Soluble Endoglin Specifically Binds Bone Morphogenetic Proteins 9 and 10 via Its Orphan Domain, Inhibits Blood Vessel Formation, and Suppresses Tumor Growth*

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Endoglin (CD105), a transmembrane protein of the transforming growth factor β superfamily, plays a crucial role in angiogenesis. Mutations in endoglin result in the vascular defect known as hereditary hemorrhagic telangiectasia (HHT1). The soluble form of endoglin was suggested to contribute to the pathogenesis of preeclampsia. To obtain further insight into its function, we cloned, expressed, purified, and characterized the extracellular domain (ECD) of mouse and human endoglin fused to an immunoglobulin Fc domain. We found that mouse and human endoglin ECD-Fc bound directly, specifically, and with high affinity to bone morphogenetic proteins 9 and 10 (BMP9 and BMP10) in surface plasmon resonance (Biacore) and cell-based assays. We performed a function mapping analysis of the different domains of endoglin by examining their contributions to the selectivity and biological activity of the protein. The BMP9/BMP10 binding site was localized to the orphan domain of human endoglin composed of the amino acid sequence 26–359. We established that endoglin and type II receptors bind to overlapping sites on BMP9. In the in vivo chick chorioallantoic membrane assay, the mouse and the truncated human endoglin ECD–Fc both significantly reduced VEGF–induced vessel formation. Finally, murine endoglin ECD–Fc acted as an anti-angiogenic factor that decreased blood vessel sprouting in VEGF/FGF–induced angiogenesis in in vivo angioreactors and reduced the tumor burden in the colon-26 mouse tumor model. Together our findings indicate an important role of soluble endoglin ECD in the regulation of angiogenesis and highlight efficacy of endoglin–Fc as a potential anti-angiogenesis therapeutic agent.

Endoglin (CD105) is a homodimeric glycosylated cell-surface protein of 180 kDa previously identified as a co-receptor belonging to the TGFβ superfamily (1). Several lines of evidence support an important role of endoglin in cardiovascular development and vascular remodeling (2). Loss-of-function mutations in endoglin are implicated in the vascular disorder hereditary hemorrhagic telangiectasia type 1 (HHT1),3 which is a bleeding disorder characterized by arteriovenous malformations in the brain, lungs, and liver and is attributed to haplinsufficiency (3–5). Homozygous endoglin knock-out mice died during early gestation due to the lack of development of normal mature blood vessels (6). Adult endoglin heterozygous mice (7–8) or mice with a conditional mutation in the endoglin gene (9) exhibited similar angiogenic abnormalities and were used as animal models for HHT1. Mutations in the endoglin gene found in numerous HHT1 patients are localized most exclusively in the extracellular domain (4). The specific role of endoglin in the vascular dysplasia observed in HHT1 patients is not known, but it is likely to be related to the role of TGFβ family signaling in angiogenesis (2, 10). Interestingly, another form of HHT, known as HHT2, which is also characterized by the presence of telangiectases as well as arteriovenous malformations in brain, lungs and liver, results from the loss of TGFβ type I receptor ALK1 (11), which suggests an interrelatedness between endoglin and ALK1 and possibly involvement of the same ligand(s) in the mechanism of action of both molecules.

A soluble form of endoglin has been observed in the serum of patients with different types of solid malignancies (12) and of pregnant women suffering from preeclampsia, a disease leading to vascular permeability (13), hypertension, and placental abruption (14). This soluble form, which reportedly results from partial shedding of the membrane-bound form of endoglin by the matrix metalloproteinase 14 (MT1-MMP) (15), a phenomenon also observed for the other type III receptor betaglycan (16), has been proposed to act as a scavenger or trap for circulating TGFβ family ligands, thus impairing binding to their physiological receptors and normal vasculature development (13). However, the specific ligands for soluble endoglin have not been directly identified.

Despite strong evidence highlighting the role of endoglin in angiogenesis, cancer (12, 17), and preeclampsia, the molecular mechanism of its action remains unclear as the data are somewhat contradictory. Originally, it was demonstrated that endoglin binds to TGFβ1 and TGFβ3 but fails to bind to TGFβ2 in cross-linking experiments performed on HUVEC....

3 The abbreviations used are: HHT, hereditary hemorrhagic telangiectasia; CAM, chick chorioallantoic membrane; ECD, extracellular domain; hEngECD, soluble human endoglin ECD; mEngECD, soluble mouse endoglin ECD; hFc, human Fc; mFc, mouse Fc; SEC, size exclusion chromatography; SPR, surface plasmon resonance; BMP, bone morphogenetic protein; TGFβ, transforming growth factor β.

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cells (18). Co-immunoprecipitation experiments with overexpressed endoglin have shown some binding of radiolabeled activin A, BMP2, and BMP7 to endoglin only in the presence of their respective type I and type II receptors (19), but the role of these complexes is not clear. Some direct binding between the membrane-bound endoglin and BMP9 has also been observed through cross-linking experiments (20). Other studies demonstrated that endoglin specifically binds to type I or II receptors of the TGFβ superfamliy (21) and that this interaction is absolutely required for the association of endoglin with the TGFβ ligand (19, 22, 23). Of particular importance are findings suggesting that type I receptors ALK1 and ALK5 interact with endoglin, promoting the TGFβ/ALK1 signaling pathway, but inhibiting the TGFβ/ALK5 pathway. We (24) and others (25) have recently demonstrated that ALK1 binds specifically and with very high affinity to BMP9 and BMP10; however, the potential role of endoglin in these interactions has not been fully addressed.

Here we describe the use of surface plasmon resonance (SPR)-based assay that allowed us to characterize binding of different TGFβ family ligands to endoglin ECD-Fc (EngECD-Fc) chimeras and assess the effect of such complex formation on the interactions with type I and II TGFβ receptors. We demonstrate for the first time that endoglin-ECD binds directly, specifically, and with high affinity to BMP9 and BMP10. We mapped the binding site of these ligands to the orphan domain of the ECD through the production and evaluation of a series of truncations of the protein and demonstrated that endoglin and type II receptors share the same binding site on BMP9. We further demonstrated the use of EngECD-Fc fusion protein to mediate a significant loss of blood vessel formation in vivo. In the oncology setting we showed that EngECD-Fc inhibits colon-26 tumor growth, suggesting its potential use as an anti-angiogenic therapeutic agent.

**EXPERIMENTAL PROCEDURES**

**Materials**—Biacore 3000, Biacore T100, research grade CM5 chips, amine coupling reagents, 10× HBS-EP buffer, and rabbit anti-mouse IgG were purchased from Biacore (GE Healthcare). Human BMP2, BMP2/7, BMP3, BMP3b, BMP4, BMP4/7, BMP5, BMP6, BMP7, BMP8b, BMP9, BMP10, BMP15, GDF11, Nodal, Artemin, Neurturin, Persephin, GDF3, GDF15, MIS, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, Activin A, Activin B, Activin AC, Activin AB, TGFβ3, and GDF9b were purchased from R&D Systems, and GDF8 was purchased from Fitzgerald Industries Int. Human TGFβ1, TGFβ2, GDF5, and GDF6 were purchased from PeproTech. mEngECD-hFc 27–581 (ECD of mouse endoglin fused to human Fc), hEngECD 26–586 (ECD of human endoglin), and type I (hALK1-hFc, hALK2-hFc, hALK3-hFc, hALK4-hFc, mALK5-hFc, hALK6-hFc, rALK7-hFc) and type II (hTGFβRII-hFc, hBMPRII-hFc, hMISRII-hFc) receptors containing the ECD of the respective receptors fused to human Fc were purchased from R&D Systems and reconstituted as suggested by the manufacturer. hActRIIA-hFc, hActRIIB-hFc, mActRIIB-mFc, mALK1-mFc, and hALK1-His were produced and purified in-house. Goat anti-human Fc-specific IgG antibody was purchased either from Biacore (GE Healthcare), The Jackson ImmunoResearch Laboratories, or Thermo Fisher Scientific. Novex 4–12% gels, Native PAGE gels, and other electrophoresis reagents were from Invitrogen.

Cloning, Expression, and Purification of mEngECD-mFc 27–581, hEngECD-hFc 26–586, and Truncation Variants Thereof from HEK 293 Cells—Mouse (27–581) and human (26–586, 26–437, 26–378, 26–359, 26–332, 26–329, 360–586, and 438–586) endoglin extracellular domain constructs were obtained by PCR amplification of the gene sequence obtained from Open Biosystems (Huntsville, AL). Purified PCR fragments were ligated into pAID4mFc or pAIDhFc vectors to create the pAID4-endoglin expression constructs. The sequences of the endoglin constructs were confirmed by DNA sequencing. mEngECD-mFc 27–581 and hEngECD-hFc 26–586 constructs and truncation variants were transiently transfected into human embryonic kidney (HEK) 293 cells (Invitrogen). Cells were seeded at a density of 0.5 × 10⁶ cells/ml into 500 ml of FreeStyle medium (Invitrogen) in a 3-liter spinner flask. Cells were grown at 37 °C overnight, and a complex of DNA/polyethyleneimine (1 μg/ml) was added to the cells. After 5–8 h, an additional 500 ml of FreeStyle medium was added. After 1 week of growth at 37 °C, the conditioned media was harvested and concentrated for purification.

The filtered conditioned media material containing mEngECD-mFc 27–581, hEngECD-hFc 26–586, or truncated variants of human endoglin was purified by affinity chromatography using mAb Select SuRe protein A chromatography. The protein mEngECD-mFc 27–581 was subsequently purified by Q-Sepharose column chromatography. hEngECD-hFc 26–437 was further purified by flow through a ceramic hydroxyapatite (CHT-II, Bio-Rad) column in 5 mM sodium phosphate, pH 7.0, to remove high and low molecular weight impurities. hEngECD-hFc 26–359 was purified by MabSelect SuRe and ceramic hydroxyapatite columns as described above for hEngECD-hFc 26–437 followed by a Q-Sepharose chromatography as for mEngECD-mFc 27–581. The purified proteins were dialyzed into 10 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.14 (modified TBS). The purity of the protein preparations was analyzed by SDS-PAGE under reducing and non-reducing conditions and visualized by SilverSNAP Stain for mass spectrometry (Pierce). Analytical size-exclusion chromatography (SEC) was performed on Agilent 1100 Series HPLC with a Tosoh column TSK gel G3000 SWxl, 7.88 (ID) × 30 cm (length), 5-μm particle size. The purity of the protein preparations was greater than 95%. The protein concentration was measured at 280 nm with NanoDrop 1000 (Thermo) using the extinction coefficient and the molecular weight calculated from the theoretical protein sequence. The N terminus of each construct was verified by N-terminal sequencing using Procie 494 Sequencer (Applied Biosystems).

Cloning, Expression, and Purification of mEngECD-mFc 27–581, hEngECD-hFc 26–586, and hEngECD-hFc 26–359 from CHO Cells—The mEngECD-mFc 27–581, hEngECD-hFc 26–586, and hEngECD-hFc 26–359 constructs were subcloned into a UCOE vector (Millipore). 25 μg of linearized UCOE construct was transfected into CHO cells using TransIT transfe-
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In alpha media (Sigma) supplemented with 10% dialyzed FBS (Invitrogen) and 10 μg/ml ADT mixture (Sigma) with one media change after 16 h. On the 2nd day post-transfection, cells were split 1:6 into selection media containing methotrexate (5 and 10 nm) and grown for 5 days before the media were changed with the appropriate methotrexate concentration. Fourteen days post-transfection, pools were selected by anti-Fc ELISA or Western blotting. Selected pools were then adapted to serum-free conditions in CHO P3 media (Irvine Scientific). The protein was purified from the filtered conditioned media in two steps (affinity and ion-exchange chromatography) as described above for mEng\textsuperscript{ECD}-mFc 27–581 from HEK293 cells.

**SPR Assay**—Receptor-ligand binding affinities were determined by SPR using Biacore 3000 and T100 instruments (GE Healthcare).

**Kinetic Assay in a Capture Format**—Goat anti-human Fc-specific IgG or rabbit anti-mouse IgG was immobilized onto research grade CM5 chip using standard amine coupling chemistry following the manufacturer’s protocol. hEng\textsuperscript{ECD}-hFc 26–586 or mEng\textsuperscript{ECD}-mFc 27–581 and truncated variants thereof were captured on the experimental flow cell. Another flow cell was used as a reference (control) to subtract for nonspecific binding, drift, and bulk refractive index. The endoglin-antibody complex was stable over the time course of each ligand binding cycle. A concentration series of BMP9 and BMP10 (0.00977–1.25 nM, with 2-fold serial dilutions) was injected over experimental and control flow cells at a flow rate of 70 μl/min over an anti-hFc IgG CM5 chip on the Biacore. The equilibrium dissociation constant \( K_D \) was determined by double referencing (subtraction of the binding reagent (Mirus); cells were incubated at 37 °C with 5% CO\textsubscript{2} in Bullock buffer, which included BMP9 and BMP10 at a final concentration of 5 ng/ml. After an overnight incubation at 37 °C, the cells were rinsed and lysed with passive lysis buffer (Promega) and frozen at −70 °C. Before the assay, the plates were warmed to room temperature with gentle shaking. Cell lysates were transferred in duplicate to a chemiluminescence plate (96-well) and analyzed in a luminometer with reagents from a Dual-Luciferase Reporter Assay system (Promega) to determine normalized luciferase activity.

**Directed in Vivo Angiogenesis Assay**—Directed in vivo angiogenesis assay (Trevenig, Gaithersburg, MD) was used as a quantitative method for assaying angiogenesis. Implant-grade silicone cylinders were filled with 20 μl of basement membrane extract premixed with or without a combination of vascular endothelial growth factor (VEGF, 600 ng) and fibroblast growth factor (FGF-2, 1.8 μg). The silicone angioreactors were implanted subcutaneously in the dorsal flank of athymic nude mice, 2 angioreactors on each side of the animal. Mice were housed under barrier conditions in specific pathogen-free (SPF) facilities. On the 5th day, angioreactors were immediately excised from the animal, photographed, and solubilized. The amount of FITC-dextran in the angioreactor was quantified at 520-nm emission and 485-nm excitation using a fluorescence plate reader (Infinite m200, Tecan, Mannedorf, Switzerland). The intensity of the signal is proportional to the amount of blood vessels in each angioreactor.

**Chick Chorioallantoic Membrane (CAM) Assay**—Nine-day-old fertilized chick embryos were maintained in a 48-place tabletop egg incubator at 37 °C and specific humidity (60%). Prominent blood vessels were visualized through the eggshell with the aid of an egg lamp. The area of the outer eggshell, where the prominent blood vessels are located, was swabbed with 70% alcohol, a small hole was made, and the air sac was displaced causing a "blister" to form between the shell membrane and the CAM; finally, a small window was cut through...
the eggshell with a hobby grinding wheel (Dremel Emerson Electric Co., Racine, WI). Small filter disks infused with the angiogenic substance VEGF (50 ng) either in the presence or absence of mEng<sup>ECD</sup>-hFc 27–581 (3 doses of 14 μg) in Bicore running buffer, pH 7.4, was then placed at the opening. For hEng<sup>ECD</sup>-hFc 26–586, 3 doses of 20 μg were used in the assay. Each group (8 eggs) was treated daily for 3 days, and CAM results were analyzed on the fourth day. Quantification of the number of resulting vessels per disk post-treatment was made visually with the help of an egg lamp. Statistical analysis of the results was performed with Excel and SigmaStat software.

Colon-26 Cancer Model—Five- to six-week-old BALB/c mice were obtained from Harlan Laboratories. They were supplied autoclaved food and water ad libitum. Colon 26 (ATCC# CRL-CL25) cells were grown in T-175 flasks at 37 °C ± 5% CO<sub>2</sub> in RPMI 1640 medium (ATCC) + 10% FBS (Invitrogen). Cells were trypsinized with TrypLE Select (Invitrogen) and counted using a CEDEX cell counter. Cells were washed once with PBS and resuspended in sterile water at a concentration of 10<sup>6</sup>cells/ml. 100 μl of the cell suspension were placed at the opening. For running buffer, pH 7.4, was then placed at the opening. For hEng<sup>ECD</sup>-hFc 26–586, 3 doses of 20 μg were used in the assay. Each group (8 eggs) was treated daily for 3 days, and CAM results were analyzed on the fourth day. Quantification of the number of resulting vessels per disk post-treatment was made visually with the help of an egg lamp. Statistical analysis of the results was performed with Excel and SigmaStat software.

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RESULTS

Cloning, Expression, and Purification of Mouse and Human Endoglin ECD—The full-length ECDs of the mouse and human endoglin were cloned and fused to the Fc portion from an IgG of corresponding species producing mEng<sup>ECD</sup>-mFc 27–581 and hEng<sup>ECD</sup>-hFc 26–586 constructs (Fig. 1, A and D). Expression of each construct was achieved by transient transfection of HEK293 cells or stably transfected CHO cells. Proteins were purified from conditioned media by column chromatography as described under “Experimental Procedures.” A silver-stained SDS-PAGE gel revealed protein bands of ~110 kDa under reducing and ~220 kDa under non-reducing conditions (Fig. 1, B and E). Analytical SEC showed that both mEng<sup>ECD</sup>-mFc 27–581 and hEng<sup>ECD</sup>-hFc 26–586 were ~95% pure with the absence of aggregates (Fig. 1, C and F) irrespective of the cell expression system used.

Endoglin ECD Does Not Bind to Either Type I or Type II Receptors in Vitro—Previous studies performed on membrane-bound receptors have shown that endoglin can directly associate with type II receptor TGF<sub>BR2</sub> (19) as well as type I receptors ALK5 and ALK1 (21, 27) without TGF family ligands present. It has been suggested that these interactions occur through the ECDs of these receptors (28). To understand what role endoglin ECD plays in modulating interactions of TGF<sub>B</sub> family ligands with their receptors, we tested binding of hEng<sup>ECD</sup>-hFc 26–586 to the ECD of 5 human type II and 7 type I TGF<sub>B</sub> family receptors fused to hFc using SPR technology (Biacore). Anti-hFc IgG was immobilized on a CM5 chip followed by capture of receptor-hFc fusion proteins. The assay design allows the receptor molecule to adopt homogeneous orientation at the surface of the chip. The construct hEng<sup>ECD</sup>-hFc 26–586 was then injected over the captured receptors. We did not observe any binding of endoglin ECD to captured receptors (supplemental Table I). Similarly, mEng<sup>ECD</sup>-mFc 27–581 captured on an anti-mFc IgG chip failed to show any binding to the ECD of mouse type II and type I receptors (data not shown). Thus, we conclude that the ECD form of endoglin is not able to associate directly with the ECD of TGF<sub>B</sub> family receptors in vitro.
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Screening for Endoglin Binding Ligands—Because previous studies suggested that endoglin might bind to TGFβ and several other ligands directly, thus affecting a variety of different signaling pathways (18, 20, 23, 25), we used SPR to screen multiple TGFβ family ligands for binding to human and mouse endoglin ECDs. In-house-generated human endoglin (hEng ECD·hFc 26–586) and the commercially available mouse endoglin (mEng ECD·hFc 27–581) were captured on an anti-hFc IgG CM5 chip. Subsequently, different TGFβ superfamily ligands (listed under “Experimental Procedures”) were injected over captured endoglin molecules at concentrations well above the physiological values usually found in serum (29, 30). Of all the ligands tested, in the absence of other receptors, only BMP9 and BMP10 showed any significant binding to both endoglin constructs, as defined by a KD value lower than 10^{-9} M (supplemental Table II). A similar experiment was performed with in-house-generated mouse endoglin (mEng ECD·mFc 27–581), which also showed binding only to BMP9 and BMP10 (data not shown). To confirm these findings and ensure that the Fc portion of the fusion protein did not interfere with ligand binding, we performed additional ligand screening with hEng ECD 26–586 that lacks any Fc. hEng ECD 26–586 was immobilized directly onto a CM5 chip and screened with the same ligands. Again, we observed only binding of BMP9 and BMP10 but not other TGFβ ligands (data not shown), suggesting that endoglin ECD is capable of binding to only two TGFβ family ligands: BMP9 and BMP10.

We further confirmed these results by preincubating the mEng ECD·mFc 27–581 construct with either BMP9, BMP10, activin A, or TGFβ1 and analyzing the potential complexes on a native PAGE gel (supplemental Fig. S1). Although we have not seen any complex formation between endoglin and TGFβ1 (supplemental Fig. S1, lane 5) or activin A (supplemental Fig. S1, lane 4), we observed decreased mobility as evidenced by a visible upward shift of the endoglin band in the presence of BMP9 and BMP10 (supplemental Fig. S1, lanes 2 and 3), which indicates formation of strong complexes between endoglin ECD and these ligands. We wanted to confirm the lack of interaction between TGFβ1 and endoglin ECD in a cell-based assay format. We performed a reporter gene assay with TGFβ1 (mixed or not with mEng ECD·mFc 27–581) as a ligand in HepG2 cells transfected with the CAGA12-luciferase plasmid (23). We attempted to mimic this system in an SPR experiment by injecting TGFβ1 over captured hTGFβRII-hFc (containing ECD of human TGFβRII fused to hFc) followed by injection of hEng ECD 26–586 (supplemental Fig. S2). Although TGFβ1 formed a stable high affinity complex with TGFβRII, no binding of hEng ECD 26–586 to the TGFβ1-TGFβRII receptor-ligand complex was observed (supplemental Fig. S2A). Additionally, the assay was reversed by capturing mEng ECD·hFc 27–581 on the Biacore chip, then injecting preformed TGFβ1·h-TGFβRII complex over captured endoglin. No binding of the preformed TGFβ1·h-TGFβRII complex was observed by SPR in this configuration either (data not shown).

To test if endoglin might alter TGFβ affinity for its receptor, we premixed TGFβ1 with hEng ECD 26–586 or buffer and injected both mixtures over captured TGFβRII-hFc receptor (supplemental Fig. S2B). No difference between the two injections was observed, suggesting that endoglin ECD does not affect TGFβ binding to the receptor. Thus, we conclude that in vitro mouse and human endoglin ECDs bound to BMP9 and BMP10 but did not associate with TGFβ1 either free or in complex with the type II receptor.

High Resolution Kinetic Analysis of mEng ECD·mFc 27–581 and hEng ECD·hFc 26–586 Binding to BMP9 and BMP10 and Potency Testing in the A204 Cell-based Assay—To characterize more fully BMP9 and BMP10 binding to endoglin, we performed a high resolution kinetic study using captured mEng ECD·mFc 27–581 or hEng ECD·hFc 26–586 proteins expressed either in HEK293 or CHO cells. A representative kinetic analysis is shown in Fig. 2 (CHO) and supplemental Table SIII (HEK293). The interaction between ligands and endoglin-Fc is partially mass transport-limited. To minimize the mass transport, we used a high flow rate (70 μl/min) and a low level of the captured endoglin; however, we were not able to remove the limitations completely. Nevertheless, by including a mass transport term into 1:1 binding model, we were able to achieve an excellent fit of the experimental data as shown by overlay of the simulated binding responses (Fig. 2, A–D, red lines). BMP9 showed high affinity binding to mouse and human endoglin constructs with the equilibrium binding constants Kd of 39 pm (HEK293-derived material) and 22 pm (CHO-derived material) for mEng ECD·mFc 27–581 and 26 pm (HEK293) and 33 pm (CHO) for hEng ECD·hFc 26–586 (Fig. 2E and supplemental Table III). The kinetic rate constants for BMP9 binding to mouse and human endoglin constructs were very similar. Interestingly, BMP10 seems to have different modes of binding to human and murine endoglin, which can be deduced from markedly different Kd values obtained for mEng ECD·mFc 27–581 (76 pm (HEK293) and 71 pm (CHO)) and hEng ECD·hFc 26–586 (241 pm (HEK293)) and 491 pm (CHO) proteins, the difference being due mainly to a faster dissociation of the BMP10-hEng ECD·hFc complex. To understand endoglin interaction with ligand changes at physiological temperature, we further performed SPR analysis at 37°C. We found that hEng ECD·hFc 26–586 (CHO) maintained its affinity for BMP9 (27.8 pm) and BMP10 (408 pm) (data not shown). Thus, the ECD of human endoglin binds BMP9 and BMP10 specifically and with high affinity at physiological temperature.

To measure the ability of mEng ECD·mFc 27–581 and hEng ECD·hFc 26–586 to antagonize Smad signaling induced by BMP9 and/or BMP10, we performed a reporter gene assay in the human rhabdomyosarcoma cell line A204 transfected with a pGL3BRE-luciferase reporter plasmid (1/5-responsve construct (25, 26) and plasmid pRLCMV-luciferase (internal control). The cells were treated with the corresponding ligands in the presence or absence of mouse and human endoglin ECDs and assayed for luciferase activity. As predicted by the binding affinities determined by SPR, mouse and human endoglin inhibit BMP9 and BMP10 signaling in a dose-dependent manner with inhibitory concentrations (IC50) in the sub-nanomolar range.
lar ~ low nanomolar range (Fig. 2E). These data demonstrate that endoglin-Fc fusion protein inhibited signaling via the Smad pathway by BMP9 and BMP10. Taken together the results from two distinct in vitro systems suggest that the extracellular domain of mouse and human endoglin bind to and efficiently inhibit BMP9- and BMP10-induced signaling.

**Truncations of hEngECD-hFc Mapped the Ligand Binding Site to the Orphan Domain 26–359**—Although no crystal structure for endoglin is available, electron microscopy modeling and sequence alignment have revealed that the endoglin ECD is composed of three domains; the N-terminal domain does not show any similarity to known proteins and was thus designated “orphan domain,” whereas the subsequent two domains show similarity to a zona pellucida (ZP) domain (31). To map the BMP9/BMP10 binding site on the human endoglin protein, we performed truncations of the ECD based on the electron microscopy model and the location of the protease cleavage site proposed for the generation of the soluble endoglin form found in preeclampsia (13, 31). As shown in Fig. 3, we constructed hEngECD-hFc proteins containing either the orphan domain (26–359), the ZP (360–586) domain, the soluble, protease-cleaved ECD suggested for the protein found in preeclampsia (26–437) (13, 31), or the remainder of the protease-cleaved endoglin ECD (438–586). Each construct was expressed by transient transfection in HEK293 cells, purified by affinity chromatography, and tested by SPR for their ability to bind BMP9 and BMP10.

To further characterize the BMP9/BMP10 binding site, we prepared human endoglin constructs with further truncations at the C terminus of the orphan domain (Fig. 3). The secondary
structure prediction for the endoglin ECD, generated by using the Psipred and Jalview software (32, 33), showed that the Ile359 residue at the terminus of the orphan domain is right at the end of an $\alpha$-helix (amino acids 352–358), which is located in a long disordered segment of amino acids (amino acids 328–367) that could impair expression and purity (Fig. 3A). Hence, we decided to prepare the constructs hEngECD-hFc 26–378 (includes an extra $\alpha$-strands and $\alpha$-helix segment compared with hEngECD-hFc 26–359), 26–332 (removal of the $\alpha$-helix at amino acids 352–358), and 26–329 (terminating the orphan domain at the end of a $\beta$-strand at amino acids 322–327 and removal of an extra Cys (amino acid 330) potentially important for correct protein folding). The constructs 26–329 and 26–332 showed no binding activity to either ligand by SPR, whereas the construct 26–378 was still able to bind to both BMP9 and BMP10 (Fig. 3B). These results highlight the potential importance of the $\alpha$-helix at amino acids 352–358 and/or the need for this potentially disordered segment of amino acids for the flexibility of the orphan domain in relation to the Fc portion of the construct to actively bind BMP9 and BMP10.

Having established that the minimal construct capable of binding to ligands was 26–359, we further tested binding specificity and potency of hEngECD-hFc 26–359 as well as other truncation constructs 26–378 and 26–437 in comparison with the construct containing full-length ECD by Biacore and A204 cell-based assays as shown in Table 1. The equilibrium dissociation constant ($K_D$) obtained in these assays was very similar to the value obtained for the full-length endoglin ECD hEngECD-hFc 26–586 with both BMP9 and BMP10. However, although BMP9 dissociates quite rapidly from the full-length hEngECD-hFc 26–586 ($k_d$ of $1.98 \times 10^{-3}$ s$^{-1}$), its dissociation is 6-fold slower for the truncated construct hEngECD-hFc 26–359 ($k_d$ of $2.99 \times 10^{-4}$ s$^{-1}$), which represents a marked improvement in the stability of the ligand-endoglin complex. The improved binding of BMP9 was somewhat reflected by an IC$_{50}$ value of hEngECD-hFc 26–359 (0.16 nM) compared with hEngECD-hFc 26–586 (0.26 nM) and was not due to the cell type used to express the material. Based on the limitations of the assay, this change in IC$_{50}$ values suggests a trend instead of a significant difference. The truncation of the C-terminal portion of the endoglin ECD appears to allow for a more specific construct.
with tighter binding to BMP9 compared with the full-length ECD in hEng^{ECD}-hFc 26–586. To further assess the specificity of hEng^{ECD}-hFc 26–359, we tested binding of this construct to other members of the TGF\(\beta\) family including TGF\(\beta\)1, -2, and -3, activin A, BMP2, and BMP7; however, no direct binding was observed (data not shown).

hEng^{ECD}-hFc 26–359, cloned into a UCOE vector and stably expressed in CHO cells (as described under “Experimental Procedures”), was used for subsequent studies. Fig. 4 shows that the hEng^{ECD}-hFc 26–359 expressed in CHO cells and purified with protein A and a Q-Sepharose purification steps yielded a protein of 91% purity as determined by SDS-PAGE gel and analytical SEC (Fig. 4, A and B). The specificity of this construct was the same as the full-length endoglin chimera, as shown by Biacore and cell-based assay data (Fig. 4, C–E) and ligand screen (supplemental Table II). This protein was used for further antiangiogenesis studies.

**Table 1**

| Endoglin construct | BMP9 | Cell-based assay | BMP10 | Cell-based assay |
|--------------------|------|------------------|-------|------------------|
| hEng^{ECD}-hFc26–586 | 5.12 \times 10^{-4} | 6.67 | 6.67 | 5.12 \times 10^{-4} |
| hEng^{ECD}-hFc26–437 | 7.33 \times 10^{-3} | 19.3 | 19.3 | 7.33 \times 10^{-3} |
| hEng^{ECD}-hFc26–378 | 5.12 \times 10^{-3} | 6.67 | 6.67 | 5.12 \times 10^{-3} |
| hEng^{ECD}-hFc26–359 | 1.43 \times 10^{-2} | 20.9 | 20.9 | 1.43 \times 10^{-2} |

a CBD, could not be determined. b 0% inhibition was observed at the maximum concentration tested (70 nM).

Endoglin-Fc Acts by Blocking BMP9 Binding to Type II Receptors—Our previous findings (24) and experiments presented here demonstrated that both endoglin ECD and type I receptor ALK1 bind to BMP9 with high affinity and specificity; thus, they could potentially either act synergistically or compete for binding to these ligands. To understand if endoglin ECD alters BMP9 binding to ALK1, we performed a solution inhibition experiment using SPR. BMP9 and mALK1-mFc were premixed at different ratios, and the mixtures were injected over captured hEng^{ECD}-hFc 26–359 at saturating concentrations followed by injection of soluble mALK1-mFc or hALK1-His (Fig. 5B). We observed a ternary complex between endoglin, BMP9, and ALK1 (Fig. 5B, insert).
The same binding exclusion experiment was run in reversed format by injecting BMP9 over hALK1-hFc followed by the injection of mEngECD-mFc 27–581 with similar results (supplementary Fig. S3). Thus, we conclude that endoglin and ALK1 bind to different sites on BMP9.

BMP9 can bind to three different type II receptors, ActRIIA, ActRIIB, and BMPRII (20, 34, 35). To understand if endoglin ECD alters BMP9 binding to type II receptor, we performed a similar series of SPR experiments. By altering the ActRIIB:BMP9 ratio we were able to show inhibition of BMP9 binding to captured hEngECD-hFc 26–359 (Fig. 5C), which suggests that mActRIIB-mFc competes with hEngECD-hFc 26–359 for BMP9 binding. Moreover, injection of BMP9 followed by mActRIIB-mFc did not produce additional binding of the receptor to the ligand and impairing the formation of the ternary complex necessary for signaling.

ECD alters BMP9 binding to type II receptor, we performed a similar series of SPR experiments. By altering the ActRIIB:BMP9 ratio we were able to show inhibition of BMP9 binding to captured hEngECD-hFc 26–359 (Fig. 5C), which suggests that mActRIIB-mFc competes with hEngECD-hFc 26–359 for BMP9 binding. Moreover, injection of BMP9 followed by mActRIIB-mFc did not produce additional binding of the receptor to the ligand and impairing the formation of the ternary complex necessary for signaling.
Soluble Endoglin Acts as a BMP9/BMP10 Trap

Multiple lines of evidence suggested that endoglin plays a crucial role in angiogenesis (1, 2), cancer (12, 17), and preeclampsia (13). In this paper we have systematically studied the structure-function relationships and interaction of the endoglin ECD with its ligands and receptors. Using endoglin ECD (hEngECD 26–586) as well as proteins containing the ECD fused to IgG Fc, we found that both forms bind specifically and with high affinity to only BMP9 and BMP10 ligands (Fig. 2). We were surprised that endoglin ECD failed to bind TGFβ1 or TGFβ3 in HepG2 cell-based assays and in SPR experiments even when complexed with TGFβRII, particularly as several publications had suggested the importance of direct interactions between endoglin and these ligands in the TGFβ signaling pathway in vivo.

Several published investigations have reported that type I receptors (ALK1 and ALK5) and type II receptor TGFβRII are needed for endoglin interactions with TGFβ ligands (19, 21–23). Thus, we initially investigated whether the receptors are able to bind directly to endoglin ECD and, secondly, whether TGFβ1 or TGFβ3 ligands bound to the receptor TGFβRII facilitate the interaction with endoglin. We did not observe any interactions between the ECD of type I or type II receptors and endoglin, in contrast to reported experiments with overexpression of these molecules on the cell surface. We cannot rule out the possibility of intracellular domains promoting the interaction (37), but our data strongly suggest that the binding cannot be attributed to interactions between the extracellular domains. Furthermore, by varying the sequence of assembly of the TGFβ complex on the surface of the Biacore chip, we explored multiple potential ways for endoglin ECD to be involved in the TGFβ ligand–receptor complex formation. However, we did not detect any effect of endoglin ECD in this system (supplemental Fig. S2). Our observation that endoglin ECD is unable to bind TGFβ1 complexed with type II receptor TGFβRII indicates that ligand-receptor association does not alter the specificity of endoglin ECD and that the balance between soluble versus membrane-bound endoglin, which could possess different specificities, might add an additional level of regulation to the system.

To further address the discrepancy between in vitro SPR data for endoglin ECD and previously published in vivo literature data obtained for membrane-bound endoglin, we performed cell-based assays (Fig. 2) that confirmed the specificity of endoglin ECD observed in the SPR experiments. Thus, we conclude that the effect of endoglin ECD is attributed to its inhibition of signaling triggered by BMP9 and BMP10 and not TGFβ1 or TGFβ3. Endoglin previously has been shown to bind BMP9 by means of a cross-linking assay with a radiolabeled ligand (20). Here we demonstrate for the first time a detailed kinetic characterization of BMP9 and BMP10 binding to endoglin ECD using SPR technology. Recent studies have established BMP9 and BMP10 to be major ligands in the angiogenic pathway of ALK1 (24, 25). Mutations in both endoglin and ALK1 result in similar disease symptoms in hereditary hemorrhagic telangiectasia HHT1 and HHT2, respectively, which supports our find-
ing that both ALK1 and endoglin act through the same ligands and are implicated in the same signaling pathway. Also, several mutations found in patients with HHT1 and HHT2 prevented endoglin and ALK1 binding to BMP9 or BMP10 ligands.4

Given the role of endoglin in vascular function and disease, the identification of ligands for soluble endoglin is of utmost importance. In fact, soluble endoglin from the placenta has been shown to be present at much lower levels in healthy pregnant women than in preeclamptic women and barely present in non-pregnant women and thus is implicated in severe vascular problems (13). Our identification of the binding site for the ligands BMP9 and BMP10 in the orphan domain of human endoglin, comprised of the amino acid sequence 26 to 359 (Figs. 3 and 4), is consistent with reports of physiological and ligand binding activity for the soluble form of endoglin in preeclampsia.4

4 T. Travis, D. Mitchell, E. G. Pobre, R. Castonguay, and A. V. Grinberg, unpublished observations.
sia containing residues 26–437. Our observation that small portions of the orphan domain of endoglin (e.g. residues 26–378 and 26–359) have higher affinities for BMP9 and BMP10 than the full-length ECD (residues 26–586) further restricts the ligand binding site and indicates an opportunity for the design of anti-angiogenic, endoglin-based therapy with improved ligand binding parameters (Table 1). The two domains contained in the remainder of the extracellular domain not required for ligand binding are members of a large class of structures, the ZIP domains, which perform highly diverse functions in other proteins, most notably filament formation (38). In endoglin these domains may act as spacers between the ligand binding site of endoglin and the cell surface and are suggested, based on published studies, to be involved in endoglin oligomerization and in ligand-independent heteromeric interactions with receptors and activin receptor-like kinase (21).

Although the exact mechanism is a subject of ongoing investigation and beyond the scope of this paper, we hypothesize that the main role of soluble endoglin may be scavenging BMP9 or BMP10 ligands, which could affect the delicate balance required for angiogenesis in normal physiological conditions.

The efficacy of Eng\textsuperscript{ECD}-Fc to block angiogenesis was demonstrated in the CAM and angioreactor assays in which endoglin dramatically inhibited tumor vessel formation induced by VEGF (Fig. 6). These data agree with previously published observations that Eng-Fc reduced spontaneous and VEGF-induced endothelial sprouting in HUVEC cells (15). Anti-angiogenic activity of endoglin ECD was further manifested as anti-tumor activity in the colon-26 model, where the mEngECD-mFc construct was effective in reducing the tumor burden. These findings can help explain the disruption of normal vasculature development in instances when soluble endoglin is present, for example in pre-eclampsia (13), and indicate potential roles for soluble forms of endoglin as anti-angiogenic factors.

In summary, the orphan domain of endoglin binds BMP9 and BMP10 with a very high affinity and specificity. It inhibits the interaction of BMP9 with type II receptors, thereby blocking multiple signaling pathways at once, which could be advantageous over other therapeutic agents targeting a single component of the TGFβ pathway. More experiments in animal models of cancer and angiogenesis are needed to establish the use of endoglin ECD as a modulator of BMP9/BMP10 activity.

Acknowledgments—We thank Tim Ahern, a scientific/technical writer supported by Acceleron Pharma, who provided editorial assistance during preparation of this manuscript. We also thank Erin Ronayne and Tiffany Travis for preparation and purification of several endoglin constructs, Eileen Pobre, Dianne Mitchell, and June Liu for assay development work, Erik Vogan for secondary structure prediction, John Knopf and John Quesin for critical review of the manuscript, and all members of Acceleron Pharma Cell Biology, Protein Biochemistry, and Preclinical Pharmacology groups for contributions in support of this work.

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