Endoglin Structure and Function

DETERMINANTS OF ENDOGLIN PHOSPHORYLATION BY TRANSFORMING GROWTH FACTOR-β RECEPTORS*

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Determination of the functional relationship between the transforming growth factor-β (TGFβ) receptor proteins endoglin and ALK1 is essential to the understanding of the human vascular disease, hereditary hemorrhagic telangiectasia. TGFβ1 caused recruitment of ALK1 into a complex with endoglin in human umbilical vein endothelial cells (HUVECs). Therefore, we examined TGFβ receptor-dependent phosphorylation of endoglin by the constitutively active forms of the TGFβ type I receptors ALK1, ALK5, and the TGFβ type II receptor, TβRII. Of these receptors, TβRII preferentially phosphorylated endoglin on cytosolic domain serine residues Ser634 and Ser635. Removal of the carboxyl-terminal tripeptide of endoglin, which comprises a putative PDZ-liganding motif, dramatically increased endoglin serine phosphorylation by all three receptors, suggesting that the PDZ-liganding motif is important for the regulation of endoglin phosphorylation. Constitutively active (ca)ALK1, but not caALK5, phosphorylated endoglin on cytosolic domain threonine residues. caALK1-mediated threonine phosphorylation required prior serine phosphorylation, suggesting a sequential mechanism of endoglin phosphorylation. Wild-type, but not a threonine phosphorylation-defective endoglin mutant blocked cell detachment and the antiproliferative effects of caALK1 expressed in HUVECs. These results suggest that ALK1 is a preferred TGFβ receptor kinase for endoglin threonine phosphorylation in HUVECs and indicate a role for endoglin phosphorylation in the regulation of endothelial cell adhesion and growth by ALK1.

In humans, there are seven type I and five type II TGFβ receptors currently known (1). TGFβ type I/II receptors are serine and threonine (S/T) kinases, although the significance of the serine- versus threonine-phosphorylating activities of the TGFβ receptors is not clear. The TGFβ type I receptors include activin-like kinase 1 (ALK1) and TβRII, also known as ALK5. ALK1 and ALK5 associate with the type II TGFβ receptor, TβRII (2). The binding of TGFβ ligands by TβRII induces the type I receptor to associate with TβRII, allowing the latter to phosphorylate the type I receptor and activate its kinase domain (3). The activated type I receptor propagates signaling by phosphorylating the Smad family of transcription corepressors and coactivators (4). Endoglin is a type III TGFβ receptor (5, 6) that is also a substrate for TβRII- and ALK5-catalyzed phosphorylation (7), although the relevance of endoglin phosphorylation and the ability of endoglin to serve as a substrate for ALK1 have not been demonstrated.

Phosphorylation of human endoglin occurs in endothelial cells and mouse fibroblasts (8, 9). These studies demonstrate that endoglin is phosphorylated on serine (9) and threonine residues (10). TβRII and ALK5 interact with endoglin, and, as a result of these associations, endoglin is phosphorylated on its cytosolic domain (CD) (7). No previous studies have identified the specific endoglin residues that were phosphorylated, examined the kinase specificities of different TGFβ receptors toward endoglin, or addressed the physiological consequences of endoglin phosphorylation.

Although endoglin associates with TβRII (6, 7) and modulates the activities of TβRII (7), ALK1 (11, 12), and ALK5 (7), the function of endoglin in the endothelium remains unclear. Constitutively active (ca) forms of the TGFβ type I receptors (13) have been widely used to examine TGFβ receptor signaling (14) and were useful in elucidating the relationship between ALK1 and ALK5 (11, 15, 16) and receptor kinase-substrate phosphorylation (17). Using this approach, we analyzed endoglin phosphorylation by TβRII and the constitutively active forms of ALK1 (Q201D) (caALK1) and ALK5 (T204D) (caALK5).

Our data indicated that threonine phosphorylation of endoglin was a distinguishing consequence of its interaction with caALK1 versus caALK5. We provide evidence that endoglin is a direct kinase substrate for caALK1, which preferentially phosphorylated mature endoglin on threonine residues, as compared with caALK5. Examination of endoglin cytosolic domain deletion mutations indicated that phosphorylation of endoglin is tightly regulated by the putative carboxyl-terminal PDZ-liganding tripeptidyl motif. Site-specific mutagenesis studies

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3 The abbreviations used are: TGFβ, transforming growth factor beta; ca, constitutively active; HHT, hereditary hemorrhagic telangiectasia; CD, cytosolic domain; ALK, activin receptor-like kinase; kd, kinase-dead; pSer, phosphorylserine; pThr, phosphothreonine; HUVEC, human umbilical vein endothelial cell; Wt, wild type; FA, focal adhesion; HA, hemagglutinin; pfu, plaque forming unit(s); MS, mass spectrometry.
revealed that serine phosphorylation is required for threonine phosphorylation and that phosphorylation occurred preferentially on endoglin residues Thr<sup>2440</sup> and Thr<sup>2454</sup>. TGFβ1 treatment of human umbilical vein endothelial cells (HUVECs) induced formation of a complex containing endogenous endoglin and ALK1, with attendant incorporation of <sup>32</sup>P from [γ-<sup>32</sup>P]ATP and threonine phosphorylation of endoglin. Finally, in HUVECs, wild-type (Wt) endoglin, but not a threonine phosphorylation-defective mutant, rescued cell detachment and the anti-proliferative effects of caALK1 and exhibited caALK1-dependent exit from a focal adhesion (FA)-associated pool of proteins.

Mutations in endoglin (18) and ALK1 (19) result in the autosomal-dominant vascular dysplasia, hereditary hemorrhagic telangiectasia (HHT). The data presented suggest the hypothesis that ALK1-dependent threonine phosphorylation of endoglin comprises a shared molecular signature of HHT1 and HHT2.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Cytokines**—TGFβ1 was obtained from R&D Systems. Anti-endoglin antibody P4A4 (20) was from the Developmental Studies Hybridoma Bank (NICHHD, National Institutes of Health), University of Iowa. Anti-endoglin clone 35 was obtained from BD Transduction Laboratories. Anti-human ALK1 was kindly provided by A. Lux (Mannheim, Germany). Rabbit polyclonal anti-phosphoserine (α-pSer) and anti-phosphothreonine (α-pThr) antibodies were obtained from Zymed Laboratories. Goat anti-TGFβ type III receptor antibody (sc-6199), and rabbit and mouse anti-hemagglutinin (HA, Y-11 clone) antibodies were obtained from Santa Cruz Biotechnology and Covance (Mono HA 11 and MMS-101P).

**Expression Constructs and Site-directed Mutagenesis**—Human HA-tagged ALK1 constructs (Q201D and K229R), HA-caALK5 (T204D), and HA-TβRII originated in the Massague Laboratory (Howard Hughes Medical Institute, New York, NY). Human endoglin in the pcDNA3.1 vector was subjected to site-specific mutagenesis using the QuikChange (Stratagene) system, according to the manufacturer’s instructions. The sequences of all mutated constructs were confirmed.

**Cell Culture, Transfection, Immunoprecipitation, and Western Blot Analysis**—HEK293T cells (ATCC) and GM7372 bovine endothelial cells were grown in Dulbecco’s modified Eagle’s medium or α minimal essential medium, respectively, supplemented with 10% fetal bovine serum. HUVECs were cultured in EBM-2 medium containing EGM-2 complete supplement (Cambrex). HEK293T cells were transiently transfected using the GeneJuice<sup>®</sup> transfection reagent (Novagen) according to the manufacturer’s instructions, and were lysed, ∼48 h after transfection, in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 300 mM sucrose, 1.0% Triton X-100, and 0.5% sodium deoxycholate 0.1% sodium dodecyl sulfate, 10 mM β-glycerol phosphate), and were supplemented with protease (Roche Applied Science) and phosphatase (Calbiochem) inhibitor cocktails. All lysates were normalized for total protein by Bradford assay (Bio-Rad) prior to immunoprecipitation. Cell lysates were immuno precipitated for 4 h at 4 °C using the P4A4 anti-endoglin antibody. The resulting immune complexes were isolated with protein A/G-agarose (Santa Cruz Biotechnology). Purification of focal adhesion-associated protein was conducted using RGD-labeled paramagnetic microspheres according to a modification (21) of the method developed by Plopper et al. (22). Protein isolations were resolved by SDS-PAGE, electrotransferred to polyvinylidene fluoride membranes, and Western blotted using the indicated antibodies. Bound antibodies were visualized by chemiluminescence (ECL, Amer sham Biosciences).

**Protein Isolation, Digestion, and Mass Spectrometry**—Immuno precipitated endoglin expressed in HEK293T cells (8 × 10<sup>7</sup> cells) was subjected to 10% SDS-PAGE. The gels were stained with Coomassie Blue (BDH Chemicals), and the band corresponding to endoglin was excised and immediately processed. All solvents used for mass spectrometry were high-performance liquid chromatography grade (Sigma). Excised gel slices were digested sequentially by trypsin, followed by Glu-C protease, and the resulting peptides were isolated using the Montage In-Gel Digest Kit (Millipore), according to the manufacturer’s recommendations. After digestion, the peptides were pooled and dried under vacuum in a SpeedVac. The peptide mixtures were desalted using a Poros R2 micro column as described (23) and eluted directly into a nanospray needle (New Objective). HUVECs (5 × 10<sup>7</sup> cells) were transduced with endoglin and caALK1 adenoviruses (below). After 48 h, endoglin was immunoprecipitated as described above. Protein elution was performed in 50 μl of 100 mM dithiothreitol, 50 mM NH<sub>4</sub>HCO<sub>3</sub>, and 1% SDS at 100 °C for 5 min. The supernatant was diluted 5× with 50 mM NH<sub>4</sub>HCO<sub>3</sub>, and incubated at 25 °C for 30 min in the dark with 100 mM iodoacetamide. Buffer exchange was performed against 50 mM NH<sub>4</sub>HCO<sub>3</sub>, on 5-kDa cut-off spin filters (Ultrafree-MC, Millipore, Bedford, MA). Proteins were digested with 10 μg of Glu-C (Roche Diagnostics, Penzberg, Germany) for 18 h at room temperature in 50 μl of NH<sub>4</sub>HCO<sub>3</sub> on top of the spin filter. The resulting peptides were eluted through the filter membrane in three steps, 30 μl each, using 20%, 50%, and 70% acetonitrile containing 0.1% acetic acid. After lyophilization peptide acidic groups were converted to their corresponding methyl esters, followed by enrichment of phosphorylated peptides via immobilized metal affinity chromatography as previously described (24). Phosphopeptides were directly eluted onto a reversed-phase microcapillary high-performance liquid chromatography pre-column (360-μm outer diameter × 100-μm inner diameter, fused silica, 8-cm bed length, 10- to 25-μm irregular-shaped C18 particles) and rinsed with 50 μl of 0.1% acetic acid. Next, the precolumn was connected directly to a reversed-phase microcapillary high-performance liquid chromatography analytical column (360-μm outer diameter × 50-μm inner diameter, fused silica, 8-cm bed length, 5-μm spherical-shaped C18 particles) and integrated with a 1-μm diameter electrospore emitter tip. Peptides were eluted online with a hybrid quadrupole time-of-flight mass spectrometer (QSTAR, MDS-SCIEX, Toronto, Canada) using a solvent gradient of 0–50%B in 30 min and then 50–90%B in 10 min, where solvent A was 0.2 M acetic acid and solvent B was 70% acetonitrile with 0.2 M acetic acid. Data acquisition on the mass spectrometer was performed in information-depend-
ent mode, whereby precursor peptides were automatically selected based on an MS-only scan and subjected to collision-activated dissociation (MS/MS) in subsequent scans. These data were searched against a protein data base using the Mascot algorithm (Matrix Science, London, UK), with manual verification of high scoring endoglin peptides.

In Vitro Kinase Assay—HEK293T cells were transfected with the indicated plasmids and, 48 h later, extracted with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM β-glycerophosphate, 300 mM sucrose, 1% Triton X-100), which was supplemented with protease and phosphatase inhibitor cocktails. The cell lysates were incubated with P4A4 anti-endoglin antibody for 3 h. The protein complexes were subsequently isolated using protein A/G-agarose (Santa Cruz Biotechnology). In vitro kinase assays were conducted in 30 µl of kinase buffer (50 mM Tris-HCl, pH 7.5, 60 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, 2 mM dithiothreitol, 0.1 mM Na₃VO₄) in the presence of 100 μM cold ATP and 10 μCi of [γ-³²P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences) for 30 min at room temperature (25). The reactions were terminated by washing with kinase buffer, followed by addition of SDS sample buffer, subjected to 10% SDS-PAGE, and the gels were dried. In vitro kinase assay of endogenous endoglin and ALK1 isolated from HUVECs was conducted as described above with or without prior TGFβ1 treatment. Incorporation of [³²P]-phosphate was visualized by autoradiography or quantitated using a Typhoon PhosphorImager (Amersham Biosciences).

Recombinant Adenoviral Vectors—The adenoviral vectors expressing caALK1, kinase-dead (kd)ALK1, Wt endoglin, and ΔThr5 endoglin were prepared from the respective cDNA clones. The coding sequences for caALK1 and kdALK1 were excised from the parental pCMV5 vector by sequential BamH1 and HindIII cleavage and ligated to BamH1/HindIII-cut pAdLox (26, 27). Wt and ΔThr5 endoglin were excised from parental pcDNA3.1 vector with EcoRI. The ends were blunted with the Klenow large fragment of DNA polymerase and subsequently cloned into BamH1/HindIII-cut and blunt-ended pAdLox. Recombinant adenoviruses expressing caALK1, kdALK1, Wt endoglin, and ΔThr5 endoglin were generated as previously described (26). For an infectious control, we used an analogous adenovirus expressing β-galactosidase.

Cell Proliferation Analysis—HUVEC (passage 10 or less) proliferation was determined by [³H]thymidine incorporation assay (28). The assays were performed by seeding 7.5 × 10⁴ cells/well in 6-well plates in triplicate in HUVEC medium, described above. Preliminary HUVEC caALK1 adenovirus infection experiments were conducted to establish the minimum plaque forming units (pfu) required to achieve 80–90% loss of HUVEC viability in the absence of other adenovirus-transduced genes. Twenty-four hours after plating, the cells were infected with adenovirus at a multiplicity of infection (based on pfu determination) of 60–300, and incubated for an additional 24 h. The cells were then given culture medium containing 0.5 μCi/ml [³H]thymidine, and incubated an additional 24 h. The cells were lysed, and the incorporation of [³H]thymidine determined by liquid scintillation counting (Beckmann).

RESULTS

Endoglin Is Constitutively Serine-phosphorylated on Residues Ser⁶３４ or Ser⁶３５—The 48-amino acid l-isofomi (29) of the endoglin CD is serine- and threonine-rich (14 Ser (29%) and 5 Thr (10%)) presenting many potential sites of phosphorylation. Therefore, we initially used a mass spectrometric approach to facilitate identification of sites of endoglin phosphorylation. Wt endoglin was expressed in HEK293T cells, immunoprecipitated, and resolved by SDS-PAGE. The band corresponding to endoglin was excised and subjected to sequential trypsin and Glu-C proteolytic digestion (30). Initial mass spectrometric analysis of endoglin tryptic/Glu-C phosphopeptides obtained from transfected HEK293T cells revealed an ion potentially matching the singly phosphorylated peptide EPVVAVAA-PASSE. This result was consistent with data suggesting that the central serine-rich ASSESS motif of the endoglin is serine-phosphorylated (Ref. 31 and data not shown). Next, a Glu-C digest of immunoprecipitated transduced endoglin in HUVECs, which were also transduced with caALK1, was used to further study the phosphorylation of endoglin. Mass spectra of putatively singly (Fig. 1A) and doubly (Fig. 1B) phosphorylated peptides were obtained. Expanded regions of the time-of-flight mass spectrum showed the triply charged ions of singly and doubly phosphorylated species of 637SSSTNHSIGSTQSTPCSTSSMA⁶⁵⁸. Analysis with mass spectrometry suggested at least double phosphorylation between serines 639 and 656, but spectra from tandem mass spectrometry were ambiguous as to the exact sites. The lack of basic residues within the sequence resulted in an incomplete fragmentation pattern. We cannot discount the presence of multiply phosphorylated isoforms, because collectively these peptides may nearly co-elute, making it difficult to resolve distinct phosphorylated species. However, these results suggested the presence of basal phosphorylation sites within the Ser⁶３４⁶３５ motif, and additional sites were phosphorylated by transduced caALK1 in the 637SSSTNHSIG-STQSTPCSTSSMA⁶⁵⁸ region. Based on these data, we undertook site-directed mutagenesis of the endoglin CD.

Serine Phosphorylation by TβRII: Regulation by the PDZ-ligating Motif of Endoglin—We employed an α-pSer antibody Western blotting strategy to independently monitor changes in endoglin phosphorylation by the TGFβ receptors. First, a diseryl-to-alanyl (634AA⁶３５) mutation was constructed by site-directed mutagenesis of endoglin residues Ser⁶３４ and Ser⁶３５. Because preliminary studies indicated that an endoglin deletion mutation lacking the carboxyl-terminal PDZ liganding motif (ΔPDZ (21)) was differentially phosphorylated, the ΔPDZ construct was similarly mutated. Table 1 lists the endoglin CD mutations used in the following experiments.

Wt endoglin, 634AA⁶３５, ΔPDZ, and a compound 634AA⁶３５-ΔPDZ construct were co-expressed with HA epitope-tagged TβRII, caALK1, or caALK5 in HEK293T cells. Western blotting with anti-HA antibody (Fig. 2, fourth panel) demonstrated that the type I/II receptors were associated with endoglin and equivalently co-immunoprecipitated, indicating that the ability of endoglin to interact individually with TβRII, caALK1, and caALK5 was independent of the endoglin cytosolic domain mutation tested.
Mass spectrometry reveals multiple sites of phosphorylation within the endoglin cytosolic domain. Quadrupole time-of-flight mass spectrometry of the peptide digest derived from sequential tryptic and Glu-C proteolytic digestion of human endoglin obtained by immunoprecipitation following expression in HUVECs. A and B, HUVECs infected with adenovirus bearing caALK1 and endoglin. Insets show mass spectra of (A) singly and (B) doubly phosphorylated peptides obtained following digestion of endoglin with Glu-C protease. Calculated (Calc.) and experimentally (Exp.) observed masses are indicated.

**TABLE 1**
Cytosolic domain sequences of endoglin mutations
Substituted residues are in boldface type.

| Name        | Protein sequence                        |
|-------------|-----------------------------------------|
| Wt, l-isoform | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| 634AA       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| 635DA       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| ΔPDZ        | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| Δ5A, Δ5ΔPDZ | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| Δ645        | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| Δ632        | WT                                      |
| ΔC          | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| T617A       | WYIYSHARSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| T640A       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| T647A       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| T650V       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| T664A       | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| 640–650     | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| 617–640–650 | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| 617–640–650–654 | WYIYSHTRSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
| ΔThr5       | WYIYSHARSPKPVAVAAPASSSTTHSNGTQSTPCSTSSMA |
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Western blot analysis demonstrated serine phosphorylation of Wt endoglin only by TβRII (Fig. 2, panel 1, lane 3). Basal and TβRII-dependent phosphorylation were restricted to the mature glycosylated extracellular form of Wt endoglin (32, 33), because no serine phosphorylation was detected on the endoglin precursor (panel 1, lanes 2 and 3). Both basal (Fig. 2, panel 1, lane 2 versus 6) and TβRII-dependent serine phosphorylation of endoglin were nearly eliminated in the 634AA635 endoglin protein (lane 3 versus 7), confirming the mass spectrometry data and demonstrating that these residues were the predominant sites phosphorylated by TβRII. Under the conditions employed, neither caALK1 nor caALK5 resulted in endoglin serine phosphorylation above basal levels, indicating that expression of these receptors did not force the phosphorylation of endoglin.

A striking increase in serine phosphorylation of endoglin was detected for all three kinases as a result of removal of the carboxyl-terminal tripeptide in the ΔPDZ construct (Fig. 2, panel 1, lanes 11–13). In contrast to Wt endoglin, phosphorylation of ΔPDZ endoglin occurred on both the low and high mobility endoglin polypeptides. Basal serine phosphorylation of ΔPDZ endoglin in the absence of added receptors was also increased. Receptor-dependent serine phosphorylation of ΔPDZ endoglin was not affected by the introduction of the 634AA635 mutation in the ΔPDZ construct (lanes 15–17), although a reduction in the basal level of phosphorylation of the 634AA635 ΔPDZ construct was evident (lanes 10 versus 14).

To obtain independent structural confirmation that the anti-pSer antibody detected phosphorylation of endoglin by the co-expressed receptors, Western blots were reprobed using an anti-TβRII antibody (α-TβRII). This antibody was raised following immunization with a non-phosphorylated synthetic peptide that encompasses a conserved β-glycan carboxyl-terminal epitope, and is highly cross-reactive with the homologous non-phosphorylated endoglin CD epitope (see legend, Fig. 3A) (34). Western blotting showed roughly equivalent α-TβRII antibody reactivity for high and low mobility forms of endoglin expressed in the absence of exogenous receptor as compared with the α-endoglin antibody (Fig. 2, panel 2, lanes 2, 6, 10, and 14). The partially glycosylated form of Wt endoglin (32) showed little or no difference in reactivity to the α-TβRII antibody, regardless of the presence of co-expressed exogenous caALK1, caALK5, or TβRII, consistent with the lack of α-pSer antibody reactivity of the high mobility form of Wt endoglin. In contrast, the mature form of Wt endoglin demonstrated nearly complete loss of reactivity with the α-TβRII antibody in the presence of caALK1 and TβRII (panel 2, lanes 3 and 4, respectively), although caALK5 caused incomplete loss of α-TβRII antibody reactivity (panel 2, lane 5). This result suggested that caALK1 phosphorylated endoglin, although not necessarily on serine residues. The antibody reactivity for Wt versus ΔPDZ endoglin,
probed with the α-TβRIII antibody, was indistinguishable (Fig. 2, α-TβRIII, lanes 2–5 versus 10–13), suggesting that the sites of phosphorylation of ΔPDZ endoglin remained within the α-TβRIII epitope (residues 642–656, Fig. 3A). However, additional nearby residues were likely phosphorylated.

Finally, Western blotting of the co-immunoprecipitate for total endoglin revealed a reduction in the level of the mature ΔPDZ endoglin protein, relative to the immature form. There was also a slight increase in the immature form of endoglin corresponding to the ΔPDZ constructs, suggesting that the ΔPDZ endoglin precursor was not as efficiently glycosylated as Wt endoglin.

Deletion Mutagenesis Indicates Phosphorylation of Distal Endoglin CD Residues—Because the endoglin CD consists of many serine residues that could be phosphorylated following loss of the PDZ-liganding domain (Fig. 3A), we constructed sequential deletion mutations to assess the contribution of blocks of residues to phosphorylation. The primary structures of the endoglin deletion mutations are depicted in Fig. 3A. Phosphoserine Western blotting of these constructs, following co-expression with either caALK1 or caALK5, is shown in Fig. 3B. As observed above, ΔPDZ endoglin demonstrated a strongly enhanced pSer signal with caALK1 and less so with caALK5. Wt endoglin showed basal serine phosphorylation, which was consistent with the previous data, indicating minimal serine phosphorylation of endoglin by either caALK1 or caALK5 under these conditions. In contrast to ΔPDZ endoglin, the Δ645 endoglin deletion mutation showed serine phosphorylation that was slightly above basal levels. This result indicated that pSer residues within the 646–655 region were sites of phosphorylation of ΔPDZ endoglin by caALK1 and caALK5. Western blotting of endoglin deletion constructs using the α-TβRIII antibody (lower panel, Fig. 4B) whose epitope (underlined, Fig. 3A) is deleted in the Δ645 construct, confirmed that serine phosphorylation of ΔPDZ involved primarily distal residues. The Δ632 endoglin mutation reduced the pSer signal to basal levels, suggesting the loss of additional pSer sites within the serine-rich ASSESSS motif. These results suggested that deletion of the three amino acids comprising the putative PDZ-liganding caused up-regulation of phosphorylation of distal endoglin serine residues Ser646, Ser649, Ser653, and Ser655.

Endoglin Is an ALK1 Kinase Substrate—Because endoglin interacted with caALK1 with consequent loss of α-TβRIII antibody reactivity, the absence of detectable serine phosphorylation of endoglin by caALK1 was surprising. To investigate this further, endoglin was co-expressed with either caALK1 or kdALK1 (K229R) (kdALK1), and the interacting proteins were purified by co-immunoprecipitation using anti-endoglin antibody. The isolated, pre-formed endoglin-ALK1 complexes were incubated with [γ-32P]ATP, separated by gel electrophoresis, and were autoradiographed. Expression of endoglin alone resulted in basal incorporation of 32P into endoglin (Fig. 4, lane 1). However, co-expression of endoglin with caALK1 resulted in a strong signal for 32P incorporation into endoglin (Fig. 4, lane 2), indicating that caALK1 contributed to endoglin phosphorylation. Endoglin phosphorylation was directly attributable to the kinase activity of caALK1, because only basal 32P incorporation was observed if endoglin was co-transfected with kdALK1. These results support the view that ALK1 interacts with and phosphorylates endoglin.

In these experiments, we consistently observed a labeled band corresponding to caALK1 that was not present in kdALK1, suggesting that caALK1 underwent autophosphorylation. Although caALK5 undergoes autophosphorylation (35), this activity has not been reported for caALK1.

caALK1 Phosphorylates Endoglin on Threonine Residues—The TGFβ receptors are protein serine and threonine kinases (36, 37). Although Smad activation following serine phosphorylation by ALK1 (38) and ALK5 (28, 39) is well understood, little is known about the relevance of threonine phosphorylation of non-Smad S/T receptor kinase substrates. Because caALK1 expression resulted in 32P incorporation but did not increase pSer levels as determined by immunoblotting, we used an α-pThr antibody to investigate the possibility of endoglin threonine phosphorylation by caALK1 and caALK5. As shown in Fig. 5A, Western blotting using α-pThr antibody revealed a strong pThr signal in response to caALK1. The pThr signal appeared to be specific for caALK1, because it was not evident for caALK5 under these conditions (Fig. 5A, lane 2 versus 1). The absence of detectable threonine phosphorylation of ΔC endoglin (Fig. 5A, lanes 5 and 6) confirmed that all pThr sites were located in the endoglin CD. We routinely noted that at equivalent levels of receptor DNA transfected, we saw an ~2-fold lower level of expression of caALK5 versus caALK1 (Figs. 5 and 6), as determined using an anti-HA antibody for Western blotting. However, densitometric analysis of blots (data not shown) consistently indicated that this difference (2-fold) was much less than the preference for endoglin threonine phosphorylation by caALK1 versus caALK5 (>20-fold,
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Figs. 5 and 6. These results indicated that caALK1 but not caALK5 phosphorylated endoglin on threonine residues.

Threonine Phosphorylation of Endoglin Requires Phosphorylation of Ser634/Ser635—An unanticipated finding was that threonine phosphorylation of endoglin by caALK1 was nearly completely abrogated in the serine phosphorylation-defective 634AA635 endoglin construct (Fig. 5A, lane 3). Site-specific mutagenesis producing either S634A or S635A mutations partially suppressed endoglin threonine phosphorylation by caALK1, with the S634A substitution being slightly more inhibitory than S635A (data not shown).

To establish whether the reduction of caALK1-dependent threonine phosphorylation in the 634AA635 endoglin construct was due to the loss of serine or phosphoserine, residue Ala634 was replaced with a phospho-mimicking aspartyl residue, resulting in 634DA635 endoglin. As shown in Fig. 5B, the 634DA635 mutation restored caALK1-dependent threonine phosphorylation, as well as basal threonine phosphorylation in the absence of added receptor. This result indicated that threonine phosphorylation of endoglin required serine phosphorylation of one or both residues Ser634 and Ser635.

ALK1-dependent Phosphorylation of Endoglin Occurs Preferentially at Thr640 and Thr654—To further investigate the differential endoglin threonine phosphorylation by caALK1 versus caALK5, threonine residues Thr617, Thr640, and Thr650 were mutated either individually or in combination. caALK1 preferentially threonine phosphorylated Wt and all mutated constructs, as compared with caALK5 (Fig. 6). For this set of mutations, reduction of the caALK1-dependent pThr signal was detected for the T640A mutation (Fig. 6, lanes 8–10). Mutation of Thr617 resulted in a slight decrease in pThr signal. Mutagenesis of residue Thr650 or combined mutagenesis Thr640 and Thr650, indicated that Thr650 was not a prominent site of phosphorylation by caALK1. The T640A mutation in combination with T617A and T650V (lanes 17–19) reduced the pThr signal from caALK1-dependent endoglin phosphorylation, suggesting that caALK1 also phosphorylated residue Thr617. These results indicated that caALK1, in contrast to caALK5, preferentially phosphorylated endoglin at multiple pThr sites.

In contrast to the 634AA635 mutation, no single threonine mutation tested (Fig. 6, shaded boxes) eliminated the caALK1-dependent pThr signal. Therefore, we undertook more extensive mutagenesis of endoglin. caALK1 was co-transfected with endoglin single-site mutations (T617A, T640A, T647A, T650V, and T654A), selected triple (T617A/T640A/T650V), quadruple (T617A/T640A/T650V/T654A) mutations, and a construct lacking all five endoglin CD threonine residues, ΔThr5. The single-threonine point mutations (Fig. 7, shaded boxes) T640A and T654A showed the greatest loss of threonine phosphorylation, suggesting that these endoglin CD threonine residues were preferentially phosphorylated by caALK1. The single point mutations T617A and T647A demonstrated weaker reduction in the pThr signal. Comparison of endoglin constructs bearing mutations at three (Fig. 7, lanes 15 and 16) or four (lanes 17 and 18) sites revealed a progressive reduction of pThr signal, supporting Thr654 as a prominent site of threonine phosphorylation, and confirmed Thr647...
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as a site of phosphorylation. Mutagenesis of all threonine residues (lanes 19 and 20) completely abrogated the pThr signal, indicating that the α-pThr antibody specifically detected pThr in the endoglin CD. Site-specific and deletion constructs suggest that specific threonine phosphorylation by caALK1 occurred principally at residue Thr654 near the carboxyl terminus of endoglin.

Endogenous ALK1 Threonine-phosphorylates Endoglin following TGFβ1 Treatment—To further characterize the role of ALK1 in the phosphorylation of endoglin, HUVECs were used because they express both endoglin and ALK1 (40). HUVECs were incubated in the presence or absence of TGFβ1, because this ligand induces ALK1-dependent phosphorylation of Smad1 in vitro (16, 38). Immunoprecipitation of endogenous endoglin following exposure to TGFβ1 was subjected to in vitro kinase analysis, as described above, to detect co-immunoprecipitated receptor kinase. The endoglin immunoprecipitate that was isolated following exposure to TGFβ1 exhibited increased radiophosphate incorporation from [γ-32P]ATP (Fig. 8A). Western blot analysis of immunoprecipitated endoglin revealed increased threonine phosphorylation in response to TGFβ1. The presence of co-immunoprecipitated endogenous ALK1 was demonstrated by Western blotting using a polyclonal antibody to human ALK1. Interestingly, the complex between endoglin and ALK1 was not isolated unless TGFβ1 was added, suggesting that the endoglin-ALK1 complex was induced by ligand in HUVECs (Fig. 8B).

Endoglin Threonine Residues Are Required for Rescue of caALK1-induced Detachment and Anti-proliferative Effects—caALK1 expression causes cell detachment and inhibition of endothelial cell growth in vitro (41). Therefore, we hypothesized that a high ratio of caALK1 activation relative to endogenous endoglin levels achieved using caALK1 in HUVECs contributed to reduced viability of endothelial cells. This hypothesis predicts that up-regulation of endoglin expression will balance ALK1 activity and restore normal HUVECs adhesive interactions and proliferation. Moreover, if threonine phosphorylation plays a role in the regulation of ALK1, then endoglin constructs that are deficient in threonine phosphorylation should be loss-of-function mutations in this context. To test these possibilities, we examined the ability of Wt and ΔThr5 endoglin to compensate for caALK1-dependent inhibition of HUVECs viability.

To confirm threonine phosphorylation of endoglin in primary endothelial cells, HUVECs were infected with pAdLox bearing Wt endoglin, with and without co-infection with pAd-Lox bearing caALK1 or β-galactosidase control. As shown in Fig. 9A, lane 2, transduced Wt endoglin showed a detectable pThr signal in the absence of exogenous receptor. This result indicated that endoglin was threonine-phosphorylated by an endogenous kinase in HUVECs, likely ALK1, which is specifically expressed in these cells (40, 42). Co-infection with endoglin and caALK1 resulted in an increased pThr signal (Fig. 9A, lane 3), indicating that caALK1 threonine phosphorylated endoglin in HUVECs. A very weak pThr signal was detected on endogenous endoglin (Fig. 9A, lane 1). The weakness of this signal is likely due to low abundance of endogenous endoglin-ALK1 complex in unstimulated HUVECs.

Additional controls were necessary to validate transduced protein expression levels. Western blot analysis of Wt and ΔThr5 endoglin expression in adenovirus-infected HUVECs (Fig. 9B) demonstrated that Wt and ΔThr5 endoglin were expressed at equivalent levels under the conditions employed. The lower panel of Fig. 9B shows the result of probing with the α-TβRIII antibody. The absence of α-TβRIIIB antibody reactivity confirmed expression of endoglin bearing the ΔThr5 mutation. To exclude the possibility that caALK1 expression altered endoglin protein levels, HUVECs were subjected to Western blot analysis following co-infection with caALK1 or kdALK1,
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A

| AdLox-Wt    | + | - | + |
| AdLox-caALK1 | - | + | - |
| AdLox-βGal  | + | - | - |
| IP: α-END   | + | - | - |
| WB: α-βEnd  | + | - | - |
| TCL: α-END  | + | - | - |
| WB: α-HA    | + | - | - |

B

| AdLox-Wt    | + | - | - |
| AdLox-ΔThr5 | - | - | + |
| AdLox-Wt    | + | - | - |
| IP: α-END   | + | - | - |
| WB: α-βEnd  | + | - | - |
| TCL: α-END  | + | - | - |
| WB: α-HA    | + | - | - |

C

| AdLox-caALK1 | + | - | - |
| AdLox-ΔThr5  | - | - | + |
| AdLox-Wt     | + | - | - |
| IP: α-END    | + | - | - |
| WB: α-βEnd   | + | - | - |
| TCL: α-END   | + | - | - |
| WB: α-HA     | + | - | - |

D

βGal  
kdALK1  
caALK1

E

βGal  
caALK1

βGal  
Wt  
ΔThr5

F

G

Cell Count / Field

| βGal   | + | + | - | - |
| AdLox-caALK1 | - | + | + | + |
| AdLox-Wt     | + | - | + | + |
| AdLox-ΔThr5  | - | - | - | + |

C.P.M. x 10^3

| caALK1  | + | - | - | - | - | - |
| AdLox-βGal | + | + | + | - | - | - |
| AdLox-Wt   | + | + | + | + | - | - |
| AdLox-ΔThr5 | - | - | - | + | + | + |
and either Wt or ΔThr5 endoglin. At the highest multiplicity of infection (pfu 300) used for infection of HUVECs with pAdLox caALK1, Western blotting confirmed that endoglin (Wt or ΔThr5) levels were not perturbed in the presence of the co-expressed protein (Fig. 9C). Western blot analysis of caALK1 and kdALK1 at similar multiplicity of infection consistently resulted in a much stronger signal for kdALK1 versus caALK1 (Fig. 9C). This effect was likely due to detachment of caALK1-expressing HUVECs. In addition, pulse-chase measurement of [35S]cysteine/methionine incorporation into immunoprecipitated endoglin revealed no difference in the half-lives of Wt and ΔThr5 endoglin (data not shown). Therefore, differences in the ability of Wt versus ΔThr5 endoglin to rescue caALK1-dependent inhibition of HUVECs growth could not be attributed to differences in Wt or ΔThr5 endoglin levels.

Infection of HUVECs with caALK1 dramatically increased HUVEC detachment as compared with the β-galactosidase control, at 28 h post-infection. This effect was due to the kinase activity of caALK1, because kdALK1 had no effect on HUVEC proliferation (Fig. 9D). We next compared the ability of Wt and ΔThr5 endoglin to rescue caALK1-dependent growth inhibition. The first column of Fig. 9E indicated that co-infection of HUVECs with β-galactosidase and either Wt or ΔThr5 endoglin had no apparent effect on cell growth or detachment at 48 h post infection. In contrast, adenoviral-mediated transduction of caALK1 reduced the number of cells at 48 h post infection (Fig. 9E, first row). In addition, caALK1-infected HUVECs exhibited a rounded morphology and were readily detached from the culture dish, suggesting that caALK1 expression caused a loss in cell adhesive capacity (41). However, co-infection of HUVECs with caALK1 and Wt endoglin restored cell numbers and cell morphology close to that demonstrated for endoglin alone (Fig. 9E, second versus first row). In contrast, co-infection of HUVECs with caALK1 and ΔThr5 endoglin failed to protect against cell rounding or loss (Fig. 9E, third row), indicating that the protective influence of endoglin required intact endoglin threonine residues.

caALK1-dependent reduction in viability of vascular endothelial cells is attributed to combined down-regulation of adhesion proteins (zyxin and paxillin) and up-regulation of cyclin-dependent kinase inhibitor p21 (41). We therefore conducted cell counting and [3H]thymidine incorporation measurements on HUVECs that were co-transduced with caALK1 and Wt or ΔThr5 endoglin. As shown in Fig. 9F, cell counting confirmed the trends seen in Fig. 9E, indicating that ΔThr5 endoglin was significantly less effective than Wt endoglin in terms of protection against the reduction in cell number resulting from co-expression of caALK1. Cell proliferation, as measured by [3H]thymidine incorporation into HUVEC DNA, was not significantly different from control, following infection with pAdLox-Wt endoglin alone (Fig. 9G, lanes 1 and 2). Consistent with cell counting data, pAdLox-Wt endoglin was significantly more effective than pAdLox-ΔThr5 endoglin at reducing the inhibition of [3H]thymidine incorporation. This effect was more pronounced at moderate and high levels of caALK1 adenovirus, indicating a dose dependence for endoglin relative to caALK1 (Fig. 9G). The upper inset of Fig. 9G shows a representative Western blot result for caALK1 corresponding to the levels of transduced pAdLox-caALK1, whereas the lower inset shows that the levels of pAdLox-Wt and -ΔThr5 endoglin were approximately equivalent. These results confirmed previous studies (41) and support the hypothesis that endoglin threonine phosphorylation by ALK1 plays a role in the modulation of ALK1-dependent adhesive and proliferative effects in vascular endothelial cells, potentially via cytosolic domain-dependent, proadhesive properties of endoglin (21, 43).

The affinity of endoglin for sites of focal adhesion involves interaction of its extracellular domain with cell surface-expressed focal adhesion-localized proteins (21), potentially including integrins (43). We hypothesize that endoglin engages cytosolic adhesion-associating proteins via cytosolic domain-mediated interactions (21) and that these interactions are modulated by phosphorylation. To test this hypothesis, we examined the ability of Wt and ΔThr5 endoglin to associate with sites of focal adhesion obtained using RGD-labeled magnetic microspheres (21, 22), under conditions that provide endogenous or constitutive (caALK1) phosphorylation. We predicted that phosphorylation would promote exit of endoglin from sites of FA. In transiently transfected GM7372 bovine endothelial cells (21), Wt endoglin associated with the FA-associated protein fraction, and this association was unaffected in the presence of kdALK1, demonstrating that minimally phosphorylated endoglin associated with FAs. In contrast, caALK1 expression resulted in loss of endoglin from the FA-associated protein fraction. As observed for cytosolic domain-deleted ΔC endoglin (21), the non-phosphorylatable ΔThr5 endoglin mutant polypeptide associated with FAs. However, the association of ΔThr5 endoglin with the FA-associated protein fraction was

**FIGURE 9.** Wt and ΔThr5 endoglin differentially affect caALK1-dependent inhibition of HUVEC proliferation. A–C, Western blots were developed 48 h post infection. A, HUVECs were infected with pAdLox adenosine bearing Wt endoglin (pAdLox-Wt), β-galactosidase (pAdLox-BGal), or caALK1 (pAdLox-CaALK1). Immunoprecipitation of endoglin and Western blot analysis were conducted as described above using the indicated antibodies. B, HUVECs were infected with pAdLox adenosine bearing either Wt or ΔThr5 endoglin, and the resulting protein lysates were subjected to Western blotting with the indicated antibodies. C, HUVECs were co-infected with pAdLox-caALK1 or pAdLox-kdALK1 (multiplicity of infection = 300), together with either pAdLox-Wt or ΔThr5 endoglin. As above, protein lysates were subjected to Western blot analysis. D and E, photomicrographs (40× magnification) of representative fields of HUVECs. D, photomicrographs taken 28 h following transduction of HUVECs with adenosine bearing β-galactosidase, kdALK1, or caALK1. E, HUVECs were transduced with adenovirus bearing either β-galactosidase or caALK1, and co-transduced with Wt or ΔThr5 endoglin. Photomicroscopy and Western blots were conducted at 48 h post infection. F and G, HUVECs were seeded at 4 × 10^4 cells per milliliter in serum-containing media, and counted daily in triplicate using a Coulter counter. F, cell counting was conducted on HUVECs remaining after 48 h of infection with adenosine (100 pfu) bearing the indicated cDNAs. Data shown is representative of three separate experiments. Error bars represent the standard deviation of the mean. G, cells were seeded at 1 × 10^4 cells/ml in 6-well plates, and treated with the indicated pAdLox adenovirus for 24 h in 10% serum-supplemented EC growth medium. pAdLox (Wt, β-galactosidase, ΔThr5) endoglin were used at 150 pfu. Increasing pAdLox-caALK1 corresponded to 60, 100, and 300 pfu. Upper inset: anti-HA antibody Western blot for caALK1 corresponding to the indicated pAdLox-caALK1 pfu. Lower inset: anti-HA antibody Western blot confirming levels of wild-type and ΔThr5 endoglin. All protein levels were normalized by Bradford assay. [3H]thymidine was added 4 h before cells were harvested. Data are representative of three independent experiments. Error bars represent the 95% confidence interval.
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| A | GM7372 |
|---|-------|
| END:Wt | + + + - - |
| END:ΔThr5 | - - + + + |
| kdALK1 | - + + - - |
| caALK1 | - - + + + |
| Mag-RGD; WB: α-END; IP-α-END; α-HA |

| B | HUVEC |
|---|------|
| AdLoxLacZ | + - - - - |
| AdLox-Wt | - + + - - |
| AdLox-ΔThr5 | - + + + + |
| AdLox-kdALK1 | - + + - - |
| AdLox-caALK1 | - + + + + |
| Mag-RGD; WB: α-END; IP-α-END; α-HA |

FIGURE 10. Focal adhesion-association of Wt but not ΔThr5 endoglin is regulated by caALK1. GM7372-bovine endothelial cells (A) or HUVECs (B) were transiently transfected with the indicated plasmids or adenoviral constructs, respectively. After an additional 48 h, FA-associated protein was isolated using RGD-labeled magnetic microspheres. RGD-microspheres (Mag-RGD) and total cell lysates (TCL) were analyzed for transfected endoglin by Western blotting using anti-endoglin antibody.

insensitive to the expression of caALK1 (Fig. 10A). To confirm this result in a primary endothelial cell, this experiment was repeated using pAdLox-encoded endoglin and receptors in HUVECs, yielding essentially the same result (Fig. 10B). These results support the hypothesis that phosphorylation of the endoglin cytosolic domain by caALK1 is anti-adhesive, because it down-regulates the association of endoglin with the FA protein fraction.

**DISCUSSION**

Mutations in the genes encoding endoglin and ALK1 cause the vascular diseases HHT1 and HHT2, respectively (18, 19). The striking similarities shared by the corresponding mouse models of endoglin (44–46) and ALK1 (47) deficiency suggest that these proteins function in the same pathway. Despite this insight, the function of endoglin and its relationship to ALK1 remain unclear. In the present study, we characterized the role of endoglin as a substrate for the ALK1 serine- and threonine-receptor kinase. Endoglin phosphorylation by ALK5 and TβRII was also studied to provide comparative data on the specificities of these S/T receptor kinases.

TβRII Phosphorylates Endoglin within the Central Serine-rich Motif—TβRII expression resulted in serine phosphorylation of endoglin. Phosphorylation of Ser^534 or Ser^635 by TβRII was confirmed by mutagenesis of the first two serines in the SASESS motif. Ser^634 and Ser^635. Recent reports indicate that TGFβ1 induces serine phosphorylation of endoglin, which was detected as a result of fortuitous recognition of the SASESS motif of endoglin by a phospho-specific NF-κB antibody (31). Because endoglin only binds TGFβ1 in the presence of TβRII (7), these results are consistent with the hypothesis that TβRII contributes to basal endoglin serine phosphorylation.

In contrast to TβRII, co-expression of Wt endoglin with caALK1 or caALK5 did not lead to serine phosphorylation above basal levels. It is important to note that co-immunoprecipitation was conducted under stringent conditions. Under these conditions, the association of endoglin with all three receptors was equivalent and unaffected by truncation or mutation of the endoglin CD. These results suggest that differences in the amount of endoglin-receptor complex formed with specific endoglin CD mutations can be ignored. This view is supported by previous findings indicating that the endoglin extracellular domain is sufficient for interaction with ALK5 and TβRII (7). Therefore, these data suggest that basal endoglin serine phosphorylation is a property of the TβRII complex with endoglin. These data do not exclude the possibility of ALK1- or ALK5-dependent pSer sites that were not detectable by Western blotting. However, the robust detection of pSer obtained in the case of ΔPDZ endoglin suggests that Western blotting of

Because TβRII is a constitutively active receptor that interacts with endoglin in the absence of ALK5 (7), phosphorylation of Ser^634 or Ser^635 may reflect constitutive phosphorylation of endoglin that occurs in the absence of ligand. The serine-rich cluster ^633ASSESSS^639 comprises a region of the endoglin CD that is non-homologous to the CD of the other type III receptor, β-glycan. Recent studies identified two related endoglin-specific CD-interacting proteins, zyxin (21) and ZRP-1 (48), that do not interact within the β-glycan CD. Therefore, future experiments will be conducted to determine if receptor-mediated phosphorylation within the endoglin serine-rich cluster is involved in the regulation of these protein-protein interactions by phosphorylation.

Endoglin PDZ-liganding Regulates Endoglin Phosphorylation—The second finding is that removal of the putative carboxyl-terminal PDZ-liganding motif greatly increased detection of endoglin serine phosphorylation mediated by caALK1, caALK5, and TβRII. In contrast to Wt endoglin, the phosphorylation of ΔPDZ endoglin occurred principally on residues outside of the ASSESSS motif, as shown by the Δ^645 mutation. It is possible that the level of ΔPDZ endoglin phosphorylation was due to the unmasking of a pSer epitope. However, several lines of evidence suggest that this interpretation is insufficient. First, the anti-pSer antibody was generated using a synthetic phosphopeptide bearing an internal pSer residue, suggesting that a carboxyl-terminal phosphoserine may not be as efficiently detected. Second, we observed a consistent, though less robust, enhancement in endoglin pThr by caALK1 (data not shown), indicating that the effect of the loss of the PDZ-liganding domain was broader than α-pSer antibody detection of pSer. Third, deletion of the neighboring block of serine and threonine residues eliminated the hyperphosphorylation, even though several distal serine residues remained that could be detected.

The data suggested that the loss of the endoglin PDZ-liganding domain resulted in additional functional consequences. Removal of the PDZ-liganding domain resulted in a shift in the detection of pSer from the mature form of Wt endoglin to both
mature and high mobility immature endoglin in the ΔPDZ construct. This result suggests that the PDZ-liganding motif controls trafficking or glycosylation of endoglin. Removal of the PDZ-liganding domain also resulted in a decrease in the level of total mature endoglin protein but not partially glycosylated endoglin. However, cell surface biotinylation experiments in both HEK293T and COS7 cells failed to demonstrate a reduction in steady-state surface expression of the ΔPDZ endoglin (data not shown). These results suggest that the endoglin carboxyl-terminal tripeptide is an important functional motif within the endoglin cytosolic domain, sequestering endoglin from phosphorylation prior to surface expression, and potentially regulating the level or rate of glycosylation. However, for Wt endoglin, phosphorylation did not appear to play a direct role in the expression or maturation of endoglin in transiently transfected HEK293T cells or in the adenovirus-transduced HUVECs.

PDZ domain-containing proteins are characterized as adapter or scaffolding proteins that interact with PDZ-liganding motifs and coordinate formation of protein-protein interactions (49). Our results suggest a model in which endoglin’s PDZ-liganding domain mediates an interaction with another protein, leading to steric repression of phosphorylation. Alternatively, this motif may participate in a protein-protein interaction that results in endoglin’s sequestration from receptor kinases. The TGFβ type III receptor, β-glycan, possesses a PDZ-liganding motif, which is required for its interaction with GIPC/Synectin (50). GIPC/Synectin is a potential candidate for the regulation of endoglin phosphorylation, because it also regulates the surface expression of β-glycan (51). Alternatively, an unidentified interacting protein may be required for endoglin sequestration from protein kinases.

ALK1 Specifically Phosphorylates Endoglin on Threonine Residues—A third finding was that receptor-mediated threonine phosphorylation of endoglin is a distinct feature of ALK1 in HUVECs. Previous phospho-amino acid labeling studies noted serine phosphorylation but little threonine phosphorylation of endoglin (8, 9). However, experimental differences may explain these potential discrepancies. In the first instance, endoglin derived from porcine endothelial cells showed predominantly serine phosphorylation. However, endoglin phosphorylation showed no significant induction by 100 ng/ml TGFβ1 (8), suggesting that the ligand-independent constitutive activity of TβRII, and not ligand-dependent ALK1 activity, accounts for the observed serine phosphorylation of endoglin. In the second instance, the prevalence of pSer was observed on endoglin expressed in fibroblasts and may reflect the absence of the endothelial cell-restricted ALK1 receptor (45, 52), though the ubiquitously expressed TβRII receptor was likely present (9). These studies, and the data presented above are consistent with the hypothesis that endoglin serine phosphorylation is constitutive, and threonine phosphorylation follows activation of ALK1.

Endoglin Serine Phosphorylation Is Required for Threonine Phosphorylation—A fourth finding is the unexpected result that mutagenesis of serine residues Ser634 and Ser635 to alanine abolished and subsequent phospho-mimicking aspartate substitution of Ser634 restored threonine phosphorylation of endoglin by caALK1. These results, along with preferential serine phosphorylation of Ser634 and Ser635 by TβRII, suggest a novel mechanism for the sequential control of endoglin phosphorylation; that is, TβRII-dependent phosphorylation of the central serine-rich motif of endoglin is a requirement for caALK1-mediated phosphorylation of more carboxyl-terminal threonine residues. Based on the exhaustive threonine mutagenesis and Western blotting results presented, it is unlikely that pThr residues were present but undetected in the 634AA635 construct. This sequential pattern of phosphorylation, as well as the propensity for the mature glycosylated endoglin to be phosphorylated, suggests a mechanism whereby threonine phosphorylation is normally blocked until endoglin becomes associated with TβRII on the cell surface. Serine phosphorylation as a requirement for threonine phosphorylation represents a potentially novel mechanism for regulation mediated by the TGFβ serine/threonine receptor kinases. Smad1 and Smad5, which are downstream effectors of ALK1, are phosphorylated on serine residues (9). The observation that ALK1 predominantly threonine phosphorylates a serine- and threonine-rich subregion of endoglin CD suggests that threonine phosphorylation is a novel and specific property of ALK1 that is utilized for Smad-independent functions mediated by the TGFβ receptors, such as cell adhesion (21) or cell detachment (43).

ALK1 Phosphorylates Endoglin on Specific CD Threonine Residues—Detailed mutagenesis mapping of the endoglin CD confirmed preferential threonine phosphorylation of endoglin by caALK1 versus caALK5, at essentially all phosphorylated endoglin threonine residues. Mutagenesis data indicated that distal endoglin residues Thr640, Thr647, and Thr654 were preferred caALK1 targets; whereas residues Thr617 and Thr650 did not appear to be major sites of phosphorylation by caALK1. The overall dominance of caALK1 versus caALK5 for endoglin threonine phosphorylation suggests that this is a functional distinguishing property of the ALK1-endoglin complex.

The interaction of endogenous endoglin and ALK1, and threonine phosphorylation in response to the ligand TGFβ1, was confirmed using immunopurified endoglin-bound components from primary HUVECs. It is interesting that ligand treatment not only increased total radiophosphate incorporation and phosphothreonine levels but appeared to facilitate the formation of the endoglin-ALK1 complex itself. We note that caALK1 was immunoprecipitated with endoglin in the absence of added ligand. This result suggests that activation of the ALK1 receptor promotes its association with endoglin, under the conditions used here. These results do not preclude the presence of another protein kinase in the immunoprecipitated endoglin complex. However, based on our other data, ALK1 is the only type I receptor tested that threonine phosphorylates endoglin. It is important to note that our results do not indicate that TGFβ1 solely mediates endoglin threonine phosphorylation in vivo. Although TGFβ1–3 are established ligands for TβRII/ALK1, it has been reported that bone morphogenetic protein 9 binds ALK1 and the bone morphogenetic protein type II receptor (53), suggesting that bone morphogenetic protein 9 or related ligands play a role in ALK1 signaling.
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To date, no HHT patients have presented with endoglin CD mutations, suggesting that this is a rare event. Mutated forms of endoglin that are associated with HHT are thought to cause instability of the endoglin protein, resulting in the reduced expression of endoglin and haploinsufficiency (32, 54, 55). With the possible exception of ∆PDZ endoglin, discussed above, none of the endoglin loss- or gain-of-function phosphorylation mutations tested here were unstable as measured by expression in vitro. This result suggests that, although mutation of the endoglin extracellular or transmembrane domains produce misfolding and loss of expression (32, 33), endoglin CD mutations may alter endoglin phosphorylation but not total protein levels.

Mutations in the endoglin CD result in loss-of-function by a different mechanism. For example, CD deletion mutations in endoglin might heterodimerize with the wild-type protein, resulting in a reduction of the glycosylated mature protein (32). An alternative mechanism is suggested by recent investigations that identified novel CD-dependent functions for endoglin in the modulation of the actin cytoskeleton and cell migration (21, 48). These studies demonstrated that the endoglin CD specifically interacts with the LIM domains of zyxin and ZRP-1, respectively. The expression of endoglin results in redistribution of zyxin from sites of cellular focal adhesion (21) and changes in the organization of actin filaments (48). Indeed, recent studies confirmed that the carboxyl-terminal domain of endoglin is required to inhibit cell detachment (43). An adhesive model of endoglin function explains the existence of a large molar excess of endoglin relative to TGFβ receptors in some endothelial cells (56). This model predicts that endoglin haploinsufficiency, which occurs in HHT1 (54), leads to TGFβ- and Smad-independent cellular effects due to the stoichiometric reduction of endoglin CD-protein interactions required for cell migration (21), maintenance of cell structure and integrity (48), or cell-cell adhesion (43).

Endoglin Rescues caALK1 Kinase-dependent Cell Detachment and Inhibition of Cell Proliferation—Overexpression of caALK1 in human microvascular endothelial cells (41) and HUVECs (present study) caused a dramatic loss of HUVEC viability. Decreased cell viability correlated with detachment of cells exhibiting a rounded cell morphology and inhibition of cell proliferation. Our data using kdALK1 demonstrated that the growth-inhibitory effect of caALK1 required an intact kinase activity. This result precluded the possibility that adenovirus-mediated expression of this protein is simply poorly tolerated in HUVECs. Our data also showed that WT endoglin expression was able to rescue the growth-inhibitory effects of increased ALK1 kinase activity in HUVECs, suggesting that a critical balance and not absolute levels of ALK1 activation and endoglin expression is required for HUVEC growth and morphology. In contrast, ∆Thr5 was significantly less effective than WT endoglin at attenuating cell detachment and antiproliferative effects of caALK1 in HUVECs.

It is not yet clear how WT endoglin protects HUVECs from caALK1 expression. Our data corroborate other studies (41) that clearly demonstrate a role for ALK1 in endothelial cell detachment and inhibition of proliferation. Our results demonstrate that endoglin opposes caALK1-dependent cell detachment and growth inhibition and the need for intact CD threonine residues for this effect. The ability of ALK1 to phosphorylate endoglin suggests that high levels of phosphorylated endoglin contribute to cell detachment. This is an appealing hypothesis because it is supported both by studies of the role of endoglin CD in the modulation of cell migration and focal adhesion-associated protein composition (21) and f-actin microfilament organization (48), as well as studies that demonstrate that cell detachment is opposed by up-regulation of endoglin, with the requirement for an intact cytosolic domain (43). A potential mechanism for these effects of endoglin is suggested by our observation of caALK1-dependent threonine phosphorylation of the endoglin CD, coupled with the observed caALK1-dependent down-regulation of the association of wild-type, but not ∆Thr5 endoglin with a FA-associated protein fraction in two endothelial cell types. Therefore, these studies support the hypothesis that increased endoglin expression in the presence of activated ALK1 rescues HUVEC detachment and growth inhibition by permitting proadhesive re-engagement of endoglin in sites of focal adhesion. We suggest that this mechanism is part of a novel feedback regulatory loop for the regulation of ALK1, because endoglin has been reported to be a transcriptional target of ALK1 (57) and because patients with ALK1 deficiency (HHT2) show decreased levels of endoglin (58).

These data suggest a model in which ALK1 activity is balanced by its participation in the direct threonine phosphorylation of endoglin. Although ALK5 associated with endoglin, it was not efficient in serine or threonine phosphorylation suggesting that ALK5-associated endoglin is less phosphorylated than ALK1-associated endoglin. This hypothesis predicts that endoglin phosphorylation is governed by the relative levels of ALK1 and ALK5. However, ALK1 and ALK5 have non-overlapping patterns of expression in vivo. ALK1 is specific to endothelial cells during development that lack ALK5 (42). Therefore, our model predicts that threonine-phosphorylated endoglin will be present in endothelial cells with activated ALK1. Conversely, because endoglin is up-regulated following vascular injury (59) or in atherosclerosis (60) in ALK5-positive and ALK1-negative (42) vascular smooth muscle cells, endoglin devoid of threonine phosphorylation may predominate in these cells. Our results suggest that cell-specific differences in the extent of endoglin phosphorylation could also arise from differences in endoglin-interactor PDZ-domain protein expression, different patterns of expression of other endoglin CD interacting proteins (21, 48), or in endoglin haploinsufficiency.

This study provides new insights into the complex relationship of endoglin structure and its function in terms of TGFβ receptor-mediated phosphorylation. The data presented are consistent with a model of endoglin post-translational modification in which serine and threonine phosphorylation occur as a multistep process involving mature endoglin. In this model, threonine phosphorylation of the endoglin cytosolic domain by ALK1 follows serine phosphorylation and plays a role in the regulation of the ALK1-dependent adhesive and proliferative effects on endothelial cells. These results suggest that either endoglin or ALK1 haploinsufficiency can lead to reduced threonine-phospho-
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rlylated endoglin, thereby constituting a potential common molecular feature of HHT1 and HHT2.

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