Assignment of Transforming Growth Factor β1 and β3 and a Third New Ligand to the Type I Receptor ALK-1*

(Received for publication, June 19, 1998, and in revised form, January 7, 1999)

Andreas Lux‡§, Liliana Attisano¶, and Douglas A. Marchuk‡**

From the ‡Department of Genetics, Duke University Medical Center, Durham, North Carolina 27710 and the ¶Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Germ line mutations in one of two distinct genes, endoglin or ALK-1, cause hereditary hemorrhagic telangiectasia (HHT), an autosomal dominant disorder of localized angiodyplasia. Both genes encode endothelial cell receptors for the transforming growth factor β (TGF-β) ligand superfamily. Endoglin has homology to the type III receptor, betaglycan, although its exact role in TGF-β signaling is unclear. Activin receptor-like kinase 1 (ALK-1) has homology to the type I receptor family, but its ligand and corresponding type II receptor are unknown. In order to identify the ligand and type II receptor for ALK-1 and to investigate the role of endoglin in ALK-1 signaling, we devised a chimeric receptor signaling assay by exchanging the kinase domain of ALK-1 with either the TGF-β type I receptor or the activin type IB receptor, both of which can activate an inducible PAI-I promoter. We show that TGF-β1 and TGF-β3, as well as a third unknown ligand present in serum, can activate chimeric ALK-1. HHT-associated missense mutations in the ALK-1 extracellular domain abrogate signaling. The ALK-1/ligand interaction is mediated by the type II TGF-β receptor for TGF-β and most likely through the activin type II receptor for the serum ligand. Endoglin is a bifunctional receptor partner since it can bind to ALK-1 as well as to type I TGF-β receptor. These data suggest that HHT pathogenesis involves disruption of a complex network of positive and negative angiogenic factors, involving TGF-β, a new unknown ligand, and their corresponding receptors.

Hereditary hemorrhagic telangiectasia (HHT),1 or Osler-Rendu-Weber disease, is an autosomal dominant disorder characterized by localized angiodyplasia (1). Mutations have been identified in two genes, endoglin and ALK-1 that can cause HHT (2, 3). The scope and variety of mutations identified in the endoglin gene (2, 4–8) and the ALK-1 gene (3, 9, 10), as well as RNA and protein expression analyses, suggest that the majority of these represent null alleles and that the disease phenotype is the result of inherited haploinsufficiency for either endoglin or ALK-1.

By sequence homologies, endoglin and ALK-1 are thought to be endothelial cell receptors for members of the TGF-β superfamily. The TGF-β superfamily (TGF-βs, activins, bone morphogenetic proteins (BMPs), and Müllerian-inhibiting substance) constitutes a family of multifunctional cytokines that regulate many aspects of cellular function such as proliferation, differentiation, adhesion, migration, and extracellular matrix formation (11–14). Signaling occurs via different ligand-induced heteromeric receptor complexes consisting of type I and type II transmembrane serine/threonine kinase receptors.

Models for TGF-β and activin signaling have been proposed by several authors (15–17). TGF-β or activin initially binds the constitutively phosphorylated type II receptor, thereby recruiting the type I receptor into the ligand-type II receptor complex. The type I receptor is subsequently phosphorylated by the type II receptor on serine and threonine residues in its cytoplasmic juxtamembrane GS domain (15, 18). The type I receptor then phosphorlates downstream signaling mediators such as members of the recently identified Smad family (reviewed in Refs. 19–21).

Currently, seven mammalian type I receptors have been cloned. For the majority of these, ligands and corresponding type II receptors have been identified. ALK-4 is an activin type I receptor, ActR-IB (22–24). ALK-3 and ALK-6 are the BMP type I receptors BMPR-IA and BMPR-IB, respectively (24, 25). ALK-2 is an activin and BMP type I receptor (13, 26). ALK-5 is the TGF-β type I receptor, TβR-1 (27). ALK-7 is predominantly expressed in the central nervous system and can complex with type II receptors for both TGF-β and activin (28, 29). However, in functional assays neither ligand was able to elicit a signal.

The ligand and corresponding type II receptor(s) for ALK-1 are also unknown. In the presence of the TGF-β type II receptor (TβR-II), ALK-1 binds TGF-β1, whereas in the presence of the activin type II receptor (ActR-II), ALK-1 binds activin A (22, 30, 31). However, neither of these complexes elicits a signal, as determined by a number of outcomes including proliferation response, an alteration in fibronectin expression, or the ability to activate a PAI-1 promoter-based reporter gene in the mink cell line Mv1Lu. Nonetheless, this does not exclude TGF-β and activin as ALK-1 ligands, as ALK-1 signaling initiated by these cytokines might activate other cellular responses. Unfortunately, the lack of a signaling assay prohibits further analysis of ALK-1 signal transduction and its role in the pathogenesis of HHT.

Signaling assays have been devised for other receptors in this superfamily. In Mv1Lu cells, TβR-1 and ActR-IB are able...
to mediate TGF-β or activin-induced activation of the p3TP-Lux reporter construct, which contains the ligand-inducible portion of the PAI-1 promoter (22, 32). By using chimeric receptors, several investigators have shown that signal specificity is determined by the intracellular domain of a chimeric type I receptor, together with the appropriate type II receptor, independent of the ligand binding specificity of the extracellular domain (33–37). We hypothesized that the same would hold true for the domains of ALK-1 if provided with the correct ligand/type II receptor combination. Therefore, we constructed chimeric receptors with the ALK-1 extracellular domain and the cytoplasmic domain of TβR-I or ActR-IB to create an ALK-1 signaling assay.

Endoglin is thought to be a TGF-β type III receptor based on its sequence homology to the proteoglycan betaglycan (38–40). Betaglycan presents the ligands to TβR-II and TβR-I increasing the signaling activity of the type I receptor (41). Because of its sequence homology to betaglycan, a similar function is proposed for endoglin. We will present here data that endoglin is the binding partner for ALK-1 suggesting a role for endoglin in modulating signaling via ALK-1.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**All cell media and fetal bovine serum (FBS) were obtained by Life Technologies, Inc. COS-1 cells, a gift by Dr. B. Cullen (Duke University, Durham, NC), were cultured in modified Dulbecco’s medium + 5% FBS. R-1B and DR-26 cells were derived in earlier studies by chemical mutagenesis of M1-Lu cells (42, 43). Cells were maintained in minimal essential medium (MEM) containing nonessential amino acids (NEAA) and 10% FBS. All cell lines were cultured at 37 °C in a 5% CO₂ environment.

**Antibodies and Cytokines—**Rabbit antiserum against endoglin and ALK-1 was prepared against part of the endoglin extracellular domain, encoded by exons 2–5, and the entire ALK-1 extracellular domain except for the leading sequence. Briefly, the corresponding cDNAs were cloned into the bacterial expression vector pET-15b (Novagen) behind a 6× His tag and expressed in bacteria. Bacteria were lysed, and recombinant proteins were purified without a nickel-nitriiotriacetic acid column (Qiagen). The column-purified recombinant proteins were then separated over a SDS-PAGE, cut out, and electroeluted. The eluted antigen was mixed with Freund’s adjuvant and used to immunize rabbits. The resulting polyclonal antibodies were purified over a nickel-nitrilotriacetic acid column (Qiagen). The column-purified recombinant proteins were then used in the activin A luciferase reporter assays. Human serum was purified over a nickel-nitrilotriacetic acid column. To create ALK-1(TM) the entire TβR-1/transmembrane/cytoplasmic domain encoding sequence was amplified using the 5’-primer, an endoglin-specific oligonucleotide (number 1–4) for the GS domains of the different receptors, containing the AccIII recognition sequence in the middle of the primer. The new AccIII sites were verified by sequencing. The constructs were then digested with the restriction enzyme AccIII and an appropriate second enzyme that allowed us to cut out the C-domain of the different type I receptors. The resulting DNA fragments were gel-purified (Qi-AEX Gel Extraction Kit, Qiagen) and used for ligations to create different chimeric receptors. These chimeras are assigned a name with the addition -GS, i.e. ALK-1-TβR-I(GS). The correct reading frame around the AccIII site was verified by DNA sequencing. The chimeric cDNAs were then cloned back into the pCMV5 vector.

A second set of chimeric receptors was constructed, containing the extracellular domain of ALK-1 or ALK-3 and the transmembrane domain and cytoplasmic domain of TβR-1, ALK-1/TβR-1(TM) (ALK-1(TM)), and ALK-3/TβR-1(TM) (ALK-3(TM)). The chimeric receptors were constructed using PCR with a 1/5 mixture of Vent polymerase (New England Biolabs)/Taq polymerase (Life Technologies, Inc.). Specifically, the TβR-I cDNA in pUC18 was used as template in the PCRs. To create ALK-1(TM) the entire TβR-1 transmembrane/cytoplasmic domain encoding sequence was amplified using, as the 5’-primer, an endoglin-specific oligonucleotide (oligonucleotide 5) containing the last 11 nucleotides of the ALK-1 extracellular domain, with the ALK-1 unique MscI site, followed by the first 23 nucleotides of the TβR-1 transmembrane domain.

To construct the chimeric type I receptors that were swapped in the extracellular domain of ALK-1 or ALK-3 and the transmembrane domain and cytoplasmic domain of TβR-1, ALK-1/TβR-1(TM) (ALK-1(TM)), and ALK-3/TβR-1(TM) (ALK-3(TM)). The chimeric receptors were constructed using PCR with a 1/5 mixture of Vent polymerase (New England Biolabs)/Taq polymerase (Life Technologies, Inc.). Specifically, the TβR-I cDNA in pUC18 was used as template in the PCRs. To create ALK-1(TM) the entire TβR-1 transmembrane/cytoplasmic domain encoding sequence was amplified using, as the 5’-primer, the oligonucleotide (number 6) was used, containing the last 12 nucleotides of the ALK-3 extracellular domain, with the ALK-3 unique BamHI site, followed by the first 21 nucleotides of the TβR-1 transmembrane domain. For the 3’-end the M13 reverse primer was used, amplifying the part of the pUC18 multiple cloning site, which allows cutting the PCR products at their 3’-end with BamHI for subsequent cloning. To create ALK-1(TM) the entire TβR-1 transmembrane/cytoplasmic domain encoding sequence was amplified using, as the 5’-primer, the oligonucleotide (number 7) containing the last 11 nucleotides of the ALK-1 extracellular domain, with the ALK-1 unique MscI site, followed by the first 23 nucleotides of the TβR-1 transmembrane domain. For the 3’-end the M13 reverse primer was used, amplifying the part of the pUC18 multiple cloning site, which allows cutting the PCR products at their 3’-end with BamHI for subsequent cloning. The resulting PCR products were purified with the QiAquick PCR Purification Kit (Qiagen), digested with MscI/BamHI or BamHI/BamHI, gel-purified, and then ligated in the extracellular domain of ALK-1 or ALK-3. The correct sequence and reading frame were verified by DNA sequencing. All constructs have a C-terminal HA tag.

**Extracellular HHT patient mutations were introduced in the ALK-1/TβR-1(TM) chimeric receptor by site-directed mutagenesis using the QuickChange™ mutagenesis Kit (Stratagene).** The constructs were made with the following oligonucleotide pair combinations: ALK-1(TM)insG140 (number 7,8), ALK-1(TM)/W50C (number 9,10), ALK-1(TM)/C51Y (number 11,12), ALK-1(TM)/R67Q (number 13,14).
Roles of ALK-1 and Endoglin in TGF-β Signaling

μl of monoclonal antibody (cell culture supernatant). After at least 3 h 20 μl of protein A-Sepharose was added for additional 3 h. The protein A-Sepharose immunocomplex was then washed three times in 500 μl of lysis buffer and resolved in 15 μl of Laemmli buffer. The precipitated proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane (BioBlot-NC, Costar) for immunodetection with the indicated antibody. Immunodetection was performed with the ECL Western blotting analysis system (Amersham Pharmacia Biotech) according to the manufacturer’s instruction.

Luciferase Reporter Assay—For luciferase expression assays, the R-1B and DR-26 cells were transiently transfected with p3TP-Lux (32), the primed receptor cDNA in pCMV5, pCMV5 alone, or both. The pSV-β-galactosidase vector (pSV-β) (Promega) was always included to normalize the luciferase activity for the different transfections. Cells were plated on 60-mm dishes and grown to 80% confluence. Prior to transfection, cells were washed once with serum-free medium. Cells were transfected in 3 ml of serum-free medium containing 6 μg of total DNA in the following combinations: (a) 2 μg of p3TP-Lux, 3 μg of pCMV5, and 1 μg of pSV-β; (b) 2 μg of p3TP-Lux with either 2 μg of type I receptors or type I chimeras, 1 μg of pCMV5, and 1 μg of pSV-β. The transfections also included 0.1 mM chloroquine/ml and 0.125 mg/ml DEAE-dextran in phosphate-buffered saline. In assays where two type I receptors were co-transfected, 1.5 μg of each receptor cDNA were used. In assays where the activity for cells co-transfected with two receptors had to be compared with the activity of single receptor transfected cells, the second receptor was substituted with 1.5 μg of pCMV5 DNA in the single receptor transfected cells. After 3 h incubation at 37°C, cells were shocked with 10% Me2SO in phosphate-buffered saline for 2 min and allowed to recover overnight in MEM/NEAA + 10% FBS. The following day, cells were trypsinized and replated into a 24-well plate and allowed to recover overnight in MEM/NEAA + 10% FBS to allow the cells to reattach. Prior to the luciferase reporter induction, cells were incubated for 2 h in MEM/NEAA + 0.2% FBS. Medium was then exchanged for fresh MEM/NEAA + 0.2% FBS with the addition of the different cytokines to induce the luciferase reporter. After 16 h incubation, cells were washed with phosphate-buffered saline and lysed for 15 min in 40 μl of lysis buffer. 10 μl were used to measure the luciferase activity using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer. The remaining 30-μl lysates were used to measure the LacZ activity as described previously (45). Luciferase activity was corrected for LacZ activity.

Primers—The following primers were used in the constructions: (1) (ALK-1Acc), 5′-GGGAGTGGCCCTCCGCACCCTCCCTCC-3′; (2) (Thr-1Acc), 5′-GGGTCTGGCCTCCGATATTCACTGG-3′; (3) (ActR-IBAcc), 5′-GGGGTCTGGCCTCCGATATTACCCCTCCCTCC-3′; 4 (ALK-1gsAcc), 5′-GTATGGGGTCCGACTACCTC-3′; 5 (ALK-1gsAccThr-1B), 5′-CAGATGGCAGTAGTGCTG-3′; 6 (ALK-3gsAccThr-1B), 5′-CAGCAGCA-TTGCACGTGCTTGCAGGACC-3′; 3 (ActR-IgsAcc), 5′-GGGAGCAGC-TGACGTGCTTGCAGGACC-3′; 7 (ALK-1gsAccTM-ACTR-IB), 5′-GGGGTCTGGCCTCCGATATTACCCCTCCCTCC-3′; 8 (Thr-1Acc), 5′-GTGACAGCAGGGCCCCGGGGTGA-3′; 9 (ActR-IgsAcc), 5′-GGGGGAGCTTGGTGGTACATGGTCTG-3′; 10 (ALK-3gsAccTM-ACTR-IB), 5′-GGGGGAGCTTGGTGGTACATGGTCTG-3′; 11 (ALK-1gsAccTM-ALK-1), 5′-GGGGGAGCTTGGTGGTACATGGTCTG-3′. The

RESULTS

Test of the Chimeric Receptor Signaling Assay—Since the ligand and type II receptor for ALK-1 are not known, a signaling assay for ALK-1 activity is not currently available. We sought to develop a signaling assay involving chimeric receptors. As an initial test, we created chimeric receptors which we surmised would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal. ALK-3 is the receptor for BMP-2 and to a lesser extent BMP-7, but does not surmise would be able to transmit a signal.
 Roles of ALK-1 and Endoglin in TGF-β Signaling

Fig. 2. BMP and activin A-induced signaling in DR-26 cells via chimeric receptors. DR-26 cells were transiently transfected with the indicated chimeric receptors or the empty expression vector pCMV5. A fixed amount of the reporter p3TP-Lux was included in all transfections. Luciferase activity in cell lysates was plotted as the average and standard deviation for transfections done in triplicate. Only data from the chimeras exchanged in the GS domain are shown. A, cells were incubated overnight with either 15 nM BMP-2 or 15 nM BMP-7 or without BMP (w/o). B, cells were incubated overnight without ligand (w/o), or with 150 µl of conditioned medium (activin) of COS cells transfected with an activin A expression construct, or with 150 µl of conditioned medium (IMD medium) of COS cells not transfected with the activin A expression construct.

pared with mock-transfected cells (Fig. 3). Despite the high background, the ALK-1/TβR-I(GS) chimera also showed a clear response to TGF-β1 and TGF-β3, although only half that of TβR-I. Significantly, the chimera did not respond to TGF-β2. The ALK-1/TβR-I(1TM) chimera also showed a similar ligand-specific response although the magnitude of the response was lower (data not shown). This response was specific to the ALK-1 extracellular domain as ALK-3/TβR-I(GS) showed no response to any of the TGF-β ligands (data not shown). These combined results indicate that TGF-β1 and -β3 can induce a specific signal via the ALK-1 chimeras. The binding specificity for the TGF-β isoforms resembles that of endoglin (46), consistent with the idea that endoglin and ALK-1 are in the same signaling pathway.

One of the controls, TβR-I/ActR-IB, did not respond to any of the TGF-βs (Fig. 3), although the chimeric protein was expressed. This was surprising, since the exchanged ActR-IB kinase domain is nearly identical to the kinase domain of TβR-I, particularly in the L45 region of kinase subdomains IV and V, which are responsible for the TGF-β specificity (47). These results suggest that the type I receptor is not solely responsible for the signaling specificity and that another requirement may be the correct combination of type I and II receptors in the complex. Consistent with this, Persson and colleagues (34) showed that the chimeric TβR-I/BMPR-IB receptor is able to signal in combination with the chimeric TβR-II/ActR-IB receptor upon TGF-β addition but not with the wild-type TGF-β type II receptor TβR-II.

Receptor Complexes with Endoglin and ALK-1—Since endoglin and ALK-1 show the same TGF-β ligand specificity, we determined whether they could form a receptor complex. We also determined whether ALK-1 can form a ligand-independent complex with either TβR-II or ActR-II, which might explain the high basal level of luciferase activity for the ALK-1 chimeras, even in the absence of any ligand. COS cells were co-transfected with endoglin and ALK-1 (HA-tagged), lysed, and immunoprecipitated with either the monoclonal HA antibody or rabbit anti-endoglin sera. The immunoprecipitations were separated by SDS-PAGE and immunostained with the reciprocal antibody. Fig. 4, A and B, shows that endoglin and ALK-1 can be immunoprecipitated in a ligand-independent complex. However, a different anti-endoglin antibody, P1D3, did not co-immunoprecipitate ALK-1, although endoglin was immunoprecipitated (data not shown). P1D3 is directed against an epitope encoded by exons 2 and 3 (48). This region may also be critical for the endoglin/ALK-1 interaction, which may be blocked by P1D3.

Similar immunoprecipitations with endoglin and TβR-I or ActR-IB demonstrate that these two type I receptors are also able to co-immunoprecipitate endoglin, although these interactions were weaker than for ALK-1 (Fig. 4A). Co-immunoprecipitations for endoglin and the type II receptors TβR-II and ActR-II did not reveal any interactions among these receptors (data not shown).

In order to identify ALK-1 interactions with type II receptors, cells were co-transfected with combinations of either ALK-1 and TβR-II or ALK-1 and ActR-II. Immunoprecipitation for ALK-1 brought down both type II receptors (Fig. 4C). Reciprocal immunoprecipitations for the type II receptors brought down ALK-1 (data not shown). These results confirm that ALK-1 and these type II receptors are in a ligand-independent receptor complex and suggest that TβR-II and ActR-II can both act as type II receptors for ALK-1.

Human Serum Contains an Unknown Ligand for ALK-1—In an effort to identify a ligand for ALK-1, we also examined human serum as a source of ligand in the signaling assay. All three ALK-1 chimeras transfected into either R-1B or DR-26 cells showed a dosage-dependent increase in induction of luciferase activity which reaches a plateau at 20% serum (Fig. 5A). In order to determine if this response was due to TGF-β present in the serum, R-1B cells were transfected with TβR-I or ALK-1/TβR-I(GS) and incubated with either TGF-β1 or 20% human serum in the presence or absence of a TGF-β1 neutralizing antibody. The neutralizing antibody reduced the level of luciferase activity by more than half for TβR-I-transfected cells.
incubated with TGF-β1 (Fig. 5B). However, it had no effect on the human serum-induced signaling activity of the ALK-1/TβRI(GS) transfected.

In addition, when DR-26 cells were transfected only with TβRII cells transfected with ActR-IB or the ALK-3 chimeras also showed no response to the human serum (data not shown). These combined data show that the serum-induced receptor activity observed with ALK-1 chimaera-transfected cells is not due to the presence of TGF-β, activin A, or BMP in the serum. Inhibin A is similar to activin, present in serum, and binds to ActRII (49–51). Since we have shown that ActRII can form in a complex with ALK-1, and DR-26 cells express the endogenous activin type II receptor, the observed serum response may be induced by inhibin A via an ActRII-ALK-1 chimaera receptor complex. However, inhibin A was also unable to induce signaling via the ALK-1 chimeras (Fig. 5D).

Effect of HHT-associated Patient Mutations on ALK-1 Signaling—To examine further the presence of an ALK-1 ligand in serum, we tested the effect of ALK-1 chimeras harboring mutations that have been identified in HHT patients. Mutations introduced include one insertion mutation, insG140 (10), and three base substitutions causing amino acid changes, W50C, C51Y, and R67Q (9, 10). These mutations were introduced into the extracellular domain of the ALK-1/TβRII(TM) chimaera, and their effect on the serum-induced signaling activity was determined. The insertion mutation served as a negative control as it introduces a frameshift early in the extracellular (ligand binding) domain, leading to a premature stop codon. Examination of serum-induced luciferase activity demonstrated that the insG140 and C51Y mutations both showed no measurable signaling (Fig. 6). The signaling activity for the W50C and R67Q substitutions were also examined for TGF-β1-dependent induction of the luciferase reporter. The signaling activity of these mutant ALK-1 chimeras was also abrogated (data not shown). These data are the first demonstration that any of the ALK-1 missense mutations identified in HHT patients have deleterious consequences for ALK-1 signaling.

ALK-1 Signaling Interferes with TGF-β-induced TβRII Signaling—Although ALK-1 and TβRII bind TGF-β1 and TGF-β3 and can form a ligand-independent complex with endoglin, they do not activate the same genes (22, 25, 31). Thus, we hypothesized that wild-type ALK-1 may have an opposing effect on TGF-β signaling through TβRII. In order to induce ALK-1 signaling independent of TGF-β addition, we designed a constitutively active form of ALK-1. For other type I receptors, the substitution of either Gln or Asp for a critical residue in the kinase domain creates a constitutively active type I receptor able to signal without ligand or corresponding type II receptor (18, 44). Therefore, we created an “activated” form of ALK-1 by replacing Gln (wild type) with Asp at the equivalent position in ALK-1 (ALK-1*Q201D). The effect of the activated ALK-1 receptor in the luciferase assay was determined.

Co-expression of TβRI and wild-type ALK-1 yielded a 20% decrease of TGF-β1-induced signaling in comparison to TβRI alone (Fig. 7A). This effect is seen both for TGF-β1 and -β2. Furthermore, when activated ALK-1 is co-expressed with TβRI, TGF-β1-induced signaling is decreased by 33% in comparison to TβRI alone. Since constitutively activated receptors do not always signal with the same intensity as the wild-type receptor when induced by ligand, we determined whether serum-activated ALK-1 might have a more potent effect on TβRII signaling through TGF-β1. R-1B cells were co-transfected with TβRII and ALK-1 and incubated with either TGF-β1 or TGF-β2 plus human serum. The resulting signaling data were compared with cells transfected with TβRI alone. Co-incubation of TβRII-transfected cells with TGF-β1 and serum shows a 31% reduction in signaling compared with TGF-β1 alone (Fig. 7B). This may be due to serum components that bind to TGF-β1 and sequester it or otherwise inhibit its function. The ALK-1 and
FIG. 5. Human serum-induced ALK-1 chimera signaling in R-1B and DR-26 cells. Luciferase activity in cell lysates was plotted as the average and standard deviation for transfections done in triplicate. A, R-1B cells were transiently transfected with p3TP-Lux and the indicated receptor or pCMV5. Cells were incubated overnight with the indicated amount of heat-inactivated human serum or without serum (w/o). B, R-1B cells were transiently transfected as in A. Cells were incubated overnight with either 4 ng/ml TGF-β1 or 20% heat-inactivated human serum, or without ligand (w/o). Neutralizing antibody to TGF-β1 was added where indicated at 1 μg/ml. C, DR-26 cells were transiently transfected with p3TP-Lux and the indicated receptors or pCMV5. Cells were incubated overnight with either 4 ng/ml TGF-β1, 20% heat-inactivated human serum, or without ligand (w/o). D, inhibin A does not induce ALK-1 chimera signaling. R-1B cells were transiently transfected with p3TP-Lux and the indicated receptor or pCMV5. Cells were incubated overnight with either the indicated amount of recombinant human inhibin A or without ligand (w/o). Luciferase activity in cell lysates was plotted as the average and standard deviation for transfections done in triplicate.
TβR-I co-transfection showed a 10% reduction in luciferase activity after TGF-β1 induction. However, the TGF-β1 plus human serum co-incubation resulted in a 70% reduction in signaling (Fig. 7B). These data demonstrate that in this system, ALK-1 signaling opposes the TβR-I-induced pathway. It is unclear whether ALK-1-mediated signaling actively inhibits the PAI-1 promoter or if the ALK-1-induced pathway is competing for components of the TβR-I-signaling pathway, such as Smad4.

**DISCUSSION**

The autosomal dominant vascular disorder HHT is characterized by the development of localized vascular arteriovenous malformations. Human genetic studies have shown that HHT can be caused by mutations in one of two genes, endoglin or ALK-1. Endoglin is a TGF-β1- and -β3-binding protein expressed primarily on the surface of endothelial cells, and ALK-1 is a type I TGF-β superfamily receptor also expressed on the surface of endothelial cells. However, the role of these receptors in the pathogenesis of HHT is unclear.

TGF-β is known to play a key role in angiogenesis, especially after injury or inflammation (56–58). In addition, the phenotypes of the TGF-β1 (ligand) and the TβR-II (receptor) knockout mice suggest a critical role in early yolk sac vasculogenesis and hematopoiesis (59, 60). Intriguingly, the vascular defects in the TGF-β1 null mice share some histological similarities to the vascular lesions seen in HHT patients (59). ALK-1 expression in the developing mouse embryo parallels that of TGF-β1 (61), and ALK-1 knock-out mice are also embryonic-lethal due to defects in yolk sac vasculogenesis and hematopoiesis. The data from these mouse mutations might suggest that ALK-1 and endoglin act in the same angiogenic pathway involving TGF-β.

However, additional data suggest complications to this simple interpretation. Although TβR-I and ActR-IB signal in mink lung cells through the PAI-1 promoter reporter system, ALK-1 does not signal in a TGF-β1- or activin A-specific manner (22). Furthermore, ALK-1 does not mediate TGF-β1-like responses such as growth inhibition or elevated expression of extracellular matrix proteins (23, 25, 31). These data suggest distinct roles for ALK-1- and TGF-β-mediated signaling.

Through the use of a chimeric receptor signaling assay based on the inducible PAI-1 reporter construct, we have shown that activin A, BMP-2, BMP-7, and inhibin A are unable to activate ALK-1 signaling. The negative result for activin A was surprising since activin A binds to ALK-1 in the presence of the activin type II receptor (22), and our data and that of others (25, 31) show ALK-1 and ActR-II present in a ligand-independent complex. However, we created two distinct ALK-1 chimeras, containing either the ActRI-B or TβR-I kinase domains, and neither exhibited any evidence of signaling in our reporter system. Furthermore, a positive control chimera, ActR-IB/TβR-I(GS), demonstrated even higher activin-induced signaling activity.

**FIG. 6.** Effect of HHT-associated mutations on ALK-1 signaling. R-1B cells were transiently transfected with p3TP-Lux and the indicated receptors or pCMV5. Cells were incubated overnight with either 20% heat-inactivated human serum or without ligand (w/o).

**FIG. 7.** Effect of ALK-1 signaling on TGF-β-induced TβR-I signaling. Luciferase activity in cell lysates was plotted as the average and standard deviation for transfections done in triplicate. A, R-1B cells were transiently transfected with p3TP-Lux and the indicated receptors or pCMV5. Cells were incubated overnight with either 4 ng/ml TGF-β1, 4 ng/ml TGF-β2, or without ligand (w/o). B, R-1B cells were transiently transfected with p3TP-Lux and the indicated receptors or pCMV5. Cells were incubated overnight with either 4 ng/ml TGF-β1, 20% heat-inactivated human serum (h.serum), or 4 ng/ml TGF-β1 and 20% heat-inactivated human serum (beta1+h.serum), or without ligand (w/o).

2 En Li, personal communication.
than the natural activin type I receptor ActR-IB. Thus, we conclude that activin A is not a signaling ligand for ALK-1.

The ALK-1 chimeras show increased signaling activity after TGF-β1 and TGF-β3 incubation but not with TGF-β2. This ligand specificity parallels that of endoglin (46). Both ALK-1 and endoglin are also expressed in trophoblasts during early stages of placenta development (61, 62). These data and our endoglin/ALK-1 and ALK-1/TβR-II co-immunoprecipitation results suggest that ALK-1 and endoglin are part of a TGF-β signaling receptor complex. The TGF-β-induced ALK-1 signaling in R-1B cells is only half that of the TβR-I controls. This might be due to a lower affinity of ALK-1 to TGF-β or due to the presence of two different type II receptor binding partners for ALK-1. The second type II receptor might be able to sequester ALK-1 resulting in reduced ligand-induced signaling activity. Our serum and co-immunoprecipitation data show that ALK-1 is able to use two different type II receptors, both present in R-1B cells.

Additional but indirect evidence for the importance of endoglin is provided by Matsuzaki et al. (64), who reported intrinsic ligand-independent interactions among type III and I TGF-β receptors and also among type II I and I receptors but not among type III and II receptors, which were ligand-dependent. Similarly, we observed ligand-independent complexes of endoglin/ALK-1 (a type III-I interaction), as well as ALK-1/TβR-II and ALK-1/ActR-II (a type III-II interaction) but not endoglin/ TβR-II or endoglin/ActR-II (a type III-II interaction). They also tested the phosphorylation status of the TβR-I/TβR-II receptor complex after TGF-β addition and found an increase in TβR-I and TβR-II phosphorylation. When betaglycan was added to this complex they observed an additional increase of phosphorylation for both. The transmembrane and cytoplasmic domain of endoglin is highly conserved among different species and shows high homology (71%) to the corresponding region of the TGF-β type III receptor betaglycan (38–40, 46, 52, 53). The combined data suggest that type III receptors (either betaglycan or endoglin) promote efficient type I receptor signaling.

TGF-β cross-linking studies in porcine aortic endothelial cells and pre-B leukemic cells have demonstrated endoglin in a complex with TβR-I and TβR-II (52, 65). In both studies the immunoprecipitation was performed with either an endoglin- or TβR-II-specific antibody after TGF-β1 cross-linking and resulted in the co-precipitation of a protein of approximately 70 kDa, the predicted size for a type I receptor. The authors concluded that this was the TGF-β type I receptor. However, since ALK-1 is expressed in endothelial cells, the co-precipitated type I receptor in porcine aortic endothelial cells may actually have been ALK-1. One possibility is that ALK-1 and TβR-I are expressed on different subsets of cells which would allow for unique and specific responses to TGF-β. This hypothesis has also been proposed by Panchenko et al. (66). They showed that the rat ALK-1 is highly expressed in adult lung, whereas TβR-I is not, and that the two receptors are also differentially expressed in the fetal lung. Therefore, it is possible that some of the well documented endothelial responses to TGF-β (56–58) are due to signaling through ALK-1 and not TβR-I.

A component of serum, which seems to be not one of the “common” TGF-β family members, can act as a third signaling ligand for ALK-1. Although TGF-β1- and β3-induced ALK-1 signaling occurs via TβR-II, signaling induced by the “serum ligand” does not, since serum-induced signaling via ALK-1 chimeras was observed in cells lacking either TβR-I or TβR-II. An extensive PCR screen with degenerate oligonucleotides for type II receptors could not identify any novel type II receptor in endothelial cells. Thus, the serum ligand may instead signal through ActR-II. Although we cannot exclude unknown candidates, these data suggest TβR-II and ActR-II are the major type II receptors for ALK-1.

The existence of a third unknown ligand for ALK-1 complicates our understanding of the function of ALK-1 in endothelial cells. Previous studies have shown that ALK-1 does not mediate growth inhibition or elevated expression of extracellular matrix proteins such as fibronectin or PAI-1 after TGF-β1 incubation (23). We have shown that ALK-1 signaling is able to oppose TGF-β-induced PAI-1 promoter activity, although we cannot conclude if this is a direct inhibitory effect of ALK-1 signaling on the PAI-1 promoter or if the TβR-I and ALK-1 pathways overlap and therefore compete for common signaling mediators. In future studies of TGF-β signaling, it will be critical to differentiate ALK-1 versus TβR-I signaling, particularly in cells that co-express both receptors.

We are still a long way from understanding the roles of ALK-1 and endoglin in angiogenesis and their role in the pathogenesis of HHT. Nonetheless, the cumulative data suggest a basic outline for the role of the two receptors in these processes. With few exceptions, the endothelial cell turnover in a healthy adult organism is very low. The maintenance of this quiescent state is thought to be regulated by endogenous negative regulators. During angiogenesis the balance between negative and positive regulators is shifted, and positive regulators dominate. Studies have shown that TGF-β (particularly TGF-β1) can be either a positive or negative regulator (reviewed in Ref. 56). A biphasic effect of TGF-β on angiogenesis is dependent on TGF-β concentration (67). Therefore, endothelial response to TGF-β may also be concentration-dependent (68). This biphasic effect may be established by the use of two different receptors such as TβR-I and ALK-1, which may have different affinities for TGF-β. Endoglin might be required as a receptor-partner for both type I receptors in order to maintain the balance between negative and positive regulation of angiogenesis. This picture is complicated by the identification of a third unknown ligand. ALK-1 antagonistic activity could be induced by the ligand at different time points. Alternatively, ALK-1 signaling via this novel ligand may result in other angiogenic responses that are TGF-β-independent. Future studies are required to identify the ligand present in serum and to dissect the ALK-1/ endoglin signaling pathway and its role in angiogenesis and the pathogenesis of HHT.

Acknowledgments—We thank D. Klaus for technical assistance; T. Stenzel (Duke University) for making the endoglin polyclonal antibody; B. Cullen (Duke University) for the gift of the COS cell line; and H. Esche (Universitätsklinikum Essen, Germany) for the gift of the monoclonal HA hybridoma. Inhibin A was obtained from A. F. Parlow (Harbor-UCLA Medical Center) through the National Hormone and Pituitary Program.

REFERENCES

1. Guttmacher, A. E., Marchuk, D. A., and White, R. I. (1995) N. Engl. J. Med. 333, 918–924.
2. McAllister, K. A., Grogg, K. M., Johnson, D. W., Gallione, C. J., Baldwin, M. A., Jackson, C. E., Heimbold, E. A., Markel, D. S., McKinnon, W. C., Murrell, J., McCormick, M. K., Pericak-Vance, M. A., Heutink, P., Oostera, B., Haitjema, T., kWesternman, C. J. J., Porteous, M. E., Guttmacher, A. E., Letarte, M., and Marchuk, D. A. (1994) Nat. Genet. 8, 345–351.
3. Johnson, D. W., Berg, J. N., Baldwin, M. A., Gallione, C. J., Marenadel, I., Yoon, S.-D., Stenzel, T. T., Speer, M., Pericak-Vance, M. A., Diamond, A., Guttmacher, A. E., Jackson, C. E., Attisano, L., Kucherlapati, R., Porteous, M. E. M., and Marchuk, D. A. (1996) Nat. Genet. 13, 189–196.
4. McAllister, K. A., Baldwin, M. A., Thukkani, A. K., Gallione, C. J., Berg, J. N., Porteous, M. E., Guttmacher, A. E., and Marchuk, D. A. (1995) Hum. Mol. Genet. 4, 1983–1985.
5. Shovlin, C., Hughes, J. M. B., Scott, J., Seidman, E., and Seidman, J. G. (1997) Am. J. Hum. Genet. 61, 66–79.
6. Pece, N., Vera, S., Cymerman, U., White, R. I., Wrana, J. L., and Letarte, M. D. W. Johnson and D. A. Marchuk, unpublished data.
