2011

Characterizing the Phosphorylation State of Tie2 using SH2 Domain Fusion Proteins

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A thesis submitted to the partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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December 2011

ACKNOWLEDGMENTS

I would like to thank my family and friends for their support throughout my studies. I would also like to thank my advisor William A. Barton for giving me the opportunity to work in his laboratory and for his guidance and expertise.

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List of Abbreviations

Ang	Angiopoietin
HEK	Human Embryonic Kidney
SH2	Src Homology 2
SYFP2	Super Yellow Fluorescent Protein
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains

ABSTRACT

CHARACTERIZING THE PHOSPHORYLATION STATE OF TIE2 USING SH2 DOMAIN FUSION PROTEINS

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The cardiovascular system develops through two distinct processes in embryogenesis: vasculogenesis, whereby the primary plexus in the heart is formed along with embryonic and extraembryonic vasculature, and angiogenesis, which begins after vasculogenesis and results in the refinement and maturation of the branched vessel system. In pathological angiogenesis, tumors expand by releasing pro-angiogenic factors in response to hypoxic conditions. The Tie receptors, Tie1 and Tie2, are receptor tyrosine kinases that are integral to angiogenic pathways. A family of Angiopoietins, Ang1-4, have been shown to act as ligands for Tie2, of which Ang1 and Ang2 are best characterized. Activation of the receptor causes dimerization and autophosphorylation,

whereby adaptor proteins recognizing the phosphorylated tyrosine activate downstream signaling via their Src homology 2 (SH2) domains. Currently there are no phosphospecific antibodies for Tie2, therefore, identifying critical residues responsible for certain pathways remains difficult. In our study, we aim to use purified SH2 domains of known binding partners to Tie2 to assess the phosphorylation state of the receptor under various cellular conditions and settings, utilizing immunoprecipitation and western blotting. Unexpectedly, we found that Tie2 can bind non-specifically to nickel sepharose when the SH2 proteins were used as antibody mimetics, and was unable to be consistently precipitated in Protein A sepharose when used in conjunction with a monoclonal YFP antibody. Under the latter conditions however we were able to precipitate the SH2 protein itself. When immunoprecipitations were used with cobalt activated IMAC beads, we were able to precipitate Tie2 in overexpressed systems using the SH2 domains of Shp2 N-C and Grb2. As expected, phosphorylation of Tie2 in the presence of its orphan receptor Tie1 was attenuated compared to wild-type levels. Based upon available data, we anticipate this method as a useful tool to assess the phosphorylation state of Tie2 and its signaling pathways in the near future.

Chapter 1

Introduction

1.1 Vasculogenesis

The field of vascular biology has blossomed in the past few years, mainly as a result of the increased interest in anti-angiogeneic adjuvants as chemotherapeutics. Similarly, many other common human diseases, such as atherosclerosis, ischemia, thrombosis, and hypertension have been attributed to a dysfunction in blood vessel function. During vertebrate embryogenesis, the cardiovascular system develops through two distinct processes: vasculogenesis and angiogenesis (Augustin et al., 2009). In vasculogenesis, endothelial precursor cells, also known as angioblasts, migrate and differentiate in response to multiple growth factors, including the vascular endothelial growth factor (VEGF) and its corresponding receptor (VEGFR). The result of this process is the formation of the primary vascular plexus in the heart, and the embryonic and extraembryonic vasculature (Harris and Black, 2010). The balance of VEGF signaling during this stage of development is crucial as demonstrated by the lethality of VEGF heterozygous mice (Thirunavukkarasu et al., 2008).

1.2 Angiogenesis

Alternatively, angiogenesis begins where vasculogenesis left off, at day 8.5 in mice, and ultimately results in the maturation and remodeling of the primitive and poorly branched vessel system. The resulting vasculature consists of a stable, highly-connected endothelial cell wall surrounded by support cells including pericytes and smooth muscle

cells. In addition to the absolute requirement for VEGF and VEGFR signaling, multiple additional growth factors are required including the Angiopoietins and Tie receptors.

1.3 Pathological Angiogenesis

While developmental angiogenesis ends shortly after birth, pathological angiogenesis can occur at any stage of development (Cai et al., 2011). For example, an expanding tumor requires new blood vessels to support an increased need for nutrient and gas exchange to grow beyond an estimated 1-2 mm^3. During tumor expansion, hypoxic conditions promote the release of pro-angiogenic factors including VEGF and Angiopoietin-1 and -2. Eventually, these newly established vessels serve as vehicles for tumor cells to spread to other parts of the organism, in a process called metastases. Among the clinical therapies that are actively pursued to target metastasis include angiogenic markers such as VEGF. Among them are Bevacizumab (Genentech), otherwise known as Avastin, which is a monoclonal antibody directed against free VEGF, and Aflibercept (Regeneron), which is a fused protein containing VEGF-R domains designed to bind to all forms of VEGF with high affinity (Cai et al., 2011). Despite the promising outlook in anti-angiogenics, signaling mechanisms in angiogenesis are still poorly understood, which may hinder progress in finding new therapies (Augustin et al., 2009).

1.4 Tie Family Receptors

The Tie receptors, Tie1 and Tie2, are endothelial-cell specific receptor tyrosine kinases (RTK) with a molecular weight of 135 and 150 kDa, respectively (Thomas and Augustin, 2009). They are 76% similar in sequence identity of the intracellular domain, and 33% identical in the extracellular domain. The extracellular ligand-binding domain

of the two receptors features three epidermal growth factor (EGF) domains flanked by 3 Ig-like domains (2 amino-terminal, 1 carboxy-terminal), and three Fibronectin type III domains. The intracellular domain features a split tyrosine kinase signaling domain. Interestingly, Tie1 and Tie2 are co-expressed in blood vascular and lymphatic endothelial cells, with neither cell type, nor any other endothelial cell having been reported to only express one of the receptors (Augustin et al., 2009). Mice deficient in Tie2 die between embryonic days 10.5 and 12.5, due to the primary capillary plexus failing to mature. Loss of Tie2 function also leads to endothelial cell apoptosis, which results in vascular hemorrhaging (Jones et al. 2001). A loss of Tie1 on the other hand does not disrupt the angiogenic cascade, but the integrity of blood vessels is lost, and the embryos are susceptible to edema and death between embryonic day 13.5 and birth (Sato et al., 1995 and Puri et al, 1995). A double knock-out of Tie1 and Tie2 showed that vasculogenesis was able to continue normally, but similar to the Tie2 knockout, these embryos die at day 10.5 with severe vascular defects, further suggesting that the Tie receptor system is essential in maintaining vessel integrity (Puri et al., 1999).

1.5 Angiopoietins

The Angiopoietins are a family of Tie2 binding ligands, of which Ang1 and Ang2 are best characterized. Figure 1 depicts the family of Angiopoietins and their interactions with Tie2. The amino acid sequence of the angiopoietin ligands suggests that they consist of an N-terminal superclustering domain, a coiled-coil domain, and a C-terminal fibrinogen-homology domain. This C-terminal domain is responsible for receptor binding, while the coiled-coil domain is responsible for multimerization. In contrast to the co-expression of their receptors, the Angiopoietins have distinct expression levels

(Augustin et al., 2009). Ang1 is mostly expressed in smooth muscle cells and pericytes, while endothelial cells and tumors produce Ang2. Interestingly, knockout studies for Ang1 and overexpression studies of Ang2 show lethality at embryonic day 11, with a phenotype less severe but similar to the Tie2 deficient mice, suggesting that these receptors are agonists and antagonists for Tie2, resepctively (Thomas and Augustin, 2009). Crystallographic studies demonstrate that Ang1 and Ang2, through the C-terminal domain, interact with the extracellular domain of Tie2 (Barton et al., 2005). Despite the fact that both ligands may bind to Tie2, Ang1 has been shown to have a more positive affect on Tie2 signaling, causing the receptor to dimerize which induces autophosphorylation in the tyrosine kinase domain and activation of downstream signaling cascades. Little is known about the activation of Tie1, however it has been shown to form a heterodimer with Tie2, which in turn is disrupted in the presence of Ang1 (Seegar et al., 2010). Activation of Tie2 by Ang1 leads to recruitment of many adaptor proteins that promote pro-survival pathways in the cell, such as AKT and others. On the contrary, Ang2 acts as an antagonist of Tie2 signaling, promoting angiogenesis and pro-apoptotic pathways, but may also act as an agonist as it is context-dependent on the presence of Tie1 (Seegar et al. 2010).

1.6 SH2 domain characteristics

Upon activation and trans-phosphorylation of Tie2 induced by its angiopoietin agonist Ang-1, adaptor proteins such as the phosphatase SHP2, the PI3K subunit p85, Grb14 and Grb2 interact with the receptor and activate various signaling cascades. This interaction is mediated through the adaptor proteins' Src homology 2 (SH2) domains, which recognize phosphorylated tyrosine residues. As shown in Figure 2, the adaptor

proteins SHP2, Grb2, Grb14, and p85 bind to Tie2 at key tyrosine residues 814, 895, 897, 1022, 1066, 1100, and 1111. These key residues were determined via a yeast-two hybrid system and surface plasmon resonance using peptides corresponding to the appropriate regions of Tie2 (Jones et al., 1999).

The three-dimensional structure of the SH2 domain shows that it is composed of two alpha-helices that surround a beta-sheet containing three or four beta strands (Schlessinger and Lemmon 2003). The phosphorylated tyrosine residue lies in a conserved binding pocket that is positively charged, forming hydrogen bonds with several residues of the beta-sheet (Figure 3). Despite the fact that the SH2 recognizes phosphorylated tyrosines, the primary amino-acid sequence to which they bind is not absolutely conserved, but instead relies on the surrounding three-dimensional presentation of the phospho-tyrosine. This is best illustrated by the fact that Grb2, for example, binds RTKs at a pYXN motif (pY, phosphotyrosine; X, any amino acid; N, asparagines), while the SH2 domain of p85 binds specifically to pYMXM (M, methionine) sequence motifs. In addition to assembling adaptor protein complexes, SH2 domains can also serve as regulators of enzymatic activity of the proteins that contain them. An example of this is the protein kinase Src, whose SH2 domain binds to a p-Tyr residue in the protein, thereby locking the tyrosine kinase domain in an inactive conformation. To relieve inhibition, other p-Tyr sequences may then compete for the SH2 domain binding, which leads to its activation. Similarly, dephosphorylation of the p-Tyr sequence by tyrosine phosphatases or oncogenic mutation of the p-Tyr site will also activate Src (Schlessinger and Lemmon 2003).

SH2 domains also play a role in transcription factor dimerization, particularly within the JAK (Janus Kinase)/STAT (Signal transducers and activators of transcription) pathway. The dimerization of phosphorylated STAT is mediated by its SH2 domain through a tyrosine phosphorylation site on the molecule. Multimerization promotes localization of STAT into the nucleus to regulate gene expression (Levy et al., 2002).

1.7 Adaptor protein characteristics

Adaptor proteins that have been widely studied that bind to Tie2 are SHP2, Grb2, Grb14, and p85. SHP2 is a cytoplasmic protein tyrosine phosphatase (PTP) that has an SH2 domain on its N-terminal and C-terminal, followed by a catalytic PTP domain. It has been shown to bind to Tie2 at residue Y1111, as mutation of this residue decreased SHP2 association with the phosphorylated tyrosine kinase domain. Interestingly though, mutation of this residue increases autophosphorylation of Tie2 and increases downstream signaling (Jones et al., 2003). This increased phosphorylation and kinase activity was investigated in a subsequent study, and suggested that residue Y1111 of Tie2 may have a negative regulatory role, as its mutation was shown to potentially relieve inhibition by the C-terminal tail of Tie2 (Sturk et al., 2010). While it is widely thought that PTPs are negative regulators on the basis that they have primarily been shown to inhibit tyrosine kinases, studies have demonstrated that they promote Ras activation in cytokines. Activation of Ras leads to activation of the Raf-MEK-MAP kinase cascade, which promotes cell proliferation, differentiation, and survival (Matozaki et al., 2009). Mutations of SHP2 have been shown to disrupt the interactions between the SH2 and PTP domains, leading to a loss of autoinhibition of PTP activity. This results in excess

activation of the MAPK pathway, which leads to the development of juvenile myelomonocytic leukemia (Matozaki et al., 2009).

Another adaptor protein of Tie2, Grb2 (Growth factor receptor-bound protein 2) is a protein that is responsible for the recruitment of Sos, the Ras exchange factor, by recognizing phosphorylated tyrosines on the EGFR (Epidermal Growth Factor Receptor). Its overexpression has been known to induce liver tumorgenesis in mice (Nioche et al., 2002). It is known to bind Tie2 at residue Y1100, as mutation of this site to phenylalanine decreased this interaction (Huang et al., 1995). In addition to recruiting Sos, Grb2 plays a role in intracellular signaling through the Map Kinase cascade (Thomas and Augustin, 2009).

p85 is the regulatory subunit of Phosphatidylinositol 3-kinase (PI3K), which phosphorylates the 3-position in the inositol ring of phosphoinositides. Similar to Shp2, it contains SH2 domains on both its amino and carboxyl-terminal. Accumulation of PIP3 leads to induction of signaling cascades that control cell survival, motility and division (Kumar and Carerra, 2007). For example, PI3K phosphorylates Akt, which leads to activation of the Forkhead Transcription factor also known as FOXO-1 (FKHR-1) (Thomas and Augustin, 2009). Akt activation also leads to the phosphorylation of pro-apoptotic proteins such as BAD and procaspase-9, thereby inhibiting their function. Conversely, the Akt pathway upregulates Survivin, which is an apoptosis inhibitor, thereby promoting survival of the cell (Papapetropoulos et al., 1999). Similar to Grb2, p85 binds Tie2 at residue Y1100, which was confirmed by mutagenesis studies that reduced this interaction (Kontos et al., 1998).

Growth factor receptor-bound protein 14 (Grb 14) is one of three members of the Grb7 family of adaptor proteins, with the other two being Grb7 and Grb10. The SH2 domain of these proteins is located on the carboxyl-terminus. Unique to these proteins is a novel interaction region called the BPS (Between PH and SH2 domain). This region has been demonstrated to play a role in Grb7 family protein interactions between the Insulin receptor and Insulin-like Grown Factor Receptor (Cariou et al., 2004). While signaling pathways involving Grb14 have not been well characterized, it's known that the protein becomes tyrosine phosphorylated in the presence of Tie2 in endothelial cells, and that residues 1100 and 1106 of the receptor are required for phosphorylation of the protein (Sturk and Dumont, 2010). This suggests that Grb14 may play a role in endothelial cell signaling pathways.

1.8 Tie2 Signaling

In an elegant study by Saharinen and Fukuhara, it was recently demonstrated that Tie2 signals down one of two distinct cellular signaling cascades that are context dependent on the presence of Tie2 on the endothelial cell surface. For example, in cells that are grown to confluency on tissue culture plates and form tight junctions, activation of Tie2 by Ang-1 leads to the formation of distinct signaling complexes, which include a complex consisting of Tie2 and VE-PTP (Vascular Endothelial Protein Tyrosine Phosphatase). This complex then induces the Akt pathway, which as previously stated promotes cell survival (Saharinen et al., 2008). Alternatively, if cells are grown sparsely and lack cell to cell contact points, the Akt pathway is not activated, but instead, Ang-1 dependent Tie2 signaling proceeds down the Dok-R, ERK 1/2, and Mitogen activated

protein kinase (MAP-Kinase) pathway, promoting cell proliferation and differentiation (Fukuhara et al., 2008).

The phosphorylation state of Tie2 is known to dictate the cellular signaling pathways that are activated in response to Ang-1 recognition and receptor clustering. However, there are no phospho-specific antibodies that would allow one to probe the role of specific residues; the antibodies probe the whole receptor itself, and hence, specific pathways that are activated under different growth conditions cannot be fully determined. This could, therefore, be detrimental to the identification of which phosphotyrosine sites are critical in downstream signaling activation. However, it has been demonstrated in the past that individual SH2 domains can function as phospho-specific antibody mimetics to help identify and clarify which sites are phosphorylated in certain receptors (Jones et al., 1999 and Huang et al., 1995).

In our laboratory studies investigating Tie2 signaling with its receptor agonist Ang-1, we have shown a proficiency in protein expression and binding assays. In this regard, we wished to explore the possibility of using SH2 fusion proteins of known adaptor proteins and investigate their binding capabilities to Tie2. We aim to use our knowledge of protein purification techniques to successfully purify the SH2 domains of Tie2 binding partners, and subsequently use them in our binding assays with endogenous and overexpressed Tie2 in different cell lines. Our main goal of this study is successfully evaluating the phosphorylation state of the receptor under various stimuli and settings using the SH2 fusion proteins, and the possibility of identifying the signaling pathways activated from each protein and the phosphorylation sites responsible for their activation via mutagenesis studies, thereby comparing the critical sites for each SH2 domain with

the residues from previous studies. If successful, the SH2 fusion proteins would be an essential tool to help assess Tie2 phosphorylation and signaling.

Figure 1: Schematic representation of the Tie and Angiopoietin family members. Ang-1 and Ang-2 have been best characterized to interact with Tie2. Ang-1 primarily acts as an agonist, while Ang-2 may act as an agonist or antagonist, as it is context-dependent on the presence of Tie1. Ang-3 and Ang-4 have not been fully studied, though it is thought that Ang-3 behaves as an antagonist, while Ang-4 behaves in an agonistic manner. Preliminary data has also suggested interactions of Tie2 with the integrin $\alpha 5\beta 1$.

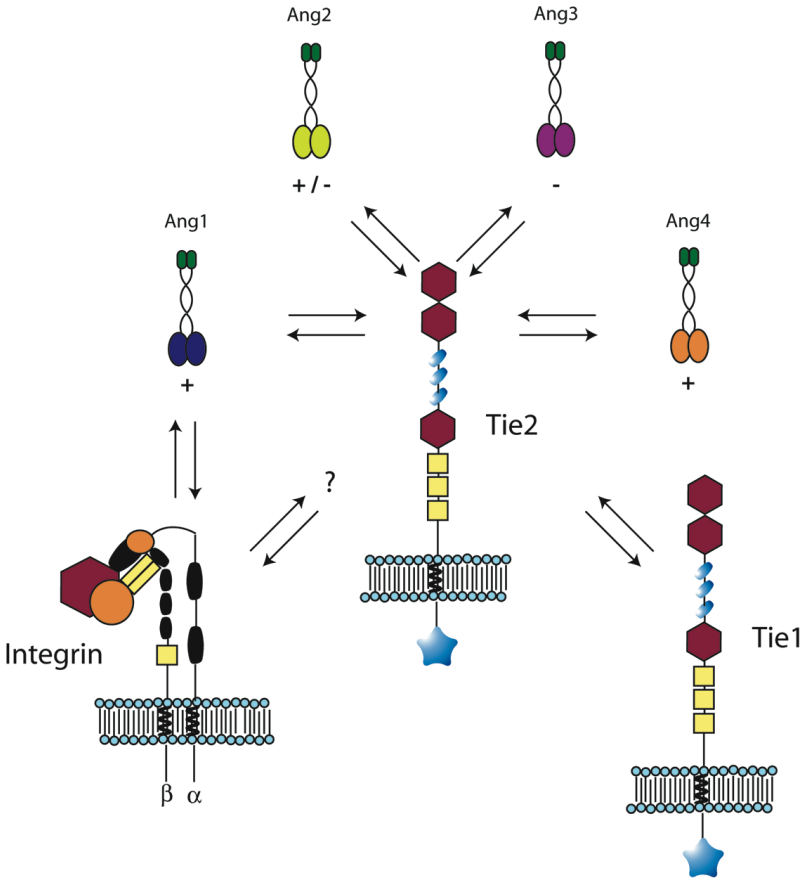

Ang2
Ang3
+ / -
-
Ang1
Ang4
+
+
Tie2
?
Integrin
Tie1
β α

Figure 2: Schematic representation of adaptor proteins binding to Tie2 via SH2 domains. Activation of Tie2 leads to autophosphorylation of the tyrosine kinase domain and recruitment of adaptor proteins binding to the phosphorylated tyrosine residues via their SH2 domain. The binding sites of the proteins were previously determined using a yeast-two hybrid system and surface plasmon resonance with phosphopeptides (Jones et al., 1999).

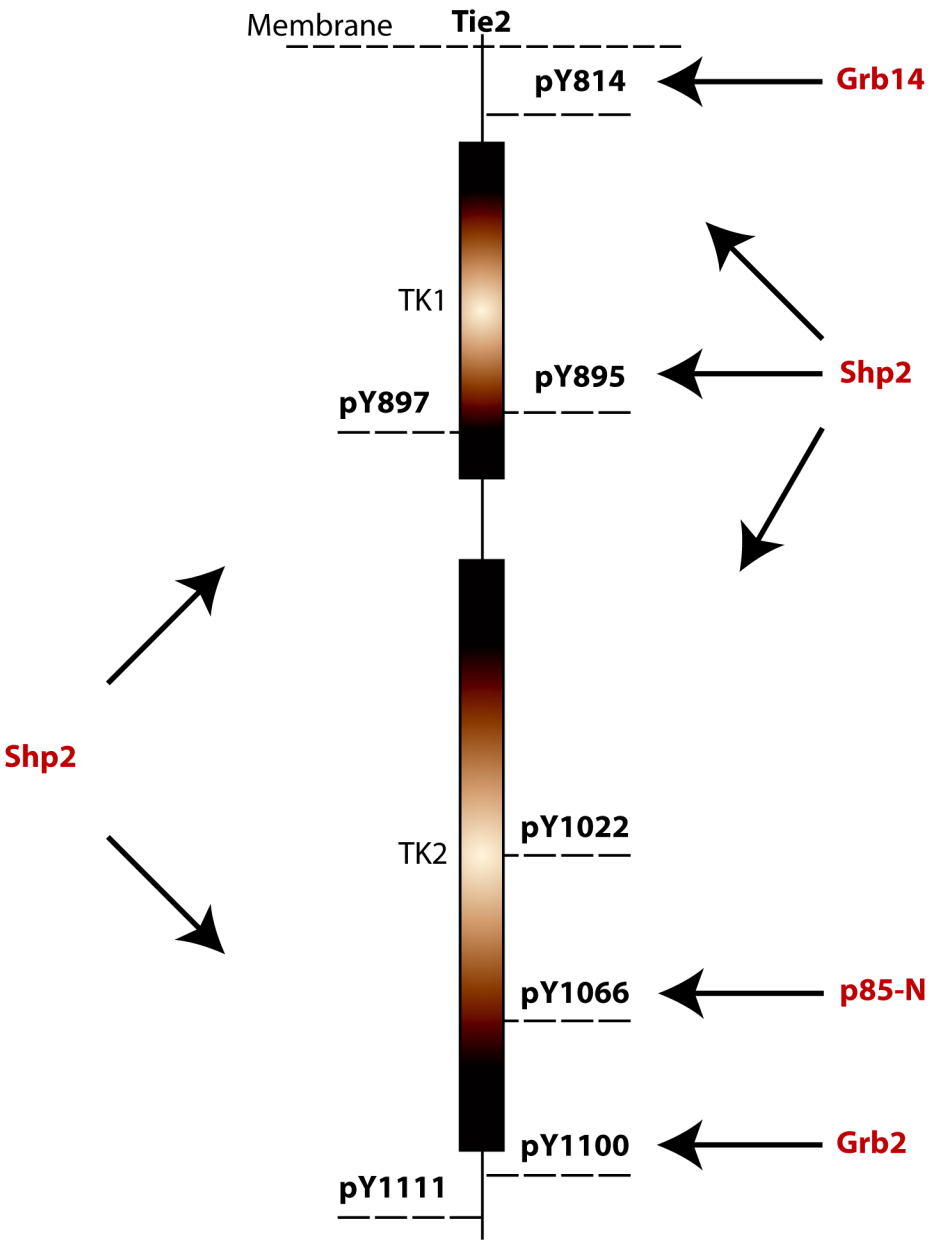
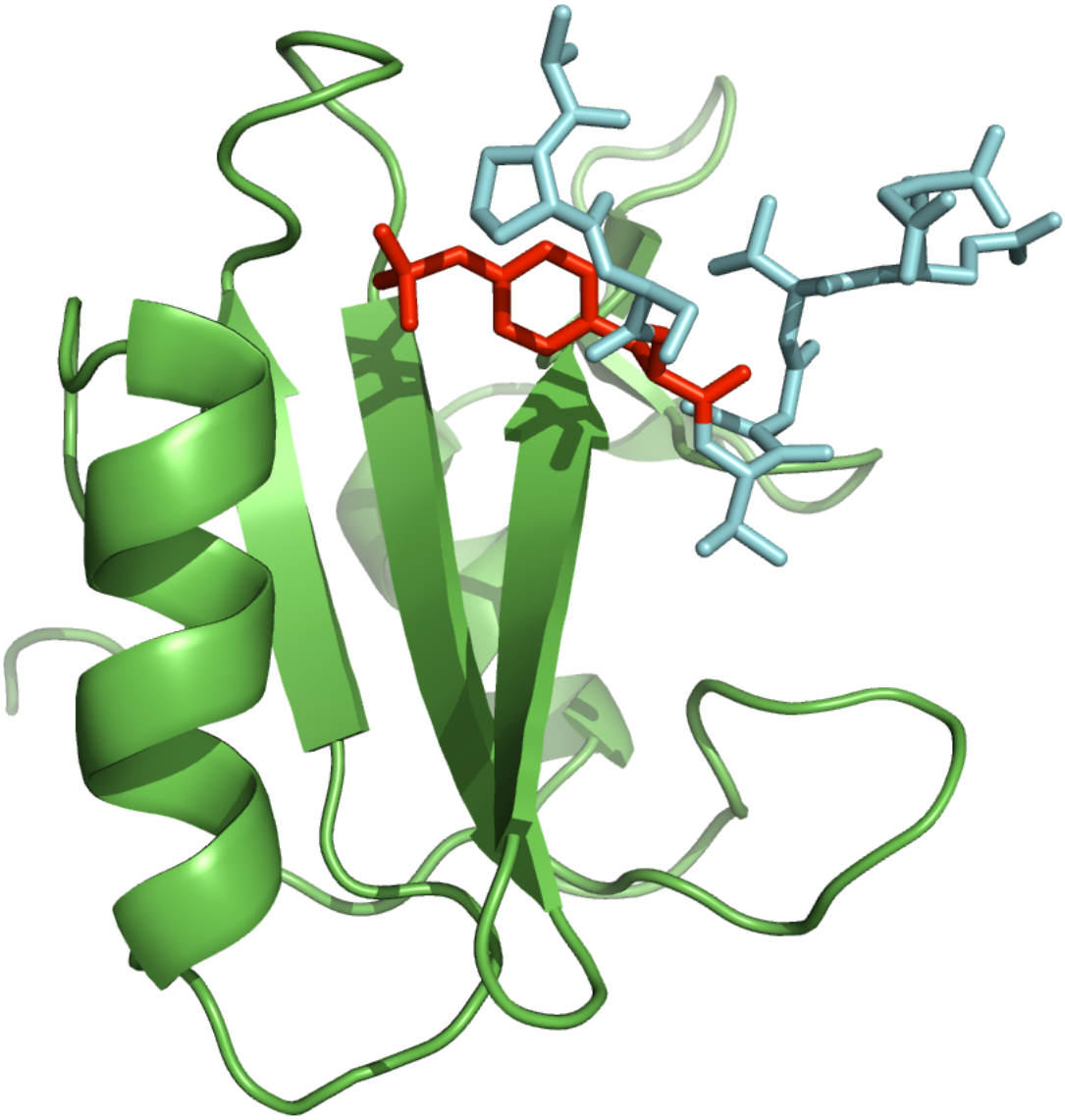

Membrane
Tie2
pY814
Grb14
TK1
pY895
Shp2
pY897
Shp2
TK2
pY1022
pY1066
p85-N
pY1100
Grb2
pY1111

Figure 3: SH2 domain crystal structure. The SH2 structure consists of a large beta sheet flanked by two alpha-helices, with the phosphotyrosine recognition site in a binding pocket forming hydrogen-bond interactions with the beta sheet. Structure generated via PyMol, using the coordinates of the Grb2 SH2 domain with a phosphopeptide.



Chapter 2

Methods

2.1 DNA Cloning and Mutagenesis

The sequences containing the SH2 domains of Shp2, Grb2, Grb14, p85 were amplified by PCR from human cDNA (Open Biosystems) and cloned into a modified pETDUET-1 expression vector (Novagen) for expression in *E.coli*. Briefly, individual SH2 domains were cloned via NcoI and BamHI (restriction enzymes supplied by New England Biolabs) restriction sites upstream of a SYFP2 (Super Yellow Fluorescent Protein) coding sequence (previously introduced into the BamHI and XhoI restriction sites). A hexa-histidine tag was introduced via PCR into the carboxy-terminus of SYFP for purification purposes. SYFP2 was obtained as a gift from the laboratory of Theodorus W. J. Gadella, Jr.

To construct individual tyrosine mutants of Tie2, overlapping mutagenic primers were designed which mutated tyrosines at residues 1111, 1100, 1066, 1022, 897, 895, and 814 to phenylalanine using a commercial mutagenesis kit (Agilent Stratagene) or overlap-extension PCR (Phusion polymerase supplied by New England Biolabs). The parameters for the mutagenesis kit were as follows: 95ºC for 30 seconds, 55ºC for 1 minute, and 68º for 10 minutes. This cycle was repeated 18 times, before laying idle at 4ºC. The resulting reaction was digested with DpnI (Agilent Stratagene) for 1 hour before transformation onto XL-1 Blue supercompetent cells and ampicillin plates. The parameters for overlap-extension PCR were as follows: 98º for 45 seconds, 58º for 45 seconds, and 72º for 10 minutes. This cycle was repeated 30 times, followed by a final extension at 72º for 5 minutes before incubation at 4ºC. The reaction was digested with

DpnI (New England Biolabs), and run via gel electrophoresis on a 1% agarose gel stained with ethidium bromide. The visualized band was excised and the DNA extracted using a Gel Extraction Kit (Qiagen). T4 DNA ligase (New England Biolabs) was added to the plasmid, after which it was transformed into MACH-1 competent cells and ampicillin plates. All mutations were confirmed via standard di-deoxy sequencing chemistry (Cornell University).

2.2 Recombinant Protein Expression and Purification

For protein expression, individual pET SH2-YFP fusion vectors were transformed into BL-21(DE3) cells and grown overnight in 50 mL of Luria broth (LB) containing 100 μg/ml ampicillin to serve as a starter culture for large-scale growth. The following morning, 10 mL of overnight culture was added per liter to three 1L aliquots of LB in shaker flasks pre-equilibrated at 37^{o} C. Culture growth was checked periodically by observing the turbidity and when an optical density of 0.6 at 37 degrees was reached, the incubation temperature was shifted to 20^{o} C and protein expression was induced with 1 mM IPTG. We found that the lower temperature was absolutely required to promote robust soluble protein production of many of the SH2 fusion proteins. Approximately, 18-24 hours post-induction, the cultures were centrifuged at 5000 rpm for 15 minutes, and the resulting cell pellets were re-suspended in a 20 mM Tris and 300 mM NaCl buffer (Buffer A). The re-suspended cells were disrupted by passing them through a cell homogenizer at ~15000 psi three times (until viscosity was significantly reduced), and the resulting crude lysate was centrifuged at 15000 rpm for 45 minutes to remove insoluble debris. The SH2-SYFP fusion proteins were purified via affinity chromatography by passing the crude lysate over a 5 mL His-trap column charged with

Co^{2+} and pre-equilibrated with Buffer A. Purified protein was eluted with a linear Imidazole gradient (Buffer A + 200 mM imidazole). Individual fractions were analyzed by SDS-PAGE and those that contained protein judged to be >90% pure (by Coomassie staining) were pooled. The proteins were subsequently concentrated to 5 mg/mL and glycerol was added to a final concentration of 10% prior to storage at -80º C. A working stock of 100 µg/mL was additionally created for binding assay studies. To prevent cross-contamination, the chelating Sepharose column was washed with a solution containing 1% (v/v) Tween 20 detergent and 0.1 M acetic acid, followed by a 70% ethanol wash between individual purifications.

2.3 Cell Culture and Manipulation

Human Embryonic Kidney 293 (HEK293) and Human Endothelial (EA.hy 926) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 10% Fetal Bovine Serum (FBS) (Serum Source International) and Pen/Strep (Invitrogen) in a 5% CO2 atmosphere at 37º C. For transfections, HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) and Opti-MEM (Invitrogen) according to manufacturers recommendations in a 10 cm dish with Tie2-TKD-myc (20 µg), or both Tie2-TKD-myc and Tie1-HA (10 µg each). Transfections were routinely performed in complete media lacking antibiotics. After 24 hours, the cells were washed once with warm Phosphate Buffered Saline (PBS) and returned to complete media containing both 10% FBS and Pen/Strep for additional 24-48 hours of incubation.

For Tie2 stimulation experiments, EA.hy cells were serum-starved by changing the media to DMEM lacking FBS and incubating for approximately 4 hours.

Recombinant Angiopoietin 1 and Angiopoietin 2 ligand was added to the media at a concentration of 0.5 μg/mL and the cells were allowed to incubate for an additional 30 minutes at 37^{o}C. To prepare crude cellular lysates, both the transfected and stimulated cells were harvested following a brief rinse with cold PBS by gentle scraping with a cell scraper. Cells were collected in a microcentrifuge tube and lysed in ice-cold lysis buffer (20 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% NP-40 detergent, 1 mM Sodium Orthovanadate, and cOmplete protease inhibitor (Roche)). Following a 10 minute incubation on ice and a brief (5 second) vortex, cellular debris was removed via centrifugation for 10 minutes at 15,000 rpm and the supernatant was collected and protein content quantified by Bradford assay (BioRad).

2.4 Far-Western Blotting, Immunoprecipitation and Western Blot studies

For analysis of protein interaction by Far-western analysis, 100 μg of crude cellular lysate was subjected to SDS-PAGE gel electrophoresis (10% polyacrylamide), and transferred to a nitrocellulose membrane (VWR). After blocking the membrane with non-fat milk for 1 hour, the membrane was probed with 0.1 mg/mL of the SH2 proteins in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) for 1 hour, and visualized on the Typhoon imager (GE Healthcare) using a 488 nm laser and 505 nm emission filter.

For most immunoprecipitation experiments, Tie2 in crude lysates was precipitated by incubation with purified SH2-SYFP proteins. Briefly, either 500 μg of EA.hy lysate or 250 μg of HEK293 lysate was mixed with 50 μL of a 1:1 (v/v) slurry of Nickel-Sepharose (GE Healthcare), and the volume was raised to 1 mL with HBST (20 mM Hepes, 150 mM NaCl, 0.1% Triton-X detergent). The mixture was rotated on a

mechanical rotisserie for one hour at 4°C prior to changing the slurry of sepharose beads, after which they were returned for incubation overnight at 4ºC, followed by extensive washing with HBST and HBS. To avoid non-specific immunoprecipitation of Tie2 on chelating sepharose, Protein A sepharose beads (Thermo Scientific) were also used under certain conditions in association with a monoclonal anti-YFP antibody (Clontech). In another attempt to avoid non-specific immunoprecipitation, we utilized Immobilized metal ion affinity chromatography (IMAC) sepharose beads (GE Healthcare) activated with 200 mM cobalt chloride. Immunoprecipitations using these beads were washed with solution containing TBS and 0.1% Triton-X100. The SH2 proteins were then eluted with 200 mM imidazole and centrifuged, and the supernatant was collected. Protein was eluted from the beads (supernatant for IMAC sepharose) by boiling in 1x Sample Buffer (10 mM Tris pH8.0, 1% SDS, bromophenol blue), prior to electrophorese in 10% polyacrylamide gels and transfer to nitrocellulose. Membranes were blocked with 4% non-fat milk for one hour, and probed with anti-Tie2 C-20 (Santa Cruz Biotechnology) for one hour. After extensive washing, the blot was incubated with an anti-rabbit-HRP secondary antibody (Promega) for an additional hour. Following three five-to-ten minute washes with TBST, the blot was developed with chemiluminesent substrate (Pierce).

Chapter 3

Results

3.1 SH2 domain expression and purification

Individual SH2 domains from Grb2, Grb14, Shp2, and p85 were expressed under control of the T7 promoter in BL21 (DE3) cells and placed amino-terminal to the superfolder variant of YFP (SYFP2). Inclusion of SYFP2 allowed us to visualize purified proteins using fluorescence and aided in the in vivo folding of SH2 domains (Kremers et al., 2006). Initial attempts to express proteins at 37º C were largely unsuccessful. Therefore, several attempts were made to optimize soluble protein expression by modifying the expression growth temperature from 37º C to either 25º C or 20º C. Ultimately, it was empirically found that 20º C allowed the greatest yield of soluble protein for almost all of the fusion proteins. As SYFP2 folds well at 37º C in E. coli, it is likely that the SH2 domains were responsible for the solubility issues, and this has been observed by other labs producing GST-SH2 domain fusion proteins. SH2-SYFP2 fusions were purified via affinity chromatography on either nickel- or cobalt-sepharose (the lab has found that fewer non-specific contaminants result from protein preparations when cobalt is used in place of nickel), and eluted with a linear imidazole gradient. SDS-PAGE and coomassie staining of the individual fractions allowed us to evaluate sample purity and pool those fractions that were greater than 90% pure. Most protein preparations were sufficiently pure to avoid further purification steps, although in some cases the fusion proteins were further purified and buffer exchanged via gel filtration chromatography. Figure 4 demonstrates that the proteins were largely free of contaminants. In addition, the proteins all migrated at close to (within 5%) their expected

theoretical molecular weights and only minor contaminating bands were observed. Interestingly, some cleavage was observed between the SH2 domain and SYFP2, and because the hexa-histidine tag was at the carboxyl terminus (close to the SYFP2), free SYFP2 was present to varying extents as a minor contaminant. If warranted in the future, the hexa-histidine tag could be moved to the amino-terminus to guarantee the inclusion of the SH2 domains during purification.

3.2 SH2 domains binding to Tie2

Having verified the expression and purification of the SH2-SYFP2 fusion proteins, we next wished to evaluate their binding to Tie2 expressed under different conditions and stimulated with different ligands. As a first attempt at observing SH2-Tie2 interactions we utilized Far-western blotting. The Far-western technique is not unlike the protein microarray approach developed by others and utilized extensively for monitoring SH2-protein interactions, yet this technique does not require the use of expensive microarray spotters and scanners. Instead, Tie2 from crude protein lysates of transiently transfected HEK293 cells was separated via conventional SDS-PAGE and electroblotted to a nitrocellulose membrane. Once immobilized, the separated proteins can be probed *in situ* by individual SH2-SYFP2 proteins. The inclusion of SYFP2 in our fusion protein constructs allows us to monitor SH2 binding to the nitrocellulose membrane using a fluorescence scanner configured to excite the fluorophore at 488nm and monitor emission at 506nm. As a control, the blot was also probed with anti-Tie2 antibody to confirm the presence of recombinant Tie2 (data not shown). Similarly, the presence of tyrosine phosphorylation was monitored in Tie2 by immunoprecipitating Tie2 with anti-phosphotyrosine (4G10) and probing with anti-Tie2 (data not shown).

Several attempts were made to detect SH2-Tie2 binding to blots containing different amounts of crude protein lysate and different concentrations of SH2 protein in the probe buffer (from 10-100 μg/mLl). Unfortunately, despite repeated attempts at altering protein concentrations and detection schemes, we were unable to detect the presence of the SH2-SYFP2 fusion proteins binding to the nitrocellulose (data not shown). We suspect there could be several possible explanations. First, in the absence of mass spectral data, it is impossible for us to know for sure that the tyrosine phosphorylation sites that we are looking for are *actually* phosphorylated. Our IP-western data with anti-pY only *suggests* that phospho-tyrosines are present, but does not tell us where. Second, based on our estimates from western blotting with anti-Tie2, we expect approximately 10-50 ng of Tie2 to be present in each lane. If we assume all of the protein is phosphorylated and will be detected in a 1:1 molar ratio by an SH2 fusion protein, this assumes between approximately 3-15 ng (~60-300 pmoles) of SH2-SYFP2 will be present on the membrane. Although this would appear to be well within the detection limit of the fluorescence scanner we have utilized (~100 amoles of fluorescein), we must note that YFP is poorly excited by a 488nm laser, and is poorly imaged using a 506nm emission filter. Indeed, the ideal excitation and emission for YFP are 514nm and 527nm respectively. To verify detection of SYFP2, we could blot various amounts of fusion protein onto nitrocellulose and scan the blot with the assumption that SYFP2 will remain folded and fluoresce equally as it does in solution.

Alternatively, we next employed a traditional immunoprecipitation and western blotting procedure, where SH2-SYFP2 protein served as an affinity purification reagent. Crude protein lysates were again prepared from Ea.hy 926 cells expressing endogenous

Tie2 or HEK293 cells transiently transfected with either Tie2 alone or a combination of Tie1 and Tie2. As shown in figure 5, when used for immunoprecipitation, the SH2 proteins are able to precipitate endogenous Tie2 in endothelial EA.hy cells (figure 5A) and in HEK293 cells (figure 5B) when Tie2 is overexpressed. This is in agreement with the previous studies that demonstrated Tie2-SH2 interactions through a yeast-two hybrid system and surface plasmon resonance with phospho-peptides (Jones et al., 1999). Interestingly, when endothelial cells were stimulated with either Ang1, or Ang2, Tie2 levels appear to be equal to that of the wild-type levels while it is known from previous studies that Ang1 induces dramatic phosphorylation of Tie2 while wild-type and Ang2 levels remain very low. Similarly, in HEK293 cells overexpressing Tie2 or Tie1/Tie2, equivalent amounts of precipitated Tie2 were observed wile it has previously been shown by our group and others that the presence of Tie1 dramatically decreases the basal phosphorylation of Tie2.

While we were testing our ability to precipitate Tie2 with SH2-SYFP2 fusion proteins, we also analyzed the optimal amount of fusion protein to use for Tie2-specific binding in these experiments. Briefly, the amount of SH2 fusion protein was titrated from 100 ng to 1 μg and incubated with a fixed amount (500 μg) of HEK293 cell lysate containing Tie2. Following a 24-hour incubation at 4^{o}C, the nickel-sepharose pull-downs were analyzed by SDS-PAGE and western blotting as usual. For these experiments we utilized the Shp2 N-C fusion protein since based on previous studies it binds to the maximum number of phosphotyrosine residues within Tie2. As illustrated in Figure 6, altering the amount of SH2 fusion protein from 100 ng to 1 μg had little effect on the

amount of Tie2 precipitated. Indeed, amounts as low as 100 ng were sufficient for precipitation of Tie2.

Combined, these results were unexpected and led us to contemplate the validity of our observations thus far. Up to this point, our experiments lacked a critical control, which was a precipitation of Tie2 in the absence of *any* SH2 fusion protein. Surprisingly, when the experiment was performed in the absence of an SH2 fusion protein, Tie2 was found in the precipitate bound to nickel-sepharose, as demonstrated by Figure 7. This finding was especially surprising since experiments such as these had been done in the lab previously with different results. However, based on this finding, we concluded that Tie2 was non-specifically binding to the nickel-sepharose.

To circumvent the non-specific binding observed by nickel-sepharose, we took advantage of the fact that our lab had considerable experience immuno-precipitating Tie2 with Protein A-Sepharose in the presence of various antibodies. Results by our group as well as others demonstrate that Tie2 does not non-specifically bind to ProteinA-Sepharose. Therefore, the pull-down experiments were repeated with anti-YFP antibody to precipitate the SH2-SYFP2 fusion proteins. Briefly, like the previous experiments 100 ng of SH2-SYFP2 fusion protein was incubated with crude cellular lysate and mixed with 1µg of anti-YFP antibody and 50 µL of a 1:1 slurry of ProteinA-Sepharose. Ea.hy lysates stimulated with Ang2 were excluded from this study, as we wanted to focus on receptor activation via its agonist Ang1. Following SDS-PAGE and electroblotting, the membrane was probed with anti-Tie2, stripped with stripping buffer and re-probed with anti-YFP to confirm the presence of the SH2 fusion protein. Unexpectedly, we have been unable to identify Tie2 in Shp2-SH2 precipitates (Figure 8A) despite repeated attempts to

modify the lysate amount and repeat the experiment and despite the clear success of the anti-YFP binding to SH2-SYFP2 fusion protein (Figure 8C). We also ran a whole cell lysate to determine the presence of Tie2 in our lysates via the myc tag (Figure 8B).

Following our immunoprecipitations using Protein-A sepharose, we attempted to use Immobilized metal ion affinity chromatography (IMAC) beads, activated by cobalt chloride, similar to the manner of our His-trap column during initial purification. We used imidazole to elute the SH2 protein, along with our sample. Figure 9 demonstrates that the SH2 domains of Shp2 N-C and Grb2 were able to precipiatate Tie2 when transiently transfected in HEK293 cells, but not in endogenous Eahy cells. Consistent with our model of Tie2 signaling, Tie2 phosphorylation was attenuated in the presence of Tie1 with both of these SH2 proteins. Assays with the other SH2 fusion proteins elicited similar results to that of nickel sepharose, in which Tie2 was found to non-specifically bind to the beads.

3.3 Mutagenesis studies

To validate the phospho-specific nature of the binding we expected to observe, we prepared several non-phosphorylatable Tie2 expression constructs. For example, non-phosphorylatable tyrosine-to-phenylalanine mutants of Tie2 at residues 1100, 1066, 1022, 897, 895, and 814 were constructed using site-directed mutagenesis. Individual mutations were confirmed via DNA sequencing, and expression constructs were transfected into HEK293 cells and checked for protein expression by SDS-PAGE and western blotting. Figure 10 demonstrates that each of the mutants expresses well and migrates at the expected molecular weight. In future experiments we plan to check these

mutants for binding to specific SH2 fusion proteins to test the overall specificity of the residues to which they are predicted to bind.

Figure 4: Purification of SH2 proteins validated. SH2 domains from the adaptor proteins Shp2, Grb2, Grb14, and p85 were expressed in *E. coli* and purified via affinity chromatography. 50 μg of purified protein was separated by SDS-PAGE gel electrophoresis and stained via Coomassie Blue. Theoretical molecular weights are as follows: Shp2 Nt – 39 kDa; Shp2 Ct – 39 kDa; Shp2 NtCt – 47 kDa; Grb2 – 37 kDa; Grb14 – 37 kDa; p85 Nt – 35 kDa.

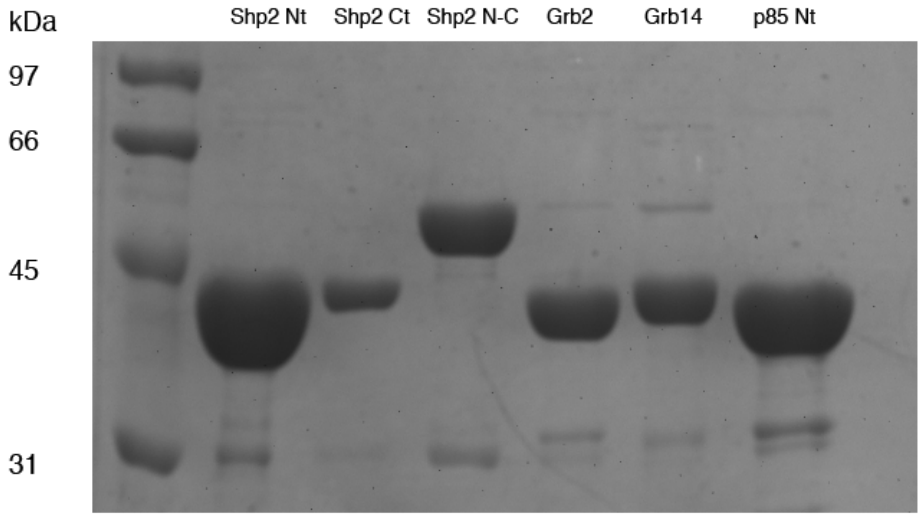

kDa
Shp2 Nt
Shp2 Ct
Shp2 N-C
Grb2
Grb14
p85 Nt
97
66
45
31

Figure 5: SH2 proteins bind to Tie2 in endothelial cells and in cells overexpressing the receptor. Tie2 was precipitated with various SH2 fusion proteins from (A) EA.hy 926 cells previously stimulated with Angiopoietin 1 and Angiopoietin 2, or (B) HEK293 cells transfected with either Tie2 or equal amounts of Tie1 and Tie2. Blots were probed with anti-Tie2 (C-20) antibody.

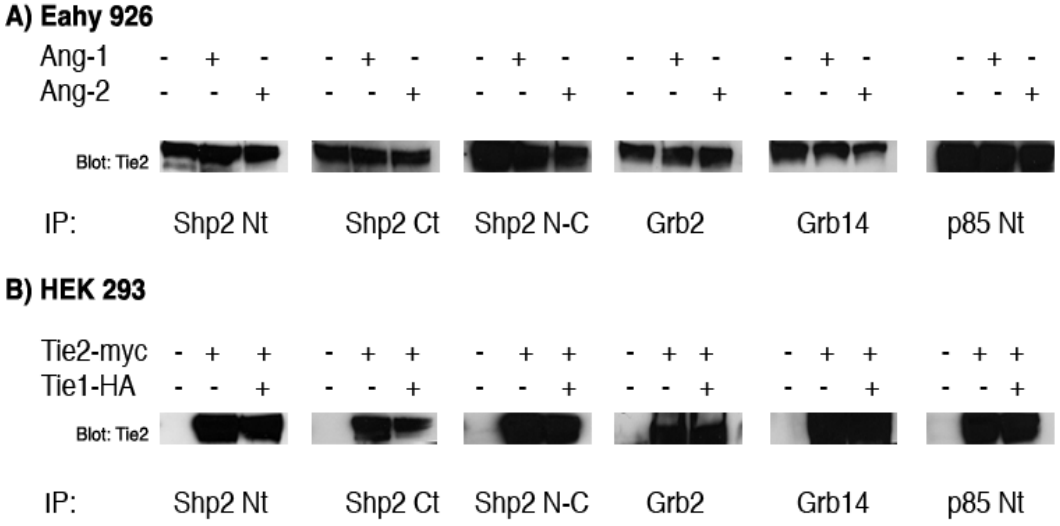

A) Eahy 926
Ang-1 - + - - + - - + - - + - - + - - + -
Ang-2 - - + - - + - - + - - + - - + - - +
Blot: Tie2
IP: Shp2 Nt Shp2 Ct Shp2 N-C Grb2 Grb14 p85 Nt
B) HEK 293
Tie2-myc - + + - + + - + + - + + - + + - + +
Tie1-HA - - + - - + - - + - - + - - + - - +
Blot: Tie2
IP: Shp2 Nt Shp2 Ct Shp2 N-C Grb2 Grb14 p85 Nt

Figure 6: Titration of SH2 domains in immunoprecipitations. HEK293 cells were transiently transfected with full length Tie2-myc alone or in conjunction with Tie1-HA. The amount of the Shp2 N-C SH2 protein used for immunoprecipitation varied from 100 ng to 1 μg. Blots were probed with anti-Tie2 (C-20) antibody.

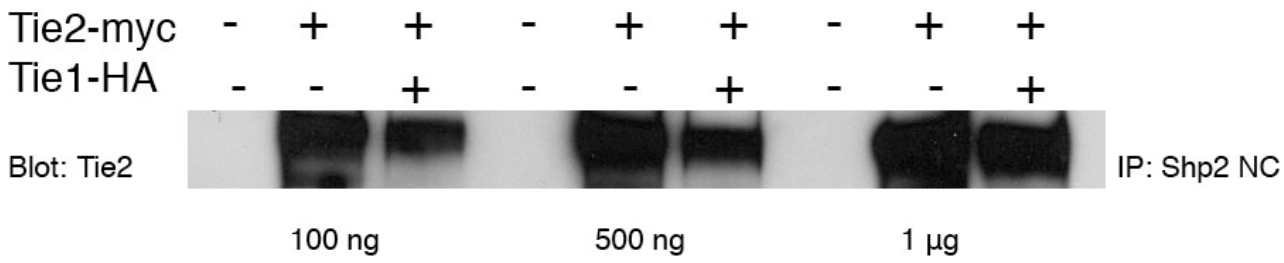

Tie2-myc - + + - + + - + +
Tie1-HA - - + - - + - - +
Blot: Tie2
IP: Shp2 NC
100 ng
500 ng
1 µg

Figure 7: Immunoprecipitation studies using a minimal amount of SH2 proteins as determined by titration. Stimulations and transfections were performed as described, as well as immunoprecipitation, with the exception that 100 ng of each SH2 protein were used as opposed to 2 μg that was used in the previous assay. A negative immunoprecipitation control was also utilized in HEK293 cells overexpressing Tie2, without the SH2 protein.

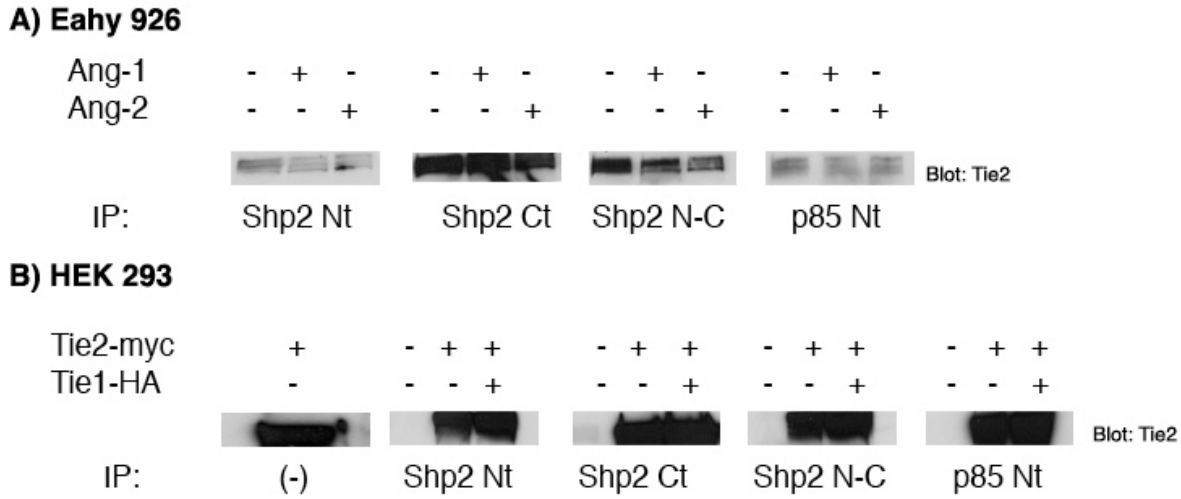
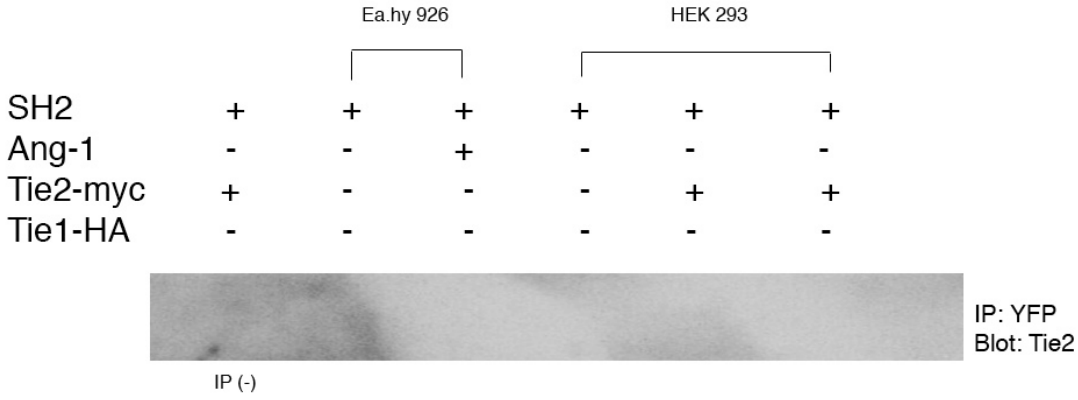
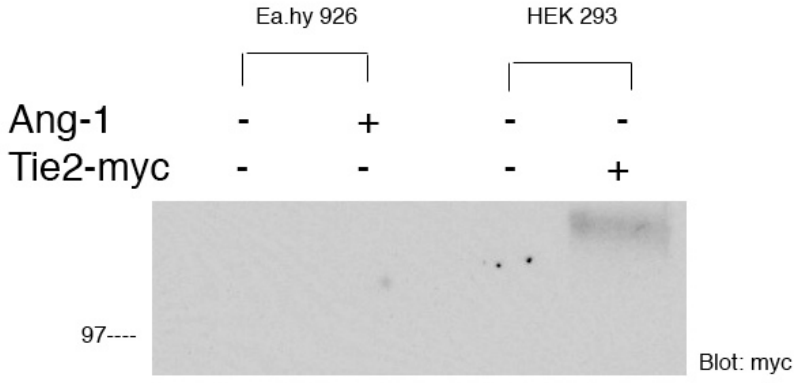

A) Eahy 926
Ang-1
Ang-2
- + - - + - - + - - + -
- - + - - + - - + - - +
Blot: Tie2
IP:
Shp2 Nt
Shp2 Ct
Shp2 N-C
p85 Nt
B) HEK 293
Tie2-myc
Tie1-HA
+ - + + - + + - + + - + +
- - - + - - + - - + - - +
Blot: Tie2
IP:
(-)
Shp2 Nt
Shp2 Ct
Shp2 N-C
p85 Nt

Figure 8: Immunoprecipitation using Protein A sepharose. Protein A beads were used for immunoprecipitation as opposed to Nickel sepharose in the previous assays. Because the SH2 proteins do not bind Protein A, monoclonal YFP antibody was added to the reaction. As with previous studies, the Shp2 N-C fusion was chosen for the immunoprecipitation. An HEK293 lysate expressing Tie2 was chosen as a negative control without addition of antibody. The blot was probed with Tie2 (A), followed by stripping and probing with anti-YFP (C). A whole cell lysate was also performed to confirm the presence of Tie2 by probing with anti-myc (B).

A


Ea.hy 926
HEK 293
SH2 + + + + + +
Ang-1 - - + - - -
Tie2-myc + - - - + +
Tie1-HA - - - - - -
IP: YFP
Blot: Tie2
IP (-)

B


Ea.hy 926
HEK 293
Ang-1 - + - -
Tie2-myc - - - +
97----
Blot: myc

C

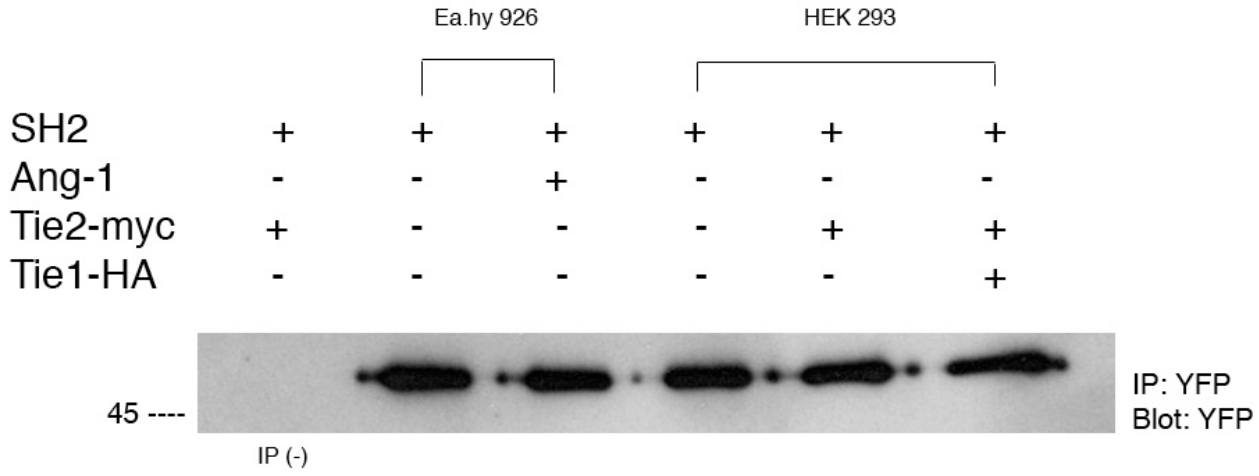


Figure 9. Immunoprecipitation using Cobalt sepharose. IMAC beads activated with cobalt chloride were used, and the SH2 proteins were eluted with 200 mM imidazole in the final wash step. Blots were probed with Tie2 as previously described.

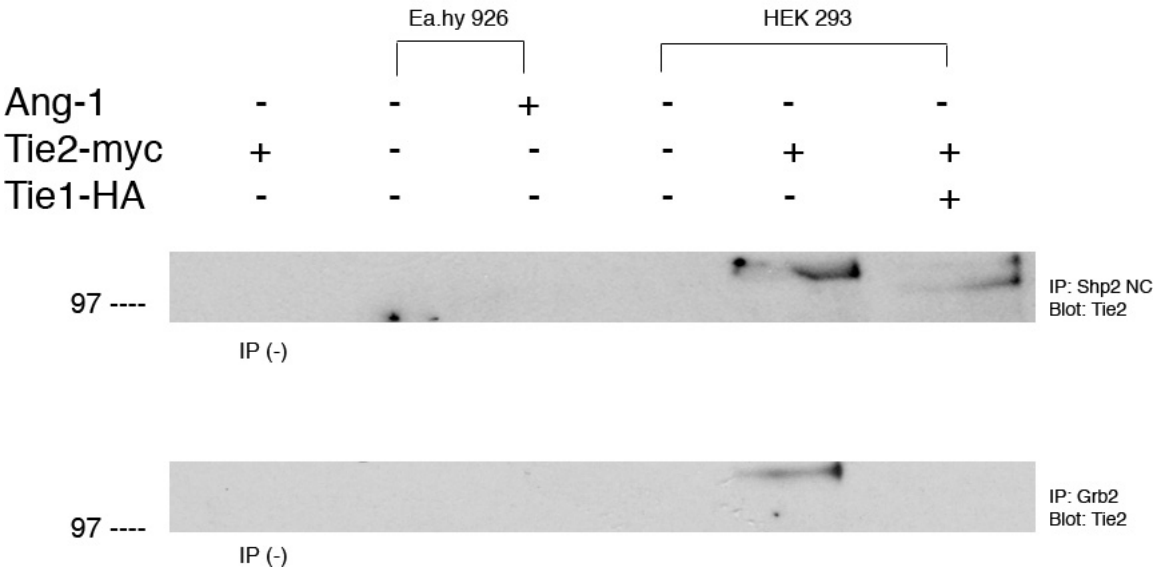
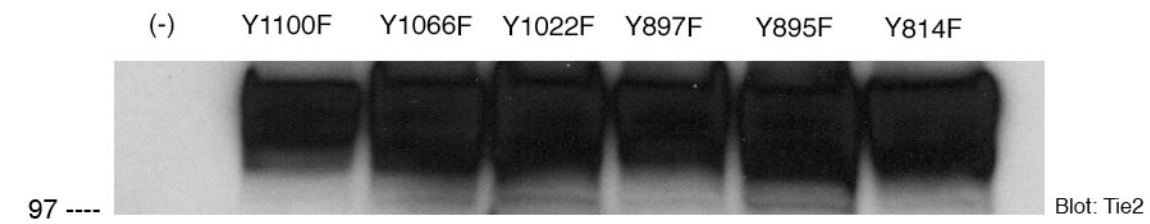

Ea.hy 926
HEK 293
Ang-1 - - + - - -
Tie2-myc + - - - + +
Tie1-HA - - - - - +
97 ----
IP: Shp2 NC
Blot: Tie2
IP (-)
97 ----
IP: Grb2
Blot: Tie2
IP (-)

Figure 10: Mutagenesis of Tie2. Tyrosine to phenylalanine mutants were transfected into HEK293 cells and lysed after 48 hours. 100 μg of the lysate was run on SDS-PAGE and probed with anti-Tie2 to visualize expression.


HEK 293
(-)
Y1100F
Y1066F
Y1022F
Y897F
Y895F
Y814F
97 ----
Blot: Tie2

Chapter 4

Conclusions

Based on previous studies, it was found that Tie2 interacts with an array of binding partners via their SH2 domains to induce signaling cascades downstream. It was also known that the phosphorylation state of Tie2 dictates which signaling cascades are activated. Since there were no means to detect specific phosphorylation sites upon Tie2 activation, we sought to determine if the phosphorylation state of Tie2 could be assessed using the SH2 fusion proteins of Shp2, Grb2, Grb14, and p85 as aids. We were able to successfully purify six different SH2-SYFP fusion proteins via affinity chromatography, which were judged to be > 90% pure (Figure 4). Our initial attempts at studying the binding of the SH2 fusion proteins to Tie2 involved utilizing the Far-Western blotting technique, however we were unable to detect the presence of the fluorophore binding to Tie2 despite repeated experiments. We then utilized a traditional immunoprecipitation and western blotting method, using the SH2 fusion proteins to precipitate Tie2, which was demonstrated in Figure 5. It was also determined that amounts as low as 100 ng of the Shp2 N-C fusion protein was sufficient enough to precipitate Tie2, shown in Figure 6. Based on the number of phosphorylation sites Shp2 was known to bind on Tie2, we determined that this amount was sufficient enough for all of our purified SH2 proteins. We repeated the immunoprecipitations with the titrated amount, along with a negative control, which was the precipitation of Tie2 in the absence of any SH2 proteins. Unexpectedly, we found that Tie2 bound to nickel-sepharose in a non-specific manner. As a means to circumvent this observation, we repeated the experiments using ProteinA-sepharose as opposed to nickel-sepharose, in conjunction with an anti-YFP antibody.

When probed for the presence of Tie2 however, it appears that the YFP-SH2 fusion complex was unable to precipitate Tie2 (Figure 8A), despite the fact that Tie2 was in fact present (Figure 8B), and that we were able to successfully pull down the SH2 protein via the YFP antibody (Figure 8C).

The fact that Tie2 bound to nickel-sepharose beads non-specifically without any SH2 protein present was surprising to us, as there do not seem to be any possible binding sites on the receptor that may have an affinity for nickel ions. There are a few possibilities for this occurrence. One of them is the possibility of cellular debris in our lysates due to improper washing. Previous attempts at altering our washing included pH changes and salt concentration changes to the buffer. We also attempted to wash the beads using 200 mM of imidazole, based on our successful purification of the SH2-YFP fusion proteins, as imidazole quenched the interaction between the Histidine tagged protein and the cobalt-charged column, and therefore its addition to our sample would possibly relieve the binding of Tie2 to the nickel beads, at the same time being outcompeted by the SH2 protein for binding of Tie2. Unfortunately after thorough imidazole washing we were unable to resolve the unspecific binding.

We were also surprised that we were unable to successfully precipitate Tie2 using Protein A sepharose, as previous immunoprecipitation studies in our lab has shown that this was possible in the presence of different antibodies. However when further investigated, Tie2 was present in our lysates due to the presence of the myc tag, and that the immunoprecipitation was successful with respect to the SH2-YFP fusion protein. This ultimately leads us back to the issue that the effectiveness of our SH2 proteins binding to Tie2 cannot yet be determined due to the unspecific binding occurrence.

However, we remain optimistic by the fact that the SH2 protein can be precipitated via the anti-YFP antibody, which leads us to believe that Tie2 can be pulled down from this complex as well.

Further attempts to resolve non-specific binding of Tie2 were taken by again changing the type of beads utilized for immunoprecipitation, this time with IMAC sepharose activated with cobalt chloride. We were able to successfully precipitate Tie2 in overexpressed systems using the SH2 domains of Shp2 N-C and Grb2 (Figure 9), however attempts to pull down Tie2 with our other fusion proteins were unsuccessful, due to the fact that Tie2 bound non-specifically to the IMAC beads. Our immediate future directions will be to continue using the cobalt activated beads for immunoprecipitation, while altering various aspects of our phosphorylation assay. We then aim to validate our studies with the Tie2 mutants we have generated as shown in Figure 10, and determining which sites of Tie2 are key in activating downstream signaling pathways.

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Vita

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