Structural basis of Tie2 activation and Tie2/Tie1 heterodimerization

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The endothelial cell (EC)-specific receptor tyrosine kinases Tie1 and Tie2 are necessary for the remodeling and maturation of blood and lymphatic vessels. Angiopoietin-1 (Ang1) growth factor is a Tie2 agonist, whereas Ang2 functions as a context-dependent agonist/antagonist. The orphan receptor Tie1 modulates Tie2 activation, which is induced by association of angiopoietins with Tie2 in cis and across EC–EC junctions in trans. Except for the binding of the C-terminal angiopoietin domains to the Tie2 ligand-binding domain, the mechanisms for Tie2 activation are poorly understood. We report here the structural basis of Ang1-induced Tie2 dimerization in cis and provide mechanistic insights on Ang2 antagonism, Tie1/Tie2 heterodimerization, and Tie2 clustering. We find that Ang1-induced Tie2 dimerization and activation occurs via the formation of an intermolecular β-sheet between the membrane-proximal (third) Fibronectin type III domains (Fn3) of Tie2. The structures of Tie2 and Tie1 Fn3 domains are similar and compatible with Tie2/Tie1 heterodimerization by the same mechanism. Mutagenesis of the key interaction residues of Tie2 and Tie1 Fn3 domains decreased Ang1-induced Tie2 phosphorylation and increased the basal phosphorylation of Tie1, respectively. Furthermore, the Tie2 structures revealed additional interactions between the Fn2 (Fn1) domains that coincide with a mutation of Tie2 in primary congenital glaucoma that leads to defective vascular development and function as a weak Tie2 agonist or as a context-dependent antagonist of Tie2 signaling, and the α5β1-integrin heterodimer enhances Ang1-induced EC adhesion and Tie2 activation (13, 19, 20). The Tie1 and Tie2 heteromeric complexes are promoted by angiopoietin stimulation, resulting in Ang1-induced activation of both Tie1 and Tie2 (13, 21–23). Several studies have indicated Tie1 as a Tie2 inhibitor (22, 24, 25), whereas recent experiments show that Tie1 association with Tie2 is required for Tie2 activation by Ang1 and Ang2 in mice (13, 26). Tie1 expression inhibits Tie2 presentation at the cell surface in sprouting endothelial tip cells, (23), but Tie1 sustains Tie2 signaling in contacting cells (13, 23). Thus, Tie1 exerts its context-dependent effects by modulating Tie2 activity (13, 22, 23, 26).

Ligand-induced dimerization is regarded as a common, but not the only, mechanism for activation of RTK signaling (27). Angiopoietin monomers form heterogeneous multimeric Tie2 ligands, and it has been suggested that Tie2 activation requires receptor clustering (28–30). Also, Tie2 clustering without ligand interactions provides new targets for modulation of Tie receptor activity.

Receptor tyrosine kinases (RTKs) expressed in the endothelial cells (ECs) of blood and lymphatic vessels control the development and function of the cardiovascular and lymphatic systems. The VEGFs and their endothelial receptors (VEGFRs) are key regulators of angiogenesis and vascular integrity (1, 2). The angiopoietin ligand/Tie receptor pathway is necessary for blood and lymphatic vessel remodeling during embryonic and postnatal development and for homeostasis of the mature vasculature (3, 4). Recently significant interest has focused on targeting the VEGFR and Tie receptor pathways in antiangiogenic and antilymphangiogenic therapies (5).

Ang1 activation of Tie2 is indispensable for embryonic cardiac development and angiogenesis, and both Ang1 and Ang2 are necessary for the development of lymphatic and ocular vasculature. In adult tissues, Ang1 is required for vessel stabilization after angiogenesis (6–8). Ang2, which is produced by ECs and stored in their Weibel–Palade bodies for rapid release, can function as a weak Tie2 agonist or as a context-dependent antagonist that inhibits Ang1-induced Tie2 activation and vascular stability (9–11). Tie2 is the major signal-transducing receptor of the angiopoietin/Tie signaling axis, and the homologous Tie1 receptor modulates Tie2 signaling (12, 13). Although Tie1, first identified in human leukemia cells (14), is an orphan receptor, mice lacking Tie1 develop severe edema around E13.5 because of compromised microvessel integrity and defects in lymphatic vasculature and die subsequently (15, 16). Furthermore, Tie1 has critical functions in vascular pathologies, e.g., in tumor angiogenesis and atherosclerosis progression (12, 17).

In EC monolayers, angiopoietins stimulate Tie receptor translocation to cell–cell junctions for Tie2 trans-association, whereas in the absence of cell–cell adhesion the Tie receptors are anchored to the extracellular matrix (ECM) by Ang1-induced Tie2 cis-association (10, 18). Integrins also have been implicated in Tie2 signaling, and the α5β1-integrin heterodimer enhances Ang1-induced EC adhesion and Tie2 activation (13, 19, 20). The Tie1 and Tie2 heteromeric complexes are promoted by angiopoietin stimulation, resulting in Ang1-induced activation of both Tie1 and Tie2 (13, 21–23). Several studies have indicated Tie1 as a Tie2 inhibitor (22, 24, 25), whereas recent experiments show that Tie1 association with Tie2 is required for Tie2 activation by Ang1 and Ang2 in mice (13, 26). Tie1 expression inhibits Tie2 presentation at the cell surface in sprouting endothelial tip cells, (23), but Tie1 sustains Tie2 signaling in contacting cells (13, 23). Thus, Tie1 exerts its context-dependent effects by modulating Tie2 activity (13, 22, 23, 26).

Significance

Tie1 and Tie2 receptor tyrosine kinases are key regulators of blood and lymphatic vessel development and of pathological processes including tumor angiogenesis, atherosclerosis, and vascular leakage, e.g., in sepsis. Tie1 is essential for the Tie2 agonist activity of angiopoietins, and the activated receptors form heteromeric complexes in endothelial cell–cell junctions. However, little is known about the activation mechanisms of the Tie receptors. Here we demonstrate that the membrane-proximal domains of Tie2 mediate homotypic interactions, which occur via intermolecular β-sheet formation and are necessary for Tie2 activation. The structural analysis suggests that Tie2/Tie1 heterodimerization occurs by the same mechanism. The crystal structures provide a model for angiopoietin-stimulated Tie2 ectodomain dimerization, clustering, and activation and insights into therapeutic targeting.
binding has been reported (31). The Tie receptors have a unique extracellular domain (ECD) for ligand binding, a single-pass transmembrane domain, a two-partite cytoplasmic protein tyrosine kinase domain, and a C-terminal tail. The ECDs consist of Ig, epidermal growth factor-like, and three fibronectin type III (Fn) domains (Fn1, Fn2, Fn3) (30, 32). The angiopoietins have an N-terminal region responsible for their multimerization, a coiled-coil domain for dimerization, and a C-terminal fibronogen-like domain (FLD) that contains the Tie2-binding region (28, 29). Cysteine structures of the angiopoietin/Tie2 ligand-binding domain (LBD) complexes demonstrate that Ang1 and Ang2 FLDs bind Tie2 in a similar manner (30, 33). Ang1 is a strong Tie2 agonist, and Ang2 a weak Tie2 agonist, suggesting that the Tie2 agonism of the native angiopoietins resides in sequences outside the FLDs. Because multimerization of Ang1 and Ang2 is critical for Tie2 phosphorylation, it has been suggested that the difference between the agonistic activities of Ang1 and Ang2 is caused by different oligomeric states (34, 35). Indeed, multiple studies indicate that the strongly activating Ang1 has higher tendency to form large oligomers than the weak agonist Ang2, although the significance of differential oligomerization is poorly understood (28, 29, 35, 36). The need for oligomerization of angiopoietin ligands suggests that Tie2 clustering is required for its activation, but the mechanism(s) mediating Tie2 dimerization or higher-order clustering are not known.

We report here the crystal structures of Tie2 and Tie1 membrane proximal domains and their structural and functional analysis, which provide mechanistic insight into ligand-induced Tie2 activation and to the mode of Tie1/Tie2 heterodimerization.

Results

Structure of Tie2 Fn-Like Domains 1–3. To better understand the mechanism of Tie2 dimerization and activation, we expressed Tie2 Fn-like domains 1–3 (Fn1–3) (Fig. L4) in insect cells and crystallized the purified protein in space groups C2 and P21. The crystal structure in space group C2 was determined at 2.9-Å resolution using multiple isomorphous replacement with anomalous scattering (MIRAS) phases, and the structure in space group P21 was determined at 2.6-Å resolution using multiple isomorphous replacement using the Tie2 LBD structure in the C2 space group as a constraint in the crystal structure of an Fn3 domain-protein (at 4.5 Å) (32), according to the analysis of the Fn1 domain of the Ang1/Fn3 LBD complex at 4.5-Å resolution (33), we observed a sequence register difference of one to three residues between the Fn1 domains (Tables S2 and S3). The Tie2 homodimers in the two crystal structures share the same overall structure, except that only one of the two Tie2 proteins in the C2 dimer has the Fn1 domain (Fig. S1). The 376 Cα atoms in the two Tie2 Fn2–3 dimers superimposed with an rmsd of 1.445 Å. The crystal packing indicates that the Fn1 domains were proteolytically removed before crystallization in space group P21, whereas in space group C2 the other Fn1 domain is either disordered or present at low occupancy, because crystal packing in C2 should accommodate it, but only weak residual density is visible (Fig. S2). Superposition of the Fn1 domain-bearing chain in space group C2 with the chain lacking the Fn1 domain creates a model of a Tie2 Fn1–3 homodimer (Fig. 1D). A recent EM analysis of full-length Tie2 ECDs did not indicate Tie2 dimerization, but the shape and length of the extended structure of Tie2 Fn-like domains 1–3 in space group C2 are consistent with those in the EM analysis (11.8 nm vs. 12 nm) (37). Although data from small-angle X-ray scattering (SAXS) of an Fn2–3 protein (at ~80 μM) indicated a dimer structure that fits best with our crystal structure (Fig. 1E), the interactions between the antiparallel β-strands are almost identical, but, because of additional interactions, the buried surface area is larger, about 700 Å² per chain (Tables S2 and S3).

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Fig. 1. Homodimerization of Tie2 Fn-like domains. (A) A schematic model of Tie2 with the LBD and the three membrane-proximal Fn-like domains labeled. (B) Comparison of the homodimer structures of Tie2 Fn-like domains in space groups C2 and P21. The structures are shown as cartoon diagrams with the Fn-like domains labeled and the N and C termini of the related labeled where applicable. (C) A surface-and-stick representation of the Tie2 Fn3 interactions. The Tie2 residues in the Fn3 surface model are colored according to the rate of evolutionary conservation from cyan (variable) to magenta (conserved). (D) A model of homodimer of the Tie2 Fn-like domains 1–3. Asn-linked N-acetylglucosamine moieties are shown as cyan spheres.
A Model of Tie1/Tie2 Heterodimerization. To study the mechanism of Tie1 and Tie2 heterodimerization, we expressed the membrane-proximal Tie1 Fn3 domain in insect cells and crystallized the purified protein for structure determination. The structure was solved at 2.6-Å resolution by molecular replacement using the Tie2 Fn3 structures, \( \text{Fig. S5} \). The model also suggests that both types of interactions exhibit natural protein contacts (Fig. S4). Combining Fn3-guided homodimerization of Tie2 with Fn2-guided interactions between homodimers creates an array of Tie2 molecules that could represent the structural basis of Tie2 clustering (Fig. S7A). The symmetrical interactions between the Fn2 domains involve multiple hydrogen bonds between highly conserved Arg-Trp-Arg motifs and the main-chain atoms of residues 582–585 in a hairpin loop of the neighboring chain (Fig. S7 B and C). Interestingly, in a primary congenital glaucoma (PCG) family, a TEK mutation (Y611C) is located close to this Fn2–Fn2′ interface of neighboring homodimers, resulting in haploinsufficiency because of the loss of Tie2 function (Fig. 5B) (38). Tyr611 is a buried residue that packs against the hairpin loop, making interactions with the Arg-Trp-Arg motif. Mutation of Tyr611 to cysteine would remove a potentially important hydrogen bond to His606 in the neighboring Fn2 loop. We next created a Q588R/V612R double mutant to target the Fn2′-Fn2‴ interface and analyzed the effect of the Fn2 mutations on Tie2 activation in Tie2 singly transfected and Tie1/Tie2 doubly transfected HeLa cells (Fig. 5 C and D and Fig. S7 D and E). The mutant showed a significant increase in basal Tie1 tyrosine mutant (3Y; V681Y, L691Y, and I693Y) in an analogous manner to the Tie2 Fn3 double mutant. Val681 was mutated to tyrosine to mimic the Tie2 Fn3 domain and to direct the side-chain conformers of I693Y and L695Y toward the interface. Another mutant (5M) with additional D689R and D696R substitutions was designed to disrupt the interactions further. The Tie1 mutants increased the basal phosphorylation of Tie1 and made Tie1 insensitive to Tie2-mediated Comp-Ang1 stimulation (Fig. 4 C and D). Interestingly, the 5M mutant did not affect Tie1/Tie2 heterodimerization according to the cell-surface crosslinking assay, and Tie1 baseline phosphorylation was not affected in Tie1 singly transfected cells, suggesting that the Tie1 Fn3 mutations affected only Tie1/Tie2 kinase crosstalk (Fig. S6 A–C).

A Model for Tie2 Clustering. In addition to the very similar interactions between the Fn3 domains in the C2 and P2′ structures, we observed structurally analogous interactions between the Fn2 domains of neighboring homodimers in the crystal, suggesting that both types of interactions exhibit natural protein contacts (Fig. S4). The Tie2 LBDs are too far apart for a dimeric angiopoietin ligand to bridge the receptors for successful dimerization and activation. Instead, a dimeric ligand would compete with multimeric angiopoietin for Tie2 binding (Fig. 3B).

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Tie2 phosphorylation in both singly and doubly transfected cells. Interestingly, the Fn2 mutant could be stimulated with Comp-Ang1 in the presence of Tie1 but not in the absence of Tie1. Alignment of Ang1-bound homodimers of the Tie2 ECDs creates a model for Tie2 clustering (Fig. 5E). Notably, multiple Tie2 homodimers can interact without steric clashes, and the model brings the neighboring LBDs close to each other.

Discussion

Although the structural features of the angiopoietin receptor-binding domain interactions with the Tie2 LBD have been described, the structural basis for Ang1-induced Tie2 activation, Tie1/Tie2 heterodimerization, and the context-dependent antagonism by Ang2 has been largely lacking. Our crystal structures and functional analysis of mutant receptors show that the membrane-proximal Fn3 domains in Tie2 and Tie1 contribute to Tie2 homodimerization and Tie2/Tie1 heterodimerization, whereas the Tie2 Fn2 domain seems to mediate Tie2 clustering. In addition, our results provide a structural explanation for the need of Ang1 oligomers in Tie2 activation.

Crystal structures of the Tie2 Fn-like domains revealed a mechanism of Fn3-mediated Tie2 dimerization in which the Fn3 domains bring their C termini close to each other in a way similar to that described for the membrane-proximal Ig-like domains in KIT and VEGFR-2 (39, 40). In VEGFR-2, the C termini are also about 25 Å apart, and in these three RTKs the membrane-proximal domains contribute symmetrical interactions around an axis with twofold symmetry (39, 40). Interestingly, the interactions between the Tie2 Fn3 domains occur via intermolecular hydrogen bonding between antiparallel β-strands. Although this binding creates an intermolecular β-sheet with only a few side-chain-mediated interactions, the interacting residues are largely conserved. Mutation of two conserved valines in the interacting β-strands in Fn3 to bulkier tyrosines was made to disorient the transmembrane and kinase domains for optimal transphosphorylation upon ligand-stimulated Tie2 activation. These mutations reduced Comp-Ang1-stimulated Tie2 phosphorylation, thus confirming the importance of the intermolecular β-sheet formation for Tie2 activation. In the accompanying study in this issue of PNAS, Moore et al. describe the same intermolecular interactions in an independent crystal structure of Tie2 Fn-like domains and show reduced Ang1-induced Tie2 phosphorylation with the Y979A mutation located in the interface (41). Receptor activation via intermolecular β-sheet formation, as seen in this dimer, is unique to RTKs, although intermolecular β-sheet formation represents a common mode of protein–protein interactions (42).

We constructed a model of the ligand-bound Tie2 ECD homodimer based on our Tie2 Fn3–3 homodimer and the published Ang–FLD/Tie2-LBD complex (33) structures. The model indicates that the ligand-binding sites are too far apart for a dimeric angiopoietin to promote the formation of a Tie2 dimer in the cis orientation. Since Ang2 is mainly a covalent dimer, whereas Ang1 is multimeric in nonreducing conditions, the model provides an interesting possible explanation why Ang2 functions as a weak agonist (28, 35, 36). In EM, both Ang1 and Ang2 exist from dimers up to hexamers, but although the majority of Ang2 showed low-order “mushroom-like” states, the majority of Ang1 existed as high-order oligomers (29, 35). Unlike the native Ang2, tetrameric Bow–Ang2 (Ang-Fc–Fc–Fc) is a potent Tie2 agonist (29). This observation underlines that the expansion of “historical” receptor-binding sites in these multimers is crucial for promoting Tie2 dimerization in cis. This dimerization may be facilitated by the flexibility of the Fn1–Fn2 junction revealed by comparing our structures with that in the accompanying paper by Moore et al. in this issue of PNAS (41). Furthermore, the recently published Ang2-binding and Tie2-activating antibody ABTAA presumably binds two Ang2 low-order oligomers, possibly homodimers, in an optimal angle for Tie2 activation (Fig. 3B) (43). Tie1 is an orphan receptor, but Ang1 activates Tie1 in Tie2/ Tie1 heteromeric receptor complexes (21–23). Promoted by our observation of Fn3-mediated homotypic Tie2 interactions, we solved the Tie1 Fn3 crystal structure for comparison. Structural comparison of the Tie2 and Tie1 Fn3 domains revealed very similar folds despite their low sequence identity, suggesting that Tie1 also interacts with Tie2 through intermolecular β-sheet formation. To analyze the role of Tie1 Fn3 in Tie2/Tie1 heterodimerization, we created the Tie1 Fn3 tyrosyl mutants 3Y and 5M in which the mutations correspond to those in Tie2. Both alterations increased the basal phosphorylation level of Tie1, making Tie1 insensitive to Comp-Ang1 stimulation in cells coexpressing Tie2. Interestingly, the Tie1 Fn3 mutations did not affect Tie1/Tie2 heterodimerization in the crosslinking assay, and the increased baseline activation was Tie2-dependent. These results indicate that the Tie1/Tie2 heterodimerization is likely to involve interactions between the membrane-proximal domains and that these interactions restrict aberrant Tie2–Tie1 cross-activation. We cannot exclude alternative interactions for Tie1 and Tie2 Fn3 domains, and additional interactions, such as putative electrostatic interactions, between the Tie2 LBD and the corresponding domain in Tie1 (25), may stabilize the heterodimerization despite the Tie1 Fn3 mutations.

In addition to Fn3-mediated Tie2 homodimerization, the two crystal structures of the Tie2 Fn-like domains revealed symmetrical interactions between the Fn2 domains that could mediate Tie2 clustering. The interactions are almost identical in
both structures, and residues around the Arg-Trp-Arg motif, which interacts with a hairpin loop of the neighboring chain, are conserved. Site-directed mutagenesis of the Fn2–Fn2 interactions increased the basal phosphorylation of Tie2, suggesting that the Fn2–Fn2 interactions may be involved in Tie2 oligomerization in the absence of ligands rather than in ligand-induced Tie2 activation. There is growing evidence that various transmembrane receptors have a preformed, but inactive, oligomeric structure on the cell surface, and Tie2 also has been shown to exist as preformed oligomers in the absence of ligands (27, 31, 44). Ligand binding then will induce a conformational change for receptor activation. The difference between the Fn2–Fn2 interface in our analysis and dimer 1 in the paper by Moore et al. (41) could represent such a change, because mutational analysis also implicates dimer 1 interactions in Ang1-stimulated Tie2 activation. Although the Fn3-mediated dimers in both papers are identical, a lateral shift of Fn3-mediated homodimers by only a few nanometers is required for the adjustment of the Fn2–Fn2 and dimer 1 interfaces [Protein Data Bank (PDB) ID codes 5MYB and 5UTK].

Interestingly, a mutation in the human TEK gene in the sequence encoding Tie2 Fn2 domain was discovered in a cohort of PCG patients who did not carry mutations in other known disease-causing genes (Fig. 5B) (38). This mutation, Y611C, occurs in the symmetrical interface between the Fn2 domains of the neighboring Tie2 homodimers in our model of Tie2 clustering and in the dimer 1 interface in the Tie2 structure by Moore et al. (41). Although the mutant Tie2 was properly localized at the plasma membrane in resting ECs, it did not undergo normal Ang1-stimulated Tie2 clustering or junctional localization (38). Although the nature of the impaired interactions remains elusive, the disease-causing mutation supports the evidence for the Fn2-mediated Tie2 clustering. Similarly, in the Eph family tyrosine kinase receptors, the membrane-proximal Fn-like domains are known to stimulate intermolecular interactions (45).

Angiopoietins provide a rare example of soluble ligands that engage receptor oligomers on the same cell in cis or bridge their receptors across cell–cell contacts in trans (10, 18). Ang1 stimulation of Tie2 in trans vs. in cis seems to induce partly different signaling pathways. Ang1 binding to Tie2 in EC–ECM contacts represents the cis association mode, which preferentially activates the Erk and DokR pathways (10, 18). In contacting ECs, both angiopoietin ligands induce Tie2 translocation to cell–cell junctions, but Ang2 induces only weak Tie2 activation (10, 18).

Tie2 association may represent distinct ligand-binding modes in trans vs. in cis. Consistent with our model of Tie2 cis association, several studies indicate that artificial angiopoietin dimers, such as Fc-tagged dimers, are inactive as Tie2 ligands (29, 30, 34). However, Oh et al. have reported a specific Tie2-activating Ang1 dimer (CA1-3), which is comprised of the linker and the FLD domains of Ang1 fused to a dimeric Comp domain (46). CA1-3 stimulation shows prominent Tie2 activation in EC–EC junctions, indicating that a dimer of Ang1 FLD and the linker domain is capable of FLD domain presentation in the correct angle for receptor in trans interaction (Fig. S7F). Comp-Ang1 stimulates strong Tie2 activation in EC–EC junctions in trans and in the rear of migrating cells in cis, although the pentameric bundle of Comp-Ang1 is only about 10 nm in diameter (10, 34, 35). Therefore, it is likely to represent a ligand-binding mode whereby the multimeric bridge may recruit neighboring Tie2 homodimers in cis in addition to Tie2 interactions in trans.

Decreased junctional Tie2 phosphorylation upon site-directed mutagenesis of the Fn3 interface suggests that Tie2 clustering in EC–EC junctions may involve arrays of Tie2 homodimers. On the other hand, Tie1 associates with Tie2 in EC–EC junctions and thereby can regulate the context-dependent differences in Tie2 signaling during angiogenesis (13, 23, 26). We show that Tie1/Tie2 heterodimerization may involve interactions between the Fn3 domains, but it is not clear how Tie1 interacts with the arrays of Tie2 homodimers in trans or how angiopoietins bridge Tie2 association across EC–EC junctions. The context-dependent differences in angiopoietin-activated Tie2 signaling pathways may depend on differences in other subcellular protein constituents, such as integrins in the ECM contacts and VE-PTP in EC–EC contacts (10, 13, 19, 47). Further structural and functional investigation is required to understand how Tie1 acts to modulate the effects of Ang1 and Ang2 on Tie2 and the mechanism of Ang2 antagonism in the Tie2 trans association.

Ligand-mediated dimerization of RTKs involves weak homotypic interactions between the membrane-proximal domains which allow precise positioning of the C-terminal regions and the transmembrane domains in the correct orientation that enables activation of the cytoplasmic tyrosine kinase domains (27, 39, 40, 48, 49). We have shown previously that targeting such interactions in VEGFR-3 with an antibody that does not block ligand binding can still block the formation of VEGFR-3 homodimers and VEGFR-3/VEGFR-2 heterodimers, and signaling (50). Similarly, antibodies targeted to the membrane-proximal domain of
KIT inhibit its activation (51). Our results here indicate that ligand-induced Tie2 activation also involves homotypic interactions of the membrane-proximal domains. Importantly, site-directed mutagenesis of the Tie2 Fn3–Fn3 interactions inhibited ligand-stimulated Tie2 phosphorylation, whereas mutagenesis of the Fn2–Fn2 interactions increased the basal phosphorylation of Tie2. Also, site-directed mutagenesis of the Tie1 Fn3 interactions in Tie1/Tie2 heterodimerization experiments increased the basal Tie1 phosphorylation. The targeting of Tie receptor membrane-proximal domains thus may provide unique therapeutic approaches for the modulation of Tie receptor activation.

Methods

Crystalization and Structure Determination. Human Tie2 Fn1–3 (residues 443–735) and human Tie1 Fn3 (residues 641–738) were expressed in insect cells, and the purified proteins were crystallized for structure determination. Full details of protein purification and X-ray and SAXS data collection and analysis are described in SI Methods.

Cell Culture and Phosphorylation of Tie1 and Tie2. WT and mutant Tie1 and Tie2 were cloned into pMXs retroviral expression vector that was used to transduce HEK cells as described by Saharinen et al. (10). Full details of cell culture and analysis are described in SI Methods.

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