Angiogenesis is essential for tissue repair and regeneration during wound healing but also plays important roles in many pathological processes including tumor growth and metastasis. The receptor protein tyrosine kinase Tie2 and its ligands, the angiopoietins, have important functions in the regulation of angiogenesis. Here, we report a detailed structural and functional characterization of the extracellular region of Tie2. Sequence analysis of the extracellular domain revealed an additional immunoglobulin-like domain resulting in a tandem repeat of immunoglobulin-like domains at the N terminus of the protein. The same domain organization was also found for the Tie1 receptor that shares a high degree of homology with Tie2. Based on structural similarities to other receptor tyrosine kinases and cell adhesion molecules, we demonstrate that the N-terminal two immunoglobulin-like domains of Tie2 harbor the angiopoietin-binding site. Using transmission electron microscopy we furthermore show that the extracellular domain of Tie receptors consists of a globular head domain and a short rod-like stalk that probably forms a spacer between the cell surface and the angiopoietin-binding site. Mutational analysis demonstrated that the head domain consists of the three immunoglobulin-like domains and the three epidermal growth factor-like modules and that the stalk is formed by the three fibronectin type III repeats. These findings might be of particular interest for drug development because Tie receptors are potential targets for treatment of angiogenesis-associated diseases.

Proper regulation of angiogenesis is required for the normal growth of embryonic and postnatal tissues as well as physiological processes in the adult such as the continuous remodeling of the female reproductive system and wound healing (1, 2). In contrast, dysregulation of angiogenesis contributes to numerous malignant, ischemic, inflammatory, infectious, and immune disorders including conditions such as diabetic retinopathy and tumor growth and metastasis (1, 2). The angiopoietin (Ang)3/Tie signaling system plays important roles in the regulation of angiogenesis (3, 4). Tie1 and Tie2 constitute a family of vascular-specific receptor tyrosine kinases (RTKs) that are expressed mainly on endothelial cells but also in certain hematopoietic cells (5–7). Angiopoietins are a family of four distinct growth factor ligands that bind to Tie2 but not Tie1 (8–10). They are unique and differ from other growth factors by having opposing effects on receptor phosphorylation. Over-expression and knock-out studies in mice have revealed that Ang1 is an agonist promoting stabilization of vessels by maximizing interactions between endothelial cells and their surrounding support cells and the extracellular matrix (11, 12). In contrast Ang2 was found to be a context-dependent antagonist that promotes angiogenic sprouting or vessel regression depending on the expression of vascular endothelial growth factor-A (13, 14). Ang3 and Ang4 also show context-dependent actions as antagonistic and agonistic ligands, respectively, and they are believed to represent widely diverged counterparts of the same gene in mouse ANGPT3 and human ANGPT4 (10, 15). Although Tie1 is considered an orphan receptor with no known ligands, it has recently been found that Ang1 and Ang4 can induce Tie1 phosphorylation (16). Furthermore, it has been shown that Tie1 and Tie2 form heteromeric complexes in cells that express both receptors, which is consistent with the observation that phosphorylation of Tie1 is significantly amplified through Tie2 (16, 17). However, the mechanistic details of how angiopoietins differentially regulate Tie receptor activity are currently not known.

Mutations in the kinase domain of Tie2 resulting in ligand-independent activation are associated with vascular anomalies such as multiple cutaneous and mucosal venous malformations and intramuscular hemangiomas (18–20). Furthermore, over-expression of Tie2 has been observed in a wide range of diseases including psoriasis (21, 22), pulmonary hypertension (23, 24), infantile hemangiomas (25), and different cancers (26). Ang1/Tie2 signaling has been studied extensively and is known to
activate several downstream pathways that ultimately result in endothelial cell survival and cell migration (4). Although the lack of a ligand has made it difficult to elucidate the function of Tie1, experiments using a chimeric receptor consisting of the extracellular domain of colony-stimulating factor-1 receptor and the intracellular domain of Tie1 show that Tie1 activation can induce phosphorylation of phosphatidylinositol 3-kinase (27). Interestingly, signaling involving phosphatidylinositol 3-kinase is also one of the major pathways activated through Tie2 phosphorylation, which indicates overlapping functions of Tie1 and Tie2.

Consistent with their expression in some other cells such as in the hemopoietic lineage, Tie receptors are found to be required specifically during postnatal bone marrow hematopoiesis (28). Recently, a critical role of the Tie2/Ang1 signaling pathway has also been demonstrated in maintaining hematopoietic stem cells in the quiescent state in the bone marrow niche (29). It has been shown that activation of Tie2 on hematopoietic stem cells promotes tight adhesion of the cells to the niche, resulting in their quiescence and enhanced survival.

Tie1 and Tie2 receptors share a high degree of homology. The extracellular regions of both receptors contain, three EGF-like modules that are flanked by two immunoglobulin (Ig)-like domains, and three fibronectin type III (FNIII) repeats (6, 7). The first Ig-like domain and the EGF-like modules of Tie2 are necessary and sufficient to bind both Ang1 and Ang2, suggesting that differential receptor binding is not likely to be responsible for the different functions of Ang1 and Ang2 (30). The cytoplasmic regions contain tyrosine kinase domains including a number of phosphorylation and protein interaction sites (31). Given the important functions of both receptors in a large number of biological processes, here we perform a detailed structural and functional characterization of the extracellular portion of Tie2 using biochemical and biophysical methods.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—The following inserts were amplified by PCR and cloned into a modified pCEP-Pu vector that contains a secretory signal sequence for BM-40/osteonectin (32): the fibronogen-like domain of human Ang1 (residues 266–498) fused at its N terminus to the coiled-coil domain of human matrillin-3 (residues 443–486, Mat3-Ang1); the extracellular domain (ECD) of human Tie2 (residues 23–745) fused to the Fc domain of human IgG1 (Tie2 ECD Fc) or a His6 tag (Tie2 ECD); the first two Ig-like domains (residues 23–210) fused to the Fc tag (Tie2 Ig12 Fc) or a C-terminal His6 tag (Tie2 Ig12); the whole extracellular domain of Tie1 (residues 21–760) fused to the Fc tag (Tie1 ECD Fc); and the extracellular domain of the Tie1 splice variant (missing the first EGF-like repeat, residues 305–347) fused to the Fc tag (Tie1ΔEGF1 ECD Fc). The Tie2 Ig12 (residues 23–210) construct was also cloned into the pHis vector, a derivative of pET-15b (Invitrogen). All mammalian vector constructs contain either a N- or C-terminal His6 tag for purification (see “Experimental Procedures” for details).

**Expression of Recombinant Proteins**—All recombinant proteins were obtained by expression in HEK293 EBNA cells using Lipofectamine according to the manufacturer’s instructions (Invitrogen). After selection with puromycin (5 μg/ml) cells were expanded for the production of the recombinant proteins. Serum-free supernatants were harvested from transfected cells, and the recombinant proteins were purified by immobilized metal affinity chromatography on Ni2+-Sepharose according to the manufacturer’s instructions (Amersham Biosciences) and dialyzed against phosphate-buffered saline. Tie2 Ig12 was expressed in bacterial strain JM109(DE3) and purified under denaturing conditions as described previously (33). The protein was refolded as described elsewhere (34).

**Sequence Analysis and Modeling**—The Ipred consensus method for protein secondary structure prediction (35) was used. The 3D-psm method for protein fold recognition by using one- and three-dimensional sequence profiles coupled with secondary structure and solvation potential information (36) was used. Residues 23–210 of the Tie2 sequence were aligned to the N-terminal Ig-like domains of the Axl receptor using ClustalW (37). The structural model was generated using the SWISS-MODEL automated modeling server (38), using the alignment between Tie2 and Axl N-terminal Ig-like domain pairs as an input and the crystal structure of the Axl Ig-like domain pair (Ref. 39; Protein Data Bank code 2c5d, chain D) as a template. Fig. 2 was prepared with the program PyMOL.4

**Electron Microscopy**—For glycerol spraying/low angle, rotary metal shadowing, 20 μl of protein samples (0.1–0.3 mg/ml in 5 mM sodium phosphate, pH 7.4, 150 mM NaCl, 30% glycerol) were sprayed onto freshly cleaved mica at room temperature and rotary-shadowed in a BA 511 M freeze-etch apparatus (Balzers) with platinum/carbon at an elevation angle of 3–5° (40). Electron micrographs were taken in a Philips Morgagni transmission electron microscope (TEM) operated at 80 kV.

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equipped with a Megaview III charge-coupled device camera at ×54,000 nominal magnification.

Circular Dichroism (CD) Spectroscopy—CD measurements were made on a JASCO J-810 spectropolarimeter equipped with a Peltier temperature controller. The cuvette pathlength was 10 mm. Data points were recorded at 1-nm intervals using a 2-nm bandwidth and 8-s response times. Ellipticities in millidegrees were converted to molar ellipticities (degree cm² dmol⁻¹) by normalizing for the concentration of peptide bonds.

ELISAs—96-well plates (Costar) were coated with Mat3-Ang1 at 5 μg/ml and incubated overnight at 4 °C. Wells were blocked with 5% bovine serum albumin for 3 h at room temperature. The appropriate ligands were added to each well at decreasing concentrations and incubated for 2 h at 37 °C. Wells were washed three times with 0.1% Tween 20 in Tris-buffered saline and incubated with a rabbit anti-Tie2 primary antibody (1:50 dilution, Santa Cruz Biotechnology) for 1 h at room temperature, washed as described above, and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:50 dilution, Santa Cruz Biotechnology) for 1 h at room temperature. The amount of ligand bound to Mat3-Ang1 was quantified by addition of 2,2-anzo-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate.

RESULTS AND DISCUSSION

The ECD of Tie Receptors Contains a Third Ig-like Domain—The first 188 N-terminal residues of Tie2 that precede the EGF-like repeats have been reported to fold into a single C2-type Ig-like domain (6, 7). Ig-like domains typically consist of about 90 residues; therefore, approximately half of the sequence has no domain assignment. We therefore analyzed the N-terminal sequence stretch in detail by several methods. Secondary structure analysis using Jpred (35) predicted the entire sequence to fold into 14 distinct β-strands (data not shown). The C2-type Ig-like fold is characterized by seven β-strands, an observation that immediately suggested the presence of a second Ig-like domain in the N terminus preceding the EGF-like repeats. Because a correlation between exon structure and domain structure is frequently found for domains composed of multiple sequence repeats (41), the exon-intron organization of the Tie2 gene was also analyzed. The exon-intron boundaries correlate very well with the secondary structure prediction and strongly support the hypothesis that the N terminus of Tie2 harbors an additional, third, Ig-like domain. The existence of an additional Tie2 Ig-like domain is further supported by sequence threading using the protein fold recognition program 3D-PSSM (36), which confidently predicts a high similarity to the tandem Ig-like repeats of the RTKs Axl-Tyro3 (39, 42) and fibroblast growth factor receptor 2 (FGFR2 (43)). According to the model shown in Fig. 2, the first and second domains of Tie2 are composed of residues 23–120 and 124–210, respectively. As expected from its high degree of homology to Tie2, the additional Ig-like domain was also predicted for Tie1 (data not shown).

The presence of a tandem repeat of Ig-like domains in Tie receptors has some potentially important functional implications. Pairs of Ig-like domains are frequently found in other RTKs and cell adhesion proteins, where they play important roles for ligand binding. A prominent example is the Axl-Tyro3 RTK, which plays important roles in angiogenesis, cancer, spermatogenesis, immunity, and platelet function (44). The extracellular domain of Axl-Tyro3 consists of two Ig-like loops followed by two FNIII repeats and therefore has a domain organization very similar to that of Tie receptors. Recently, the crystal structure of the two Ig-like domains of the Axl-Tyro3 in complex with the natural ligand Gas6 has been determined (39). Gas6 comprises two laminin G-like domains, but interestingly in the crystal structure only the first domain binds two Axl-Tyro3 tandem Ig-like repeats at distinct and opposite sites, indicating how the receptor becomes activated through dimerization. Both contacts to Gas6 involve edge β-strands and are required for signaling. The structural similarity to Axl-Tyro3 strongly suggested that the Ig-like domain pair of Tie2 harbors the Ang-binding epitope.

This hypothesis is supported by a recent study, although the authors reached a different conclusion. In an attempt to map the Ang-binding site in Tie2, Fiedler et al. (30) expressed a series of Tie2 truncation constructs as fusion proteins to aFc tag in the baculovirus system and analyzed their binding properties to Ang1 and Ang2 by immunoprecipitation and ELISA. Consistent with our findings they showed that the first Ig-like loop of Tie2 in combination with the EGF-like repeats (amino acids 1–360) is required and sufficient to bind both Ang1 and Ang2, suggesting that differential receptor binding is not likely to be responsible for the different functions of Ang1 and Ang2. In contrast, the first Ig-like domain (residues 1–199) or the EGF-like repeats alone (residues 211–360) are not capable of binding Ang1 and Ang2. Closer inspection of the construct encoding the first Ig-like domain of Tie2, however, revealed that part of the second predicted Ig-like domain is missing. In fact, the construct lacks the last 11 C-terminal residues, which essentially removes strand G of the second Ig-like domain. It is therefore very unlikely that the truncated domain folds properly into a stable structure. Misfolding of the second domain would also result in a structural disruption of a putative ligand-binding site at the interface of the domain pair, providing a plausible explanation for the inability of the truncated protein to interact with angiopeptins. Further evidence for the importance of the pair of Ig-like domains of Tie2 in Ang binding is provided by the finding that knock-out mice lacking exon 2 of Tie2 do express a truncated Tie2 protein that misses the first Ig-like domain (30). The mutant receptor missing the first Ig-like domain is functionally inactive as shown by the lack of
ligand binding and receptor phosphorylation. Together these findings demonstrate that both domains of the Ig-like tandem repeat of Tie2 are required to bind to angiopoietins.

Analysis of the EGF-like repeats suggests that these domains are unique to the Tie2 receptors. From the presence of eight cysteine residues the Tie EGFLike repeats have the greatest similarity to the laminin epidermal growth factor-like (LE) module, which is found in the extracellular matrix proteins laminins, agrin, and perlecan (45). Consecutive LE domains mainly act as spacers but can also be involved in protein-protein interactions. The LE module shows little secondary structure and has disulfide bridge connections Cys1–Cys3 (loop a), Cys2–Cys4 (loop b), Cys5–Cys6 (loop c), and Cys7–Cys8 (loop d), the first three being identical to EGF although with differences in the spacing of cysteine residues. Because of the close proximity of Cys2 and Cys4 the arrangement of disulfide bridges seen in LE modules is very unlikely for Tie EGF-like repeats, suggesting different disulfide bridge connections in these domains.

The ECD of Tie Receptors Has a Lollipop-like Structure—As a first step toward a structural characterization of the extracellular domain of the Tie2 receptor, we expressed the full-length ectodomain of the human protein fused to the constant part of human IgG1, in HEK293 EBNA cells (see “Experimental Procedures” for details; see also Fig. 1). Analysis of purified recombinant fusion protein by SDS-PAGE is shown in Fig. 3. Inspection of the Tie2 ECD Fc molecules by TEM after glycerol spraying and rotary metal shadowing yielded uniformly appearing particles with the expected structures were observed by TEM. The migration of marker proteins is indicated.

FIGURE 3. Recombinant Tie2 fragments (A) and CD analysis of the N-terminal pair of Ig-like domains (B). A, SDS-PAGE under reducing conditions. Lane 1, Tie1 ECD Fc; lane 2, Tie2 ECD Fc; lane 3, Tie2 ECD; lane 4, Tie2-(23–442); lane 5, deglycosylated Tie2-(23–442), lane 6, bacterially expressed Tie2 Ig12. The migration of marker proteins is indicated. B, CD spectrum of Tie2 Ig12 at 5 °C (13 μg/ml in phosphate-buffered saline, pH 7.4).

In summary, these finding demonstrate that the ECD of Tie RTKs folds into a globular head structure and a short rod-like stalk. The globule is formed by the three Ig-like domains and the three EGF-like modules, and the stalk consists of the three FNII repeats.

The EGF-like repeats of the globular head domain of Tie receptors appear to play an important role in protein stability. There are two known splice variants of Tie1 that lack either the first or the third EGF-like repeat (Fig. 1) (7). When we tried to express the first splice variant, Tie1 ΔEGF1 ECD Fc, we were not able to obtain significant amounts of proteins from HEK293 EBNA cells. The hypothesis on the importance of the Tie EGF-like repeats was further supported when we tried to express the tandem repeat of Ig-like domains in the mammalian system in order to demonstrate that the two domains contain the Ang-binding site. For both constructs shown in Fig. 1, no protein expression was observed. The failure to obtain protein from these three constructs may possibly be explained by a necessity to stabilize the second Ig-like domain through interactions with

Many ECDs bind calcium, and therefore we also analyzed the structures of the ECDs of Tie receptors in the presence of EDTA. Particles indistinguishable from those described above were observed for both receptors (data not shown), demonstrating that calcium is not required for the formation of the globular domain structure consisting of the Ig-like domains and the EGF-like repeats.

We also analyzed the structure of the ECD of the homologous Tie1 receptor by TEM. Although Tie1 can be activated directly by Ang1 and Ang4, no direct interaction with the ligands has been demonstrated. In endothelial cells that express both receptor types, activation of Tie1 is thought to occur through heteromerization with Tie2. As seen in Fig. 4C, particles that are indistinguishable from Tie2 ECD Fc were obtained for Tie1 ECD Fc specimens demonstrating that both receptors share the same overall structure.

Many ECDs bind calcium, and therefore we also analyzed the structures of the ECDs of Tie receptors in the presence of EDTA. Particles indistinguishable from those described above were observed for both receptors (data not shown), demonstrating that calcium is not required for the formation of the globular domain structure consisting of the Ig-like domains and the EGF-like repeats.
the first EGF-like repeat. Although a direct comparison of the mammalian and the insect systems is difficult, the Tie2(1–199) Fc fusion protein was successfully produced in the baculovirus expression system despite our expectation that the portion of the protein corresponding to the second domain would be misfolded (30). It is known, however, that expression in baculovirus can result in unglycosylated and nonfunctional proteins (47). Therefore, the most likely explanation is that the truncated protein can be produced in the insect but not the mammalian system. The integrity of the head domain structure appears less critical for protein stability because mutant mouse endothelial cells have been shown to express a truncated and inactive form of the receptor that lacks the first Ig-like domain (30).

The First Two Ig-like Domains of Tie2 Harbor the Ang-binding Site—To test our hypothesis that the tandem repeat of Ig-like domains of Tie2 contains the Ang-binding epitope, we expressed the protein fragment in bacteria (Fig. 3A). The majority of Tie2 Ig12 protein accumulated as an insoluble form in inclusion bodies, an observation that is frequently made when expressing Ig-like domains. However, it has been shown previously that functionally active pairs of Ig-like domains can be produced in Escherichia coli after refolding from inclusion bodies and that glycosylation is not required for ligand binding (34, 42, 48). Successful refolding of Tie2 Ig12 was confirmed by CD spectroscopy (Fig. 3B). The spectrum of the protein was consistent with Tie2 Ig12 folding into a structure that is rich in \( \beta \)-sheet content.

The binding properties of the pair of Ig-like Tie2 domains was assayed by ELISA and compared with those of Tie2 ECD Fc, Tie2 ECD, and Tie2(23–442). In these assays a variant form of Ang1, Mat3-Ang1, was used in which the multimerization domains of Ang1 were replaced by the short four-stranded coiled-coil domain of matrilin-3. This engineered protein was recently shown to have binding and activation properties virtually identical to recombinant full-length Ang1, but its solubility is substantially increased when compared with native Ang1 (49). As shown in Fig. 5, Tie2 Ig12 binds to Ang1 with an affinity similar to the monomeric mammalian proteins. This result shows that the Tie2 Ig12 molecules can be refolded successfully, and it suggests that high affinity binding to the ligand does not require a distinct conformation of the Ig-like domain pair. Tie2 ECD Fc shows stronger affinity to Mat3-Ang1 than the monomeric Tie2 ECD or Tie2(23–442). In contrast Tie1 ECD Fc did not bind significantly to the Ang1 variant (data not shown), which is consistent with previous findings (49).

Tie2(23–442) contains four potential N-glycosylation sites, two of which are found in the second Ig-like domain. Increased binding upon removal of the carbohydrate moiety has been observed for antibodies (50) and other proteins (51). Deglycosylation of the protein using PGNase F resulted in no significant increase in affinity to Ang1, suggesting that the N-linked carbohydrates are not in close proximity of the binding site.
strategy for treating patients with solid tumors (2). Therefore Tie2 Ig12 or derived peptides may have potential applications in the treatment of cancer and other angiogenesis-associated diseases. Furthermore, the domain pair represents a potent tool to dissect the molecular mechanisms involved in the Tie2 pathway.

When we submitted this paper, the crystal structure of a complex between Tie2 and the fibrinogen-like domain of Ang2 had been reported (53). The crystal structure confirms the compact shape of the globular head domain of Tie2 including the presence of the third Ig-like domain. Consistent with our findings the second Ig-like domain harbors the Ang-binding site. Moreover, a unique disulfide bridge pattern of the Tie2 EGF-like repeats is seen in the structure as suggested in this study. Clearly, more high-resolution structural information is now required to clarify how the activity of the receptor is differentially regulated by its ligands.

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