO MEECs expressing empty vector (EV), WT endoglin or endoglin mutants S646A, S649A or S6/9A were treated with 100 pM TGF-β1 (A) or 0.5 nM BMP-9 (B). Smad1/5/8 phosphorylation and total Smad levels were assessed by western blot analysis. Asterisk denotes non-specific band. Data are representative of three independent experiments.

Endoglin phosphorylation regulates endothelial cell migration

Activation of the ALK1/Smad1/5/8 pathway in endothelial cells has been associated with decreased migration (35,36), whereas endoglin and the cytoplasmic domain of endoglin have functional roles in mediating inhibition of endothelial cell migration (23–25). To determine whether endoglin phosphorylation affects endothelial cell migration, we investigated the migration of WT MEECs and KO MEECs expressing empty vector (neo), WT endoglin, S646A or S649A (Figure 6A). Although the KO MEECs nucleofected with empty vector exhibited an increase in migration compared with WT MEECs, expression of WT endoglin or S649A in KO MEECs led to a reduced rate of migration, rescuing the endoglin-mediated inhibition of migration observed in WT MEECs (Figure 6B). In contrast, S646A was not able to inhibit the migration of KO MEECs (Figure 6B), despite being robustly expressed (Figure 6A), supporting an essential role for phosphorylation of endoglin on S646 in endoglin-mediated inhibition of endothelial cell migration.

**Fig. 6.** Phosphorylation of endoglin on Ser646 regulates endothelial cell migration. WT MEECs and KO MEECs expressing empty vector (neo), HA-tagged WT endoglin (end) or endoglin mutants S646A or S649A (A) were plated on transwells coated with 50 mg/ml fibronectin and assessed for migration 6 h later (B). Cells were fixed and stained for their nuclei, and sample images were obtained. The number of migrated cells was counted and normalized to WT-neo MEECs and graphed (C). Expression of endoglin was assessed by cell surface biotinylation, immunoprecipitation and detection by streptavidin–horseradish peroxidase (A). Data are representative of four independent transwell migration experiments.

Discussion

Endoglin has a short serine/threonine-rich cytoplasmic domain that is phosphorylated constitutively and by the TGF-β1 superfamily kinase receptors ALK1, ALK5 and TβRII (27–29). Although endoglin has
been reported to be phosphorylated on serines by TβRII and on threonines by ALK1, specific sites of ALK5 phosphorylation have not been identified (29). Here, we have shown that endoglin is phosphorylated by ALK5 on serines 646 and 649. Mutation of serine 646 to alanine nearly abrogates endoglin phosphorylation in COS-7 cells and KO MEECS (Figures 2D and 3B), supporting serine 646 as a major site of phosphorylation. Functionally, serine 646 phosphorylation by ALK5 was required for endoglin-mediated induction of Smad1/5/8 phosphorylation in endothelial cells in response to TGF-β1, BMP-9 and endoglin-mediated inhibition of endothelial cell migration. We also showed that S649 is a target for ALK5 phosphorylation; however, loss of S649 does not impair phosphorylation as drastically as does loss of S646 (Figure 2C and 3B). Interestingly, although the loss of phosphorylation at serine 649 exhibited no loss of function in TGF-β-induced signaling or endoglin-mediated inhibition of migration, phosphorylation of S649 was required for endoglin-mediated induction of Smad1/5/8 phosphorylation in endothelial cells stimulated by BMP-9 (Figure 5). Taken together, this suggests that phosphorylation of S649 has functional consequences specifically downstream of BMP-9. This possibility is currently being explored.

In this study, we observed that endoglin is constitutively phosphorylated and that the majority of endoglin phosphorylation is on serines, consistent with previous reports (30,37). Koleva et al. (29) reported that serine phosphorylation occurs within the region spanning S646–S655. We also observed that most serine phosphorylation occurs within this region, more specifically on serines 646 and 649. In this study, we also report that a GST-endoglin cytoplasmic domain fusion protein expressed in bacteria was not phosphorylated by HA-caALK1 in an in vitro kinase assay (Figure 4A). Although this may appear to be at odds with results obtained previously, the endoglin substrate in this prior report was isolated from HEK293 cells and could have already been basally phosphorylated by ALK5 in HEK293 cells. As we have demonstrated here, ALK5 phosphorylation is required for efficient ALK1-mediated phosphorylation of endoglin (29). Koleva et al. (29) also reported that ALK1-mediated phosphorylation of endoglin required prior TβRII-mediated phosphorylation on serine residues. We have observed that, in addition, ALK1-mediated phosphorylation of endoglin and downstream phosphorylation of Smad1/5/8 require ALK5-mediated phosphorylation on serines 646 and 649. The dependence of ALK1 on ALK5 has been shown previously by studies demonstrating that loss of ALK5 expression induced a reduction in ALK1 signaling (5). Although the small molecule ALK5 inhibitor SB431542, does not inhibit the kinase activity of ALK1 (33), here, we have demonstrated that SB431542 both inhibits basal ALK5 phosphorylation of endoglin and impairs the ability of ALK1 to phosphorylate endoglin (Figure 4B). In addition, we observed that loss of endoglin phosphorylation by ALK5 leads to loss of ALK1 induction of Smad1/5/8 phosphorylation (Figure 5). These observations support a model in which ALK1, ALK5 and endoglin are in a single complex, with ALK1-mediated phosphorylation of endoglin dependent on prior ALK5-mediated phosphorylation and both these phosphorylation events regulating subsequent phosphorylation of Smad1/5/8. The role of endoglin phosphorylation by ALK5 in ALK1 signaling is currently under investigation.

Phosphorylation of proteins often leads to a conformational change that alters enzymatic activity or alters the ability to interact with other proteins. As the cytoplasmic domain of endoglin lacks catalytic activity, the phosphorylation of endoglin probably regulates the interaction of endoglin with other proteins. In addition to interacting with the cytoplasmic domains of ALK1, ALK5 and TβRII, the cytoplasmic domain of endoglin has also been reported to interact with zyxin, zyxin-related protein-1 and β-arrestin2 (23–25). As multiple studies have linked ALK1 activation to inhibition of endothelial cell migration (35,36), the phosphorylation of endoglin on serine 646 might facilitate ALK1 activation resulting in inhibition of migration, consistent with our observation that endothelial cell migration is increased when serine 646 is mutated. Phosphorylation of serine 646 might also alter the conformation of the cytoplasmic domain of endoglin promoting interaction with ALK1 and subsequent ALK1-mediated phosphorylation. Consistent with this hypothesis, the interaction between the cytoplasmic domain of endoglin and the type I TGF-β receptors, ALK1 and ALK5, has been reported to be dependent on the kinase activity of the type I receptor, suggesting that a phosphorylation event regulates endoglin’s interactions with these receptors (27,28). Alternatively, as the interaction of the cytoplasmic domain of endoglin with zyxin, zyxin-related protein-1, GAIP-interacting protein, C-terminus or β-arrestin2 have all been reported to be important for endoglin-mediated inhibition of endothelial cell migration (23–25), the phosphorylation of serine 646 might be important in terms of mediating interactions of endoglin with these scaffolding proteins. The effect of endoglin phosphorylation at serines 646 and 649 with these endoglin-interacting proteins is currently being explored.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/.

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References


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