Endoglin Is an Accessory Protein That Interacts with the Signaling Receptor Complex of Multiple Members of the Transforming Growth Factor-β Superfamily*

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Endoglin (CD105) is a transmembrane glycoprotein that binds transforming growth factor (TGF)-β1 and -β3, and coprecipitates with the Ser/Thr kinase signaling receptor complex by affinity labeling of endothelial and leukemic cells. The present study shows that in addition to TGF-β1 and -β3, endoglin interacts with activin-A, bone morphogenetic protein (BMP)-7, and BMP-2 but requires coexpression of the respective ligands binding kinase receptor for this association. Endoglin cannot bind ligands on its own and does not alter binding to the kinase receptors. It binds TGF-β1 and -β3 by associating with the TGF-β type II receptor and interacts with activin-A and BMP-7 via activin type II receptors, ActRII and ActRIIB, regardless of which type I receptor partner is coexpressed. However, endoglin binds BMP-2 by interacting with the ligand binding type I receptors, ALK3 and ALK6. The formation of heteromeric signaling complexes was not altered by the presence of endoglin, although it was coprecipitated with these complexes. Endoglin did not interact with BMP-7 through complexes containing the BMP type II receptor, demonstrating specificity of its action. Our data suggest that endoglin is an accessory protein of multiple kinase receptor complexes of the TGF-β superfamily.

The TGF-β superfamily of structurally related peptides includes the TGF-β isoforms, β1, β2, β3, and β5, the activins and the bone morphogenetic proteins (BMPs). TGF-β-like factors are a multifunctional set of growth and differentiation factors conserved among flies, frogs, and mammals (reviewed in Refs. 1–4). These factors control biological processes such as embryogenesis, organogenesis, morphogenesis of tissues like bone and cartilage, vasculogenesis, wound repair and angiogenesis, hematopoiesis, and immune regulation (reviewed in Refs. 2 and 4–8). Signaling by ligands of the TGF-β superfamily is mediated by a high affinity, ligand-induced, heteromeric complex consisting of related Ser/Thr kinase receptors divided into two subfamilies, type I and type II (3). Formation of this high affinity complex is essential, as the type II receptor transphosphorylates and activates the type I receptor in a Gly/Ser-rich region (9–11). The type I receptor in turn phosphorylates and transduces signals to a novel family of recently identified downstream targets, termed Smads (12, 13).

Although the cooperativity between two kinase receptors is a general signaling mechanism for the TGF-β superfamily, where the type I receptors are considered the signal transducing receptors, ligand binding ability is not restricted to receptor type. For TGF-β and activin, the type II receptors TβRII and ActRII or ActRIIB, respectively, are known to bind ligand independently (14–16), while the corresponding type I receptors ALK5 (activin receptor-like kinase; TβRII or ALK4 (ActRIIB) require the coexpression of the appropriate type II receptors (9, 17–21). The BMP family differs in this respect as the BMP type I receptors,ALK3 (BMPRI) and ALK6 (BMPRIIB), can bind BMP-2 and BMP-4 efficiently in the absence of the type II receptor, yet require a type II receptor for transducing a transcriptional response (22–26). In the case of BMP-7, the type II receptor BMPRII binds ligand weakly, and cooperates with the type I receptors ALK3, ALK6, and ALK2 (ActRII) to generate high affinity receptor complexes (24). Furthermore, BMP-7 can also bind to the activin type II receptors and form functional complexes with BMP type I receptors (27). This cross-talk between the activin receptor system and the BMP receptors suggests BMPs may have a broader function in vivo than first recognized.

Endoglin (CD105) is a homodimeric integral membrane glycoprotein composed of disulfide-linked subunits of 90–95 kDa. In human, it is expressed at high levels on vascular endothelial cells and on syncytiotrophoblast of term placenta (28–30). It is transiently expressed on extravillous cytotrophoblasts and in cells and on syncytiotrophoblast of term placenta (28–30). It is transiently expressed on extravillous cytotrophoblasts and in-
Endoglin is the target gene for the dominantly inherited vascular disorder hereditary hemorrhagic telangiectasia type 1 (HHT1) (36). HHT is characterized by frequent nose bleeds, mucocutaneous telangiectases, and the development of arteriovenous malformations predominantly in lung, brain, and the gastrointestinal tract that lead to recurrent hemorrhage and shunting (37). We have recently shown that mutant forms of endoglin are degraded intracellularly and that HHT1 is associated with reduced levels of surface endoglin on endothelial cells and activated monocytes (38).

Endoglin was shown to bind TGF-β1 and -β3 with high affinity, but not -β2 (39), suggesting it mimics the isofrom specificity of TβRII. Endoglin was coprecipitated with TpRII and a type I receptor in endothelial and leukemic cells (38, 40–42). Human umbilical vein endothelial cells (HUVEC) were shown to have a single high affinity binding site representative of TpβRII complexes (39). Furthermore, when overexpressed in U937 monocytes, endoglin did not alter binding (42). These previous studies suggested that endoglin alone may not bind TGF-β. We tested this using a COS1 transfection system and now demonstrate that endoglin requires the coexpression of TpβRII to bind TGF-β1 and -β3. In addition, it binds activin-A, BMP-2, and BMP-7 only in the presence of their respective ligand binding receptors. We also demonstrate that endoglin associates with heteromeric signaling receptor complexes of multiple members of the TGF-β superfamily.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—Endothelial cells were derived from HUVEC of newborns by previously published procedures and maintained as described (28). NCTC2071 fibroblasts were cultured as published (43). COS1 cells were maintained and transiently transfected with expression constructs using the DEAE-dextran-chloroquine method as reported (44, 45). Assays were performed 2 days after transfection.

**Expression Vectors**—The EcoRI fragment of human full-length endoglin in pEXV-1 (46) was subcloned into pCMV5 (47) and used for transient transfection, as all other cDNA used were already subcloned into this mammalian expression vector. The pCMV5 expression constructs containing cDNAs for TpβRII, ALK5/HA (tagged at the COOH terminus with the influenza hemagglutinin epitope, HA), ALK1/HA, ActRII/HA, ActRIIIB2/HA, ActRIIIB2, ActRII/His (tagged at the COOH terminus of human type II TGF-β receptor), was used (Santa Cruz Biotechnology Inc.). For immunoprecipitation of TpβRII, the polyclonal antisera (pAb) C16, which was raised in rabbits by immunization with a synthetic peptide corresponding to amino acids 550–565 of the highly conserved carboxy terminal of human type II TGF-β receptor, was used (Santa Cruz Biotechnology Inc.). For immunoprecipitation of HA- and FLAG-tagged TGF-β superfamily receptors, monoclonal antibodies 12CA5 (Boehringer Mannheim) and M2 (IBI, Eastman Kodak), respectively, were used.

**Binding and Affinity Labeling**—TpβRII and TpβRII were from R&D Systems. Recombinant human activin-A, BMP-2, and BMP-7 were generous gifts from Y. Eto (Ajinomoto Co. Inc.), V. Rosen (Genentech Institute), and K. Sampath (Creative Biomolecules) respectively. TGF-β3, activin, and BMPs were iodinated with 125I using chloramine-T as described previously (16, 51–53). For binding assays with and without affinity labeling, HUVEC or transiently transfected COS1 cell monolayers were incubated with 200 pM 125I-TGF-β1 for 4 h, washed, treated with or without disuccinimidyl suberate (DSS; Pierce), and solubilized with lysis solution containing 1% Triton X-100, and a type I receptor in endothelial and leukemic cells (38–42). Human umbilical vein endothelial cells (HUVEC) were shown to have a single high affinity binding site representative of TpβRII complexes (39). Furthermore, when overexpressed in U937 monocytes, endoglin did not alter binding (42). These previous studies suggested that endoglin alone may not bind TGF-β. We tested this using a COS1 transfection system and now demonstrate that endoglin requires the coexpression of TpβRII to bind TGF-β1 and -β3. In addition, it binds activin-A, BMP-2, and BMP-7 only in the presence of their respective ligand binding receptors. We also demonstrate that endoglin associates with heteromeric signaling receptor complexes of multiple members of the TGF-β superfamily.

**Antibodies**—P3D1 and P4A4 hybridoma to human endoglin were provided by E. A. Wayner (Seattle, WA) and were described and characterized extensively (50). Murine IgG1 (Coulter Electronics) was used as an isotype control for these two monoclonal antibodies (mAb). For immunoprecipitation of TpβRII, the polyclonal antisera (pAb) C16, which was raised in rabbits by immunization with a synthetic peptide corresponding to amino acids 550–565 of the highly conserved carboxy terminal of human type II TGF-β receptor, was used (Santa Cruz Biotechnology Inc.). For immunoprecipitation of HA- and FLAG-tagged TGF-β superfamily receptors, monoclonal antibodies 12CA5 (Boehringer Mannheim) and M2 (IBI, Eastman Kodak), respectively, were used.

**Metabolic Labeling and Western Blot Analysis**—Endoglin and TGF-β expression in transfected COS1 cells were quantitated by metabolic labeling in Fig. 1. Briefly, transfected COS1 were treated in parallel to affinity labeling by incubation with 100 μCi/ml [35S]methionine (Met) (Tran[35S]ible; ICN Pharmaceuticals Canada Ltd.) in [Met]-free Dulbecco’s modified Eagle’s medium (low glucose; Life Technologies, Inc.) for 4 h, solubilized in lysis solution containing 1% Triton X-100, and immunoprecipitated with saturating amounts of antibodies, and quantitated using a PhosphorImager and Image Quant Software (Molecular Dynamics) according to published procedures (38). In other experiments, endoglin protein levels in transfected COS1 cells were monitored using a PhosphorImager and Image Quant Software (Molecular Dynamics) according to published procedures (38). In other experiments, endoglin protein levels in transfected COS1 cells were monitored using a PhosphorImager and Image Quant Software (Molecular Dynamics) according to published procedures (38).
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Table I
Effect of chemical cross-linking on 125I-TGF-β1 binding to endoglin and TβRII transiently expressed in COS1 cells

| pCMV5 | -DSS | +DSS | END | -DSS | +DSS | TβRII | -DSS | +DSS | END + TβRII |
|-------|------|------|-----|------|------|-------|------|------|-----------|
| Total lyate | 402,719 | 201,309 | 416,956 | 289,225 | 876,649 | 680,456 | 857,186 | 661,511 |
| αTβRII | 1964 | 3274 | 1858 | 3528 | 67,084 | 61,499 | 47,761 | 53,759 |
| αEND | 0 | 46 | 0 | 309 | 0 | 0 | 2888 | 12,297 |

Table II
Effect of chemical cross-linking on 125I-TGF-β1 binding to HUVEC monolayers and receptor complexes using different detergents for solubilization of membrane proteins

| Triton | CHAPS | Digitonin |
|--------|-------|----------|
| DSS | +DSS | Ratio | -/+ | DSS | +DSS | Ratio | -/+ | DSS | +DSS | Ratio | -/+ |
| αTβRII | 111,526 | 105,056 | 60 | 159,256 | 124,856 | 122 | 76,600 | 46,611 |
| αEND | 4111 | 6886 | 4 | 14,622 | 12,033 | 12 | 935 | 2052 |

RESULTS
Endoglin Requires the Coexpression of TβRII to Bind TGF-β1—Previous studies have shown that endoglin interacts with the TGF-β binding complex. To investigate the nature of this complex, we transiently expressed endoglin in COS1 cells in the presence or absence of TβRII. Cell monolayers were affinity-labeled using 125I-TGF-β1, chemically cross-linked using DSS, and cell lysates were solubilized with SDS-PAGE. Endoglin co-immunoprecipitated with TβRII in HUVEC, which express both TGF-β1 and endoglin. In Triton X-100, cpm eluted from anti-TGF-β1 immunoprecipitates showed little dependence on the presence of TβRII. In contrast, when endoglin was coexpressed with TβRII, affinity-labeled endoglin showed little dependence on the presence of TβRII. These data suggest that the binding of TGF-β1 to endoglin is unstable in the absence of TβRII. In contrast, when endoglin was coexpressed with TβRII, affinity-labeled endoglin showed little dependence on the presence of TβRII. These data suggest that the binding of TGF-β1 to endoglin is unstable in the absence of TβRII. In contrast, when endoglin was coexpressed with TβRII, affinity-labeled endoglin showed little dependence on the presence of TβRII. These data suggest that the binding of TGF-β1 to endoglin is unstable in the absence of TβRII. In contrast, when endoglin was coexpressed with TβRII, affinity-labeled endoglin showed little dependence on the presence of TβRII.
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![Fig. 2](image)

**Fig. 2.** The interaction of endoglin with TGF-β1 and TβRII is more stable in digitonin. HUVEC were affinity-labeled with 200 pCi 125I-TGF-β1, treated with DSS, and solubilized with 1% Triton X-100 (T), CHAPS (C), or digitonin (D) plus protease inhibitors. Aliquots of total lysates were analyzed as in Fig. 1 (lanes 1–3). Detergent extracts containing equivalent total protein content were immunoprecipitated with pAb C16 (αTβRII; lanes 4, 6, and 8) and mAb P3D1 (αEND; lanes 5, 7, and 9) and fractionated non-reduced. Arrows indicate the affinity-labeled endoglin (END) dimers, oligomers (OLIGO), and the type II receptor (RII). Fractionation of these samples using reducing conditions (lanes 10–15) shows that the affinity-labeled monomeric endoglin product(s) migrate slightly above RII. Traces of receptor I (RI) are also noted.

![Fig. 3](image)

**Fig. 3.** Endoglin interacts with TGF-β3 and TβRII and requires coexpression of TβRII for association with TGF-β3. A, HUVEC were incubated with 250 pCi 125I-TGF-β3, cross-linked with DSS, solubilized with 1% Triton X-100, and analyzed as described in Fig. 1. Total lysates containing 15 μg of total protein were fractionated non-reduced (lane 1). Total binding was specific, as binding in the presence of 40-fold excess competing unlabeled ligand was 4000 cpm compared with 25,000 cpm/100-mm dish and revealed no detectable receptors (lane 2). Cell extracts containing equivalent total protein content were immunoprecipitated with pAb C16 (αTβRII; lane 3), mAb P3D1 and P4A4 (αEND; lanes 4 and 5, respectively), and fractionated non-reduced. Arrows indicate the affinity-labeled endoglin (END) dimers, oligomers (OLIGO), and the type II receptor (RII). Fractionation of these samples using reducing conditions (lanes 6–8) shows monomeric endoglin affinity-labeled with TGF-β3 migrating slightly above affinity-labeled TβRII. B, COS1 cells were transiently transfected as in Fig. 1 with pCMV empty vector, pCMV-END, and/or pCMV5-TβRII as indicated. Two days after transfection, cells were incubated with 250 pCi 125I-TGF-β3, cross-linked with DSS, solubilized with 1% Triton X-100, and analyzed as described in Fig. 1. Total lysates that were fractionated reduced are shown (lanes 1–4). Endoglin expression was analyzed by Western blotting of an aliquot of these total lysates, fractionated non-reduced, and probed using anti-endoglin mAb P4A4 (lower panel, lanes 1–4). Aliquots of total lysates were immunoprecipitated with mAb P3D1 (αEND; lanes 5–8) and pAb C16 (αTβRII; lanes 9–12), and fractionated reduced. Arrows indicate the affinity-labeled monomeric endoglin (END) and TβRII (RII). Fractionation of these samples using non-reducing conditions (lanes 13–15) reveals endoglin dimers, oligomers (OLIGO), and TβRII.

and digitonin known to better preserve some protein/protein interactions (55). Of the three detergents tested, CHAPS was most effective in preserving TGF-β1 interaction with TβRII. In CHAPS, we also recovered 12% of the radiolabeled TGF-β1 in the anti-endoglin immunoprecipitates in the absence of the cross-linker versus 4% recovery in Triton X-100 (Table II). In digitonin, however, we recovered 36% of the radiolabeled TGF-β1 in anti-endoglin immunoprecipitates relative to cross-linked samples, which was comparable to 46% in the anti-TβRII immunoprecipitates, despite an overall reduced efficiency of lysis. These data suggest that TGF-β1 interaction with endoglin can be preserved in mild detergents.

Immuno precipitation of TβRII-endoglin complexes using pAb C16 directed to the COOH terminus of TβRII consistently yielded efficient coprecipitation of endoglin (see Fig. 1). In contrast, anti-endoglin coprecipitated little TβRII that required overexposure of autorads for visualization. Since these experiments were routinely performed in Triton X-100, we determined whether CHAPS or digitonin might preserve endoglin/TβRII complexes in anti-endoglin immunoprecipitates. HUVEC were affinity-labeled with 125I-TGF-β1 and lysed in Triton X-100, CHAPS, or digitonin, and the lysates subjected to immunoprecipitation with antibodies directed against TβRII or endoglin (Fig. 2). The profile of endogenous receptor complexes immunoprecipitated from Triton X-100-solubilized HUVEC (Fig. 2, lanes 4 and 5) was similar to that previously reported (38, 39) and was comparable to that observed in COS1 cells coexpressing TβRII and endoglin (see Fig. 1A). Similar results were obtained in CHAPS-solubilized cells (lanes 6 and 7). However, in the presence of digitonin, we observed efficient coprecipitation of affinity-labeled TβRII with the anti-endoglin (lanes 9 and 15). Furthermore, the relative intensities and profile of digitonin solubilized receptor complexes were similar in the anti-endoglin and the anti-TβRII immunoprecipitates (compare lane 8 with lane 9, and lane 14 with lane 15). Thus digitonin preserved endoglin association with both TβRII and TGF-β1. We have shown that detergents which disrupt TβRII association with endoglin in anti-endoglin immunoprecipitates also disrupt coprecipitation of TGF-β with endoglin in the absence of cross-linker. Furthermore digitonin, which preserved association of TβRII with endoglin, also led to coprecipitation of TGF-β1 with endoglin in the absence of cross-linker. Thus, the ability of endoglin to maintain interactions with TGF-β1 is dependent on its association with TβRII. Based on these results, we propose that endoglin is not itself a TGF-β receptor, but rather is cross-linked with the ligand through association with the TGF-β type II receptor.

**Endoglin Interacts with TGF-β3 and TβRII and Requires Coexpression of TβRII for Association with TGF-β3**—To determine if endoglin could interact with TβRII bound to TGF-β3, HUVEC were affinity-labeled using 125I-TGF-β3 and analyzed by immunoprecipitation, SDS-PAGE, and autoradiography (Fig. 3A). Analysis of total cell lysates and immunoprecipitates revealed specific cross-linking of 125I-TGF-β3 to TβRII and to endoglin dimers (180 kDa) and oligomers (>200 kDa). This confirms observations that showed competitive inhibition of TGF-β1 interaction with endoglin by the TGF-β3 isoform (39). Endoglin dimers and oligomers were coprecipitated by anti-TβRII, demonstrating that endoglin and TβRII form a complex with TGF-β3 (Fig. 3A, lane 3). In COS1 cells, we next estab-

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lshed that endoglin required the coexpression of TβRII for binding to TGF-β3 (Fig. 3B). No binding of TGF-β3 to COS1 cells transfected with endoglin alone was observed despite high levels of endoglin expression as measured by Western blotting (Fig. 3B, lanes 1–4). However, when coexpressed with TβRII (Fig. 3B, lanes 5–8), endoglin bound to TGF-β3 and could be immunoprecipitated with anti-TβRII, best seen under non-reducing conditions (lanes 13–15). Thus, endoglin interacts with either TGF-β1 or -β3 and requires coexpression of TβRII to associate with these ligands.

Endoglin Interacts with Heteromeric Receptor Complexes Containing ALK5 and Does Not Disrupt Their Formation—ALK5 (TβRI) preferentially interacts with ligand-bound TβRII to generate a TGF-β receptor signaling complex. ALK5 is unable to bind TGF-β on its own, but does so when coexpressed with TβRII (9). Having established that endoglin recognizes ligand-bound type II receptors, we investigated whether it could modulate binding and subsequent formation of heteromeric complexes between ALK5 and TβRII (Fig. 4). No binding is observed when endoglin and ALK5 are coexpressed (Fig. 4, lanes 1 and 2), but cotransfection of TβRII leads to binding of TGF-β1 to both ALK5 and endoglin (lanes 3–5). Anti-endoglin immunoprecipitated ALK5 and TβRII with endoglin (lanes 6 and 7). The anti-HA immunoprecipitates showed that the heteromeric complex between ALK5 and TβRII is not affected by endoglin expression (lanes 8 and 9). Together, these data show that endoglin can interact with kinase receptor complexes containing ALK5, but does not enhance overall binding, nor modulate the association of receptor I with receptor II.

Endoglin Binds Activin-A or BMP-7 When Coexpressed with ActRII or ActRIIB2—As endoglin was cross-linked to TGF-β1 and -β3 through its association with TβRII, we tested whether it could interact with other type II receptors of the TGF-β superfamily. We first examined activin-A, which binds to two related type II receptors, ActRII and ActRIIB, and signals through a mechanism similar to that defined for TGF-β receptors (44). COS1 cells were transiently transfected with endoglin alone or together with ActRII or ActRIIB2 (a ligand binding functional isoform of ActRIIB; Ref. 16) and were then affinity-labeled using 125I-activin-A (Fig. 5A). When transfected alone, endoglin did not bind activin-A, despite efficient coexpression of the protein (Fig. 5A, lanes 1 and 2). However, in COS1 cells coexpressing type II receptors, anti-endoglin immunoprecipitated 125I-activin-A cross-linked to endoglin dimers and oligomers (lanes 12 and 13), and coprecipitated activin type II receptors (lanes 10–13). Thus endoglin can form complexes with ActRII or ActRIIB2 bound to activin-A. As these receptors can also bind BMP-7 (27), we determined whether endoglin might also associate with activin type II receptors bound to BMP-7 (Fig. 5B). Endoglin alone did not bind BMP-7, but did so in the presence of coexpressed ActRII and to a lesser degree with ActRIIB2 (bottom panel). Reduced efficiency of cross-linking of ligands to ActRII versus ActRIIB has been reported (16).

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Fig. 4. Endoglin interacts with heteromeric receptor complexes containing ALK5. COS1 cells were transiently transfected, affinity-labeled with 200 pM 125I-TGF-β1, and analyzed as in Fig. 1. ALK5 was tagged at the carboxyl terminus with HA. All samples were fractionated reduced. Shown are total lysates (lanes 1–5) and eluates from immunoprecipitates with mAb P3D1 (αEND) and with mAb 12CA5 (αHA) in lanes 6 and 7 and lanes 8 and 9, respectively. Arrows indicate the positions of the affinity-labeled products, endoglin (END), TβRII (RII), and the type I receptors (RI).

Fig. 5. Endoglin binds activin-A or BMP-7 when coexpressed with activin type II receptors. A. COS1 cells were transiently transfected with various combinations of pcMV5 empty vector, pcMV5-END, and HA-tagged pcMV5-ActRII or pcMV5-ActRIIB2, affinity-labeled with 500 pM 125I-activin-A, and analyzed as in Fig. 1. Total lysates that were fractionated reduced (R) are shown in lanes 1–6; affinity-labeled type II receptors (RII) are indicated by left bracket. Endoglin expression was analyzed by Western blot of an aliquot of this total lysate as in Fig. 3B (lower panel, lanes 1–6). Eluates from immunoprecipitates with mAb P3D1 (αEND) were fractionated reduced in lanes 7–11. Right brackets indicate the positions of monomeric affinity-labeled endoglin (END) and type II receptor (RII). Corresponding eluates from lanes 10 and 11 were fractionated non-reduced (NR) in lanes 12 and 13, respectively, and lane 14 represents the negative control for these conditions. Arrows indicate the position of endoglin dimers and oligomers (OLIGO) and ActRII affinity-labeled with activin-A. B. COS1 cells were transiently transfected with various combinations of pcMV5 empty vector, pcMV5-END, and/or pcMV5-ActRII/HA, pcMV5-ActRIIB2/HA, or Flag-tagged BMPRII as indicated, affinity-labeled with 1 nM 125I-BMP-7, and analyzed as in Fig. 1. Total lysates fractionated under reducing conditions (top panel) reveal affinity-labeled type II receptors (RII). Endoglin expression was analyzed by Western blotting of an aliquot of these total lysates as in Fig. 3B. BMPRII/FL was also analyzed by Western blotting of total lysates using mAb M2 (αFLAG). Eluates from immunoprecipitads with mAb P3D1 (αEND) were fractionated non-reduced in lower panel. Arrows indicate the position of endoglin (END) dimers and oligomers (OLIGO) affinity-labeled with BMP-7.
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Complexes—In these studies, BMP-7 was unable to interact with BMPRII alone despite efficient expression of this receptor in COS1 cells (Fig. 5B). Coexpression of endoglin did not alter this binding. Since binding of BMP-7 to BMPRII was shown previously to be dependent on the coexpression of the type I receptors ALK6 or ALK2 (24), we investigated the ability of endoglin to interact with these complexes. Fig. 6 (A and B) demonstrates binding of BMP-7 to ALK6-BMPRII or ALK2-BMPRII complexes and is similar to that observed with ActRII or ActRIIB (upper panels). However, endoglin could not associate with the BMPRII complexes, while it could with the ActRII and ActRIIB complexes (middle panels). Indeed, under reducing conditions anti-endoglin immunoprecipitates revealed coprecipitating affinity-labeled proteins corresponding to the type II receptors, ActRII or ActRIIB, together with the type I receptor, ALK2 (Fig. 6B, lanes 9–12), but it did not alter the formation of any of these complexes as seen in the anti-HA immunoprecipitates (lower panels). Thus, endoglin is not found associated with BMP-7-BMPRII complexes containing ALK6 or ALK2.

Endoglin Associates with BMP-2 When Coexpressed with the Type I Receptors ALK3 and ALK6—Unlike TGF-β, activin, and BMP-7, BMP-2 initiates signaling by first interacting with the type I receptors ALK3 or ALK6 and then recruits type II receptors into a signaling complex. Since endoglin interaction with ligands appears to require expression of the ligand-binding component of the heteromeric Ser/Thr kinase receptor complex, we determined whether endoglin might interact with BMP-2 in the presence of type I receptors. Endoglin alone was unable to bind BMP-2, but did so upon coexpression with ALK3 or ALK6 (anti-endoglin (αEND) panels, lanes 1 and 3 in Fig. 7, A and B). Interestingly, when endoglin was coexpressed with either ALK3 or ALK6 and BMPRII, we observed a substantial decrease in the association of endoglin with BMP-2 (Fig. 7A, compare lane 5 with lane 3; Fig. 7B, compare lane 6 with lane 3, anti-endoglin (αEND) panels). This occurred despite efficient formation of BMP-2 binding receptor complexes in these cells as seen in total lysates and anti-Flag and anti-HA immunoprecipitates, and may reflect the inability of endoglin to associate with BMPRII, as noted above.

We also tested for the association of endoglin with BMP-2 in the presence of ActRIII and ActRIIB. Consistent with previous observations on the Drosophila type II receptor, punt (56), we were unable to observe any binding of BMP-2 to ActRIII (Fig. 7A, lane 8) or ActRIIB (data not shown) when these receptors were expressed alone. However, in the case of cells coexpressing ALK3 and either ActRIII or ActRIIB, the association of endoglin with BMP-2 was comparable to that observed with ALK3 alone (Fig. 7A, lanes 7 and 10 compared with lane 3). Similar results were obtained in the case of cells coexpressing ALK6 and ActRIII or ActRIIB (Fig. 7B, lanes 8 and 10 compared with lane 3), although coexpression of ActRIIB yielded lower overall levels of BMP-2 binding (seen in total lysates; expression was analyzed by Western blot of an aliquot of these total lysates as in Fig. 3B, shown just below top panel. Eluates from immunoprecipitates with mAb P3D1 (αEND) were fractionated non-reduced in middle panel. Arrows indicate endoglin (END) dimers and oligomers (OLIGO) affinity-labeled with BMP-7. Immunoprecipitation analysis of ALK6/HA (αHA) or BMPRII/FL (αFLAG) is shown in the lower panel, and serves as the control for binding to and formation of heteromeric complexes. Affinity-labeled type II receptors (RII) co-precipitating with ALK6 (RI) are indicated with arrows. B. COS1 cells were transiently transfected with pCMV5-ALK6/HA, with or without pCMV5-END, and/or ActRII/His, ActRIIB, or BMPRII/FL as indicated and affinity-labeled with 2 nM 125I-BMP-7. All samples were processed as in A. In addition, anti-endoglin immunoprecipitates were fractionated reduced (R) showing coprecipitation of endoglin (END) with RII and ALK2 (RI) (right panel, lanes 9–12).
lanes 7–10); this may account for the reduced level of BMP-2 bound to endoglin observed in these transfectants. We also confirmed that endoglin could associate stably with BMP-2 receptor complexes containing the cotransfected ALK3 or ALK6 as they were coprecipitated with anti-endoglin as seen under reducing conditions (Fig. 7, A and B, lanes 11–13).

Endoglin Associates with the Ligand Binding Receptors—Since our data suggest endoglin interacts with ligand binding receptors, we next tested whether endoglin could directly bind these receptors in the absence of exogenously added ligand (Fig. 8). In COS1 cells that were transfected with type II receptors, with or without endoglin, we analyzed receptor interactions by surface biotinylation, solubilization in digitonin, and specific immunoprecipitation. We found TbRII and ActRII coprecipitated with anti-endoglin as seen under both non-reducing and reducing conditions (Fig. 8A, lanes 3–6). This was not observed in BMPRII-expressing cells (lanes 7 and 8) nor in the controls (lanes 1 and 2). When the same transfectants were analyzed with anti-TbRII (lanes 9–12), anti-HA (lanes 13–16), or anti-FLAG (lanes 17–20), endoglin coprecipitated with TbRII, ActRII, but not BMPRII, respectively. Endoglin also interacted with ActRIIB2 (data not shown) in the absence of added ligand. In a similar series of experiments, we found that the BMP type I receptors, ALK2, ALK3, and ALK6, did not coprecipitate with endoglin (Fig. 8B, lanes 3–16). Furthermore, in COS1 cells expressing endoglin alone, we found no evidence for interaction with endogenous receptors (lane 2). These data demonstrate that endoglin interacts specifically with the ligand binding type II receptors TbRII, ActRII, and ActRIIB2.

However, association with type I receptors may require ligand and/or chemical cross-linkers for detection by coimmunoprecipitation. Furthermore, these latter interactions are not likely dependent on the expression of endogenous type II receptors, since the interaction of endoglin with the low level of any endogenous type II receptors was undetectable both by affinity labeling and cell surface labeling.

We also investigated the interaction between endogenous endoglin and TbRII, which are both expressed in endothelial cells and NCTC2071 fibroblasts (43). For this, the cells were surface-labeled by biotinylation and solubilized in digitonin prior to immunoprecipitation (Fig. 8C). We observed that under non-reducing and reducing conditions, endoglin coprecipitated with TbRII in the absence of added ligand (compare lanes 2, 5, 8, and 11 with lanes 1, 4, 7, and 10). However, as with the affinity labeling experiments (Fig. 2), this interaction was disrupted in Triton lysates (Fig. 8C, lanes 13–18). Together, these data suggest that endoglin associates with the type II receptors in the absence of ligand.

**DISCUSSION**

The present studies show that endoglin can interact with TGF-β1, β3, activin-A, BMP-7, and BMP-2, but requires the coexpression of the respective ligand-binding kinase receptor partner for binding and specificity. For TGF-β1, its association with endoglin is better demonstrated with the use of weak detergents that do not disrupt the interaction of endoglin with TbRII. These results strongly suggest that endoglin binds TGF-β secondarily to its association with TbRII already bound by TGF-β.
to ligand. This would explain why the specificity and affinity of endoglin for TGF-β isoforms mimics that of TβRII. It is likely that this is true for all ligands that interact with endoglin.

Endoglin was first defined as a component of the TGF-β receptor system when betaglycan was sequenced and found to be similar to endoglin in particular, in the cytoplasmic tail where these two proteins are 71% identical (39). Betaglycan is a proteoglycan (>200 kDa) also called a type III receptor that is required for presenting TGF-β2 to the kinase receptor complex TβRII-ALK5, and promoting signaling by this isoform (57). Betaglycan also binds other isoforms, β1 and β3, on its own and potentiates binding to cells ultimate enhancing the response of cells to these ligands. Betaglycan acts as a dual modulator of ligand access to the signaling receptors, as it can be released from the cell membrane and binds ligand in soluble form; thus, it is clearly defined as a receptor (58). No known signaling domains have been identified in its structure; however, like endoglin, its short cytoplasmic tail is highly conserved among species (43, 59). Endoglin has often been compared with betaglycan and postulated to function in a similar fashion by affecting the binding of TGF-β1 and TGF-β3 to the signaling receptors (35, 39, 40, 60, 61), and it has also been described as an auxiliary receptor (42). Our results clearly demonstrate that endoglin does not function like the type III receptor betaglycan, as it needs coexpression of a ligand binding receptor to interact with ligand. Endoglin cannot bind ligand on its own; it does not alter overall binding to the kinase receptors, but mimics the specificity of the ligand binding receptor it interacts with. Indeed, previous studies have shown that overexpression of full-length functional endoglin in U937 does not alter the binding affinity of receptor complexes (42), and we have obtained similar results when endoglin is expressed in 3T3 fibroblasts. As we show endoglin is not a true receptor, we define it as an accessory protein that interacts with the ligand binding receptors of multiple members of the TGF-β superfamily.

We demonstrate that endoglin not only interacts with ligand binding receptors, but also associates with multiple heteroergic receptor complexes (summarized in Fig. 9A). For TGF-β1, β3, activin, and BMP-7, the type II receptors bind ligand and recruit the type I receptor partners into a high affinity complex. Endoglin can interact with the ligand binding type II receptors, TβRII, ActRII and ActRIIB, regardless of which type I receptor partner is coexpressed, and associates with these type II receptors in the absence of exogenous ligand. Endoglin does not disrupt the formation of the signaling receptor complexes, and can be coprecipitated with these complexes. It could not, however, interact with BMP-7-BMPRII complexes demonstrating specificity of endoglin action, nor did it interact with BMPRII in the absence of ligand. However, the type I receptors ALK3 and ALK6 bind BMP-2 and recruit the type II receptor partners into a high affinity complex. Endoglin also interacts with these ligand binding type I receptors and their respective type II receptors (Fig. 9A). However, the association of endoglin with ALK-3 and ALK-6 was only detectable in the presence of ligand and a chemical cross-linker, which suggests

\[\text{FIG. 7—continued}\]
these associations might be more transient. Interestingly, we observed a reduction in BMP-2 binding to endoglin when BMP-PR1 was coexpressed, yet the ligand binding to BMPRII was not altered. Since endoglin probably binds ligands secondary to its associations with the ligand binding receptors, these data suggest that BMP-PR1 may compete with endoglin for ALK-3 or ALK-6 and that endoglin may be excluded from these complexes. Thus, endoglin is an accessory protein that interacts with multiple heteromeric receptor complexes containing TGF-βs, activins, and BMPs.

We have shown that endoglin interacts with specific ligand binding receptors, and we postulate it is recruited into active receptor complexes in this way (see model in Fig. 9B). What is the significance of these findings? There are several levels of receptor function that could be affected by the presence of endoglin. It could modulate the activity of the receptor kinase complex. However, we have found that endoglin does not alter the transphosphorylation of ALK5 by TpR1. The receptor complexes that endoglin interacts with contain the signal transducing receptors ALK2, 3, 5, or 6. Since endoglin interacts with activin type II receptors, it is likely to be found in heteromeric complexes containing ALK4 (ActRII) (Fig. 9). As multiple signal transducing type I receptors interact with endoglin, it may function to modulate receptor activation of downstream events, such as Smad signaling (Fig. 9). For instance, endoglin might regulate BMP signaling through Smads 1, 5, and 8 or TGF-β/activin signaling through Smads 2 and 3. Although

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The expression of endoglin (1, 64) in cells, stromal fibroblasts, and mesenchymal cells where endoglin might modulate signals, by acting on known Smads and altering the association of RI with RII and thus interacts with a heteromeric complex that can initiate downstream events. We postulate that endoglin does not alter the association of RI with RII and thus interacts with a heteromeric complex that can initiate downstream events. We postulate that endoglin might modulate signals, by acting on known Smads and altering transcriptional responses or by functioning in alternate pathway(s) leading to specific transcriptional responses.

Endoglin expression has no effect on the induction of the plasminogen activator inhibitor promoter by TGF-β or activin-A in mink lung epithelial cells, a more comprehensive look at downstream events may be warranted. Furthermore, endoglin could recruit other proteins or a novel Smad into the signaling complex, thereby inducing a specific nuclear response (Fig. 9).

Previous studies have shown that endoglin plays a role in the TGF-β pathway, as overexpression of endoglin modulates some but not all TGF-β1 responses in U937 monocytes (42). Endoglin has also been implicated in the regulation of trophoblast differentiation, a process stimulated by activin and inhibited by TGF-β1 and -β3 (62, 63). As we now show that endoglin can interact with activin type II receptors, it might be functioning in activin as well as TGF-β receptor complexes during placentation development. Furthermore, activin and TGF-β exert multiple effects on many cell types including erythrocytes, endothelial cells, stromal fibroblasts, and mesenchymal cells where endoglin is expressed (1, 64).

A major role for endoglin in the vasculature was inferred by the finding that it is mutated in HHT1 (36). It is currently unclear what molecular mechanisms underlie HHT pathology; however, our studies now implicate pathways involving activins and BMPs, as well as TGF-βs. Both TGF-β and activin are known to inhibit the proliferation of endothelial cells in culture (65, 66). TGF-β is directly implicated in vascular development and thought to control interaction between endothelial cells and smooth muscle cells (67, 68). BMPs may also be involved in these processes. BMP-2 and -7 can act on vascular smooth muscle cells to inhibit their proliferation without stimulating extracellular matrix synthesis, whereas activin-A has a growth-stimulatory effect (69). The BMP-like factor GDF-5, which binds ALK6, induces angiogenesis while BMP-2 does not (70). This is not surprising, as vascular endothelial cells do not have specific binding sites for BMP-2 (71). Together, these studies suggest a role for BMPs in regulating the maintenance and/or formation of vasculature involving both endothelial and smooth muscle cells where their specific response to these ligands depends on the receptors they express. The downstream effectors responsible for mediating these responses have yet to be identified. In this context, it is interesting to note that ALK1 is the target gene for HHT2 (72). Recently, ALK1 was shown to signal BMP-like responses, yet its ligand in endothelial cells is unclear (73). Furthermore, a class of inhibitory Smads were recently shown to be expressed at high levels in endothelial cells during laminar shear stress (74–77). As stress to blood vessels has been implicated in the development of arteriovenous malformations (78), vascular Smads might also play a role in the pathology of HHT. These findings, together with the demonstration that endoglin is an accessory protein interacting with multiple receptor kinase complexes, support the notion that HHT could involve altered responses of the vasculature in pathways additional to TGF-β. Elucidating the mechanisms of how endoglin functions as an accessory molecule in the TGF-β superfamily is critical to understanding the molecular mechanisms underlying the development of HHT and biological processes where endoglin is expressed.

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