TIED TOGETHER: A MOLECULAR ROLE FOR TIE1 IN ANGIOPOIETIN TIE2 SIGNALING

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TIED TOGETHER: A MOLECULAR ROLE FOR TIE1 IN ANGIPOIETIN TIE2 SIGNALING

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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April, 2010
The primary function of the vascular system is the maintenance of oxygen homeostasis for all metazoan tissue. Angiogenesis, the remodeling and maintenance of new blood vessels from an existing vessel, is primarily controlled through the endothelial specific receptor tyrosine kinase Tie2, and the orphan receptor tyrosine kinase, Tie1. Although these receptors share highly conserved, genetic and biochemical analysis has shown these receptors have distinct and essential roles in angiogenesis. Tie2 activation typically results in vessel stability and quiescences and has further been shown to interact with all four sub-types of the angiopoietin signaling factors, Ang1-4. Conversely, Tie1 is involved in vascular remodeling and has no known ligands. The aim of this study is to resolve the molecular mechanism in which Tie1 modulates Angiopoietin-induced Tie2 signaling. Using biophysical, structural, and biochemical assays we show Tie1 directly
interacts with Tie2 via electrostatic interactions housed within the extracellular domains. The binding of Tie1 to Tie2 attenuates Tie2 phosphorylation. We further show the constitutive agonist of Tie2, Ang-1, is capable of excluding Tie1 initiating Tie2 activation. Whereas the antagonist, Ang-2, is incapable of excluding Tie1. Finally, we identify a region within the angiopoietin receptor-binding domain that is capable of including or excluding Tie1 from Tie2. Based upon the available data, we provide a model for Angiopoietin-induced Tie2 signaling.
ACKNOWLEDGMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ang</td>
<td>angiopoietin</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>DTSSP</td>
<td>3,3’-Dithiobis[sulfosuccinimidylpropionate]</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescences resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IP</td>
<td>immuno-precipitation</td>
</tr>
<tr>
<td>r.m.s.</td>
<td>root mean square</td>
</tr>
<tr>
<td>Tie</td>
<td>tyrosine kinase with immunoglobulin-like and EGF-like domains</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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Chapter 1

Introduction

1.1.1 Vasculogenesis and Angiogenesis

All metazoan species have developed a means in which oxygen homeostasis can be maintained. Simple metazoans consisting of a few thousand cells rely on simple oxygen diffusion where more complex organisms such as Drosophila melanogaster, in which diffusion is not possible, have developed a specialized tracheal tube network to maintain oxygen homeostasis (Gorr et al., 2006). In larger bodied vertebrates, a significantly more complex closed vascular system has developed to maintain oxygen homeostasis while simultaneously serving as a means to traffic nutrients and metabolic waste. Proper development and maintenance of the vascular system is essential to the organisms’ health.

Development of the cardiovascular system during embryogenesis is divided into two sequential processes: vasculogenesis and angiogenesis (Figure 1.1). Vasculogenesis occurs first with its onset centered on embryonic day 3 in mice. Initially, endothelial progenitor cells, angioblasts, differentiate from embryonic stem cells. Angioblasts sequentially migrate to sites of vascularization, where they continue their differentiation to form the primordial heart and the primary plexus. The vascular endothelial growth factor receptor (VEGFR) and its cognate ligand, VEGF, are essential for this process. Mouse knockout studies of the VEGFR2 tyrosine kinase are embryonic lethal as a result of improper angioblast migration to sites of neovascularization (Shalaby et al., 1997). This process is essential for formation of the primary plexus, a honeycomb like network of
Figure 1.1: Vascular Development. (A.) The vascular development proceeds through vasculogenesis with the formation of the primary plexus. (B.) Secondly, angiogenesis occurs with the remodeling of the primary plexus into a mature vascular system. (C.) Vascular sprouting requires cooperation between both Tie and VEGF family receptors, whereas regression only utilizes Tie family signaling.
A. Vasculogenesis
   +VEGF
   Arterial  Venous

B. Angiogenesis
   +VEGF
   +Ang1, -2
   +Tie1, -2

C. Vascular Regression
   -VEGF
   Vascular Sprouting
   +VEGF

   +Ang2
differentiated cells that express the endothelial specific markers: vascular endothelium growth factor receptor (VEGFR2), Tie1, Tie2, and vascular endothelium (VE)-cadherin (Li et al, 2009).

The onset angiogenesis begins at approximately embryonic day 10 in mice. During this stage remodeling of the primary plexus begins, forming a mature vascular bed into vessels. The vessels are lined by a layer of connective endothelial cells that are surrounded by a supportive cellular coating, consisting of smooth muscles cells and pericytes. Angiogenesis is continual throughout development and persists in adulthood. Vascular sprouting and regression require proper coordination of multiple cellular signals, most notably through the endothelial specific VEGF and Tie family receptor tyrosine kinases (RTK). Vascular homeostasis, sprouting, and regression all require changes in Tie signaling whereas the VEGF signaling only appears to be involved in vessel sprouting (Figure 1.1). While there is a wealth of information on the molecular mechanisms of VEGFR family, the mechanistic roles of Tie family receptors and the cognitive angiopoietin ligands remain controversial and poorly understood (Augustin et al., 2009). Therefore, mechanisms affecting angiopoietin induced Tie family receptor signaling remains the focus of the following study.

1.2.1 Pathological Angiogenesis

The influential studies of Judah Folkman, were the first to describe the necessity of the vascular system in tumor growth and metastasis (Folkman, 1971). As such, he hypothesized an avascular tumor growing devoid of vascular support would not exceed 1-2mm in size, limited by the diffusion rate of nutrients and waste across the spherical
mass. This proposed limit results from equivalent rates of cellular proliferation and death to become equal resulting in no net change in tumor size. Therefore, blood vessel recruitment could potentially increase tumor volume via efficient removal of cellular waste and nutrient replenishment. These observations further suggest that anti-angiogenic compounds could have considerable therapeutic potential by limiting tumorigenesis.

In contrast to developmental angiogenesis, pathological angiogenesis, as depicted in Figure 1.2, typically begins with the formation of an avascular tumor growing in the subcutaneous space detached from the vasculature. Over expression of VEGF and other angiogenic factors promote tumor growth and development. Rapid cellular growth of the tumor results in hypoxia, causing the release of pro-angiogenic factors, such as VEGF and Ang-2, which diffuse to the vascular endothelium. Initial affects of these growth factors include the disintegration of the vascular basement membrane by matrix metalloproteinase’s. Commonly, these pro-angiogenic factors consist of VEGF-A binding to its endothelial cell receptor VEGFR2 and Ang-2 binding to its receptor Tie2 (Shibuya, 2008; Gale et al., 1999). Deactivation of Tie2 by Ang2 leads to induction of additional chemoattractants that promote endothelial cell migration and mitogenesis (Holash et al., 1999). Interestingly, in the absence of VEGF stimulation through VEGFR2, newly forming vessels retract, when endothelial cells undergo apoptosis in the absence of the pro-survival signaling from VEGFR2. Conversely, in the presence of VEGF continued infiltration of vessels cause the tumor to become hypervascularized.
Figure 1.2: Pathological Angiogenesis. (1.) The avascular tumor growing off of the vascular between 1-2mm in size undergoes a (2.) hypoxic switch releasing pro-angiogenic factors. (3.) Sub sequentially, robust angiogenesis occurs allowing the tumor to increases in size an all a means for (4.) tumor cell shedding.
This process leads to a continual supply of nutrients for the growing tumor and cellular access to the vascular system for transplantation from one part of the body to another, metastasis. Furthermore, many tumorigenic cells are not only capable of eliciting angiogenesis through angiogenic factor release, but themselves utilize angiogenic receptors signaling to elicit anti-apoptotic phenotypes. Therefore anti-angiogenic therapies are widely sought in addition to traditional chemotherapeutics (Bergers et al., 2008; Holash et al., 2008). There has been mild success in the development of anti-VEGF therapies by Regeneron, although further development of effective anti-angiogenic therapies is hindered by the apparent lack in understanding of the mechanistic roles of the Tie family receptors in conjunction with their angiopoietin ligands (Augustin et al., 2009; Yancopoulos et al., 2000).

1.3.1 Tie Family Receptors

The Tie family receptors along with the VEGF receptors are the only known endothelial cell-specific RTKs. Studies with dominant negative and null mice revealed the loss of Tie2 function results in embryonic death because due to the failure of the vasculature system to mature from the primary plexus (Dumont et al., 1994; Sato et al., 1995). Thus, it was proposed that Tie2 is not required for the differentiation of endothelial cells from angioblast, but rather for their maintenance and proliferation (Dumont et al., 1994). Mice lacking TIE1 also die in utero, most likely a result of pulmonary edema (Puri et al., 1995). Although the vasculature remains intact, the integrity of vessel endothelial cells is compromised. Accordingly, TIE1 null mice display defects in vessel integrity demonstrated by localized hemorrhaging and the presence of an
underdeveloped heart. However, the exact role of Tie1 in angiogenesis remains unknown. Considerable evidence also identifies the angiopoietins and the Tie2 receptor as important regulators of tumor-induced angiogenesis and, therefore, cancer growth and metastasis (Lin et al., 1998; Lin et al., 1997; Oliner et al., 2004).

1.2.2 Tie2 Extra-Cellular Domain Structure

Tie1 and Tie2 are type 1 transmembrane protein receptor tyrosine kinases (RTKs) (Ward and Dumont, 2002; Yancopoulos et al., 2000). The two receptors are remarkably similar sharing 36.7% sequence identity. Moreover, the human, mouse, rat, and bovine Tie2 receptor is almost identical, sharing 92% sequence identity. Based upon the sequence of Tie2, the extracellular domain was predicted to contain two immunoglobulin-like (Ig) loops separated by three epidermal growth factor-like (EGF) repeats, all followed by three tandem fibronectin type III repeats (FNIII) (Dumont et al., 1993). Further structural analysis revealed by transmission electron micrographs, the extracellular portion resembled an arrow with the Ig and EGF domains making up the head and the stalk encompassing the FNIII repeats (MacDonald et al., 2006). Although binding studies originally identified both the Ig and EGF domains of Tie2 as necessary and sufficient for angiopoietin binding (Barton et al., 2005; Fiedler et al., 2003), more recent mutagenesis and crystallographic data revealed the ligand-binding site is within the Ig2 domain (Barton et al., 2006). Furthermore, as depicted in Figure 1.3A the molecular structure of the Tie2 extracellular domain it was found to contain not two Ig-like folds, but three Ig folds. The most striking feature of Tie2 observed in these studies was the lack in conformational change within the Tie2 receptor in it ligand bound and
Figure 1.3: Ribbon diagram of the key structural domains of the Tie2 receptor.

(A.) The extracellular domain of the Tie2, PDB 2GY5. The Ig1, Ig2 and Ig3 domains are color coordinated as red, green, and blue, respectably. Between the Ig2 and Ig3 domains are the tandem EGF1-3 domains colored yellow, purple, and orange, respectably. (B.) The Tie2 tyrosine kinase domain rotated 90° on the y-axis, PBD 1FVR. The two conserved lobe domains; N-terminal and C-terminal are colored blue and red, respectably. The three catalytic loops are labeled and colored yellow. The extended C-terminal tail, containing the substrate tyrosines 1101, 1107, and 1112 is labeled cyan.
unbound state. These observations tend to lean towards the mode of activation to be ridged body clustering of the Tie2 receptor by its cognitive angiopoietin ligands.

1.3.2 Tie2 Kinase Domain Structure and Function

In general, receptor tyrosine kinase signaling and activation is initiated by ligand binding which induces receptor clustering and proximity of the cellular tyrosine kinase domain. This activation leads to an increase in tyrosine phosphorylation, where upon multiple tyrosines can be phosphorylated, inducing recruitment of additional complexes, and initiation of downstream cellular signaling cascades. Determining the structures of tyrosine kinase domains has illuminated the structural elements that govern their activity (Johnson et al., 1996).

The structure of the Tie2 TKD has been determined to 2.2Å and not surprisingly appears to be similar in architecture to others, most notable the fibroblast growth factor receptor (FGFR) (Shewchuk et al., 2000). In Figure1.3B, a Cα trace of the Tie2 TDK reveals the presence of the two commonly observed N-terminal and C-terminal lobes found in virtually all kinases. The N-terminal lobe (residues 808-904) folds into a five-stranded anti-parallel β-sheet. Within the N-terminal lobe are two acidic residues (K855 and E872) and a glycine rich nucleotide binding loop (residues 831-836) responsible for proper coordination of the substrate α, β, and γ phosphates of ATP. The C-terminal lobe (residues 905-1124) is primarily α-helical consisting of seven α-helices, four β-strands, and an extended C-terminal tail. Between the N- and C-terminal lobes is the kinase active site consisting of the catalytic (residues 962-968) and activation loops (residues 982-1008). The activation loop in Tie2 contains one tyrosine (Y992) and begins with the
highly conserved sequence Asp-Phe-Gly. The end of the activation loop has single residue replacement form the conserved sequence, Ala-Phe-Glu, to end with Ala-Ile-Glu. The sequence of the catalytic loop, containing the catalytic aspartic residue (D964), is identical to other kinase domains: His-Arg-Asp-Leu-Ala-Ala-Arg-Asn (Hubbard et al., 1994; Mohammadi et al., 1996; and McTigue et al., 1999).

The C-terminal tail exist in an extended conformation and has three tyrosine substrate residues: Y1101, Y1107, and Y1112. Tyrosine phosphorylation serves to recruit intracellular signaling molecules with Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Specifically, Y1101 has been shown to be the major site of phosphorylation, recruiting growth factor receptor-bound protein 2, Grb2, and the p85 subunit of phophatidylinositol 3-kinase, PI3-K (Huang et al., 1995; Kontos et al., 1998). The recruitment of PI3-K initiates cell motility and downstream survival signaling events through the PKC and Akt pathways, respectively. It has recently been shown that Ang1 treated endothelial cells are capable of initiating either trans Tie2 signaling in cell-cell junctions or cis Tie2 signaling at cell-substratum junctions (Fukuhara et al., 2008; Saharinen et al., 2008). At cell-cell junctions, Tie2 signaling is induced by the bridging of multimeric Ang1 between neighboring cells, leading to a quiescent phenotype. Primarily through cellular signaling cascades involving the Akt-eNOS pathway. Conversely, in cells with limited cell-cell contacts the phenotype changes from a resting to mobile state. This change is reflected in the utilization of Tie2 signaling in cis, primarily through the PKC-MAPK pathway. Further studies inhibiting the activity of PI3-K have shown the effect of Y1107 in mediating cell mobility by recruitment of the downstream-of-kinase-related docking protein, Dok-R (Jones et al., 2003; Master et al.,
Once phosphorylated, Dok-R is capable of recruiting Nck along with the p21-activating kinase, Pak1, restoring cell mobility in a PI3-K independent mechanism. Phospho-tyrosine 1112 has been reported to be a regulatory mechanism in the recruitment of a protein tyrosine phosphatase, SH-PTP2 (Huang et al., 1995). Recruitment of SH-PTP2 is not fully understood but believed to act as an attenuator to Tie2 signaling by site-specific phosphate removal.

Tie2 appears similar in architecture to FGFR TKD, although appears to function slightly different in activation. Sequence alignment and structural overlays of Tie2 TKD and FGFR TDK reveals a sequence identity of 45% with an rmsd for the C-terminal and N-terminal lobes of 0.76Å and 0.58Å, respectively (Shewchuk et al., 2000). Activation of many tyrosine kinase domains is thought to be in trans due to the dimerization or clustering of the kinase domains by extracellular ligand binding. Unlike FGFR, endogenous Tie2 activation requires receptor clustering, although kinase dimerization has been shown to be sufficient for trans auto-phosphorylation (Kontos et al., 1998; Davis et al., 2003). Activation typically involves phosphorylation of a tyrosine residue in the activation loop by base-catalyzed transfer of a γ phosphate from ATP to the hydroxyl moiety in the tyrosine side chain. As in the case of FGFR TDK, this phosphorylation leads to a conformation change allowing the phospho-tyrosine to interact with basic residues, stabilizing the active conformation. Phosphorylation of the active loop leads to a 100-fold increase in tyrosine kinase activity (Furdui et al., 2006). Conversely, the Tie2 TDK activation loop appears to take on a phospho-tyrosine independent “active conformation.” Control of auto-phosphorylation for Tie2 appears to lie in the inhibitory conformation of the ATP coordination residues in the N-terminal lobe. The acidic K and
E residues are not properly aligned to coordinate ATP as well as nucleotide-binding loop is in a closed conformation blocking the active site. Furthermore, the x-ray structure of Tie2 TDK shows the extension of the C-terminal tail into the active site, possibly acting as a substrate mimic. Fundamentally, deletion of the last 15 residues shows a drastic increase auto-phosphorylation, as compared to wild type Tie2 (Niu et al., 2002). In situations with kinase domains that require increases in localization to elicit activation, control over enzymatic activity may not be as stringent as seen with other kinases that are always in close proximity (Taylor et al., 1995). As is the case for Tie2, the inhibitory elements are due to conformations of highly flexible loop regions, typically with high B-factors. This leads to the possibility of other proteins that could modulate kinase phosphorylation. This hypothesis has been further supported by the tight control of an endothelial specific phosphatase, VE-PTP, on Tie2 phosphorylation and the possibility of Tie1 as an inhibitory co-receptor (Fachinger et al., 1999).

1.4.1 Angiopoietin Ligand Function

Initially described as an orphan receptor (Dumont et al., 1992), Tie2 was subsequently shown to interact with all four of the angiopoietins (Ang1-Ang4) (Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The different angiopoietins, although having high sequence homology, elicit different responses from the receptor tyrosine kinase Tie2. Loss-of-function and gain-of-function genetic studies in mice have been beneficial in understanding the physiological effects of Ang1 and -2 on the vascular system. Mice lacking Ang-1 expression die *en utero*, displaying the same phenotype of mice lacking TIE2. Vascular development progresses through vasculogenesis but fails to
proceed through angiogenesis with a loss of tubular formation in the primary plexus. As such, the over expression of Ang-1 in mice leads to hypervascularization with many stabilized branching vesicles, supporting the agonistic mechanism of Ang1 for Tie2 (Suri et al., 1996). Furthermore, the over expression of Ang1 led to an attenuation of the chemotatic signaling by VEGF and a decrease in vessel leakiness (Thurston et al., 1999). The agonistic action of Ang1 on Tie2 signaling appears to be the primary phenotype of vascular quiescence.

Conversely, Ang2 acts as an antagonist to Tie2 signaling promoting angiogenesis. Contradictorily to the ANG1 null mouse, ANG2 knockout mice develop past embryonic angiogenesis but suffer from severe vascular dysmorphogenesis in adulthood causing mice to die about 2 weeks post birth. ANG2 null mice are hypervascularized and resistant to vascular remodeling as seen in the postnatal development of the retinal lens. Upon birth Ang2 expression is robustly increased in the retinal lens, which undergoes vascular regression that is obligated in the ANG2 null mouse (Gale et al., 2002). Furthermore, the expression of Ang2 appears to be up regulated to vascular beds undergoing angiogenesis. Vessel sprouting relies on proper coordination of Ang2 and the hypoxia induced release of VEGF. Alternatively, when endothelial cells only receiving pro-angiogenic signaling from Ang2 the vessel regresses. As such, the over expression of Ang2 during developmental angiogenesis causes the mice to die en utero, mimicking the phenotype of the TIE2 and ANG1 null mice (Maisonpierre et al., 1997). Taken together these observations depict Ang2 as an antagonist of Tie2 signaling in that its angiogenesic phenotype is to promote vessel permeability making endothelial cells susceptible to chemotatic signaling.
Indeed, Ang1 is a constitutive agonist to Tie2 while Ang2 is a context-dependent one (Davis et al., 1996; Gale et al., 2002; Maisonpierre et al., 1997). The hypothesis that differential presentation and binding of the various angiopoietins to Tie2 is responsible for their distinct biological effects appears unlikely in light of previous evidence that Ang1 and Ang2 bind to the same region of Tie2 (Barton et al., 2005; Barton et al., 2006; Maisonpierre et al., 1997).

Now, it appears more likely that other cell-specific surface receptors exist that could help transduce the angiopoietin signals and modulate their functional potential. This hypothesis was recently supported by several reports defining a potential role for Tie1 in Tie2 signaling (Kim et al., 2006; Saharinen et al., 2005; Yuan et al., 2007). Tie1, although a close sequence homologue of Tie2, does not interact directly with the angiopoietins and its in vivo ligands have yet to be identified (Maisonpierre et al., 1997). Nevertheless, the angiopoietins may affect Tie1 function (Kim et al., 2006; Saharinen et al., 2005; Yuan et al., 2007). As observed by co-immunoprecipitation analysis, Tie1 and Tie2 appear to associate on the cell surface with receptor phosphorylation correlating with activation. A recent study using catalytically inactive Tie2 demonstrates that Tie1 phosphorylation can occur in trans and is dependent on a functional Tie2 (Kim et al., 2006; Yuan et al., 2007).

1.4.2 Angiopoietin Structure

The Angiopoietins are a small set of growth factor ligands for the Tie2 endothelial-specific receptor tyrosine kinase. They are unique in that the prototypic family member, Ang-1, is a Tie2 agonist while the highly homologous Ang-2 is a
context-dependent agonist. Ang-1 expression is primarily observed in vessel supporting cells such as smooth muscle cells, pericytes, and fibroblast. Conversely, Ang-2 expression is primarily observed on endothelial cells and transcriptionally unregulated by hypoxia, shear stress, and chemotactic response signaling (Augustin et al., 2009). The primary sequence of the angiopoietins can be divided into three distinct domains with independent functions. The first ~50 amino acids folds into an angiopoietin specific super-clustering domain responsible for higher order ligand assembling. Transmission electron microscopy experiments of Ang-1 and -2 show the amino terminal domain is responsible for creating a mixture of oligomeric states, with hexameric being the predominate multimerization state (Dais et al., 2003). In this regard Ang-1 and -2 are indistinguishable from each other. Following the super clustering domain is ~215 amino acids which resembles a coiled coil domain, capable of forming ligand dimers. The remaining ~220 carboxyl terminal amino acids fold into a fibrinogen domain, shown to interact with the Tie2 extracellular domain (Barton et al., 2005; Barton et al., 2006). Initial studies by Davis and colleagues identified the receptor-binding fibrinogen domain as necessary and sufficient for their unique functional activities. Closer examination of the angiopoietin fibrinogen domain reveals it folds into the three commonly observed A, B, and P fibrinogens. Binding studies of the Ang-2 to Tie2 revealed the amino acids responsible for receptor recognition or encompassed in the P-domain (Barton et al., 2005; Barton et al., 2006). Interestingly the multimerization state of the angiopoietins directly affected their ability to transduce Tie2 activation in endothelial cells. In their monomeric forms, Tie2 activation was abrogated. Pretreatment of endothelial cells with the monomeric fibrinogen domains was capable of competitively inhibiting Tie2 activation.
by higher angiopoietin multimeric states (Davis et al., 2003). More recently, the rational for higher multimeric states have been proposed in the differential signaling from Tie2 on two adjacent cells at tight junctions or at none cell junctions; initiating quiescence or migration, respectively, further supporting the means in which the angiopoietins are capable to maintaining vascular stability (Saharinen et al., 2008; Fukuhara et al., 2008). The requirement for multimerized ligand presentation to Tie2 is currently under active research.
Chapter 2
Tie1-Tie2 complex formation

2.1 Pre-existent Tie1-Tie2 Complexes Observed By FRET Imaging On The Cell Surface

To examine the potential role of Tie1 as a co-receptor and evaluate Tie1-Tie2 interactions on the cell surface, we monitored receptor-receptor interactions in vivo by tagging the individual proteins with CFP and YFP, and following their localization and association by confocal microscopy coupled with fluorescence (Förster) resonance energy transfer (FRET). Specifically, for assessing Tie1 and Tie2 receptor spatial proximity during angiopoietin signaling, we exploit the FRET methodology recently utilized by the Tsien and Springer groups to analyze the role of lipid modifications in membrane partitioning and Integrin signaling, respectively (Kim et al., 2003; Zacharias et al., 2002). As depicted in Figure 2.1, we fused the monomeric enhanced green fluorescent protein (eGFP) variants mCFP and mYFP to the carboxyl termini of Tie2 and Tie1 respectively in place of the catalytic tyrosine kinase domain. As energy transfer between donor (mCFP) and acceptor (mYFP) only occurs over short distances (≤ 10nm), emission of mYFP following excitation of mCFP is observed only when Tie1 and Tie2 are in close proximity, presumably as a receptor/co-receptor complex. The FRET efficiency is calculated by subtracting the background FRET (determined by the acceptor-photobleaching method) from the experimental FRET efficiency (Wouters et al., 2001).

Using our FRET-based proximity assay, we clearly observe Tie1-Tie2 association on the cell surface indicating the two receptors are within less than 100Å of one another.
Figure 2.1: Schematic Representation of the Tie1-Tie2 FRET proximity assay.

Ribbon diagrams of the Tie1 (Blue) and Tie2 (Red) extracellular domains are fused to the monomeric GFP variants mCFP (Cyan) and mYFP (Yellow), respectively. As the receptors come in close proximity to each other, as depicted on the right, upon excitation of mCFP at 458nm, non-radiative energy transfer occurs to mYFP, resulting in YFP emission. In the event the receptors distance is >10nm, as depicted on the left, upon mCFP excitation, no energy transfer to YFP occurs.
by measuring a FRET efficiency and visualized by an increase in mCFP emission after photo-disruption of mYFP. Interestingly, our observations are in the absence of angiopoietin ligand, further demonstrating that Tie1 and Tie2 are in a pre-existent complex prior to ligand recognition. Figure 2.2A-C illustrates a representative image of the fluorescence intensity and membrane localization of Tie2-CFP/Tie1-YFP, which we will denote as wild-type for our discussions. Both receptors localize with uniform diffuse staining predominantly on the plasma membrane. Using the acceptor-photobleaching technique for measuring FRET efficiency, we observed Tie1-Tie2 association with an average overall efficiency of 30%, 27%, and 19% under optimal conditions in HEK293, U2OS, and EA.hy 926 cells, respectively. In accordance with the variables identified by Springer and colleagues (Kim et al., 2003), the length of linker between the receptor transmembrane domain and amino-terminus of the fluorescent protein was varied to identify the optimal combination of receptor length, and fluorophore-receptor pair (data not shown). Furthermore, for our experiments, HEK293 and U2OS cells were chosen in addition to EA.hy 926 endothelial cells, based upon their lack of endogenous Tie receptors as well as ease of transfection and cellular imaging (Yuan et al., 2007).

We found, as expected, that increased linker lengths significantly decreased overall FRET efficiency (Kim et al., 2003). Among several constructs tested, one pair with eleven and ten residues for Tie1 and -2 respectively, within the linker, consistently yielded reliable results in all three independent cell lines (EA.hy 926, HEK293, and U2OS). Importantly, both CFP and YFP variants carry the A206K, L221K, and F223R mutations thought to significantly decrease the chance of fluorescent protein multimerization (Zheng et al., 2002). As a control, we have also co-expressed each
**Figure 2.2: In vivo FRET imaging of Tie1-YFP and Tie2-CFP on the cell surface.**

HEK293 (A), U2OS (B), and EaHy.926 (C) cells were transiently transfected with the chimeric Tie2-CFP (cyan) and Tie1-YFP (yellow) receptors. Cellular fluorescences images for CFP and YFP were acquired prior to and post acceptor photobleaching. FRET efficiency was calculated within the region of interest (green box) and depicted in an absolute range from high (red-1.0) to low (purple-0.0) as a colorimetric overlay image. Light gray bar in each image indicates 10 µm.
Figure 2.3: *In vivo* FRET imaging of chimeric PlexinA1 receptors co-expressed with chimeric Tie1 and -2 receptors. HEK293 cells co-transfected with (A) Tie2-CFP (cyan) and PlexinA1-YFP (yellow) or (B) PlexinA1-CFP (cyan) with Tie1-YFP (yellow). Cellular fluorescences images for CFP and YFP were acquired prior to and post acceptor photobleaching. FRET efficiency was calculated within the region of interest (green box) and depicted in an absolute range from high (red-1.0) to low (purple-0.0) as a colorimetric overlay image. Light gray bar in each image indicates 10 μm.
Figure 2.4: Graphical representation of average FRET efficiencies for combinations of Tie receptors and plexinA1 controls in different cell lines. The data is represented as mean +/- SEM.
receptor with both fluorophores to account for any non-specific interactions that may occur between the tagged fluorescent proteins (Zacharias et al., 2002). No FRET signal is observed under these circumstances (data not shown). Similarly, to evaluate possible effects of over-expression, Tie receptors were co-expressed with the functionally unrelated receptor plexin-A1 (both mCFP and mYFP variants). As seen in Figure 2.3, under these circumstances we observed between 0 and 2% FRET efficiency. The five experimental conditions are graphically compared in Figure 2.4. Together, these findings validate the overall specificity of the FRET proximity assay and that that protein overexpression and nonspecific interactions between the fluorescent proteins does not contribute significantly to the observed FRET efficiency. Interestingly, the proximity assay allowed us to visualize in vivo the association of a Tie1-Tie2 co-receptor complex within both epithelial and endothelial cell lines. From this observation we further hypothesized the interaction is mediated by the receptors extra-cellular domains.

2.2 Identification Of Residues Involved In Tie1-Tie2 Interactions

To understand the potential structural differences between Tie1 and Tie2 that mediate their distinct biological properties and identify structural elements that may contribute to Tie1-Tie2 interactions, we modeled the structure of Tie1 using the experimentally-determined 2.5Å Tie2 structure (Barton et al., 2006) and the program MODELLER (Marti-Renom et al., 2000). Tie1 and Tie2 are highly homologous – sharing 39% amino acid identity – and, not surprisingly Tie1 can be easily modeled on the structure of Tie2. A schematic representation of the molecular surfaces of Tie2 and of the Tie1 model, color coded according to electrostatic potential and hydrophobicity of
the exposed amino acid side chains, is presented in Figure 2.5. The hydrophobic surface features of Tie2 and Tie1 are very similar overall. Interestingly, two patches of exposed hydrophobic residues, indicated with arrows, are present at the tip of Tie2, but are absent in the equivalent Tie1 region. Indeed, these overlap with the binding site of the Tie2-specific ligand Angiopoietin-2 (Ang2) indicated with a green circle (Barton et al., 2006), providing the structural explanation for the distinct ligand-binding properties of the two Tie receptors.

More importantly, comparison of the surface electrostatic potentials of Tie1 and Tie2 as seen in Figure 2.5C and D reveals that Tie2 has a slight negative overall charge with a theoretical pI value, if isolated in solution, of 6.9. Tie1, on the other hand has a positive overall charge with a corresponding pI value of 9.3. Tie2 has one expansive negatively-charged surface area, which results from the approximation of several exposed aspartic and glutamic acids in the Ig1 and EGF1 domains, including D25, E53, D60, E109, D236, D239, D252 and D283 (Figure 2.5C, right). The corresponding Tie1 surface area if Ig1 and EGF1 is overall neutral in charge. Tie1, on the other hand, contains one large positively charged area on its surface, located on the opposite side of the molecule (Figure 2.5D, left). Its electrostatic surface potential results from the approximation of several arginines and lysines in Ig1, EGF2, and Ig3, including R38, R82, K95, R91, R260, R279, R263, R349, R388, R427, R437, and R438.

The presence of large patches of oppositely charged molecular surfaces in Tie1 and Tie2 suggest that these areas might be involved in Tie1-Tie2 recognition. To evaluate their roles in mediating receptor-receptor interactions, we utilized site-directed mutagenesis in combination with our Tie1-Tie2 FRET proximity assay. Various regions
Figure 2.5: Extracellular surface feature comparisons of the experientially determined Tie2 and the modeled Tie1 structure. The left and right panels are related by 180° rotation about the y-axis. (A) The hydrophobicity surface rendering of Tie2 and Tie1, with the hydrophobic side chains colored green and the hydrolytic side chains in grey. Arrows point to the previously determined ligand-binding region within the tip of the second immunoglobulin domain in Tie2. (B) Color-coded electrostatic surface potential of Tie1 and Tie2 with Red and blue represent electrostatic potentials in the range of $-11$ to $+11$ $k_B T$, where $k_B$ is the Boltzman constant and $T$ is the temperature (293 K). Amino acids responsible for the ionic interface are labeled by their single letter code and position.
within the charged patches were targeted by mutagenesis through the construction of Tie variants containing multiple amino acid substitutions and analyzed for their ability to associate via FRET. Our experience has shown that single site mutations often have little or no effect on interactions involving relatively large protein-protein interfaces (Barton et al., 2003; Barton et al., 2005; Barton et al., 2006). Therefore multiple mutations within the charged surface were simultaneously made following a charge reversal strategy, replacing basic residues with acidic amino acids and vice versa. A list of all the point mutations is schematically represented in Figure 2.6. All of these charge reversal mutations are in surface exposed residues; as such, we expect the mutations will not affect the overall folding of the proteins. In agreement, upon transfection into HEK293 cell, all mutant receptors are well expressed, processed, and localize correctly to the cell surface. Representative images of the mutant receptors can be seen in Figure 2.7A and B.

Using the FRET proximity assay to evaluate the ability of the mutant Tie receptors to interact with the corresponding wild type Tie receptor, it is clear we have identified several charged surface residues involved in their association. Three of the four Tie1 mutants (B1, C1, D1) and all three Tie2 variants (A2, B2, C2) exhibit significantly lower FRET efficiencies than the pairing of wild-type receptors. A representative image of the receptor pair mut-B1-Tie1-YFP/wild-type-Tie2-CFP, is displayed in Figure 2.7A. Even under ideal conditions, we failed to observe significant FRET when these two receptors are co-expressed in either HEK293 or U2OS cell lines (0% FRET efficiency). Similarly, mut-A2-Tie2-CFP exhibits proper localization to the membrane, yet fails to associate with wild-type-Tie1-YFP to any significant extent (5% FRET efficiency - Figure 2.7B). Together, the data suggest a complex interaction between Tie1 and Tie2
Figure 2.6: Molecular surface representation of Tie2 and modeled Tie1 structure color-coded according to their corresponding mutations. (A) The amino acids mutated in Tie1 are colored in blue: A1: Tie1(K95E, R260E, R263E), B1: Tie1(R260E, R437E, R438E), C1: Tie1(R437E, R438E), D1: Tie1(R91E, K95E, R427E). (B) The corresponding acidic interface mutations in Tie2 are labeled red: A2: Tie2(E53K, D236K, E239K), B2: Tie2(E53K, D60K, D389K), and C2: Tie2(E53K, D60K, E109K, D236K, E239K, D389K).
Figure 2.7: *In vivo* FRET imaging of the mutant Tie receptors co-expressed with a corresponding wild type Tie receptor. Co-expression in HEK293 cells of (A) wild type Tie2-CFP (cyan) with mutant B1(R260E, R437E, R438E)-Tie1-YFP (yellow) and (B) mutant A2(E53K, D236K, E239K)-Tie2 CFP with the corresponding wild type Tie1-YFP receptors. Cellular fluorescences images for CFP and YFP were acquired prior to and post acceptor photobleaching. FRET efficiency was calculated within the region of interest (green box) and depicted in an absolute range from high (red-1.0) to low (purple-0.0) as a colorimetric overlay image. Light gray bar in each image indicates 10 µm.
Figure 2.8: Graphical representation of average FRET efficiencies for combinations of wild type Tie receptors to the corresponding mutant Tie receptors in HEK293 cells. The date is represented as mean +/- SEM.
involving a broad surface area within their basic and acidic regions, respectively. Indeed, several different mutant receptor combinations with a wild-type co-receptor result in the loss of receptor-receptor association as illustrated by low or absent FRET efficiencies, demonstrating that the charged surface patches play a role in the Tie1-Tie2 association (Figures 2.8). Moreover, analysis of the Tie1 mutants suggests that residues R437 and R438 in the Ig3 domain play a central role in receptor association (compare mutants A1, B1, and C1 in Figure 2.8). Finally, the identification of mutant-wild-type receptor pairs that fail to demonstrate appreciable FRET further demonstrates the exquisite specificity of this proximity assay.

2.3 Tie1 And Tie2 Interactions Are Direct

To validate the notion that Tie1-Tie2 interactions are direct, and not mediated by an unidentified binding partner, we assayed for receptor complementation - as illustrated by the restoration of wild-type FRET efficiencies - between two individual mutant receptors. HEK293 cells were cotransfected with all nine possible combinations of mutant Tie1 and Tie2 receptors. Given the size and complexity of the potential interface involved, only a subset of receptor combinations would be predicted to have molecular surfaces of sufficient complementarity to rescue the FRET efficiency to near wild-type levels. Indeed, from all tested receptor combinations we identified only two mutant receptor pairs; mut-B1-Tie1-YFP/mut-C2-Tie2-CFP, and mut-C1-Tie1-YFP/mut-A2-Tie2-CFP, which display significant receptor association. Representative FRET efficiencies for each mutant receptor pair are shown in Figure 2.9. Individually, each of these mutated receptors is incapable of interacting with their counterpart wild-type
Figure 2.9: Tie1 and Tie2 form a direct interaction as demonstrated by allelic suppression. HEK293 cells were co-transfected with the following mutant receptor pairs (A) mutant B1(R260E, R437E, R438E) Tie1-YFP (yellow) and mutant C2(E53K, D60K, E109K, D236K, E239K, D389K) Tie2-CFP (cyan) or (B) mutant C1(R437E, R438E) Tie1-YFP and mutant A2(E53K, D236K, E239K) Tie2-CFP. Cellular fluorescences images for CFP and YFP were acquired prior to and post acceptor photobleaching. FRET efficiency was calculated within the region of interest (green box) and depicted in an absolute range from high (red-1.0) to low (purple-0.0) as a colorimetric overlay image. Light gray bar in each image indicates 10 µm.
Figure 2.10: Graphical representation of average FRET efficiencies for pairs of mutant Tie1-Tie2 receptors demonstrating allelic suppression. The data is represented as mean +/- SEM.
Mean (% FRET Efficiency)

Tie1: WT  B1  B1  C1
Tie2: WT  A2  C2  A2
receptor or other corresponding mutant receptors, yet when co-expressed, display FRET efficiencies nearly identical to that of wild-type receptors (Figure 2.9 and 2.10). Averaged FRET efficiencies from several independent experiments for each receptor pair are graphed and compared to wild-type in Figure 2.10. FRET efficiencies between wild-type receptors and the two mutant pairs are not statistically different. This demonstration of “allelic suppression” excludes the possibility of an unknown binding partner, and provides strong support for a direct interaction between Tie1 and Tie2 involving the complementary charged surface areas within the receptor ectodomains.

2.4 Conclusions

Understanding the precise role of Tie1 in angiopoietin Tie signaling has been controversial since its discovery 15 years ago. Furthermore, identifying Tie1 as a co-receptor to Tie2 is still under much scrutiny. Using a powerful in vivo proximity assay, we have begun to identify a role for Tie1 in Tie2 signaling by demonstrating a direct interaction between these receptors. We further identify, via structure-based site-directed mutagenesis, the precise molecular surface regions of the Tie1 and Tie2 ectodomains, which mediate this critical interaction.

The utility of the FRET proximity assay allowed us to monitor the interaction in real time between Tie1 and Tie2 on the cell surface. Upon co-expression Tie1 directly interacts with Tie2 primarily through ionic interactions with in the extra-cellular domains. This observed interaction between Tie1 and Tie2 is supported by other groups in the ability to co-IP out of crude HUVEC lysate the heterogeneous receptor complex in the absence of angiopoietin stimulation (Saharinen P. et al., 2005 and Kim L.K. et al., 2006).
Most interestingly, previous attempts to co-IP Tie1 with Tie2 were dependent on stringent cross-linking with DTSSP of cellular proteins. Typically most immunoprecipitations are performed with stringent washing of protein-coupled sepharose beads, capable of disrupting weak protein-protein interactions. As seen in the molecular surface charge rendering of Tie1 and Tie2, the interaction between the receptors is primarily ionic and not hydrophobic. Furthermore, disrupting this interaction required only a small set of clustering point mutations within the co-receptor interface. These observations indicate the hetero receptor complex has adopted its minimum energy state; only a small amount of energy is required to disrupt Tie1 from interacting with Tie2. As such, we further hypothesize the angiopoietins have different properties affecting the Tie1-Tie2 hetero receptor complex.

It is interesting to note the interaction between Tie1 and Tie2 differs from others (Macdonald et al., 2006). Using Tie1 truncation mutants and a TrkA/Tie2 fusion protein, Marron and colleagues, for example, suggest an association between Tie1 and Tie2 that is mediated by their cytoplasmic kinase domains (Marron et al., 2000), while we observe a dynamic interaction between these receptors in the absence of the catalytic kinase domains and any subsequent down-stream signaling events. Moreover, our structure-based receptor mutagenesis, combined with localization and FRET analysis strongly supports receptor interactions through their extracellular domains. Interestingly, despite having documented receptor association, Marron and colleagues did not observe significant phosphorylation of Tie1 (Marron et al., 2000). However, during the course of Tie2 activation, others have observed Tie1 phosphorylation (Saharinen et al., 2005; Yuan et al., 2007). It appears in some circumstances Tie1 phosphorylation correlates with Tie2
activation, although it remains unknown if this is as a result of stable or of transient interactions caused by high local concentrations of Tie2, i.e. within signaling clusters (Milner et al., 2008; Saharinen et al., 2005). Furthermore, the physiological significance of Tie1 phosphorylation remains unknown.

2.5 Methods

2.5.1 Cloning and Gene Expression

The sequences encoding the human Tie1 (IMAGE 5767075) and Tie2 (IMAGE 5228999) genes were cloned as interchangeable receptor-fluorophore fusion cassettes in both pcDNA3.1(+) hygromycin and neomycin resistance vectors for expression in human embryonic kidney 293 cells and human osteosarcoma cell line U2OS. Briefly, Nhel and EcoRI sites were appended to the amino and carboxy-terminus via PCR using the following oligonucleotides: Tie1-Nhe; gctagcATGgtctggcgggtgcc, Tie2-Nhe; gctagcATGgactctttagccagcttag, Tie1-EcoR1; gaattcggctgctcgtgccagcagc and Tie2-EcoRI; gaattctctctttgcacatttgccctc (restriction sites are underlined and initiating methionine is in capital letters). After insertion of receptor DNA into the pcDNA vectors, GFP cassettes were cloned down-stream and in-frame with EcoRI and XhoI yielding an open reading frame consisting of Tie receptor fused to either C- or YFP. Monomeric CFP and YFP variants (containing dimeric suppression mutations-obtained from Dr Timothy Springer) were utilized as templates for fluorophore incorporation via PCR using the following oligonucleotides; C/YFP-EcoRI; gaattcatgtgagcaagggcgaggag, C/YFP-XhoI; ctcgagtatagatcgggtagtcc. Although several initial constructs were constructed and tested for optimal linker length between receptor and fluorophore, ultimately, one
receptor pair was chosen for further study in which the Tie receptors were fused to either CFP or YFP after residues 796 and 780 of Tie1 and Tie2 respectively, leaving the transmembrane domain intact as well as ten or eleven additional cytoplasmic residues, but eliminating the carboxyl-terminal tyrosine kinase catalytic domain. Altering fluorophores on individual receptors did not appear to make any significant difference and were, therefore, used interchangeably. Mutations within Tie1 and Tie2 coding regions were introduced by site directed mutagenesis (Quikchange Multi, Stratagene) following manufacturers recommendations. To confirm the presence of the desired mutations, both DNA strands were sequenced using standard di-deoxy sequencing chemistry (Cornell University Bioresource Center).

2.5.2 Cell Manipulations and Transfections

HEK293, U2OS, and EA.hy 926 (a gift from Dr Cora-Jean Edgell) cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100 µg/mL streptomycin. Cells were consistently transected at 80-90% confluence in 35mm glass bottom culture dishes (MatTek) using Lipofectamine 2000 (HEK293) or FuGENE HD (U2OS) or FuGENE 6 (EA.hy 926) according to manufacturer’s recommendations (Invitrogen and Roche). For co-expression experiments, equimolar concentrations of Tie1 and Tie2 vector DNA were used.

2.5.3 Cellular Imaging

The spatial proximity assay was based upon the work of Dr Roger Tsien (Zacharias et al., 2002) and Dr Timothy Springer and colleagues(Kim et al., 2003). Live
cell imaging was performed 24-48 hour post-transfection on a Leica TCS-SP2 AOBS confocal laser scanning microscope equipped with blue diode (405nm), Argon (458, 476, 488, 514nm), green HeNe (543nm), orange HeNe (594nm), and red HeNe (633nm) lasers, an HCX PI Apo 63x/1.3 n.a. glycerin-immersion objective lens, a motorized XY stage (Märzhäuser), and an environmentally controlled (temperature, humidity, and CO₂) stage incubator (PeCon). Fluorophore-receptor fusions were imaged using excitation and emission wavelengths of 458nm and 514 nm for CFP and YFP respectively and fluorescence emissions were detected with SP window settings of 465-505nm and 525-600nm (for CFP and YFP, respectively). The AOTF’s for 458nm and 514nm were set to 92% and 19% respectively to eliminate cross talk between the two SP window channels. FRET efficiencies were determined using the acceptor photobleaching methodology using Leica software (ver. 2.61). Briefly, regions of interest were chosen for analysis based on extent of fluorophore expression, localization, and uniformity. For acceptor photobleaching, YFP within the region of interest (ROI-green box within images) was consistently photobleached (with the AOTF ramped up to 100% transmission of the 514nm laser line) from 50% to 70% reduction in fluorescence intensity as monitored by Leica software. Care was taken to exclude cells for analysis that displayed significantly higher, or lower, fluorescent intensity than the “average” cell. To eliminate bias, cells were also chosen based on similar levels of CFP and YFP fusion protein. Similar to the work by Kim et al. (Kim et al., 2003), cells that displayed significant drift in the x-y focal plane were discarded for FRET analysis. Due to significant cellular drift, discrete regions, rather than whole cells were subjected to photobleaching to accelerate FRET analysis. Pre- and post-bleach images were recorded for both donor (CFP) and acceptor (YFP) and
FRET efficiency was calculated as: \( \text{FRET}_{\text{Eff}} = \frac{(D_{\text{post}} - D_{\text{pre}})}{D_{\text{post}}} \) for all \( D_{\text{post}} > D_{\text{pre}} \) where \( D_{\text{pre}} \) and \( D_{\text{post}} \) is the donor intensity before and after photobleaching respectively. JMP Software (SAS) was used for all statistical analysis.

### 2.5.4 Homology model of Tie1

The Tie1 homology structure was modeled using the program MODELLER (Marti-Renom et al., 2000) and the experimentally determined 2.5Å Tie2 ligand-binding domain crystal structure (PDB 2GY5) (Barton et al., 2006). Although initial alignments between Tie1 and Tie2 were prepared using CLUSTALX (Larkin et al., 2007), use of the sequence/secondary structure alignment implementation in MODELLER yielded better results and was therefore adopted for final calculations. Four distinct models were calculated which satisfied basic spatial and stereochemical restraints, however, the model used for interpretation and illustration purposes had the lowest discrete optimized protein energy (DOPE) assessment score and MODELLER objective function (Shen and Sali, 2006). Stereochemical analysis via PROCHECK (CCP4, 1994) revealed main chain parameters better than or within the typical range of values for experimentally determined protein structures at 2.5Å resolution.
3.1 Different Angiopoietins Differentially Modulate The Tie1-Tie2 Interaction

Despite the high level of sequence identity between Tie2 and Tie1, only Tie2 can form high-affinity complexes with all four known angiopoietins, while Tie1 does not bind any of them (Davis et al., 2003; Ramsauer and D'Amore, 2002). As described in chapter 2 with the identification of pre-existing Tie1-Tie2 complex, suggests that Tie1 plays a role in angiopoietin Tie2 signaling. Interestingly, our crystal structure of Tie2 bound to Ang2 (Barton et al., 2006) and to Ang1 show that all angiopoietins bind Tie2 in a similar conformation, excluding the possibility of differential Tie2 activation resulting from alternate Tie2/angiopoietin structural arrangements. However, our structural analysis does suggest that the different angiopoietin ligands could present distinct molecular surfaces outside of the receptor-binding interface that could influence the Tie1-Tie2 receptor complexes (Barton et al., 2005). Based upon our findings we hypothesized that the observed direct Tie2/Tie1 interactions are inhibitory and the ability, or inability, of individual angiopoietins to effectively destabilize the Tie1/Tie2 complexes at the cell surface would define their respective agonistic or antagonistic roles.

To assess the role of Tie1 in Tie2 clustering and activation following angiopoietin exposure, receptor association, localization, and activation was monitored in the presence of Angiopoietin-1 or -2. Specifically, Tie1-CFP and Tie2-YFP were expressed in U2OS and EA.hy 926 cells and stimulated with varying concentrations of Angiopoietin-1 or –2, and followed by confocal microscopy over the course of 30 or 60 minutes. As shown in
to that observed for Ang-1. Therefore, our data demonstrates a unique difference between individual angiopoietins to affect Tie1–Tie2 association and subsequent Tie2 clustering.

3.2 Tie1 is an Inhibitory Co-Receptor

To further assess the physiological role of Tie1, particularly with its capability to manipulate Tie2 activation, we followed Tie2 phosphorylation in the presence or absence of Tie1 and Angiopoietin in both HEK293, and HUVEC’s. Initially, HEK293 cells, which lack endogenous Tie receptors, were transiently transfected with full-length myc-tagged Tie2, full-length HA-tagged Tie1, Tie1-CFP lacking a tyrosine kinase domain, or a combination of the three. As seen in Figure 3.5, western blotting of whole cell lysates with anti-Tie1 and anti-Tie2 antibodies, respectively, clearly demonstrate robust Tie1 and Tie2 expression. Interestingly, ectopically expressed Tie2 displays a low level of basal activation in 293 cells as observed by the anti-phosphotyrosine 992 Tie2 antibody. However, addition of Tie1 dramatically decreased Tie2 basal activation by ~50%, relative to Tie2 alone. Interestingly, addition of the chimeric Tie1-CFP, lacking an intracellular tyrosine kinase domain, also decreases Tie2 activation to levels comparable to those observed with full-length Tie1. This finding further demonstrates that Tie ectodomain interactions are necessary and sufficient to attenuate Tie2 phosphorylation.

Using an alternative approach to examine the role of endogenous Tie1, HUVEC’s were infected with recombinant lentivirus encoding a non-specific (control) or Tie1-specific miR-shRNA. A Stable cell lines were selected with puromycin and knock down of Tie1 receptor expression was determined to be ~70% by western blotting (Figure 3.6). Sub sequentially, Tie2 receptor activation was analyzed following Ang-1 or Ang-2
Figure 3.1A, addition of Ang1 to the culture media led to a drastic reduction of Tie1-Tie2 association in U2OS cells within 10 minutes as measured by a loss in FRET between CFP and YFP. This timeframe corresponds to previously determined rates of Tie2 activation (Bogdanovic et al., 2006; Yuan et al., 2007). Furthermore, the loss in FRET signal was followed by a dramatic change in Tie2 localization after 20 minutes of incubation. Specifically, the majority of the membrane-associated Tie2 migrates and forms discrete foci, reminiscent of the Tie2 punctate staining observed by others in human lung microvascular endothelial cells (Fukuhara et al., 2008; Saharinen et al., 2008). Thirty minutes post Ang1 addition cells that contain discrete Tie2 foci are still observed. As expected from our FRET analysis, Tie1 localization remains undisturbed following Ang-1 stimulation. EA.hy 926 endothelial cells under similar conditions behave correspondingly. In these cells Tie2 readily localizes to the membrane, yet transforms to punctate staining with similar kinetics to HEK293 cells upon addition of Ang-1 (Figure 3.2). FRET efficiency rapidly decreases (from 19% to 3%) further demonstrating disruption of Tie1-Tie2 interactions.

Alternatively, when Tie1 and Tie2 are co-expressing in U2OS or EA.hy 926 cells and treated with similar concentrations of Ang-2, no changes in the Tie1-Tie2 association and/or Tie2 localization are observed (Figure 3.1B). FRET efficiencies and receptor localization do not significantly change over a thirty-minute period as graphically illustrated in Figure 3.3. This is in sharp contrast with the ability of Ang-2 to induce changes in Tie2 localization in the absence of Tie1, as seen in Figure 3.4. Under these conditions, Tie2 localization changes to punctate staining within 10-20 minutes, identical
Figure 3.1: Ang-1, not Ang2, promotes Tie2 clustering from the Tie1-Tie2 coreceptor complex. U2OS cells were transfected with both Tie2-YFP and Tie1-CFP and monitored by confocal microscopy over a period of 30 minutes. At time=0, 500ng/ml of (A) Ang-1 or (B) Ang-2 was added to the growth media. Listed below each representative image are averaged FRET efficiency values correlating to the listed time point. Arrowheads indicate regions of clustered Tie2. Light gray bar in each image indicates 10 µm.
Ang1 (500ng/mL)

Tie1 CFP

Tie2 YFP

FRET EFF.%

0 min. 10 min. 20 min. 30 min.

23% 8% 10% 8%

Ang2 (500ng/mL)

Tie1 CFP

Tie2 YFP

FRET EFF.%

0 min. 10 min. 20 min. 30 min.

27% 27% 33% 30%
Figure 3.2: Effect of Ang1 and Ang2 on the Tie1-Tie2 co-receptor complex in Ea.hy 926 cells. EA.hy 926 cells were transfected with both Tie2-YFP and Tie1-CFP and monitored by confocal microscopy over a period of 30 minutes. At time=0, either 500ng/ml of Ang-1 (middle panels), or 500 ng/ml of Ang-2 (right panels) was added to the growth media. Listed below each representative image are averaged FRET efficiency values correlating to the listed time point. Arrows indicate regions of clustered Tie2. Light gray bar in each image indicates 10 µm.
Figure 3.3: Graphical representation of the average FRET efficiencies between Tie1 and Tie2 after the addition of Ang1 or Ang2. Green and Orange lines represent Ang1, or Ang2 addition for U2OS cells, respectively, while closed Green circles and closed Orange squares represent Ang1, or Ang2 addition for EA.hy 926 cells, respectively. Data are represented as mean +/- SEM.
Figure 3.4: Ang2 inducing Tie2 clustering in the absence of Tie1. U2OS cells were singly transfected with Tie2-YFP and monitored by confocal microscopy over a period of 30 minutes following addition of 500 ng/ml of Ang-2 to the growth media. Light gray bar in each image indicates 10 µm.
Figure 3.5: Tie1 extracellular is sufficient for attenuating Tie2 phosphorylation.

HEK293 cells were transfected with full-length Tie2-myc alone or in combination with full-length Tie1-HA, or Tie1-CFP, harvested and lysates probed with anti-Tie1, anti-Tie2, and anti-phosphotyrosine 992 Tie2. The relative ratio of phosphorylated Tie2 (top panel) to total Tie2 (middle panel) was determined and the calculated value was arbitrarily set equal to 1.0 for Tie2 expressed alone. Values are graphically displayed below each respective lane, representative of 3 independent experiments. *Samples are from non-adjacent lanes of the same blot.
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**Blot**

- α-p992Tie2
- α-Tie2
- α-Tie1

**Graph**

Height of bars represent the ratio of pTie2 to total Tie2 (pTie2/tTie2).
Figure 3.6: Tie1 shRNA knockdown sensitizes Tie2 to Ang1 and Ang2. Human umbilical vein endothelial cells were infected with an shRNA control or Tie1 knockdown lentivirus and selected with puromycin for stable integration. Asynchronous cultures were subjected to four hours of serum withdrawal, followed by stimulation with vehicle (buffer), 500ng/ml Angiopoietin-1, or 500ng/ml Angiopoietin-2. Fifteen minutes following addition of ligand, cells were harvested and subjected to western blotting with anti-Tie2 (middle panel), anti-Tie1 (bottom panel), and the phosphotyrosine specific anti-Tie2 (992) (top panel), as indicated. The relative ratio of phosphorylated Tie2 (top panel) over total Tie2 (middle panel) was determined and the calculated value was arbitrarily set equal to 1.0 for unstimulated Tie2 from shControl cells. Values are graphically displayed below each respective lane, representative of 3 independent experiments.
addition by means of western blot analysis. In the shRNA control cell line, which displays wild-type levels of Tie1, Tie2 activation can be readily observed within 15 minutes following the addition of Ang-1 (Figure 3.6). In contrast, and in agreement with other reports, addition of Ang-2 to the control cell lines fails to significantly alter receptor phosphorylation and activation (Davis et al., 2003; Maisonpierre et al., 1997).

Alternatively, HUVEC Tie1-shRNA stable cell lines, which expresses significantly less Tie1 as seen in control cells, behave considerably different. In the absence Tie1, and in agreement with our findings in epithelial cells, Tie2 displays approximately 2 fold greater basal activation than within control cells (Figure 3.6). Furthermore, despite the observation that Tie2 appears partially active in Tie1 silenced cells, addition of Ang-1 also leads to ~3.5 fold greater receptor activation. However, in the absence of Tie1, Ang-2 is now also capable of stimulating Tie2 activation. Indeed, despite the observation that Tie2 appears partially active in Tie1 silenced cells, addition of Ang-1 or Ang-2 leads to greater receptor activation (~3.5 and 2 fold, respectively). Cumulatively, these observations are consistent with an inhibitory role for endogenous Tie1 and indicate its presence is necessary to distinguish the agonist/antagonist role for Ang-2.

3.3 The Tie2 Kinase Domain is Unnecessary for Receptor Clustering and Tie1 Interaction

Despite a clear demonstration of Tie receptor ectodomain interactions by FRET analysis, it remained unclear if the intracellular kinase domain could influence receptor localization and clustering in response to ligand. Therefore, to evaluate the role of the
Figure 3.7: Tie1 is able to block Tie2 clustering by Ang2, but not Ang1. (A) Control (top panels) or Tie1 knockdown (bottom panels) stable EA.hy 926 cells were transiently transfected with full-length Tie2 fused to mCherry and imaged via confocal microscopy forty-eight hours post transfection. Cells were stimulated with 500ng/mL of Angiopoietin-1 or -2 and mCherry expression was followed over the course of sixty minutes. GFP expression (green) indicates stable viral integration and was overlaid onto mCherry (red) for orientation purposes. Arrows indicate areas of punctate Tie2 staining. Light gray bar in each image indicates 10 µm. (B) Crude cell lysates were analyzed by western blot for total Tie1 and Tie2 protein in control and Tie1 knockdown cell lines.
Tie2 tyrosine kinase domain in Tie interactions, a full-length version of Tie2 with a carboxyl terminal mCherry domain was transfected into control and Tie1 silenced EA.hy 926 cells. Similar to the HUVEC Tie1 knockdown experiments, the recombinant lentivirus was also capable of attenuating Tie1 expression (Figure 3.7B). Under these conditions, endogenous Tie1 will interact with, and influence Tie2-mCherry localization, if indeed the tyrosine kinase domains are essential. Accordingly, Tie2-mCherry localization was monitored by confocal microscopy following both Angiopoietin-1 and -2 addition. Figure 3.7A includes images of cells prior to, 30, and 60 minutes post ligand addition. As observed with Tie2-YFP chimeras lacking the tyrosine kinase domain (Figure 3.1A and 3.2), full-length Tie2-mCherry exhibits punctate staining within 30 minutes in the presence of Ang-1 in both control and Tie1 silenced cells. Similarly, Tie2 clustering is observed within 60 minutes addition of Ang-2 in the absence of Tie1. A slight difference in time between the observed clustering in Ang-1 and -2 induced cells is likely due to the residual Tie1 (~25% of wild-type levels) that remains in Tie1 knockdown cells. Although these studies do not rule out other potential roles for the Tie tyrosine kinase domains, these findings demonstrate that kinase domain interactions do not significantly influence the clustering and localization of Tie2.

3.4 Conclusions

Until now, the molecular basis for the Tie1 and the differential angiopoietin functions has remained elusive. We have now identified a clear role for Tie1 in Tie2 signaling, and demonstrate a direct, inhibitory interaction between these receptors. We reveal that this interaction is dynamic and differentially modulated by the different
angiopoietin ligands thereby providing a molecular mechanism for the observed differences in angiopoietin function.

Based upon previous studies and the data presented here, we propose a model for Tie2 signaling as illustrated in Figure 3.8. Specifically, in cells expressing both Tie1 and Tie2, the receptors form heterodimers within endothelial cells via ectodomain electrostatic interactions that inhibit Tie2 activation and clustering. Binding of Angiopoietin-1 to Tie2 promotes heterodimer dissociation, Tie2 clustering, and signaling initiation. On the contrary, Angiopoietin-2, is unable to dissociate the inhibitory Tie2/Tie1 complexes upon binding Tie2 and, therefore, does not induce Tie2 clustering and signaling, yet behaves as a competitive antagonist by blocking further binding of Ang-1. Alternatively, for cells that do not express Tie1, all angiopoietins promote Tie2 clustering and activation. Our model explicitly proposes that the balance of Tie1 and Tie2 expression modulates the functional potential of Angiopoietin-2, and by analogy, vascular homeostasis.

While it is known that Tie2 is expressed ubiquitously throughout the vascular endothelium (Dumont et al., 1994; Dumont et al., 1992), Tie1 expression is significantly restricted to vascular bifurcations and branching points following embryogenesis (Porat et al., 2004). Furthermore, Tie1 is transcriptionally up-regulated by hypoxia, VEGF stimulation, and in areas of wound healing and tumor growth and development (i.e. sites of neovascularization) (Korhonen et al., 1992; McCarthy et al., 1998). Therefore, the adult vasculature is composed of regions of alternating (high and low, or absent) Tie1 expression. Regions that are actively involved in angiogenesis, and require the antagonistic function of Ang-2, are, interestingly, the very same regions where Tie1
expression is observed. Under these conditions, the majority of Tie1 and Tie2 form signaling-incompetent heterotypic complexes on the cell surface (Figure 3.8). Their association is dynamic and mediated via an electrostatic interaction between charged residues within their ectodomains, as well as the presence or absence of activating ligand (i.e. Ang-1). Cells expressing both receptors are responsive to the survival, migration, and chemotactic cues caused by activation and inactivation of Tie2 via Ang-1 and Ang-2 and are, therefore, able to promote the required vessel branching and sprouting necessary for angiogenesis.

Alternatively, Tie1 is absent in vascular regions that are stable and quiescent, such as mature vessels. The absence of Tie1 negates the functional differences between Ang-1 and Ang-2 and allows endothelial cells to respond to either ligand in a similar fashion (both as agonist), and foster the same phenotypic response—quiescence and survival (Figure 3.8). In this regard, Tie1 serves as a selectivity factor, designating when and how Ang-2 functions. In general, this could provide greater cellular adhesion with the underlying vessel support cells, and provide yet another mechanism to stabilize the adult vasculature and prevent aberrant vessel sprouting and branching that could lead to pathogenesis.

Consistent with our proposed model, in the absence of Tie1 (Figure 3.5), Tie2 exhibits basal phosphorylation and remains partially activated despite the absence of ligand, yet it can become further clustered and activated by both the agonist Ang-1 as well as antagonist, Ang-2 (Davis et al., 2003; Maisonpierre et al., 1997). Alternatively, in the presence of Tie1, the ligand-independence and basal Tie2 activity are attenuated (Figure 3.5). In agreement, loss of Tie1 (via shRNA), eliminates the functional
differences between Ang-1 and Ang-2 in HUVEC (Figure 3.6). Under these conditions, endothelial cells respond to both ligands similarly. In agreement, Yuan et al. observed that siRNA toward Tie1 significantly increased the downstream activation of Tie2 signaling components as well as its basal phosphorylation of Y992, therefore concluding that Tie1, in its normal role, antagonizes Tie2 function (Yuan et al., 2007). An analogous conclusion was drawn by Patan following histological assessment of Tie1 and Tie2 knockout mice phenotypes (Patan, 1998). In addition, Kim et al. demonstrate Ang2-induced Tie2 activation following down-regulation of Tie1 via siRNA in HUVECs (Kim et al., 2006). Similarly, Nguyen et al. observed differential responses of lymphatic versus venous or arterial endothelial cells to Angiopoietin-1 and -2 (Nguyen et al., 2007). In line with this observation, our model would predict that lymphatic endothelial cells, thought to be the primary target of the Ang-2 agonist (as shown by knockout and over-expression experiments), would display lower levels of Tie1 protein, although this has yet to be investigated.

Some have observed Tie2 to preferentially localize at sites of cell-cell contacts (Fukuhara et al., 2008; Saharinen et al., 2008). Indeed, our expression constructs with and without the intracellular tyrosine kinase domain demonstrate related localization patterns, and we, therefore, conclude that clustering to cell-cell junctions is likely mediated by ectodomain interactions and not through the Tie2 kinase domain. While Tie2 clustering to cell junctions presents an appealing means to localize specific signaling events, the exact mechanism by which this occurs remains unclear.

Finally, it should be noted that our studies also contrast with those by Saharinen et al. in which Tie1 and Tie2 association is proposed to occur in the presence of Ang1 as
Figure 3.8: Model for Angiopoietin-mediated Tie2 signaling. Expression of Tie2 in the absence of Tie1 at sites of vessel quiescence and maturity. In the absence of Tie1, Tie2 can be activated with either Ang-1 or Ang-2. Both ligands stimulate receptor clustering, tyrosine kinase activity, and down-stream signaling events, effectively become unresponsive to vessel sprouting and branching cues. Within sites of active angiogenesis, Tie1 and Tie2 associate to form a complex prior to ligand stimulation. Upon addition of Ang-2, Tie1 and Tie2 association and localization remains unchanged. Under these conditions, Ang-2 fails to activate the Tie2 receptor and opposes the activation of down-stream signaling generated by Ang-1. However, upon addition of Ang-1, the opposite is observed. Ang-1 stimulates Tie2 clustering, tyrosine kinase activity, and down-stream signaling events similar to that observed in the absence of Tie1. *Angiopoietins are depicted as dimers for illustration purposes although they are known to exist as higher-order clusters.
Stable Vessels  Stable Vessels  Regression and/or Sprouting
suggested by co-localization experiments (Saharinen et al., 2008). Previous reports by the same group indicate stimulation of Tie1-Tie2 co-immunoprecipitation following Ang-1 exposure (Saharinen et al., 2005). Although we observe dramatic changes in Tie2 localization, clustering, and association with Tie1 upon binding of Ang-1, we do not see a concomitant change in Tie1 localization. Furthermore, the extent of Tie2 clustering we observe, is significantly greater, and FRET analysis reveals nearly immediate dissociation of the Tie1-Tie2 complexes upon receptor stimulation. Since our analysis of receptor complex formation is at a significantly higher resolution (<10nm versus >1µm) and more direct, we feel the lack of change in Tie1 localization and loss of FRET documents Ang-1 induced Tie2-Tie1 complex dissociation rather than association. Furthermore, our data and proposed function of Tie1 correlate with the observed phenotypes of Tie and Ang knock-out mice, Tie receptor expression patterns; confirming the inhibitory effects of Tie1 on Tie2 activation (Kim et al., 2006; Patan, 1998; Yuan et al., 2007). Collectively, our results indicate that the balance of expression and dynamic interaction between Tie1 and Tie2 provides an effective means of controlling receptor activation, and by analogy vascular homeostasis, using a single set of structurally similar ligands. The exquisite level of molecular control clearly highlights the importance of restricting Tie2 activation in order to maintain vascular homeostasis and to prevent pathogenesis; therefore, the Tie1-Tie2 interface may serve as an attractive therapeutic target and may be more relevant than the currently scrutinized Ang2-Tie2 interaction.

3.5 Materials and Methods

3.5.1 Cloning and Gene Expression
Full-length Tie2-mCherry was constructed by fusing the fluorophore to the carboxy terminus of Tie2 via PCR stitching using the partially complementary oligonucleotides mCherry/TKD; gttctgctgaagaagcggccggtgcatctgtgcatctatggccatcatcaaggag, TKD/mCherry; ctccttgatgatggccatagaaccagatgcaccggccgcttcttcagcagaac, and mCherry Xho; gctcactcgacctgcttccttcagcagaac, and mCherry Xho; gctcactcgacctgcttccttcagcagaac. The final construct was cloned as an Nhel-XhoI fragment into pcDNA3.1(+) hygromycin.

3.5.2 Cell Manipulations and Transfections

Human umbilical vein endothelial cells (HUVEC) were grown in EBM II media according to recommendations (Clonetics). Cells were consistently transfected at 80-90% confluence in 35mm glass bottom culture dishes (MatTek) using Lipofectamine 2000 (HEK293) or FuGENE HD (U2OS) or FuGENE 6 (EA.hy 926) according to manufacturer’s recommendations (Invitrogen and Roche). For co-expression experiments, equimolar concentrations of Tie1 and Tie2 vector DNA were used.

3.5.3 shRNA Knockdown of Tie1

Recombinant lentivirus encoding a Tie1 or control shRNA were constructed according to manufacturers recommendations (Open Biosystems). Stable EA.hy 926 or early passage HUVEC’s were selected in the presence of 0.8 ug/ml puromycin 48 hours post viral infection. After seven days, stable cells were monitored for Tie1 and Tie2 expression via western blot analysis.

3.5.4 Tie2 Activation Assays
For analysis of Tie2 activation in HEK293, cells were transfected with combinations of full-length Tie1-HA, and/or Tie2-myc tagged vectors. Forty-eight hours post-transfection, cells were lysed in HBST (20mM Heps pH 7.4, 150mM NaCl, 0.1% Triton X-100) in the presence of 0.5mM sodium orthovanadate and subjected to western blotting. For Tie2 activation in HUVEC and EA.hy 926, post-confluent cells were serum starved for 2-4 hours prior to the addition of 500ng/ml Angiopoietin-1, -2, or vehicle (PBS). Fifteen minutes prior to ligand addition, sodium orthovanadate (Sigma) was added to 1mM in the culture medium (Teichert-Kuliszewska et al., 2001). Fifteen minutes following ligand addition, cells were harvested. Endogenous Tie1 and -2 were analyzed with anti-Tie2 (Santa Cruz Biotechnology), anti-phosphotyrosine-specific 992 Tie2 (R&D Systems), or anti-Tie1 (R&D Systems and Santa Cruz Biotechnology). Quantitative values for band intensities were obtained from western blots using the program ImageJ. Briefly, the integrated pixel intensity was determined for each band of interest using an identically sized rectangular masking box. The background was similarly determined from an identical region of the blot from lanes lacking the protein of interest - except in cases where no such lane control could be used. Under these conditions, background was calculated from a blank region above each band of interest. Finally, the background was subtracted uniformly from the experimental values to obtain the final raw values. All statistical calculations were determined using JMP software version 7.0 (SAS).

3.5.5 Cellular Imaging
Live cell imaging was performed 24-48 hour post-transfection on a Leica TCS-SP2 AOBS confocal laser scanning microscope equipped with blue diode (405nm), Argon (458, 476, 488, 514nm), green HeNe (543nm), orange HeNe (594nm), and red HeNe (633nm) lasers, an HCX PI Apo 63x/1.3 n.a. glycerin-immersion objective lens, a motorized XY stage (Märzhäuser), and an environmentally controlled (temperature, humidity, and CO₂) stage incubator (PeCon). mCherry-receptor fusions and GFP were imaged with the following excitation wavelengths; 594nm and 488nm, respectively, and fluorescence emissions were detected with SP window settings of 605-700nm and 500-560nm (for mCherry and GFP, respectively).
Chapter 4
Function difference between Angiopoetin-1 and -2

4.1 Structural comparison of Tie2 bound to Ang-1 or Ang-2

We have previously identified the differential ability of the angiopoietins to effectively exclude Tie1 from Tie2, affecting the phosphorylation state of Tie2. Specifically, Ang1 is capable of excluding Tie1 from Tie2, causing Tie2 clustering on the cell surface. Alternatively, Ang2 was incapable of excluding Tie1, causing no observed change in Tie2 phosphorylation and localization. This led to the hypothesis that Ang-1 and Ang-2 differentially bound to Tie2, affecting the inclusion of Tie1. This however seems unlikely with the observation that the binding of Ang1 to Tie2 does not induce conformational changes within the extra-cellular domain (personal correspondence Dr Dimitar Nikolov). As expected, Ang-1 bound to the same region of Tie2 with the same affinity as observed with the Ang2-Tie2 structure (Barton et al., 2006; Davis et al, 2005). The specificity of Ang2 for Tie2 involves 13 residues (S417, K432, I434l, D448, A449, P452, N467, K468, F469, K473, Y475, Y476, S480) in the P-domain close to the calcium-binding site (Barton et al., 2006). Mutagenesis studies revealed K468, F469, K473, Y475, and Y476 attenuated the binding of Ang-2 to Tie2 (Barton et al., 2005). Of these residues, 3 are conserved (Ang2 K468-Ang1 K468, Ang2 K473-Ang1 K475, Ang2 Y476-Ang1 Y478) with an additional 1 being homologous (Ang2 F469-Ang1 Leu471). Tyr475 in Ang-2 makes van der Waals contact with P166 in Tie2. The corresponding position in Ang1, H477 could make similar contacts. As such, this minor change is not likely the differential function between the angiopoietins with Tie2 mutant F161A/S164E.
unable to bind to either Ang-1 or Ang-2 (Barton et al., 2006). These observations lead us to hypothesize the ligands differential functions involved their ability to exclude Tie1 from Tie2 and not their ability to structurally modify the Tie2 receptor.

### 4.2 Identification of Angiopoietin residues involved in altering Tie1-Tie2 formation

Analysis of the crystal structures of Ang1 or -2 bound to the same region of Tie2, utilizing similar contact points lead us to examine amino acids outside of those used to recognize Tie2. The angiopoietin family members, 1-4, share high sequence homology within the receptor-binding domain allowing for identification of amino acids that can alter the Tie1-Tie2 interaction to be identified. Figure 4.1 is a surface rendering homology map of the receptor-binding domain of Ang-2 (Barton et al., 2005). Regions colored in red are considered to be 100% conserved across all four angiopoietins whereas regions colored in blue, green, and grey share 75%, 50%, 0% conservation, respectively. The yellow circle represents the surface area buried by the Ang-Tie2 interaction. As indicated by the structural analysis of Ang-1 or -2 bound to Tie2, there is a great deal of sequence conservation among these residues involved in mediating this interaction. By observing Ang-1 or -2 bound to Tie2 with similar folds, we only took surface residues into consideration and hypothesized these residues will not cause large conformational changes but support the inclusion of Tie1 by either ionic or van der Waals interactions. Interestingly, a region of low conservation and a change in ionic residues was identified in the P-domain outside of the receptor ligand interface, as highlighted in Figure 4.1 by the arrow. Furthermore, this region appears *cis* to the co-receptor interface identified in our earlier studies, Chapter 2 Figure 2.5B. Sequence alignment of the
Figure 4.1: Homology model of Angiopoietin Receptor Binding Domain. Surface residues on the angiopoietin receptor-binding domain were color-coded based upon their sequence conservation across Ang1-4, with 100%, 75%, 50%, 0% depicted as red, blue, green, and grey, respectively. The yellow ring indicates the amino acids responsible for Tie2 receptor binding. The arrow indicates the none conserved PQR ionic loop.
angiopoietins identified the residues as P460/Q461/R462 in Ang-2 and T462/A463/G464 in Ang-1. The presence of basic residues in Ang-2 would suggest, binding of Ang-2 to Tie2 would allow these residues to interact with Tie1, stabilizing the hetero-Tie receptor complex.

4.3 Phenotypic swapping of Ang2

To test the plausibility of Ang-2 in stabilizing the Tie1-Tie2 complex, we utilized site-directed mutagenesis in combination with the proximity assay utilized in Chapter 2 (Figure 2.1). It has been our experience in modulating protein-protein interactions; multiple simultaneous mutations should be utilized (Barton et al., 2003). As such, multiple mutations were made in an Ang2-Fc fusion tagged protein to resemble Ang-1 in its corresponding positions, swapping the P460/Q461/R462 to T460/A461/G462. The angiopoietin ligand requires clustering for its utility in endothelium cell based assays to elicit Tie2 phosphorylation (Davis et al., 2005; Kim et al., 2005). Although many attempts have been made in the past, we are unable to produce full-length angiopoietin using standard protein production methods. To avert this issue we adopted methodology from the Yancopoulos group, by producing the angiopoietin receptor-binding domain as an Fc fusion protein, capable of clustering by means of exogenously added anti-Fc antibody (Davis et al., 2005). As measured by the phosphorylation of tyrosine 992 in the activation loop in Tie2, our Ang1-Fc fusion protein was capable of activating Tie2 when clustered by the addition of an anti-Fc antibody whereas the clustered Ang2-Fc acted as an antagonist (Figure 4.2, lanes 7-10). The phosphorylation levels were in agreement when HUVECs were challenged with native Ang1 ligand (Compare lanes 3 and 8). In
Figure 4.2: Phosphorylation of Tie2 by the Fc angiopoietin ligands. Asynchronous HUVECs were subjected to four hours of serum withdrawal, followed by the listed ligand. After 30 minutes, cells were harvested and subjected to western blotting with anti-Tie2 (middle panel) and the phosphotyrosine specific anti-Tie2 (992) (top panel), as indicated. The relative ratio of phosphorylated Tie2 (top panel) over total Tie2 (middle panel) was determined and the calculated value was arbitrarily set equal to 1.0 for unstimulated Tie2. Values are graphically displayed below each respective lane.
agreement with other reports on the ligand-clustering requirement for angiopoietin induced Tie2 signaling, in the absence of the clustering antibody, neither Ang1-Fc nor Ang2-Fc was capable of activating Tie2 (Compare lanes 5-6 and 7-10) (Davis et al., 2005; Kim et al., 2005). As such, these observations validate the usage of the angiopoietin Fc fusion proteins in monitoring their effect on Tie2 and Tie1. Furthermore, we now have a platform in which mutant angiopoietins can be created allowing us to test these mutant variants on their effects on Tie2.

4.4 Angiopoietin modulation of Tie1-Tie2 measured by FRET

To further evaluate the angiopoietin-Fc fusion proteins ability to modulate the Tie1-Tie2 complex, we utilized our FRET based proximity assay from Chapter 2. In brief, transient transfections of epithelial cells were performed using our chimeric Tie1-CFP and Tie2-YFP receptors. Epithelial cells were utilized for their ease in transfection and the lack of endogenous Tie receptors. We have previously shown the Tie1-Tie2 interaction is not differentially modified in response to cell type (Figure 2.2). As expected, proper membrane localization of these receptors was observed (Figures 4.3 and 4.4). As previously observed, in the absence of ligand Tie1 readily interacts with Tie2 and the average initial FRET efficiency was determined by the acceptor photo bleaching method to be 24.8% (Figure 4.5). Observing the antibody clustered Ang1-Fc and not the clustered Ang2-Fc was capable of inducing Tie2 phosphorylation, we hypothesized only the clustered Ang1-Fc was capable of causing a loss in FRET efficiency between Tie1 and Tie2. As such, no statistical change in FRET was observed after 30 minutes when cells expressing both Tie1 and Tie2 were challenged with either the clustering antibodies
Figure 4.3: The clustering antibodies and clustered Ang2-Fc do not affect the Tie1-Tie2 receptor complex. U2OS cells were transiently transfected with the chimeric Tie1-CFP and Tie2-YFP receptors. At (A) time=0 either (B) clustering antibodies or (C) clustered Ang2-Fc was added to the growth media and imaged after 30 minutes. FRET was determined by acceptor photobleaching by bleaching YFP within a region of interest, green box, localized to the cellular membrane.
A.

Pre-Photobleach | Post-Photobleach

Tie1 CFP

Tie2 YFP

B.

Pre-Photobleach | Post-Photobleach

Tie1 CFP

Tie2 YFP

C.

Pre-Photobleach | Post-Photobleach

Tie1 CFP

Tie2 YFP
or the clustered Ang2-Fc. Average FRET efficiencies were determined for the antibody control and clustered Ang2-Fc to be 24.2% and 15.2%, respectively (Figure 4.5). Furthermore, Tie2 localization remains unchanged under these conditions; representative images can be seen in Figure 4.3B-C. The inability to induce Tie2 phosphorylation and stabilize the Tie1-Tie2 co-receptor complex allows us to conclude the clustered Ang2-Fc fusion protein behaves similar to the native Ang2 ligand.

In contrast to the sustained FRET efficiency measured between Tie1 and Tie2 with the clustered Ang2-Fc, a dramatic loss in FRET between Tie1 and Tie2 is observed when treating cells with the clustered Ang1-Fc. After 30 minutes of treatment with the clustered Ang1-Fc fusion protein, a dramatic change in Tie2 localization was observed, changing from confluent membrane localization to more punctate, as indicated by the arrows in Figure 4.4A. The change in Tie2 localization was similar to that observed localization of Tie2 in epithelial and endothelial cells challenged with native Ang-1 (Figure 3.1 and 3.2, respectively). Similar to our prior studies, Tie1 localization remained unchanged with continued confluent localization to the cellular membrane. Consequently, with the change in Tie2 localization a loss in FRET efficiency between Tie1 and Tie2 was observed after 30 minutes. The average FRET efficiency measured after 30 minutes was equal to 0.49%. These observations led us to conclude the Ang1-Fc fusion protein was capable of inducing Tie2 phosphorylation in a similar manner as the native Ang-1 ligand. Both ligands appear to induce Tie2 activation by clustering the Tie2 receptor and further excluding Tie1.

We next sought to test the effect our clustered mutant Ang2-TAG-Fc protein would have on modulating the Tie1-Tie2 co-receptor complex. Treatment of epithelial
Figure 4.4: The clustered Ang1-Fc and clustered mutant TAG Ang2-Fc affect the Tie1-Tie2 receptor complex. U2OS cells were transiently transfected with the chimeric Tie1-CFP and Tie2-YFP receptors. At time=0 either (A) clustered Ang1-Fc or (B) clustered mutant TAG Ang2-Fc was added to the growth media and imaged after 30 minutes. FRET was determined by acceptor photobleaching by bleaching YFP within a region of interest, green box, localized to the cellular membrane.
Figure 4.5: Graphical representation of average FRET efficiencies after 30 minutes of stimulation with AngFc variants. The data is represented as mean +/- SEM with an n=3.
cells expressing Tie1 and Tie2 with the clustered mutant Ang2-TAG-Fc fusion protein resulted in a similar Tie receptor localization when treated with the clustered Ang1-Fc fusion protein. As seen in Figure 4.4B, Tie2 localization is altered on the cell surface and results in a corresponding loss in FRET efficiency between Tie1 and Tie2. It should be noted the extent of Tie2 localization was not as dramatic as compared to cells treated with Ang-1. More importantly, the average FRET efficiency after 30 minutes was observed to equal 3.6% (Figure 4.5). This loss in FRET upon treatment is statistically different from the Ang2-Fc and antibody control values and similar to values obtained upon Ang1 treatment. These observations led us to conclude the mutant Ang-TAG-2 had switched its phenotype, causing it to act as an agonist to Tie2.

4.5 Structural Determination of the mutant Ang-TAG-2

The observed phenotypic change in the mutant Ang-TAG-2 led us to evaluate the effect TAG point mutations had on the globular structure. The mutant Ang-TAG-2 structure solved at 1.9Å by molecular replacement and refined with an R factor 20.0% (R_free=22.5%). The structure folds into the expected fibrinogen domain observed for Ang-1 and -2 (Figure 4.6A). Superimposition of the mutant Ang-TAG-2 structure onto the wild type Ang-2 reveals two almost identical structures with an r.m.s. deviation of 0.58 Å of equivalent Cα positions, well into the range of experimental error (Figure 4.6B). The most prominent difference between corresponding Cα positions was observed for the arginine (R462) to glycine (G462) mutation. The r.m.s. deviation of this position was 1.76 Å moving the loop outward from the Tie2 receptor binding interface (Figure 4.6C). The similarity between these two structures reveals, the PQR/TAG mutants does
**Figure 4.6: Structural determination of the mutant TAG Ang2.** (A) Ribbon diagram of the mutant TAG Ang2 fibrinogen domain. The TAG loop is highlighted in green with the Ca$^{2+}$ in the P-domain depicted as a black sphere. (B) Cα traces of mutant TAG Ang2 (Blue) overlaid the wild type Ang2 (orange) fibrinogen domain. (C) A magnified view of the TAG loop.
not induce any large conformation changes to account for the difference in Tie2 activation. This was also expected as we have previously observed the Ang-1 and Ang-2 structures to adopt similar folds. The high similarity between the mutant Ang-2 structure with the wild type Ang-2 structure depicts the importance of the ionic interaction of the QR in Ang-2 with keeping Tie1 bound to Tie2.

4.6 Conclusions

We have previously demonstrated that the functional differences among the angiopoietin ligands are attributed to their ability to modulate the Tie1-Tie2 receptor complex. However, our previous research failed to highlight the molecular mechanisms within Ang-1 and -2 that could manipulate the Tie1-Tie2 interaction. The Angiopoietins share significant sequence homology, and we have previously demonstrated with structural and mutagenesis data that differential binding to Tie2 appeared unlikely to mediate this mechanism (Barton et al, 2005; Barton et al., 2006). Therefore, in an attempt to further understand Angiopoietin differences at the atomic level, we structurally characterized Ang-1 and the Ang-1/Tie2 complex in addition to our previously determined Ang-2 and Ang-2/Tie2 complex structures. As expected, the ligands share significant structural similarity within the fibrinogen subdomains; A, B, and P. Furthermore, Ang-1 and Ang-2 form similar complexes with Tie2 despite their functional differences (Davis et al., 2003). Residues involved in receptor-ligand interactions are fairly conserved; as such, ligand binding to Tie2 is not substantially different between Ang-1 or Ang-2. Therefore, it appears more likely that residues outside of the receptor-binding site must influence Tie1-Tie2 interactions and mediate their functional
differences. Indeed, analysis of ligand conservation and molecular modeling suggested only a few possibilities that could be utilized to determine the functional difference between Ang-1 and -2. Analysis of the Ang-2 fibrinogen domain cis to the Tie1-Tie2 binding interface revealed an ionic loop of low sequence conservation we hypothesized was capable of interacting with Tie1. The corresponding loop in Ang-1 was considerably less ionic. Cellular studies using our FRET based proximity assay successfully identified this ionic loop of Ang-2 as the major contributing force in stabilizing the Tie1-Tie2 co-receptor complex on the cell surface. Based on our knowledge of the Tie1-Tie2 interaction, which is dominated by electrostatic contacts, it was not unexpected that a charged surface residue is among those involved in ligand differentiation. Indeed, the TAG mutant replaces an arginine with the unbranched glycine residue, suggesting Ang-2 further stabilizes the Tie1-Tie2 complex via electrostatic interactions. Unfortunately, without further structural characterization of the Tie1-Tie2 receptor complex, identification of surfaces residues in Tie1 that mediate this interaction are not apparent. Another explanation could be the involvement of yet another unidentified co-receptor to Tie2 when bound by Ang-1. Tie2 has been shown to interact with the integrins α5β1 (personal communication with Dr Dimitar Nikolov). Therefore, it is possible these mutations in Ang-2 could perturb further binding to these co-receptors, affecting the inclusion of Tie1 to Tie2. Experiments are currently being conducted to addresses these possible interactions.

In conclusion, we demonstrate that only three critical residues within the Angiopoietin fibrinogen domain are necessary to differentiate a receptor agonist from antagonist, thus highlighting the significance of subtle interactions in nature. In light of
these findings, we would predict that therapeutics targeting this region within Ang-2 would be both highly specific and beneficial in blocking Tie2 induced angiogenesis.

4.7 Materials and Methods

4.7.1 Cloning and Mutagenesis

The human Ang-1 and Ang-2 receptor-binding domains (RBD) (residues Ang-1: 278-498, and Ang-2: 276-496) were cloned as an IgG fusion protein into a modified pcDNA3.1 vector (Invitrogen) for constitutive over-expression in a human embryonic kidney 293 (HEK293) cell line as previously described (Barton et al., 2005). Human recombinant Ang-1 and Ang-2 were purchased from R&D Systems. The TAG mutations within Ang-2 coding regions were introduced by site directed mutagenesis (Quickchange Multi, Stratagene) following manufacturers recommendations. The primer sequence: GGAATGTACTATACTGCAGGCCAGAACAAATAAG. To confirm the presence of the desired mutations, both DNA strands were sequenced using standard di-deoxy sequencing chemistry (Cornell University Bioresource Center).

4.7.2 Protein Expression and Crystallization

Large-scale protein expression was performed with stable HEK293 cells expressing the angiopoietin receptor binding domain Fc-fusion proteins in a BioFlo310 bioreactor (New Brunswick Scientific) or roller-bottle culture with typical yields averaging 10 mg/L. Protein was purified from conditioned media by affinity chromatography on Protein-A sepharose, cleaved off the Fc-fusion tag via thrombin proteolysis, and further purified by gel-filtration chromatography. The following nine
vector-derived residues remain at the C-terminus following thrombin cleavage: GSASGLVPR. All of the human angiopoietin receptor-binding regions were expressed and purified as described (Baton et al., 2005). In brief, angiopoietin receptor binding domains were purified from conditioned media by affinity chromatography on Protein-A sepharose. Following elution and buffer exchange from the Protein-A column, Fc-fusion tag was cleaved off via thrombin proteolysis, and the angiopoietin receptor-binding domain was further purified by a sepharose SD200 gel-filtration column. The following nine vector-derived residues remain at the C-terminus following thrombin cleavage: GSASGLVPR. For angiopoietin receptor binding domains not requiring cleavage of the Fc-fusion tag, further purification required only the utility of the sepharose SD200 gel-filtration column to yield pure material for cell based assays.

For the mutant Ang2 structural determination, purified protein was concentrated to 15mg/mL in buffer containing 20 mM HEPES pH 7.4 and 150 mM NaCl and crystallized by hanging drop vapor diffusion at room temperature (20°C). Crystals were initially observed grown out of 1.0 M K/Na Tartate, 0.1M Tris pH 7.0, and 0.2 M Li2SO4. This condition was optimized to yield larger crystals by growing in 0.9 M K/Na Tartate, 0.1M Tris pH 7.0, and 0.2 M Li2SO4. Crystals were harvested by quickly transferred to a cryo-buffer consisting of mother liquor supplemented with 20% ethylene glycol as a cryoprotectant and flash frozen in liquid nitrogen.

4.7.3 Structural determination

The structural data for Ang-2 TAG was collected on the X-ray Operations and Research beamline 23-ID-B at Advanced Photon Source, Argonne National Laboratory.
Initially, the program AMORE was used to identify the location of the fibrinogen domain using the Ang-2 RBD (PDB Id 1Z3S) as a search model. Subsequent refinement proceeded with iterative rounds of model adjustments (using the molecular graphics program O), molecular dynamics, and energy minimization in CNS (Brunger et al., 1998; Jones et al., 1991). In an attempt to keep the model unbiased within the loop containing the TAG mutations, residues 467 - 476 were omitted in the initial search model. The model was subsequently subjected to additional rounds of refinement within the CNS suite prior to manually building the missing region using the graphical program O. Stereochemical analysis of the refined models using PROCHECK (CCP4 Suite) revealed main chain and side chain parameters better than or within the typical range of values for protein structures.

4.7.4 Cell Imaging

For FRET cell stimulations with the angiopoietin Fc-fusion proteins, 2ug/mL of the angiopoietin Fc-fusion protein were allowed to incubate for 30 minutes at room temperature (20°C) in 1mL of DMEM supplemented with a 2-fold molar excess of rabbit anti-human Fc-fragment specific (Jackson Immuno-Research) and goat anti-rabbit IgG (Jackson Immuno-Research) antibodies. The condition media was then placed over the cells and FRET images were obtained after 20 minutes of stimulation. FRET was determined between the Tie2-YFP and Tie1-CFP chimeric receptors as described in Chapter 2. All statistical calculations were determined using JMP software version 7.0 (SAS).
4.7.5 Tie2 activation Assays

For Tie2 phosphorylation analysis, post-confluent HUVECs were serum starved for 2-4 hours prior to the addition of 500ng/ml Angiopoietin-1, -2, or vehicle (PBS). Fifteen minutes prior to ligand addition, sodium orthovanadate (Sigma) was added to 1mM in the culture medium (Teichert-Kuliszewska et al., 2001). Fifteen minutes following ligand addition, cells were harvested. For HUVECs stimulated with the angiopoietin Fc-fusion proteins, 2ug/mL were added to the cells and allowed to equilibrate for 5 minutes. The clustering antibodies, rabbit anti-human Fc fragment specific and goat anti-rabbit IgG, were sub sequentially added to the media in a 2-fold molar excess of the angiopoietin Fc-fusion proteins. The cells were allowed to incubate at 37°C for an additional 15 minutes then harvested by in-dish lyses with 10% NP-40, HBS, 1mM sodium orthovanadate, and Completen (Fisher Scientific) protease cocktail inhibitor. Phosphorylation of Tie2 was performed as described in Chapter 3.
Chapter 5
Conclusions

Prior to this study, understanding the molecular role Tie1 had in angiopoietin-Tie2 signaling remained at the forefront of vasculature research. As such, the aim of this research was to identify a molecular role for Tie1 in angiopoietin signaling of Tie2. The Tie receptors share high sequence homology and have both been shown through genetic mouse studies to be essential for developmental angiogenesis. Both receptors appear to have conflicting roles in angiogenesis with the activation of Tie2 leading to vessel quiescence and the up regulation of Tie1 observed at sites of angiogenesis. Initial structural analysis of Tie1 based on Tie2 revealed the presence of basically charged surface area capable of mediating an interaction to an acidicly charged region of Tie2. To evaluate this hypothesis, we utilized a powerful biophysical proximity FRET assay to evaluate the potential interaction between the extracellular portions of Tie1 and Tie2. We found in the absence of ligand, these two receptors readily localized and formed a hetero-complex on the cells surface. Mutation analysis within the binding interface revealed the importance of this interface in mediating a direct interaction between the Tie1 and Tie2 receptors. We further evaluated the ability of the hetero-receptor complex to persist upon stimulating cells with the angiopoietin ligands, Ang-1 and Ang-2. Angiopoietin-1 acted as a constitutive agonist to Tie2, eliciting receptor clustering and phosphorylation independent of Tie1 expression. Conversely, angiopoitin-2 only acted as an agonist to Tie2 in the absence of Tie1. Structural analysis of either Ang-1 or -2 bound to Tie2 revealed similar binding mechanisms. As such we identified a loop in the P-domain of
the angiopoietins' receptor recognition domain that could account for the inclusion or exclusion on Tie1 from Tie2. From this research we were able to develop a unified model in which Tie1 attenuates Tie2 activation. In the absence of Tie1 the angiopoietin ligands are indistinguishable to the endothelium acting as an agonist to Tie2, leading to vessel stability. Although, in the presence of Tie1, Ang-1 continues to act as an agonist to Tie2 but Ang-2 now acts as an antagonist causing the vessel to increase its permeability. The attenuation of Tie2 signaling causes the vessel to become preamble to chemotatic signaling leading to either vessel regression or sprouting. Through this study we have identified a molecular mechanism for Tie1 in angiopoietin Tie2 signaling and identified novel targets for drug design to combat tumor growth.
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Vita

Tom Conrad Maugans Seegar was born on April 7, 1982, in Baltimore, Maryland. In 1998 he began molecular training under the direction of Dr Peter Emanuel for the U.S. Army Edgewood Chemical and Biological Center in Aberdeen Proving Ground, Maryland, working on developing biological agent detection procedures. He attended college at Ursinus College in Collegeville, Pennsylvania where he developed an interest in structural biology and chemical synthesis under the direction of Dr Rebecca Roberts. After graduation in 2004 with a B.S. in biochemistry, Tom enrolled in a graduate program in the Department of Biochemistry at Virginia Commonwealth University in Richmond, Virginia under the direction of Dr Darrell Peterson. After 3 years of learning vast array of biochemical techniques, Tom transferred to the laboratory of Dr William Barton for the completion of his doctoral thesis.