Angiopoietin-1 and Angiopoietin-2 Share the Same Binding Domains in the Tie-2 Receptor Involving the First Ig-like Loop and the Epidermal Growth Factor-like Repeats*

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Ulrike Fiedler†, Tanja Krissl‡, Stefanie Koidl¶, Cornelia Weiss§, Thomas Koblizek∥, Urban Deutsch∥∥, Georg Martiny-Baron∥∥∥, Dieter Marmé‡, and Hellmut G. Augustin§§§

From the †Department of Vascular Biology and Angiogenesis Research, Tumor Biology Center, 79106 Freiburg, Germany and the §Max-Planck-Institute for Physiological and Clinical Research, D-61231 Bad Nauheim, Germany

Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) have been identified as ligands with different effector functions of the vascular assembly and maturation-mediating receptor tyrosine kinase Tie-2. To understand the molecular interactions of the angiopoietins with their receptor, we have studied the binding of Ang-1 and Ang-2 to the Tie-2 receptor. Enzyme-linked immunosorbent assay-based competition assays and co-immunoprecipitation experiments analyzing the binding of Ang-1 and Ang-2 to truncation mutants of the extracellular domain of Tie-2 showed that the first Ig-like loop of Tie-2 in combination with the epidermal growth factor (EGF)-like repeats (amino acids 1–360) is required for angiopoietin binding. The first Ig-like domain or the EGF-like repeats alone are not capable of binding Ang-1 and Ang-2. Concomitantly, we made the surprising finding that Tie-2 exon-2 knockout mice do express a mutated Tie-2 protein that lacks 104 amino acids of the first Ig-like domain. This mutant Tie-2 receptor is functionally inactive as shown by the lack of ligand binding and receptor phosphorylation. Collectively, the data show that the first 104 amino acids of the Tie-2 receptor are essential but not sufficient for angiopoietin binding. Conversely, the first 360 amino acids (Ig-like domain plus EGF-like repeats) of the Tie-2 receptor are necessary and sufficient to bind both Ang-1 and Ang-2, which suggests that differential receptor binding is not likely to be responsible for the different functions of Ang-1 and Ang-2.

The first vasculogenic formation of a primitive embryonic vascular plexus occurs by in situ differentiation of angioblasts. Vasculogenesis is followed by angiogenesis, the sprouting and subsequent remodeling from a pre-existing vasculature. The vasculature in the adult is quiescent with a very low turnover rate of the lining endothelial cell layer with a physiological proliferative turnover within months to years (1, 2). However, it can rapidly respond to angiogenic stimuli supporting a complex morphogenetic program that leads to vascular remodeling and induction of neo-angiogenesis. The balance between neovessel formation and homeostasis of the resting vasculature is maintained by a finely tuned balance of proangiogenic and antiangiogenic mediators. The Tie/angiopoietin receptor ligand system is critically involved in regulating both processes, maintaining vascular homeostasis and vessel maturation as well as vascular destabilization and remodeling (1, 3).

Two members of the Tie-receptor family with strong sequence homology, Tie-1 and Tie-2, have been identified so far. Both molecules are preferentially expressed by endothelial cells and were originally isolated as orphan receptors (4). They are composed of three EGF1 homology repeats flanked by two Ig-like loops. The second Ig-like loop is followed by a fibronectin type III domain adjacent to the transmembrane domain. The intracellular domain contains a split tyrosine kinase domain (Fig. 1).

Four Tie-2 ligands have been identified, Ang-1, Ang-2, Ang-3, and Ang-4 (5–7). Among these, Ang-1 and Ang-2 are characterized in most detail. Both bind the Tie-2 receptor with similar affinity (5, 6). Ang-1 is primarily expressed by pericytes, fibroblasts, and smooth muscle cells (5). Upon Ang-1 binding, Tie-2 becomes autophosphorylated, promoting endothelial cell migration and survival (5, 8, 9). Ang-1- and Tie-2-deficient mice have similar phenotypes characterized by embryonic lethality with severe vascular remodeling defects, insufficient vessel stabilization, and perturbed vascular maturation (4, 10).

In contrast, Ang-2 is not capable of inducing Tie-2 autophosphorylation in endothelial cells upon short term treatment (6). Instead, it appears to act as a natural antagonist of Ang-1, which was inferred from the observation that Ang-2 transgenic embryos exhibit a phenotype largely similar to the embryonic lethal phenotype of Ang-1- and Tie-2-deficient mice (6, 10). Ang-2 is expressed by endothelial cells at sites of vascular remodeling and apparently acts by destabilizing the contacts between endothelial cells and surrounding mural cells, most notably pericytes (6). As such, it acts as a facilitator of vascular morphogenesis and remodeling, acting proangiogenic in the presence of angiogenic stimulators, such as VEGF (11), and antiangiogenic and vessel regression inducing in the absence of

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† Present address: Ares Serono, Geneva, Switzerland.
‡ Present address: Artemis Pharmaceuticals, Tübingen, Germany.
§§ Dept. of Vascular Biology and Angiogenesis Research, Tumor Biology Center, 79106 Freiburg, Germany.
¶¶ Present address: Max-Planck-Institute for Cellular Biology, Münster, Germany.
∥∥∥ Present address: Novartis, Basel, Switzerland.
∥∥∥∥ Depart of Vascular Biology and Angiogenesis Research, Tumor Biology Center, Breisacher Str. 117, D-79106 Freiburg, Germany.

1 The abbreviations used are: EGF, epidermal growth factor; VEGF, vascular EGF; sVEGF, soluble VEGF; Ang, angiopoietin; CHO, Chinese hamster ovary cells; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; aa, amino acids; WT, wild-type; RT, reverse transcriptase.
Angiopoietin Binding of Tie-2

**EXPERIMENTAL PROCEDURES**

**Cells**—Immortalized endothelioma cell lines were generated by infecting primary cells from several litters of embryonic day 9.5 embryos derived from matings of Tie-2+/− mice with a retrovirus coding for the Polyoma virus middle T-antigen as described previously (16, 17). The cells were characterized by fluorescence-activated cell sorter analysis and express the endothelial markers PECAM-1, ICAM-2, CD34, endoglin, Meca-32, and Flk-1. Endotheliomas were grown in Dulbecco’s modified Eagle’s Medium, high glucose supplemented with 10% fetal calf serum, 3 mM l-glutamin, 5 μM β-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids, and penicillin and streptomycin.

CHO cells were cultured in α-MEM with ribonucleosides and deoxyribonucleosides (Invitrogen) supplemented with 10% fetal calf serum. For selection of clones, 10 μg/ml blasticidine S was added. Insect cells (Sf9) were cultured in Ex-Cell 400 with 1-glutamine (JHR Bioscience) without further supplements.

**Angiopoietin and Tie-2 Expression Plasmids**—Human Ang-1 and Ang-2 were cloned by RT-PCR from mRNA isolated from human melanoma cells (A375) for Ang-1 and human umbilical vein endothelial cells for Ang-2 using standard protocols. The amino-terminal signal sequences were replaced by the signal sequence of human IgG1 fused to a myc tag using the following linker: AACGGGTTATCCGACCGACGCGACCGCGACGTGTGCTGACCTGGCGCTCATTTCTCCTGAGATTGGATCTGGCGCATCCTCTTCCTCGTCGGCGCTGCT.

**Fig. 1.** Domain structure of the Tie-2 receptor and schematic diagram of the soluble Tie-2-Fc truncation mutants in these studies. The extracellular domain of Tie-2 consists of an amino-terminal Ig-like domain followed by EGF-like repeats, a second Ig-like domain, and fibronectin type III domains. Fc fusion proteins were generated by replacing the transmembrane and kinase domain of Tie-2 by human Fc (constant part of IgG1). Different portions of the extracellular domain of Tie-2 were fused to Fc (the numbers denote the amino acids of the extracellular Tie-2 domain of each of the constructs). See “Experimental Procedures” for details of construction.

*U. Fiedler, unpublished data.

*G. Siemeister, unpublished data.*
pended Tie-2 mutant cell lines were cloned into pCRII using the TA cloning kit (Invitrogen) yielding pCRII mTie-2 ΔE2. Sequencing with an automated sequencer (ABI 373) revealed that the actual size of the PCR fragment from the Tie-2 mutant cell lines is 1330 bps and that the sequences coded for by exactly the second exon (312 bps, corresponding to 104 aa) are missing.

Production of Expression Vectors for Tie-2 WT and Mutant Proteins—First, the complete mouse Tie-2 open reading frame sequence was cloned into pBSII (Stratagene) as a BglII/partialBamHI fragment and then isolated from the pBSII subclone as a XbaI/Bsp120I fragment, which was then ligated into XbaI NotI-digested pEF14 (18) (a gift from Regeneron Pharmaceuticals) to create pEF14 mTie-2. pEF6 mTie-2 was created by releasing the Tie-2 open reading frame sequence fragment from pEF6 mTie-2 by digestion with EcoRI and Clal and ligation into EcoRI/BglII-digested pEF6 (Invitrogen). To produce pEF14 mTie-2 ΔE2, a fragment harboring the Tie-2 deletion was isolated from pCRII mTie-2 ΔE2 by digesting with BglII, filling in with Klenow fragment to produce a blunt end, and recutting with SpeI. This fragment was ligated into pEF14 mTie-2 that had been linearized with XbaI, blunt-ended as above, and recut with SpeI. pEF6 mTie-2 ΔE2 was assembled by three-fragment ligation of an EcoRI/SpeI fragment derived from pEF14 mTie-2 ΔE2, a SpeI/BglII fragment from pEF6 mTie-2, and a pEF6 vector fragment derived from pEF6 mTie-2 by cutting with EcoRI and BglII.

Expression and Purification of myc-Ang-1, myc-Ang-2, and the Truncated sTie-2-Fc Protein—The cDNA-containing plasmids were used for transfection of Sf9 cells along with linearized wild-type baculovirus DNA. Recombinant baculoviruses were obtained using the BacculoGold™ transfection kit following standard protocols (Pharmingen). For protein production, Sf9 cells grown in serum-free medium at a density of 2 x 10⁶ cells/ml were incubated with a multiplicity of infection of 10. Myc-tagged Ang-1 and Myc-tagged Ang-2 were purified from Sf9 supernatants 72 h after infection. The supernatants were filtered and bound to Concanavalin A-Sepharose (Amersham Biosciences) (1 ml of Sepharose/100 ml Sf9 supernatant) overnight at 4 °C. The beads were washed 10 times with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MnCl₂, the supernatants 72 h after infection. Individual preparations had a purity of up to 50%. Further purification led to aggregation and inactivation upon repeated freeze-thaw cycles.

Different truncated sTie-2-Fc molecules were purified from Sf9 supernatants 96 h after infection. Cells were grown in medium without fetal calf serum supplementation. Supernatants were filtered and bound to protein-A-Sepharose CL-6B (Amersham Biosciences) (1 ml of Sepharose/100 ml Sf9 supernatant) overnight at 4 °C. The Sepharose beads were added to an empty PD-10 column and washed with 10 column volumes of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM CaCl₂, 0.5 mM MnCl₂, then eluted with biotinylated Ang-1 or Ang-2 (100 μg/ml Sf9 supernatant overnight at 4 °C. The Sepharose beads were spun down at 14 000 × g for 2 h at room temperature. The Sepharose beads were spun down and washed three times with TBS containing 0.1% Nonidet P-40. The beads were boiled in sample buffer, and samples were loaded on a 7.5% SDS-PAGE. Western blots were analyzed with an anti-Myc antibody (anti-Myc 9E10, ATCC). Immunochemical Analysis—Cultured CHO cells and Tie-2-expressing CHO cells (80,000/well) were seeded in a 24-well plate (Greiner) and exposed to 5 μg/ml Myc-Ang-1 or Myc-Ang-2 for 5 min. Cells were washed twice with PBS and fixed with cold methanol. Fixed cells were washed twice with PBS and blocked with 250 μl of buffer (PBS containing 1% fetal calf serum and 0.2% Tween) for 30 min. After blocking, 250 μl of first antibody in blocking buffer was added for 1 h at room temperature. The anti-Myc antibody 9E10 (1 mg/ml; ATCC) was used for the detection of Myc-Ang-1 and Myc-Ang-2. Likewise, the anti-Tie-2 antibody (clone AB33) (4 μg/ml; Upstate Biotechnology) was used for the detection of Tie-2. The cells were washed three times for 10 min with PBS before the addition of the biotinylated anti-mouse IgG antibody (DAKO, 1:500) for 30 min at room temperature. Cells were washed three times and incubated with streptavidin-fluorescein isothiocyanate (1:50) or streptavidin-RPE (1:50) (DAKO) and Hoechst dye (1:5000) for an additional 30 min.

RESULTS

Purification of myc-tagged Ang-1 and myc-tagged Ang-2—Full-length Ang-1 protein has not yet been generally available so far due to inherent difficulties in purifying the bioactive molecule. Instead, a modified variant, designated as Ang-1*, in which the 73 amino-terminal amino acids of hAng-2 are fused to hAng-1 at residue 77, has been used in angiopoeitin bioassays (6, 8, 9, 19). Thus, to map the angiopoietin binding sites in the Tie-2 receptor, we first set up experiments aimed at purifying full-length bioactive Ang-1 and Ang-2 as well as the different truncation mutants of sTie-2. Expression of Myc-tagged human Ang-1 and Myc-tagged human Ang-2 in insect cells (Sf9) using a baculovirus expression system followed by ConA affinity purification and storage in the presence of 100 μg/ml BSA yielded functional Ang-1 and Ang-2 in purities up to 50% (Fig. 2A). Western blot analysis of Ang-1 and Ang-2 under non-reducing conditions showed that Ang-1 is a oligomeric, most likely hexameric protein, whereas Ang-2 runs as a dimeric molecule (Fig. 2B). Both Myc-tagged angiopoietins bind to Tie-2 with an affinity of 3 nM (data not shown) as described previously (5, 6). Both Ang-1 and Ang-2 bind uniformly to cultured endothelial cells and are rapidly internalized as evidenced by the intracellular granular staining (Fig. 2C). Functional activity of the angiopoietins was tested by Tie-2 phosphorylation in nonendothelial cells (see Fig. 6) as well as in a three-dimensional spheroidal in gel angiogenesis assay (Ref. 14 and data not shown).

Truncated sTie-2-Fc for the Mapping of the Angiopoietin Binding Sites—The following amino-terminal truncation mutants of the extracellular domain of Tie-2 were expressed as Fc fusion proteins: (i) sTie-2-(1–199)-Fc consisting of the first Ig-like loop of Tie-2 (aa 1–199); (ii) sTie-2-(1–360)-Fc consisting of
Bioactive recombinant Ang-1 and Ang-2 (purity up to 50%) can be expressed in Sf9 cells and purified by ConA affinity chromatography of Myc-tagged full-length Ang-1 and Ang-2. The angiopoietins were a soluble Tie-2-Fc truncation mutants.

The first Ig-like loop plus the EGF-like repeats (aa 1–440) (Fig. 3, A) consisted of the first Ig-like loop plus the EGF-like repeats plus the second Ig-like loop (aa 1–730) (Fig. 1). All fusion proteins could be purified to greater than 95% purity (Fig. 2F).

Quantitative ELISA-based Mapping of the Angiopoietin Binding Sites within the Tie-2 Receptor—Angiopoietin binding was studied in a competition ELISA-based assay in which binding of immobilized Ang-1 and Ang-2 to the biotinylated full-length extracellular Tie-2 domain (sTie-2-(1–730)-Fc) is inhibited by increasing concentrations of the different amino-terminal truncation mutants of soluble Tie-2-Fc. Positive control experiments (inhibition of angiopoietin binding by biotinylated full-length extracellular Tie-2-Fc with competing nonbiotinylated full-length extracellular Tie-2-Fc) revealed the sensitivity of the experimental approach: a concentration of 10 pmol of competing soluble Tie-2-Fc was capable of inhibiting binding of biotinylated sTie-2-Fc to Ang-1 and Ang-2 to less than 25% (IC$_{50}$ of Ang-1: 2.2 ± 1.1 pmol; IC$_{50}$ of Ang-2: 3.3 ± 1.1 pmol) (Fig. 3, A and B). In turn, competition of sTie-2 binding to Ang-1 and Ang-2 by soluble VEGF-R2 was used as a negative control, demonstrating the specificity of the competition assay and showing that even higher concentrations of sVEGF-R2 quenched sTie-2-Fc binding to Ang-1 and Ang-2 by less than 20% (Fig. 3, A and B). Analysis of the different truncation mutants of the extracellular domain of Tie-2 in this assay revealed that sTie-2-(1–440)-Fc and sTie-2-(1–360)-Fc are similarly capable of inhibiting binding of Ang-1 and Ang-2 to Tie-2. Surprisingly, sTie-2-(1–440)-Fc containing the second Ig-like domain was less effective in inhibiting Ang-1 binding than the shorter sTie-2-(1–360) variant, which lacked the second Ig-like domain (sTie-2-(1–440)-Fc: IC$_{50}$ of Ang-1, 9.1 ± 1.3 pmol; IC$_{50}$ of Ang-2: 27.5 ± 1.4 pmol; sTie-2-(1–360)-Fc: IC$_{50}$ of Ang-1, 4.2 ± 1.3 pmol; IC$_{50}$ of Ang-2, 3.8 ± 1.2 pmol). Moreover, sTie-2-(1–440)-Fc was more effective in inhibiting Ang-1 binding (to 30%) than Ang-2 (to 58%), indicating that the second Ig-like domain in the Tie-2 receptor is capable of modulating angiopoietin binding and may confer some specificity of Ang-1 over Ang-2 binding. Lastly, increasing concentrations of the shortest sTie-2-Fc form consisting just of the amino-terminal Ig-like domain of Tie-2 (sTie-2-(1–199)-Fc) quenched sTie-2-Fc binding to Ang-1 and Ang-2 to 65%, albeit not in a classical sigmoidal inhibition curve, indicating that the first Ig-like domain is not sufficient to effectively bind Ang-1 and Ang-2.

Analysis of Angiopoietin Binding to Tie-2 by Immunoprecipitation Analysis—Based on the results of the ELISA-based angiopoietin binding experiments, we further studied binding of Ang-1 and Ang-2 to the extracellular domain of Tie-2 in angio-
Angiopoietin Binding of Tie-2

Fig. 4. Co-precipitation of Myc-Ang-1 and Myc-Ang-2 with the different sTie-2-Fc truncation mutants. The different truncated sTie-2-Fc molecules were incubated with myc-Ang-1 (A) and Myc-Ang-2 (B), precipitated with protein-A-Sepharose, and probed with an anti-Myc antibody following Western blotting after 7.5% SDS-PAGE. Input, positive control of input Ang-1 (A) and Ang-2 (B); unbound, supernatant following immunoprecipitation; precipitated, sTie-2-Fc co-precipitated Myc-Ang-1 (A) and Myc-Ang-2 (B); no Ang-1, no Ang-2, and no sTie-2, negative control immunoprecipitations omitting Ang-1, Ang-2, or sTie-2-Fc. The sTie-2-(1–730)-Fc, (1–440)-Fc, and (1–360)-Fc are similarly capable of co-precipitating Ang-1 and Ang-2, whereas sTie-2-(1–199)-Fc binds neither Ang-1 nor Ang-2. All experiments were performed at least twice.

Fig. 5. Co-precipitation of Myc-Ang-1 and Myc-Ang-2 with sTie-2-(1–730)-Fc, sTie-2-(211–360)-Fc, sTie-2-(211–730)-Fc, and sTie-2-(341–730)-Fc. The different truncated sTie-2-Fc molecules were incubated with Myc-Ang-1 (A) and Myc-Ang-2 (B), precipitated with protein-A-Sepharose, and probed with an anti-Myc antibody following Western blotting after 7.5% SDS-PAGE. Input, positive control of input Ang-1 (A) and Ang-2 (B); unbound, supernatant following immunoprecipitation; precipitated, sTie-2 co-precipitated Myc-Ang-1 (A) and Myc-Ang-2 (B); no Ang-1, no Ang-2, and no sTie-2, negative control immunoprecipitations omitting Ang-1, Ang-2, or sTie-2-Fc. None of the additional truncated sTie-2-Fc molecules are capable of binding Ang-1 and Ang-2. All experiments were performed at least twice.

Angiopoietin Binding of Tie-2

FIG. 4. Co-precipitation of Myc-Ang-1 and Myc-Ang-2 with the different sTie-2-Fc truncation mutants. The different truncated sTie-2-Fc molecules were incubated with myc-Ang-1 (A) and Myc-Ang-2 (B), precipitated with protein-A-Sepharose, and probed with an anti-Myc antibody following Western blotting after 7.5% SDS-PAGE. Input, positive control of input Ang-1 (A) and Ang-2 (B); unbound, supernatant following immunoprecipitation; precipitated, sTie-2-Fc co-precipitated Myc-Ang-1 (A) and Myc-Ang-2 (B); no Ang-1, no Ang-2, and no sTie-2, negative control immunoprecipitations omitting Ang-1, Ang-2, or sTie-2-Fc. The sTie-2-(1–730)-Fc, (1–440)-Fc, and (1–360)-Fc are similarly capable of co-precipitating Ang-1 and Ang-2, whereas sTie-2-(1–199)-Fc binds neither Ang-1 nor Ang-2. All experiments were performed at least twice.

Angiopoietin Binding of Tie-2

FIG. 5. Co-precipitation of Myc-Ang-1 and Myc-Ang-2 with sTie-2-(1–730)-Fc, sTie-2-(211–360)-Fc, sTie-2-(211–730)-Fc, and sTie-2-(341–730)-Fc. The different truncated sTie-2-Fc molecules were incubated with Myc-Ang-1 (A) and Myc-Ang-2 (B), precipitated with protein-A-Sepharose, and probed with an anti-Myc antibody following Western blotting after 7.5% SDS-PAGE. Input, positive control of input Ang-1 (A) and Ang-2 (B); unbound, supernatant following immunoprecipitation; precipitated, sTie-2 co-precipitated Myc-Ang-1 (A) and Myc-Ang-2 (B); no Ang-1, no Ang-2, and no sTie-2, negative control immunoprecipitations omitting Ang-1, Ang-2, or sTie-2-Fc. None of the additional truncated sTie-2-Fc molecules are capable of binding Ang-1 and Ang-2. All experiments were performed at least twice.

Angiopoietin Binding of Tie-2

The different truncated sTie-2-Fc molecules were incubated with Myc-Ang-1 (A) and Myc-Ang-2 (B), precipitated with protein-A-Sepharose, and probed with an anti-Myc antibody following Western blotting after 7.5% SDS-PAGE. Input, positive control of input Ang-1 (A) and Ang-2 (B); unbound, supernatant following immunoprecipitation; precipitated, sTie-2 co-precipitated Myc-Ang-1 (A) and Myc-Ang-2 (B); no Ang-1, no Ang-2, and no sTie-2, negative control immunoprecipitations omitting Ang-1, Ang-2, or sTie-2-Fc. None of the additional truncated sTie-2-Fc molecules are capable of binding Ang-1 and Ang-2. All experiments were performed at least twice.

Angiopoietin Binding of Tie-2

To further corroborate these findings, we generated additional truncated sTie-2-Fc mutants that lacked varying amino-terminal parts of the Tie-2 receptor (Fig. 1): (i) sTie-2-(211–360)-Fc, consisting of the EGF-like repeats; (ii) sTie-2-(211–730)-Fc, being composed of the entire extracellular domain of Tie-2 just lacking the first Ig-like domain; and (iii) sTie-2-(341–730)-Fc, consisting of the carboxyl-terminal extracellular domains of Tie-2 lacking the first Ig-like loop as well as the EGF-like repeats. The first set of experiments had indicated that the EGF-like repeats might be involved in angiopoietin binding, suggesting that sTie-2-(211–360)-Fc and sTie-2-(211–730)-Fc might be capable of binding Ang-1 and Ang-2. Surprisingly, none of the additional Tie-2 truncation mutants, sTie-2-(211–360)-Fc, sTie-2-(211–730)-Fc, and sTie-2-(341–730)-Fc, were able to bind Ang-1 or Ang-2 in precipitation experiments (Fig. 5) as well as in the ELISA-based competition assays (data not shown). Collectively, the data show that neither the first Ig-like domain alone nor the EGF-like repeats are sufficient to bind Ang-1 and Ang-2 but that both domains together (sTie-2-(1–360)-Fc) are necessary and sufficient to bind Ang-1 and Ang-2.

Angiopoietin Binding of Tie-2

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Angiopoietin Binding of Tie-2

Angiopoietin Binding of Tie-2
Fig. 6. Detection of mutant Tie-2 protein in endotheliomas derived from mouse embryos with a targeted mutation of Tie-2. As shown in A, Polyoma middle T immortalized endothelioma cells were generated from mouse embryos homozygous for a targeted insertion into exon 2 of the Tie-2 gene (cell lines 9, 25, 32, and 43); heterozygously Tie-2-targeted mouse embryos (cell line 27); and wild-type mouse embryos (cell line 38). Cell lysates were separated by 7.5% SDS-PAGE, blotted, and probed with an anti-Tie-2 antibody. Wild-type endothelioma cells express full-length 166-kDa Tie-2. In contrast, all homozygous Tie-2 mutant endothelioma cells express a smaller Tie-2 band with an apparent molecular size of around 133 kDa. Heterozygous mutant endothelioma cells express the wild-type 166-kDa band as well as the mutant 133-kDa band. As shown in B, CHO cells were transfected with either a plasmid coding for full-length mTie-2 (lanes 1–3) or a plasmid that codes for the mutant murine Tie-2 (Δ aa 1–103) (lanes 4–6). Cells were starved for 12 h and stimulated with 5 μg/ml Myc-Ang-1 or Myc-Ang-2 for 15 min. Cell lysates were immunoprecipitated with an anti-Tie-2 antibody, separated by SDS-PAGE, blotted, and probed with an anti-P-tyrosine antibody (upper panel) and reprobed with an anti-Tie-2 antibody. Both Ang-1 and Ang-2 are capable of inducing Tie-2 phosphorylation. In contrast, the mutant Tie-2 lacking aa 1–103 is not capable of becoming phosphorylated upon Ang-1 or Ang-2 stimulation.

To study the signal transduction properties of full-length mTie-2 receptor and the mutant mTie-2 receptor (mTie-2(Δ aa1–103)), we stably overexpressed both molecules in CHO cells and stimulated the cells with Ang-1 and Ang-2. Stimulation of full-length Tie-2-expressing CHO cells results in rapid autophosphorylation upon Ang-1 as well as Ang-2 addition (Fig. 6B). In contrast, mTie-2(Δ aa1–103) does not become phosphorylated upon Ang-1 or Ang-2 stimulation (Fig. 6B). Corresponding experiments with wild-type and mTie-2(Δ aa1–103)-expressing endothelioma cells showed that Ang-1 is capable of inducing Tie-2 phosphorylation in WT endothelioma but not in mutant endothelioma cells (data not shown).

The experiment with WT and mutant Tie-2 suggested either that the mutant mTie-2(Δ aa1–103) receptor lacking parts of the first Ig-like domain is not able to bind Ang-1 and Ang-2 or that mutant Tie-2 can bind the angiopoietins but cannot undergo the conformational change that leads to autophosphorylation. To address these alternative possibilities, we studied the binding of Myc-Ang-1 and Myc-Ang-2 by CHO cells expressing either full-length Tie-2 or the mutant mTie-2(Δ aa1–103) and traced binding by cytochemical detection using anti-Myc antibodies. These experiments showed that full-length Tie-2-expressing CHO cells bind Ang-1 as well Ang-2, whereas mTie-2(Δ aa1–103)-expressing cells are not capable of binding Ang-1 and Ang-2 (Fig. 7). Collectively, these experiments show that the first 104 amino acids of the first Ig-like domain of the Tie-2 receptor are critically required for binding of Ang-1 and Ang-2 to the Tie-2 receptor in vivo.

DISCUSSION

The present study was aimed at mapping the binding sites of Ang-1 and Ang-2 in the extracellular domain of the Tie-2 receptor. The experiments revealed that binding of both angiopoietins involves the first Ig-like domain in combination with the EGF-like repeats. Neither domain alone is capable of effectively binding the angiopoietins. Despite the fact that the definition of specific binding motifs requires further experimentation including the mutational analysis of individual candidate amino acids, the essentially identical binding properties of both angiopoietins in all experimental settings strongly suggest that Ang-1 and Ang-2 bind to the same site in the Tie-2 receptor. However, it is difficult to mechanistically explain how Ang-1 can act agonistically and Ang-2 can act antagonistically if both molecules compete for the same binding site in the Tie-2 protein. One would expect that both molecules should elicit the same conformational changes in the Tie-2 receptor, leading to tyrosine autophosphorylation and subsequent signal transduction if they have identical binding properties. However, confirming previous reports (5, 6), our experiments have also shown that full-length Ang-1 is presented as oligomeric protein, whereas Ang-2 is synthesized as a dimeric molecule. Thus, different oligomeric states of Ang-1 and Ang-2 may cluster Tie-2 in a different manner despite sharing of the same binding sites. Accordingly, different presentations of the angiopoietins may well contribute to regulating differential responses of the Tie-2 receptor upon binding.

Recent experiments have shown that Ang-1 and Ang-2 may not just exert opposing functions on the Tie-2 receptor, but...
Angiopoietin Binding of Tie-2

have not been studied. Our experiments showed that the mutant mTie-2 (Δ aa 1–103) receptor is not capable of binding Ang-1 and Ang-2 and has consequently no signal transducing capacity.

Taken together, our results indicate (i) that the first Ig-like loop in combination with the EGF-like repeats is necessary and sufficient for proper binding of both Ang-1 and Ang-2 to Tie-2, (ii) that the first 104 amino acids of the first Ig-like domain are necessary but not sufficient for binding of Ang-1 and Ang-2, (iii) that the second Ig-like domain in the Tie-2 receptor may confer some differential binding activity of Ang-1 versus Ang-2, and (iv) that the identical binding properties of Ang-1 and Ang-2 in all experiments strongly suggest that both molecules share the same binding site in the first 360 extracellular amino acids of Tie-2 (although the elucidation of individual binding motifs is required to conclusively substantiate identical binding properties). Collectively, our data support the concept that differential target cell properties and differential presentation of the angiopoietins to the target cells are responsible for the differential contextually regulated effects of the angiopoietins rather than inherent differences in ligand binding to the receptor.

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Additionally, that in certain conditions, Ang-2 is also able to activate the Tie-2 receptor. This conclusion is supported by the observation that long term Tie-2 stimulation with Ang-2 leads to Tie-2 phosphorylation in endothelial cells (13) and is correspondingly capable of inducing sprouting angiogenesis in 24–48 h experiments (14). Likewise, the complex phenotype of Ang-2-deficient mice with defects in retinal angiogenesis and vascular regression as well as in the lymphatic vasculature cannot exclusively be explained by opposing functions of Ang-1 and Ang-2 (15). If indeed the angiopoietins are capable of exerting opposing functions as well as being able to both act agonistically in different contextual frameworks, it is unlikely that differential presentation and binding of the ligands is responsible for mediating different biological effects. Instead, it appears more likely that differential activities are controlled at the level of the target cell, e.g., blood vessel endothelial cells versus lymphatic endothelial cells. This is also supported by the observation that Tie-2-transfected nonendothelial cells elicit the same Tie-2 activation and phosphorylation response upon stimulation with Ang-1 as well as Ang-2. Endothelial cell-specific proteins shown to interact with Tie-2 include Tie-1 (4, 20) and the vascular endothelial receptor phosphotyrosine phosphatase (VE-PTP) (21). Tie-1 and VE-PTP are attractive candidate molecules to serve as modulators of the angiopoietin/Tie-2 signaling. Alternatively, another hitherto unidentified Ang-2 receptor may be responsible for the agonistic or antagonistic functions of Ang-1 and Ang-2.

Contextuality of ligand presentation to the Tie-2 receptor is likely another important factor that regulates the differential interaction of the angiopoietins with their Tie-2 receptor. Ang-1 is primarily produced by pericytes and other nonendothelial cells (5, 10) and acts constitutively on endothelial cells in a paracrine manner (5, 10). In turn, Ang-2 is primarily produced upon stimulation by endothelial cells and, thus, acts as an autocrine vascular regulator (6, 23, 24). Ang-2 expression is down-regulated in the quiescent vasculature and is strongly induced upon endothelial cell activation (22–24). Endothelial Ang-2 expression has been reported during vascular remodeling and pathological angiogenesis, including tumor angiogenesis (25). The autocrine mode of action of Ang-2 and the induction upon endothelial cell activation are controlled by the unique properties of the Ang-2 propeptide.4 Moreover, the effects of Ang-2 on endothelial cell Tie-2 activation are strongly regulated by contextual cues and vary significantly if Ang-2 is acting in a paracrine or autocrine manner.5

In line with the finding that the first Ig-like domain in concert with the EGF-like repeats is necessary for Tie-2 binding of Ang-1 and Ang-2, we made the surprising observation that loss of the first 104 amino acids to the first Ig-like domain completely disrupts angiopoietin binding and Tie-2-mediated signal transduction in vitro and in vivo. Careful analysis of mice with a targeted mutation of exon 2 of the Tie-2 gene revealed that the Tie-2 receptor gene in these mice is not completely inactivated but that endothelial cells derived from mutant embryos express an amino-terminally truncated 133-kDa Tie-2 receptor that lacks 104 amino acids of the first Ig-like domain. This receptor is signaling-deficient, as evidenced by the early embryonic lethal phenotype of mice homozygously expressing this truncated Tie-2 receptor. However, the binding and activation properties of the mutant receptor

4 A. Hegen, H. G. Augustin, and U. Fiedler, manuscript in preparation.
5 M. Scharpfenecker, U. Fiedler, and H. G. Augustin, manuscript in preparation.