ENGINEERING NOVEL ANGIPOIOETIN RECEPTOR LIGANDS

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Abstract

Angiopoietin-1 is a multimeric glycoprotein which signals through the vascular endothelial Tie2 receptor to protect against inflammation and leakage, an effect antagonised by the selectively upregulated antagonist angiopoietin-2. In many pathologies this destabilisation by angiopoietin-2 is excessive or inappropriate, and here angiopoietin-1 has a promising role in therapeutic application. However angiopoietin-1 is difficult to purify and administer, its large multimeric structure rendering it prone to aggregation and insolubility. In this work the abilities of two small heptameric Tie2 binding peptides, VTSRGNV and NLLMAAS, multimerised using the established oligomeric scaffold cartilage oligomeric matrix protein (COMP), to bind and activate Tie2 were investigated. cDNAs for synthetic ligands were created by PCR, and protein synthesis was carried out in mammalian and bacterial expression systems. Ligands were expressed as stable, soluble pentamers and tetramers which showed similar abilities to bind Tie2 in vitro. Both ligands activated Tie2 similarly in Eahy926 and HUVEC endothelial cells. Both ligands were also able to activate two important downstream signalling mediators of Tie2 in HUVECs, namely Akt and ERK, in a dose-dependent fashion. However, the kinetics of ERK appeared different between the two ligands, implying possible differences in signalling of the two ligands through Tie2. This work is proof of principle and is among the first work to demonstrate that Tie2 binding elements other than the ang1 FRD can be used to activate Tie2. Additionally, kinetics work suggests that the two ligands, presumed to bind to different areas on Tie2, might induce slightly different patterns of receptor activation.
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List of Abbreviations

ABIN-2 A20 Binding Inhibitor of NFκB
CCOD Coiled-coil Oligomerisation Domain
ECM Extracellular matrix
ERK Extracellular Ligand-regulated Kinase
FAK Focal Adhesion Kinase
FKHR Forkhead Transcription Factor
FGF Fibroblast Growth Factor
FLD Fibrinogen-like Domain
HMEC Human Microvascular Endothelial Cell
HUVEC Human Umbilical Vein Endothelial Cell
ICAM Intercellular Adhesion Molecule
Ig Immunoglobulin
LPS Lipopolysaccharide
MAPK Mitogen-activated Protein Kinase
NFκB Nuclear Factor Kappa B
PAK p21-activating Kinase
PI3K Phosphatidylinositol 3-Kinase
rtp Room temperature and pressure
SCD Superclustering Domain
SH2 Src Homology 2 Domain
Smac Second Mitochondrial Activator of Caspases
TNF-α Tumour Necrosis Factor alpha
VCAM Vascular Cell Adhesion Molecule
VEGF Vascular Endothelial Growth Factor
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1: Introduction
Angiopoietins are secreted glycoproteins which function as ligands for the Tie2 receptor. The latter is found mainly upon cells of the vascular endothelium (Dumont, 1992; Partanen, 1992). The angiopoietin family consists of angiopoietin-1 (ang1) (Davis, 1996), angiopoietin-2 (ang2) (Maisonpierre, 1997), angiopoietin-3 (Kim, 1999) and angiopoietin-4 (Valenzuela, 1999) thus far. Ang4 is considered to be the human counterpart of mouse ang3 (Valenzuela, 1999). Ang1 and ang2 are better characterised.

The importance of both Tie2 and ang1 in embryonic vascular morphogenesis is paramount, as is illustrated by the lethal embryonic vascular defects which occur in transgenic mice lacking either angiopoietin-1 or Tie2 (Sato, 1995; Dumont, 1994).

The characteristic features of ang1 and ang2 are opposing actions on intracellular signalling through Tie2. Available evidence suggests strongly that ang1 functions as a constitutive signal to maintain the structural integrity of the vascular endothelium whereas ang2 acts as an antagonist at Tie2. Ang2 therefore destabilises the endothelium as required, for example, in situations where inflammation or angiogenesis occur (Brindle, 2006; Eklund, 2006; Scharpfenecker, 2005; Jones, 2001; Maisonpierre, 1997; Davis, 1996). The complementary actions of other vasoactive growth factors, such as vascular endothelial growth factor, are also incorporated into the final effect on the vascular endothelium (Hanahan, 1997).

1.1. Formation and Maintenance of the Vasculature

1.1.1 Mechanisms of Vessel Formation

Formation of the vasculature is a coordinated process which is regulated tightly. There are two main processes. In the first, vasculogenesis, mesenchymal precursors establish an initial vascular network (Choi, 2002). In the second,
angiogenic remodelling modifies this primitive set of structures into a branched and organised network, which is observed subsequently in the embryonic and mature vasculature. Most of this process occurs by branching and vessel enlargement (Yancopoulos, 2000). Subsequently, and as occurs in most adult tissues, new blood vessels arise by sprouting from pre-existing blood vessels (Yancopoulos, 2000) and vessels grow into previously avascular tissue (Risau, 1997).

The initial event in angiogenesis is endothelial cell activation, initiated in response to factors such as hypoxia (Carmeliet, 2005). Cytokines are upregulated under these conditions, particularly vascular endothelial growth factor (VEGF) (Forsythe, 1996). The latter arises mainly from macrophages and hypoxic tumour cells (Brown, 1997). Subsequently, activated endothelial cells initiate the secretion of proteolytic enzymes, in conjunction with related cells such as fibroblasts and macrophages (Forsythe, 1996). This allows loosening of endothelial cell contacts with their associated tethering vascular basement membrane (Forsythe, 1996), as well as selective degradation of the extracellular matrix (Griffioen, 2000), ultimately leading to vascular wall destabilisation. This leads to the release of fibrin which has been suggested to serve as a scaffold for endothelial cell migration (Dvorak, 1987). The endothelial cells migrate towards chemotactic stimuli, proliferating and forming tubular structures as they progress, with the aid of locally produced plasmin (Patan, 2000). The latter aids in the migration of endothelial cells by degrading fibronectin, laminin and proteoglycans in the ECM. Additionally it aids in the liberation of other ECM-bound angiogenic factors such as FGF. Endothelial tubes coalesce to form closed vascular loops. Finally the formation of stable vessels requires interaction of endothelial cells with the extracellular matrix and mesenchymal cells (Griffioen,
2000). In small vessels these cells are known as pericytes, whereas in larger vessels smooth muscle cells surround the vessel.

1.1.2. Vascular Growth Factors and Angiogenesis

Tie2 and ang1 are essential for normal vascular development, along with the related vascular endothelial growth factor (VEGF) and its receptors (Hanahan, 1997). The structure, interactions and signalling of ang1 and Tie2 are discussed in more detail below. VEGF was identified as the first selective angiogenic factor for endothelial cells (Alon, 1995). Thus far six members of the VEGF family with distinct roles have been identified, namely VEGF A-E and Placental-Derived Growth Factor (PDGF). They interact with and signal through three subsets of receptor, named VEGFR 1-3 (Byrne, 2005). VEGF is a powerful endothelial cell mitogen and survival factor, induces microvascular permeability, and stimulates endothelial cell migration (Breen, 2007; Byrne, 2005) Studies with knockout mice have shown that the actions of VEGF are critical at a primary stage in blood vessel formation. Mice engineered to lack VEGFR2 simply fail to develop any type of vasculature, and also lack a haematopoietic system. They die at around day 8 (Shalaby, 1995). This is consistent with VEGFs critical role in the initiation of both vasculogenesis and angiogenesis (Hanahan, 1997), via the initial differentiation of angioblast precursors into endothelial cells.

Tie2 knockout mice show a different picture. The mice also die relatively early in embryogenesis, but later than VEGFR2 knockout mice, at around day 10 (Hanahan, 1997). Strikingly, endothelial cells are present in normal numbers and are assembled into tubes. However the vessels lack branching and are not properly organised into large and small vessels (Sato, 1995; Dumont, 1994). Furthermore, and
most demonstrable in the developing heart, the vessels are not encapsulated by supporting pericytes. Endothelial cells are not correctly adherent to the underlying basement membrane and extracellular matrix (Sato, 1995; Dumont, 1994). The phenotypes of ang1 null mice are similar to those of Tie2 knockout mice, consistent with the former’s role as agonist via the latter in blood vessel formation (Suri, 1996). Interestingly, the phenotype resulting from overexpression of ang2 is similar to that of Tie2 knockout, consistent with the antagonistic role of ang2 in Tie2 signalling (Maisonpierre, 1997).

In adult animals VEGF inactivation does not have such a profound consequence, seemingly affecting those vessels where angiogenic remodelling occurs frequently, for example, bone growth plates or corpus lutei (Gerber, 1999; Ferrara, 1998). However there is evidence that VEGF is required for the maintenance of some vascular beds that are not remodelling, particularly those in which significant permeability is actually desirable. This includes the fenestrated glomerular endothelium and the hepatic sinusoidal endothelium (Maharaj, 2006). VEGF expression in most quiescent vascular beds in the adult is, perhaps unsurprisingly, low. Indeed exposure of such vascular beds to exogenous high levels of VEGF leads to angiogenesis, but with haemorrhagic and leaky vessels (Yancopoulos, 2000). On the other hand, withdrawal of VEGF seems only to be important in those tissues in which angiogenesis is occurring, such withdrawal leading to regression not of established vessels but of neoangiogenic vessels. This is most clearly demonstrable in tumour vasculature (Holash, 1999).

In stark contrast, in the adult Tie2 is seen to be active constitutively in quiescent vasculature, suggesting a maintenance function (Wong, 1997). Consistent with this, blocking Tie2 signalling in vivo by the overexpression of ang2 leads to
profound vascular leakage and endothelial cell apoptosis in some studies (Roviezzo, 2005). Furthermore, overexpression of ang1 in adult mice by adenoviral delivery seemed to maximise interactions between endothelial cells and their supporting pericytes and extracellular matrix, with the resultant effect that the vessels of the mice were resistant to agents which promote vascular leakage (Thurston, 2000).

The evidence, taken cumulatively, supports the proposal of a model for the roles of VEGF and the angiopoietins in blood vessel formation and maintenance (Hanahan, 1997). Ang1-Tie2 signalling promotes vessel maturation and elaboration, in addition to a continual role thereafter in maintaining vessel interactions with surrounding support cells and matrix. Ang2 blocks this to destabilise the vessel, but the expression of ang2 alone does not induce angiogenesis, rather allows it to occur by decreasing endothelial-matrix interactions. In the presence of a further inducer of angiogenesis, such as VEGF, endothelial cell sprouting, tube formation, migration and reorganisation can occur. In the absence of a supporting angiogenic growth factor vascular regression seems to occur. The latter suggestion is highlighted particularly by the study of Maisonpierre et al (1997) who noted that, in the developing ovarian follicle, whilst high levels of ang2 and co-associated VEGF were associated with angiogenesis, non-productive ovarian follicles showed only ang2, and endothelial cell apoptosis. A similar situation is also seen in the vasculature of some tumours (Holash, 1999).

1.1.3. Pathological Vascular Destabilisation

There are many conditions, discussed in section 1.8, where evidence is emerging for the critical role of vascular destabilisation as a pathological foundation for disease. Examples include systemic inflammatory response syndrome (SIRS),
diabetic retinopathy, transplant rejection and stroke (Brindle, 2006). Additionally, there are conditions where vascular regression plays a fundamental role in disease processes, of which examples include poor wound healing and radiation side-effects on bone marrow and intestine (Brindle, 2006). This had led to the question of whether angiopoietin-1 might have potential therapeutic uses, given its role in blood vessel formation and maintenance.
1.2 Structural Characteristics Of Angiopoietins

Ang1 and ang2 have a similar structure. The two structures have amino acid sequence homology of approximately 60% (Maisonpierre, 1997). Each structure has a carboxyl terminal fibrinogen-like domain (FLD) of about 200 amino acids. This region contains the Tie2 receptor binding region for both ang1 and ang2 (Procopio, 1999). The structure of this FRD region has been characterised particularly well for human ang2 (Barton, 2005). It is composed of three domains, known as A, B and P domains (Barton, 2005). The P domain is small and, in contrast to the A and B domains, has little secondary structure. It consists mainly of coiled-coiled regions, with 2 short α helices and 2 short β strands. It packs underneath the B domain on the undermost surface of the FLD domain. It is this domain which is necessary for Tie2 binding (Barton, 2006; Barton, 2005). The coiled-coil domain mediates the formation of disulphide-linked homo-oligomers from individual monomers (Kim, 2005; Procopio, 1999), whilst the N-terminal superclustering region links these oligomers together into higher order multimers (Kim, 2005; Davis, 2003; Procopio, 1999). In the case of ang1 50-60% of structures are higher order (Kim, 2005; Davis, 2003). Contrastingly ang2 exists mainly as dimers, with a significantly smaller proportion of higher order structures (<20%) (Kim, 2005; Davis, 2003; Procopio, 1999). This multimerisation difference may have a role in the differential activities of ang1 and ang2 (Kim, 2009). The linker region allows secreted ang1, but not ang2, to bind to the extracellular matrix (Xu, 2001).
1.3 Distribution of Angiopoietins

1.3.1 Angiopoietin-1

Angiopoietin-1 is produced widely and constitutively by the periendothelial cells of the vasculature (Brindle, 2006; Davis, 1996). Secreted ang1 is initially adherent to the extracellular matrix via its linker region (Xu, 2001), a form in which it seems not to be available to activate Tie2 (Xu, 2001). However, adhesion to vascular endothelial cells leads to the release of the biologically active ang1 (Xu, 2001). The mechanisms and factors controlling regulation of ang1 synthesis and secretion are poorly understood. Ang1 expression has been demonstrated to be increased in some inflammatory conditions such as rheumatoid arthritis, possibly via the actions of tumour necrosis factor-α (TNF-α) or interleukin-1β on the ESE-1 transcriptional activating factor (Brown, 2004). Also, ang1 has been reported in vitro to be upregulated by hypoxia and VEGF in bovine pericytes (Park, 2003). In systemic inflammatory response syndrome expression of ang1 may be reduced (David, 2010, Giuliano, 2007) However in other inflammatory conditions such as cerebral ischaemia-reperfusion injury there is no evidence that ang1 expression is altered (Hansen, 2008).

1.3.2 Angiopoietin-2

In contrast to ang1, ang2 is not detected in a range of tissues in the adult. It is detected predominantly at sites of vascular remodelling such as the ovary, placenta, uterus and healing wounds (Maisonpierre, 1997). In such tissues it is also observed to be expressed earlier than ang1, preceding the sprouting of new vessels, whereas in these tissues ang1 coincides with or follows vessel growth (Maisonpierre, 1997). This
is consistent with the antagonistic function of ang2, allowing a blockage of ang1-mediated vessel stabilisation during the period of remodelling.

Ang2 is produced and stored in vascular endothelial cells themselves, in cytoplasmic granules known as Weibel-Palade Bodies (Fiedler, 2004). This means that ang2 is readily available for rapid vascular homeostatic reactions, since it can be released within a few minutes of stimulation (Fiedler, 2004), with subsequent effects of ang2 demonstrable clearly within 30 minutes (Scharpfenecker, 2005). A range of stimuli has been shown to modulate ang2 release, including thrombin (Fiedler, 2004), histamine (Fiedler, 2004), angiotensin II (Otani, 2001), prostanoids (Otani, 2001), VEGF (Goettsch, 2008; Pichiule, 2004; Zhang, 2003; Oh, 1999; Mandriota, 1998), blood flow (Goettsch, 2008) and hypoxia (Oh, 1999; Mandriota, 1998). In humans ang2 expression is elevated in severe sepsis (Davis, 2010; Kumpers, 2010; Kranidioti, 2009; van der Heijden, 2009; Parikh, 2006).

1.4 Structure and Distribution of the Tie2 Receptor

Tie2 is a Tie (‘tyrosine kinase with immunoglobulin and EGF-like homology domains’) receptor tyrosine kinase identified on the basis of structural homology with other tyrosine kinases (Dumont, 1992; Partanen, 1992). It is a 150kDa transmembrane protein with a 730 amino acid extracellular domain binding the angiopoietins (Davis, 1996; Dumont, 1992; Partanen, 1992), a short transmembrane region and a split intracellular tyrosine kinase domain (Fiedler, 2003; Dumont, 1992; Partanen, 1992). The latter transphosphorylates tyrosine residues on adjacent Tie2 intracellular domains when they are in close proximity. This leads to signal amplification. Subsequently further tyrosine residues on the intracellular domain are phosphorylated
and then act as docking sites for downstream mediators of signalling to the cell (Hubbard, 2000).

1.4.1. Expression of the Tie2 Receptor

Tie2 is expressed mainly on vascular endothelial cells and their precursors (Dumont, 1995; Schnurch, 1993). It is also expressed to a lesser and variable extent on non-endothelial cells such as fibroblasts, mural cells, keratinocytes, neutrophils, monocytes, eosinophils, synovial membrane cells, cancer cells and neurones (Brkovic, 2007; Kosacka, 2005; Nakayama, 2005; Voskas, 2005; Metheny-Barlow, 2004; Nakayama, 2004; Iurlaro, 2003; Uchida, 2000), either in normal physiological states or disease states.

Tie2 is not only expressed in tissues in the adult where active angiogenesis is occurring, for example ovarian follicular maturation, during uterine development, and in healing skin wounds (Kampfer, 2001; Wong, 1997), but is also expressed widely in quiescent vascular beds where no remodelling is occurring (Wong, 1997). Furthermore Tie2 is phosphorylated and active in this quiescent vasculature (Wong, 1997).

Though Tie2 is expressed constitutively its expression has been reported to be upregulated in vitro by VEGF, hypoxia, TNF-α, IL1-β (Park, 2003; Christensen, 2002; Willam, 2000), and under conditions of ischaemia in vivo (Lin, 2001; Willam, 2000). Tie2 expression has also been reported in one study to be increased relative to the basal state in healing wounds (Wong, 1997). Also, increased Tie2 expression has been noted in several tumours (Moon, 2006; Hata, 2004; Yu, 2001), including some tumour cells themselves (Martin, 2008).
1.4.2. Structure of the Tie2 Ectodomain

The ectodomain of Tie2 consists structurally of two N-terminal immunoglobulin (Ig)-like domains (Ig1 and Ig2) of about 200 amino acids (Barton, 2006; Macdonald, 2006) followed by three epidermal growth factor homology domains, after which is a further approximately 100 amino acid Ig-like domain (Ig3). Following this are three fibronectin-like structural repeats. The crystal structure of the Tie2 ectodomain has recently been determined in a complex also containing bound ang2 FLD (Barton, 2006). This shows clearly that there is an extensive amount of area from Ig1, Ig2 and the three EGF repeats which is folded into itself and buried. The resultant structure is an arrowhead-like shape with the sides formed by Ig1 and EGF3. At the tip of the arrowhead lie exposed surface loops of Ig2. It is this surface, almost at its tip in fact, where the P domain of ang2 binds (Barton, 2006).
1.4.3. Tie2 Structure and Angiopoietin Binding

Most of the current structural information on binding of the angiopoietins to Tie2 derives from the crystallographic and mutagenesis studies of Barton and colleagues (Barton, 2006; Barton, 2005), and relates to ang2. They identified residues in the ang2 FRD which were in good positions to make van der Waals interactions, hydrophobic interactions and salt bridges, in a crystal complex of ang2 FRD with Tie2 ectodomain (Barton, 2006). From residues 149-168 on the Tie2 Ig2 domain the authors identified 11 amino acids involved in interactions with ang2, with a further lysine at 194 on the Ig2 domain also involved. Correspondingly 13 amino acids from residues 417-480 on the ang2 FRD were seen to be involved in interactions with Tie2 (Barton, 2006). The binding of ang2 to the Tie2 ectodomain was suggested by these studies to proceed via a lock and key mechanism, with
Though the structure of the ang1-Tie2 complex is not known presently, structurally it is likely that ang1 binds Tie2 itself in a similar fashion (Barton, 2006). This is based on the observation of similar binding affinities of ang1 and ang2 for Tie2 (Maisonpierre, 1997). Additionally, homology sequence comparison between ang1 and ang2 reveals six of the thirteen amino acid residues in ang2 which interact with Tie2 to be the same in ang1 (Barton, 2006). Furthermore, the ang2-Tie2 binding mechanism proceeds with minimal structural change in either component on binding (Barton, 2006). Also in support of this, Tie2 receptor mutants for several key residues in the binding residues for ang2, created by Barton (2006), also abrogated ang1 binding. The question of how the apparently similar binding characteristics of ang2 and ang1 can lead to almost opposite outcomes is still unclear. The higher order multimeric state of ang1 might influence activation, over the mainly dimeric ang2 (Brindle, 2006), particularly as discussed above ang1 must be at least tetrameric to activate endothelial Tie2 (Kim, 2005; Davis, 2003). This is supported by the observation in one study that similar multimeric constructs of both the ang1 and ang2 FRD had almost identical activity at Tie2 (Kim, 2009). However, in direct opposition to this, Davis (2003) also engineered tetrameric forms of ang1 and ang2 FRD domains, using a different multimerisation motif. They observed that whilst the ang1 tetramer was able to activate Tie2 receptors in endothelial cells, the similar tetramer of ang2 was not. Therefore it is likely that multimerisation differences do not account fully for the observable differences in activity. Indeed though ang2 is mainly seen to be antagonistic to ang1 with regard to endothelial Tie2, a more accurate description of ang2 is as a partial agonist. For instance, in nonendothelial cells transfected to express
Tie2, dimeric ang2 can induce Tie2 (Davis, 2003; Maisonpierre, 1997). Also, if applied for long periods or at high concentrations in vitro, ang2 can activate endothelial Tie2 and signal for downstream functional effects known to be associated with ang1 (Mochizuki, 2002; Korff, 2001; Teichert-Kuliszewska, 2001; Kim, 2000). One additional explanation might be the presence of an inhibitory co-receptor which inhibits a response to ang2 signalling versus ang1 by some method. Indeed Tie2 associates closely with the related Tie1 receptor in vivo (Marron, 2000). Until recently the role of Tie1 was not known. However, recent evidence suggests that it exerts a modulatory role in Tie2 signalling, appearing to inhibit Tie2 signalling via ang1 without affecting that of ang2 (Hansen, 2010; Seegar, 2010; Marron, 2007).

1.5. Angiopoietin Signalling and Effects

Dimerisation of the Tie2 receptor, dependent on ang1 binding, leads to activation of the kinase domain (Hubbard, 2000). Subsequent autophosphorylation of specific tyrosine residues serves to create docking sites for effectors which couple receptor activation to intracellular signalling (Hubbard, 2000; Murray, 2001).

Angiopoietin-1 induces survival of endothelial cells in vitro, as well as endothelial migration, sprouting and tube formation. Ang1 also has vascular endothelial anti-inflammatory and anti-permeability effects. However, current prevailing evidence does not support conclusively any mitogenic role for ang1 (Brindle, 2006). In vitro actions mediated by ang1 through Tie2 are summarised in figure 1.6.
1.5.1. Endothelial Cell Survival

Angiopoietin-1 inhibits apoptosis in several endothelial cell types (Harfouche, 2002), an action dependent on signalling through Tie2. (Papapetropoulos, 2000; Kontos, 1998). This has been noted in response to irradiation; serum deprivation; and TNF-α (Chen, 2004; Kwak, 2000).

Phosphorylation of the Tie2 receptor leads to recruitment of the regulatory p85 subunit of phosphotidylinositol 3-kinase (PI3k), via its SH2 domain, to the docking site phosphorylated tyrosine 1101 (Kontos, 1998). Downstream, subsequent activation of Akt occurs, a major influence in protection against cell apoptosis (Harfouche, 2002; Kim, 2000; Papapetropoulos, 2000). Further downstream the transcription factor FKHR, which is involved in control of several genes concerned with vessel remodelling and pro-apoptotic cellular consequences, is inhibited by phosphorylation (Daly, 2004). The end results include inhibition of the apoptosis enzymes caspases-3, -7 and -9, the upregulation of the inhibitor of apoptosis Survivin, and the downregulation of the caspase–activating protein Smac (Harfouche, 2002). Modulation of phosphorylation of the ERK1/2 pathway is also likely to contribute to the protective downstream effects of Akt (Harfouche, 2003).

Ang1 also has pro-survival effects on some nonendothelial cells. An anti-apoptotic effect dependent on Tie2 has been observed in mouse cortical neurones (Valable, 2003), in addition to cardiac and skeletal myocytes (Dallabrida, 2005). In the case of the latter cells no Tie2 is expressed, and the signalling pathways are unknown, but may be mediated by the association of Ang1 with cellular integrins (Dallabrida, 2005).
1.5.2. Anti-Inflammatory Actions

Angiopoietin-1 mediates a reduction in the activation state of the endothelium in response to a number of pro-inflammatory agents. Ang1 reduces the upregulation of E-selectin, ICAM-1 and VCAM-1 which occurs in response to VEGF, with a resultant decrease in leucocyte adhesion to endothelial cells (Kim, 2001). This effect can be blocked by soluble Tie2 receptor, and also by the use of wortmannin, an inhibitor of the PI 3-kinase/Akt signalling pathway. Furthermore ang1, signalling through Tie2, was able to abolish transmigration of leucocytes across endothelial monolayers in response to TNF-α (Gamble, 2000). Ang1 has also been found to block the VEGF-induced expression of tissue factor in HUVECS (Kim, 2002). The signalling pathways have yet to be demonstrated, but in part likely involve suppression of NFκB, a transcription factor involved pro-inflammatory gene expression in response to VEGF and TNF-α (Hughes, 2003). The observed activation of ABIN-2 downstream of PI 3k is likely to play a role in the inhibition observed in ang1 on TNF-α and VEGF-mediated leucocyte adhesion, transmigration and the upregulation of inflammatory molecules (Brindle, 2006; Hughes, 2003).
Figure 1.6: Schematic analysis of identified and potential signalling pathways for angiopoietin 1 through Tie2. The miR-96 phosphotyrosine P1, P2 and P3 represent residues 1101, 1106 and 1113 respectively, known docking sites for regulators of downstream intracellular pathways. Downstream mediators are abbreviated as discussed in the Abbreviations section (p.5) and are discussed in detail in the text of section 1.5.
1.5.3. Inhibition of Endothelial Barrier Permeability

Angiopoietin-1 inhibits the permeability of endothelial monolayers in vitro. Gamble et al (2000) demonstrated a decrease both in basal endothelial layer permeability, and also in response to the pro-permeability stimuli VEGF and thrombin, in HUVECs and human umbilical arterial cell layers. At the glomerular filtration barrier podocyte-expressed ang1 has been observed to inhibit the permeability induced by VEGF in human glomerular endothelial layers in vitro (Hori, 2004; Satchell, 2004).

The mechanisms for the anti-permeability effects of ang1 are not entirely clear, but are mainly via signalling through Tie2 (Gamble, 2000). Ang1 enhances PECAM-1 localisation to interendothelial junctions and decreases its phosphorylation, as well as decreasing phosphorylation of VE cadherin. Phosphorylation of these proteins which promote endothelial barrier integrity is associated with an enhanced permeability of endothelial layers in vitro, in response to histamine, thrombin and VEGF (Gamble, 2000), with or without frank disruption of intercellular adherens junctions. In fact it has been demonstrated directly that ang1 abrogates the phosphorylation and internalisation of VE cadherin in response to VEGF (Gavard, 2008). A downstream mediator of Tie2, mDia, binds and inactivates a downstream signal of the VEGF receptor VEGFR2, known as src, which ultimately leads to downregulation of VE cadherin in the absence of ang1. This action of VEGF induces endothelial layer permeability (Gavard, 2008), at least in part by disrupting the interaction of VE cadherin with β-catenin and the cell cytoskeleton (Schnittler, 1998). Ang1 may also interfere with VEGF-induced PLC (phospholipase C)-dependent Ca\(^{2+}\) influx (Jho, 2005). Furthermore the dissociation of VE cadherin from intracellular components secondary to VEGF may require PKC-β, which is inactivated by ang1
The ang1 induced downregulation of activated PKζ seems also to have a role in reducing thrombin-mediated endothelial permeability (Li, 2004).

In vitro studies also suggest ang1 has a potent anti-permeability effect in the blood brain-barrier. It has been shown to increase expression of the tight junction protein occludin (Hori, 2004), a protein known to be suppressed by VEGF-induced phosphorylation (Antonetti, 1999). Additionally, in vitro ang1 also enhances the expression of a protein closely associated with occludin, ZO-2, leading to a resistance to VEGF-induced hyperpermeability directly (Lee, 2009).

1.5.4. Endothelial Cell Migration

The stimulation of endothelial cell migration by ang1 requires Tie2 (Jones, 2003). One pathway involves the recruitment of Dok-R protein to the phosphorylated tyrosine residue 1106 on the intracellular domain of Tie2 (Jones, 2003; Jones, 1998). Dok-R is itself then phosphorylated and recruits a downstream kinase, PAK (Master, 2001) to induce a pro-migratory effect. Other pathways are less well defined, but one likely involves recruitment of the adaptor protein ShcA to Tie2 at phosphotyrosine 1101 (Iurlaro, 2003), with downstream activation of the MAPK pathway having a role in migration (Iurlaro, 2003). Additionally PI3K has a role in ang1-mediated motility via activation of FAK (Cascone, 2003) and RhoA/Rac1 GTPases (Kim, 2000).

Additionally ang1 can promote chemotaxis of nonendothelial cells. This has been observed for rat smooth muscle cells which express Tie2 (Metheny-Barlow, 2004; Iurlaro, 2003), as well as cells such as fibroblasts which do not express Tie2 (Carlson, 2001). Indeed in some ang1-mediated migration has been observed to occur through intermediary chemotactic molecules (Nishishita, 2004; Iivanainen, 2003). Such observations shed a small amount of light on potential mechanisms by which
Ang1 might not only recruit endothelial cells in the remodelling vasculature, but also mediate interactions between endothelial cells and pericytes, consistent with the role of Ang1 in vascular maturation and stabilisation.

1.5.5. Reorganisation and Sprouting

Ang1 induces endothelial sprouting and the reorganisation into tubule-like structures in vitro (Chen, 2004; Kim, 2000; Hayes, 1999; Koblizek, 1998), phenomena seemingly requiring Tie2 (Koblizek, 1998). Downstream intracellularly the adaptor protein ShcA (Audero, 2004), FAK (Kim, 2000), PI3k (Chen, 2004) and endothelial NO synthase (Chen, 2004) have been suggested experimentally as involved mediators. Effectors of reorganisation observed in vitro as a result of Ang1 are matrix metalloproteinases (Das, 2003; Kim, 2000) and plasmin (Kim, 2000).

1.6. Angiopoietin-1 Vascular Protection in Vivo

Although angiopoietin-1 is a promoter of angiogenesis in the embryo its main role in the adult is the maintenance of the vasculature in a quiescent state. Evidence currently supports strongly a model in which angiopoietin-1, expressed widely in the periendothelial cells of the vasculature (Davis, 1996), provides a constitutive signal to promote quiescence of the endothelium via Tie2 (Maisonpierre, 1997). In areas of endothelial activation and angiogenesis upregulation of angiopoietin 2 occurs (Hanahan, 1997), in conjunction with other pro-angiogenic cytokines, to destabilise the vasculature. This allows remodelling. Inhibiting destabilisation is crucial to the protective effects of Ang1, and is achieved by the inhibition of apoptosis, vascular leakage and inflammation in vivo, via mechanisms reflective of its in vitro actions discussed in section 1.5. Examples in preclinical studies of inhibition of endothelial
cell apoptosis include microvascular endothelial cells of the rat intestine (Cho, 2004), the retinas of diabetic mice (Joussen, 2002) and mouse sepsis models (Childs, 2008; Huang, 2008). Examples of significant in vivo anti-permeability effects of ang1 include reducing retinal oedema and urinary microalbuminuria in diabetic mice (Lee, 2007; Joussen, 2002), reducing blood-brain barrier breakdown in mouse models of focal cerebral ischaemia (Zhang, 2002), and promotion of generalised resistance to vascular leak in response to challenge with inducing agents such as PAF and 5-HT (Thurston, 2000). Examples of anti-inflammatory effects in vivo include reductions in expression of leucocyte adhesion molecules, as well as reductions in the actual adhesion and extravasation of leucocytes, in diabetic retinopathy (Joussen, 2002), diabetic nephropathy (Lee, 2007b), sepsis (Huang, 2008; Witzenbichler, 2005) and cardiac allograft rejection (Nykanen 2003), all in mouse models. Evidence for these protective effects of ang1 is discussed in more detail in section 1.8.

1.7. Roles of Angiopoietin-2 and Vascular Endothelial Growth Factor

Angiopoietin-2 is a natural antagonist of ang1 at the Tie2 receptor (Maisonpierre, 1997). There is significant evidence that it is a partial agonist, as discussed in section 1.4.3. In adult mice the expression pattern of ang2 is limited to areas of the vasculature which are undergoing vascular remodelling, or where there is inflammation. In fact ang2 is crucial in this regard, since mice deficient in ang2 cannot generate sufficient protective inflammatory responses (Fiedler, 2006). In quiescent vasculature it is expressed at significantly lower levels than ang1, the latter being expressed widely throughout the vasculature (see section 1.3.2.). The evidence above suggests that ang1 serves as a constitutive signal to the endothelium to remain
‘quiescent’, reflected by the baseline level of Tie2 phosphorylation observed in vivo (Wong, 1997). At sites of vascular remodelling or inflammation ang2 is upregulated and the ang2:ang1 ratio becomes greater, favouring destabilisation of the vessel, probably a prerequisite for angiogenesis. This is thought to be a context-dependent process whereby ang2 might sensitize endothelial cells to the actions of other growth factors, such as VEGF (Hanahan, 1997) in vascular remodelling and angiogenesis, as well as TNF-alfa (Fiedler, 2006) in inflammatory responses. In the presence of VEGF a high ang2:ang1 ratio leads to remodelling, whereas in its absence there is evidence this favours vascular regression (Scharpfenecker, 2005; Holash, 1999; Hanahan, 1997).

This relationship is also evident in disease. Scharpfenecker and colleagues showed that ang2 disturbs the integrity of endothelial monolayers in a three-dimensional model in vitro, leading to detachment and leakiness. This effect could be blocked by ang1 (Scharpfenecker, 2005). In vivo, in a mouse paw model, administration of recombinant ang2 induced oedema in a dose-dependent manner (Roviezzo, 2005). The oedema was associated with extravasation of neutrophils into the tissue. This effect was blocked by coadministration, again in a dose-dependent manner, of soluble Tie2 receptor or recombinant ang1. In humans the vascular destabilisation occurring in ARDS in critically-ill patients is at least in part secondary to ang2 upregulation and consequent alteration of ang2/ang1 ratios in favour of leakage (Parikh 2006, and see section 1.8.1.).
1.8. Potential Clinical Applications of the Protective Effects of Angiopoietin-1

The evidence above suggests a unique potential for ang1 in therapy to combat diseases in which regression of the vasculature, leakiness and excessive inflammatory responses are causative. Below the evidence is reviewed with respect to different pathologies.

1.8.1. Systemic Inflammatory Response Syndrome

In the Systemic Inflammatory Response Syndrome (SIRS), there is acute, diffuse damage to multiple vascular beds, particularly the alveolar-capillary interface in the lungs (Gropper, 2008; Piantadosi, 2004), where adult respiratory distress syndrome (ARDS) often results. Damage to the endothelium (Zimmerman, 1999) with consequent disruption of its integrity, resulting intrapulmonary oedema, and inflammatory cell infiltration, occur (Gropper, 2008). The result is significant ventilation-perfusion mismatch, with a high mortality of around 30% (Milberg, 1995). The causes are numerous and heterogeneous, and include sepsis; lung contusion; pancreatitis and massive transfusion (Gropper, 2008). Cytokines released activate and damage the vascular endothelium, particularly TNF-α, IL-1β, IL-6 and IFN-γ (Pittet, 1995). As the pathological causes and inflammatory mediators are multiple, and probably heterogeneous, this may explain the lack of success of approaches to blocking particular cytokines (Gropper, 2008, Piantadosi, 2004).

Since ang1 has in vitro and in vivo anti-inflammatory, anti-apoptotic and anti-permeability actions, it may have an advantage since it acts on the vascular barrier itself. Supporting this, there are a number of human studies of critically-ill patients
which suggest strongly that ang2 levels rise in SIRS and ensuing ARDS and correlate with resulting organ dysfunction and mortality (Parikh, 2006), independent of other comorbidities such as cardiac failure or chronic renal disease, and reducing in survivors. This has been corroborated for ARDS arising with (Mankhambo 2010; Ebihara, 2009; van der Heijden, 2008; Gallagher, 2008; Orfanos, 2007) or without sepsis (van der Heijden, 2008) as a cause.

There are at least eight preclinical studies in mice suggesting that ang1 reduces the pathogenesis and severity in SIRS/ARDS. Six of these assessed the response to SIRS induced by challenge with LPS (Hwang, 2009; Kim DH, 2009; Huang, 2008; Guo, 2007; McCarter, 2007; Witzenbichler, 2005), which is similar to the syndrome of endotoxic shock in humans (Gutierrez-Ramos, 1997), whilst one assessed the response to hypovolaemic ischaemia-reperfusion in the small intestine (Childs, 2008). Another study observed the effects of ischaemia-reperfusion injury, as occurs during SIRS, on the kidney (Jung, 2009). Studies varied from using adenoviral or cell-mediated overexpression of ang1 (Jung, 2009; Huang, 2008; McCarter, 2007; Witzenbichler, 2005), to the use of native ang1 or an analogue (Hwang 2009; Childs, 2008; Guo, 2007). All studies noted effects consistent with ang1 vascular protection. These include reduced CD31 in lung tissue (Huang, 2008) and reduced caspase 3 activation in intestinal endothelium (Childs, 2008), suggesting reduced apoptosis. Additionally, there was evidence of reduced pulmonary vascular leakage of fluid and leucocytes in several studies (Huang, 2008; McCarter, 2007; Witzenbichler, 2005), with one group demonstrating inhibition of downregulation of an intercellular junction molecule, PECAM-1 in vivo by COMPang1 (Hwang, 2009). Mice treated with ang1 also showed significantly lower expression of VCAM-1, ICAM-1 and E-selectin, in response to LPS challenge, than controls (Witzenbichler, 2005), as well as
inhibition of lung architectural damage (Huang, 2008; McCarter, 2007). These effects could not occur without Tie2 (McCarter, 2007). The protective effects of ang1 were not confined to the lungs, but could also be observed in the kidney (Kim DH, 2009) and small intestine (Childs, 2008). Importantly, studies have also demonstrated substantial reductions in mortality to challenge with LPS after pretreatment with ang1. Witzenbichler (2005) described up to 50% reduction in mortality, whilst Huang (2008) noted that pre-treated mice had a survival of 63% at one week, compared with only 18% of untreated mice.

Naturally there are limitations in what we are able to derive from animal models, particularly physiologically healthy mice. Additionally, too many studies use adenoviral expression of ang1, a situation which would be less desirable clinically than the use of a soluble mediator. Also, in only two studies as highlighted above, was ang1 administered after challenge with LPS, and the studies are inconclusive with regard to protection here (Huang, 2008; Guo, 2007). However human studies of the ang1/2-Tie2 system in SIRS are emerging. Parikh and colleagues (Parikh, 2006) showed a potent effect of ang1 in counteracting the vascular leakage in 22 patients with defined SIRS secondary to sepsis. They found that peak ang2 levels correlated with reduced gas exchange, a suggestion of worsening intrapulmonary vascular leakage from ARDS (ang2 levels did not however correlate with cardiac failure or chronic lung disease, other comorbidities in the study which could also lead to impaired gas exchange). Moreover the high ang2 levels resolved as improvement occurred. Serum from these patients showed an ability to increase the permeability of HMEC monolayers in vitro. Furthermore this effect was correlated directly with circulating ang2 levels in patients at the time of sampling. As patients improved clinically and their ang2 levels fell to lower levels their serum lost this ability.
Exogenous Ang1 was able to reverse directly the profound vascular leakage which occurred when the most ill patients’ sera were applied to HUVEC monolayers in vitro, implying strongly that the raised serum ang2 levels were not just a circulating marker of sepsis, but also a causative factor in the endothelial disruption.

1.8.2. Retinopathy of Diabetes Mellitus

Retinal oedema is the single most important cause of visual loss in diabetes, principally by inducing neovascularisation (Moss, 1988). Overexpression of ang2 has been demonstrated to lead to vascular abnormalities very similar to diabetic retinopathy in mice (Pfister, 2009). Joussen et al (2002) showed that a single unilateral intraocular injection of 800ng of a recombinant analogue of ang1 induced a 50% suppression of retinal oedema in streptozocin-induced diabetic mice. Additionally, retinal leucocyte adhesion was inhibited. This method of delivery is also attractive since drug can be delivered to the site of interest with minimal or no effects in other areas of the body (Avery, 2006b). Certainly, intraocular administration of becavizumab has been reported to very well tolerated by human patients. It has been administered repetitively at short intervals, in order to reduce symptoms and disease in diabetic retinopathy and age-related macular degeneration (Avery, 2006a; Avery, 2006b).

However, there are several limitations of the data. There is only one study addressing ang1 use in animals. Also, there are no human studies. Additionally the model of induction of diabetes here was similar to a type-1 model of human diabetes, therefore not accounting for type-2 models (Rees, 2005). Also, it is not clear whether ang1 itself is capable of promoting neoangiogenesis, which would be a disastrous side-effect here. Indeed this has been observed, and in some studies induced
deliberately, though the mechanisms are unknown, and tend to be more associated with high expression of ang1 or the potent recombinant analogue COMPangiopoietin-1 (see section 1.9).

1.8.3. Stroke

Nearly 80% of strokes result from an embolus or local thrombosis causing decreased cerebral perfusion with ensuing ischaemic damage to the neuronal compartment (Suwanwela, 2007). This is accompanied by vascular leakage, microvessel inflammation and endothelial apoptosis (Carden, 2000; del Zoppo, 2003). Vasogenic cerebral oedema is a major cause of neurological deficit and death in large infarcts (Ayata, 2002). Additionally, reperfusion of hyperpermeable cerebral vessels can result in haemorrhage and secondary brain injury (Suwanwela, 2007), and this limits the time window for attempts at reperfusion (Maier, 2006).

Vascular leakage after stroke compromises neurological function by increasing the volume of the infarct (Schoch, 2002). The tendency of ang1 to inhibit vascular leakage might therefore be protective. It is becoming clear that hypoxic VEGF-mediated disruption of the blood-brain barrier, together with an increase in the ang2/ang1 ratio in the injured region and vascular destabilisation, are two very important caused of oedema and haemorrhage (Hansen, 2008). Reversing the ang2/ang1 ratio, in addition to counteracting the deleterious effects of VEGF, is a rational approach to solving this problem. Ang1 might be beneficial for both effects. Indeed ang1 and the ang1 variant, COMPang1, have both been demonstrated to reduce stroke infarct volumes in mice (Shin, 2010; Zhao, 2010). In fact application of the latter was observed to reduce the severity of resulting ischaemic neurological deficits (Shin, 2010). Administration of high levels of recombinant ang1 to mice had
the capacity to inhibit VEGF-induced vascular permeability after ischaemic stroke, with concomitant reductions in the size of the infarcts (Zhang, 2002). In addition it has been suggested by experimental observation that ang1 can promote endothelial stability in vivo in the presence of exogenous VEGF, without compromising the pro-angiogenic potential of VEGF. This has been observed in the blood-brain barrier after stroke in mice (Valable, 2005; Zhao, 2010), as well as in the peripheral vasculature (Thurston, 2000). A combined approach involving the use of both mediators might thus maximise the potential for inhibition of cerebral oedema, whilst at the same time promoting the parallel neovascularisation which has been suggested to be beneficial in improving the morbidity of human stroke patients (Krupinski, 1994).

Despite the potential of such an approach, and several supportive preclinical models, there are no human studies thus far. Furthermore, in Zhang’s study (2002) of the protective effects of ang1, ang1 was either delivered as either a tetrameric construct of ang1 receptor binding domains 12h prior to inducing stroke, or as adeno-virally-induced ang1 overexpression. In Shin et al’s (2009) and Zhao et al’s (2010) studies rats were pre-treated with vectors encoding overexpression of ang1 or an ang1 variant. In the latter case ang1 was overexpressed eight weeks before the induction of ischaemia. This does not reflect a timing of administration which is likely to be practical in acute human stroke.

1.8.4. Diabetic Nephropathy

The renal vascular bed is analogous to other microvascular beds, with podocytes, expressing ang1 and VEGF (Satchell, 2002), taking the place of pericytes. The glomerular endothelium expresses Tie2 (Satchell, 2002).
Diabetic nephropathy is the most common cause of end-stage renal failure worldwide (Ritz, 1999). Clinico-pathologically this is manifest first as microalbuminuria, a reflection of increased permeability of the renal filtration barrier (Forbes, 2007; Raptis, 2001). The degree of albuminuria is a good indicator of future mortality from renal failure in human type-2 diabetes (Stephenson, 1995). Higher levels of circulating ang2 have been correlated directly with increasing urinary microalbuminuria in human diabetics (Lim, 2004). Loss of ang1 from damaged podocytes and the upregulation of ang2 by the endothelium favours the destabilisation of the latter (Satchell, 2004). A recent study showed that application of COMPangiopoietin-1 has a strong effect on suppression of urinary microalbuminuria in a db/db mouse model (Lee, 2007b). Vascular ICAM-1 and monocytic chemoattractant protein (MCP-1), both markers of ongoing inflammation, were expressed to a much greater degree in the glomerular and peritubular capillaries than controls, but were reduced substantially by COMPang1. Furthermore thickening of the basement membrane of the glomerular endothelium on histological analysis was also reduced. This is encouraging because the authors used a db/db mouse model, which is the closest available model to a human model of type-2 diabetes, and bears many of the features of the latter (Rees, 2005). However corroborating evidence is required.

1.8.5. Asthma

Asthma is a chronic inflammatory lung disorder mediated via a Th2 helper immune response, and characterised by elevated serum IgE levels, airway hyperresponsiveness and hypersecretion of mucus (Elias, 2003). Remodelling of the microvasculature occurs (Wilson, 2006). The changes which have been observed
include angiogenesis (Hoshino, 2001) and microvascular leakage (McDonald, 2001), in addition to inflammatory cell adhesion, transmigration and tissue infiltration (von Andrian, 2000). The latter are predominantly eosinophils, with a smaller number of macrophages and lymphocytes (Wilson, 2006). Vascular leakage contributes to airway obstruction and hyperresponsiveness (Van de Graaf, 1991).

In one study improvement in asthma control and the reduction in airway vascular permeability index after fluticasone or montelukast therapy correlated with a decreasing ang2/ang1 ratio after therapy in humans (Kanazawa, 2007), which may reflect a decreasing tendency of ang2 to destabilise the vascular endothelium.

Two recent studies have used native ang1 and the recombinant, potent ang1 analogue COMP-angiopoietin-1, in mouse models of allergic airway hyperresponsiveness (Simoes, 2008; Lee, 2007). Both authors sensitised mice to ovalbumin by injection for two weeks prior to induction of airway hyperresponsiveness. Simoes et al (2008) challenged mice after pre-application of 1μg ang1 intranasally. They observed a pronounced reduction in inflammatory migration of eosinophils into the lung airways, together with reduced levels of the vascular adhesion molecule VCAM-1, and reduced levels of eosinophil chemotaxins, such as eotaxin, IL-5 and IL-13. Additionally, clinical improvement was observed, with significantly reduced airway hyperresponsiveness in response to methacholine challenge in the ang1-treated mice versus controls. Simoes’ work (Simoes, 2008) is particularly interesting, since they used only small amounts of ang1 applied nasally. This has implications for the potential avoidance of side effects. Whilst COMP-ang1 also demonstrated benefit against asthma in mice, substantially greater amounts were administered (Lee, 2007). Since asthma is chronic, such an approach might have potentially deleterious
consequences longer term, particularly regarding unwanted airway angiogenesis (Byun, 2007; Cho, 2006; Cho, 2005; and see section 1.9).

1.8.6. Allograft Rejection

Part of the process of chronic cardiac allograft rejection involves atherosclerosis and endothelial cell injury and death in the intima of large and medium-sized vessels of the graft (Weis, 1997; Turunen, 1995). Coronary artery injury and perivascular inflammation play a key role in progression of neointimal lesions (Libby, 2001), and vessel injury relates to upregulation of pro-inflammatory adhesion molecules, with subsequent extravasation of leucocytes into the graft (Turunen, 1995). Nykanen and colleagues (Nykanen, 2003) demonstrated that overexpression of ang1, encoded adenovirally, reduced significantly the influx of monocytes and lymphocytes into the graft. There was a corresponding reduction in graft interstitial fibrosis and improved survival of cardiac allografts (100% at 8 weeks compared to 73% in controls).

1.8.7. Side-Effects of Radiotherapy on Blood Vessels

A study on microvascular apoptosis in a rat radiation colitis model (Cho, 2004b) suggests that this may be a potential application for ang1 therapy in humans. The dosing in abdominal radiotherapy for cancer is limited predominantly by deleterious effects on the gastrointestinal tract, the primary lesion being extensive apoptosis of microvascular endothelial cells in the lamina propria (Paris, 2001).

Myelosuppression is one of the major side-effects of chemoradiotherapy. Haematopoietic stem cells (HSCs) express Tie2, and these cells are most affected by radiotherapy (Iwama, 1993). Again one of the prime lesions is apoptosis of the stem
cells themselves, as well as vascular sinusoidal regression in the spleen. Pre-treatment with adenovirally-overexpressed COMPang1 reduced depletion of HSCs and the sinusoidal vascular regression which occurred in response to whole-body irradiation (Lee, 2008).

A potential problem is that COMPangiopoietin-1 has shown potency for promoting neovascularisation in several studies (Byun, 2007; Cho, 2006; Cho, 2005; and see section 1.9). The application of such approaches may therefore have deleterious consequences in cancer, unless vascular protective actions and angiogenic actions can be applied selectively.

1.9. Angiogenic Actions of Angiopoietin-1: Potential Applications

In vitro ang1 stimulates vascular endothelial cell migration and reorganisation into capillary tubule sprouts in vitro, at least in part through Tie2 (see section 1.5). In vivo ang1 is essential for developmental angiogenesis and vascular remodelling (Brindle, 2006). Suri et al (1998) and Thurston (1999) found that, in transgenic mice overexpressing ang1, vessel branching and number were increased. However, ang1 overexpression in adult mice did not lead to these effects, at least for the duration of study, with the emphasis of ang1 more in vascular protection. However there is evidence indicating that ang1 can induce angiogenesis in vivo, and that this may have potentially useful effects. In most studies ang1 is applied at high levels or for sustained periods. Lubiatowski and colleagues noted that ang1 overexpression, by adenoviral gene transfer, on a cremaster muscle flap model in Sprague-Dawley rats (Gurunluoglu, 2002), led to an increase in capillary density. In a slightly different model they also showed that ang1 can promote enhanced skin flap
survival (Jung, 2003). Moreover, they found ang1 alone to be as effective in promoting capillary density and blood flow as the combination of ang1 and VEGF, at seven days (Lubiatowski, 2002). Cho et al (Cho, 2004a) demonstrated that an ang1 variant, COMP-angiopoietin-1, with similar in vitro actions to native ang1 induced angiogenesis strongly in the mouse corneal micropocket angiogenesis assay. In vivo, short term and intermittent administration of COMP-angiopoietin-1 systemically promoted enlargement and enhancement of blood flow in vessels, predominantly postcapillary venules, capillaries and terminal arterioles (in decreasing order of potency) in tracheal mucosa (Cho, 2005). This occurred via endothelial cell proliferation, rather than hypertrophy.

Thus, ang1 and COMPang1 might have a role in conditions where therapeutic enhancement of angiogenesis and blood flow would be desirable, for example, the diabetic wound. Here healing is impaired as a result of a combination of impaired cutaneous blood flow, excessive inflammation and oedema (Martin, 2003). There is persistent and significant upregulation of ang2 such that the ang2:ang1 ratio is much higher for much longer compared with non-diabetic wounds (Kampfer, 2001). There is also a deficiency of local VEGF production. The result is vessel destabilisation, vascular regression and impaired healing (Kampfer, 2001). Wound closure in normal and diabetic mice was significantly more rapid when treated with adenovirally-encoded COMP-ang1 (Cho, 2006), as well as locally applied COMPang1. This was associated with a greater density of blood vessels, as well as lymphatic vessels, at the wound margins.

The combination of ang1 and VEGF might be advantageous, combining the significant mitogenic and angiogenic capacity of VEGF with the significant protective effects of ang1. Coadministration of VEGF and ang1 by adenoviral transfer in a rabbit
model of peripheral limb ischaemia resulted in a significantly greater formation of collaterals as well as a significantly greater actual blood flow, than either alone (Chae, 2000). This combination could prove to be applicable to the wide variety of potential therapies in which VEGF has been implicated, including myocardial and peripheral limb ischaemia and wound healing (Pandya, 2006).

1.10. Recombinant Angiopoietin Derivatives

1.10.1. Problems With Using Native Angiopoietin-1

Some of the potential effects of angiopoietin-1 discussed above have generated interest in its therapeutic potential. However, generation of native angiopoietin-1 is difficult, since the activity of the protein varies frequently after purification. The latter is mainly as a result of the tendency of ang1 to form higher order multimers, resulting in precipitation and loss of activity (Cho, 2004a; Maisonpierre, 1997). Some authors have reported undetectable levels shortly after intravenous administration (Davis, 2003). Indeed many studies to date have used adenoviral vectors to overexpress angiopoietin-1 reliably where systemic high expression therapy was required (Huang, 2008; Witzenbichler, 2005; Thurston, 1999), although some authors have observed successful use of native angiopoietin-1 when administered systemically (Childs, 2008). The use of adenoviruses leads to the potential problems of host immune responses and tumour induction. McCarter et al (2007) adopted a different approach to deliver ang1, using host fibroblasts transfected with a plasmid encoding native ang1, and thus engineered to generate ang1 in vivo. After administration they conferred substantial protection against vascular inflammation in a rat model of ARDS, a protection ablated in Tie2-null mice. However this approach still involves foreign DNA insertion and the potential for excessive or prolonged effects.
1.10.2. Modifications of Angiopoietin-1

A number of other authors have attempted to modify the structure of ang1 for ease of generation. Yancopoulos and colleagues at Regeneron pharmaceuticals have converted the cysteine at position 245 to a serine, and substituted the N-terminal domain of ang2 for that of ang1. This facilitates purification and improves solubility (Davis, 2003). The resulting structure, known as recombinant ang1, or ang1*, has been reported to have been used successfully in several studies. In diabetic mice ang1* administered intravitreally suppressed apoptotic endothelial cell death, vascular leakage and the expression of pro-inflammatory vascular adhesion molecules on the endothelium (Joussen, 2002). Additionally in a study in which developing rat retinal blood vessels had been deprived of mural cell cover, rendering them immature and prone to leakage, ang1* was able to rescue this (Uemura, 2002). Interestingly ang1* induced this effect even in the absence of mural cell association, usually a key event marking vessel stabilization.

Davis et al (2003) engineered ang1-FRD-Fc-FRD, a structure consisting of an IgG1 Fc domain fused to two ang1 FRD domains. The Fc portion dimerizes, so that the overall construct developed was precisely tetrameric, as demonstrated by the authors. The species bound Tie2 with comparable affinity to native ang1, and indeed activated endothelial Tie2 phosphorylation. The precise tetrameric nature, rather than variably-sized multimers, may explain its solubility and excellent pharmacokinetics when it was administered to mice. The authors were able to measure plasma levels reliably, which lasted for at least seven days. Furthermore ang1-FRD-Fc-FRD induced tissue Tie2 phosphorylation. That this led to downstream signalling associated with Tie2 was suggested by the protection conferred upon the mice when challenged with mustard-oil, a potent inducer of vascular leakage. In fact the
protection conferred was comparable with adenovirally-delivered ang1 in the same study. There may be advantages conferred by a precise multimeric stoichiometry where activating Tie2 is concerned, since this might allow predictable modification of intracellular signalling, in order to achieve a precise effect. Though it is not at all known whether heterogeneous multiples of ang1 above tetramer alter intracellular signalling profiles substantially, there is some evidence that this may be the case for the related clustering receptor tyrosine kinase EGF (Warren, 2006; Sweeney, 2000). It is certainly an area worth consideration of further study. The main potential problem with ang1-FLD-Fc-FLD is the potential for immune sensitisation, which may become more apparent after repeated administrations. This may be less relevant in the treatment of ARDS than chronic processes, for example transplant arteriosclerosis or diabetic retinopathy, where several administrations may be required.

1.10.3. COMP-angiopoietin-1

Cho et al (2004) have overcome some of the problems highlighted in the previous sections by the creation of a significantly shorter version of ang1. Here, the C-terminal receptor-binding FRD domain (residues 284-498) of ang1 is fused to a new oligomerization domain. Specifically, residues 28-77 of the rat cartilage oligomeric matrix protein (COMP), which forms a pentameric short ring (Malashkevich, 1996), has been used (see figure 1.7). The authors observed that COMPang1 is soluble, stable and does not aggregate over time (Cho, 2004a) and is also significantly more potent than native ang1 in stimulating endothelial migration, sprouting and apoptosis inhibition in vitro. In vivo the authors have tested COMPang1 on a variety of pre-clinical models to elucidate its efficacy to inhibit apoptosis (Lee, 2008; Cho, 2004b), its anti-inflammatory and anti-leakage effects on the vasculature
(Lee, 2007), its ability to induce angiogenesis (Cho, 2005), and its accelerating effects on wound healing through angiogenesis and lymphangiogenesis (Cho, 2006). By what mechanism COMP-angiopoietin-1 it is more potent compared with native ang1 is not clear, but might be attributable to enhanced solubility or smaller size increasing access to Tie2 receptors.

![Diagram of COMPangiopoietin-1](image)

**Figure 1.7: Schematic representation of COMPangiopoietin-1.** The short coiled-coil oligomerisation domain of rat COMP (residues 28-77, shown in red) is fused to the FRD receptor-binding C-terminal domain of human ang1 (shown in yellow).

There are several potential problems with COMPang1. Firstly it acts at the site where ang1 binds to Tie2. This means that it is expected to be antagonised by ang2 at the Tie2 receptor, though this has not been tested formally. This has not prevented the observed beneficial effects discussed in the above studies. However it is likely to mean that a greater ‘dosing’ of COMPang1 would be needed for in vivo therapy. It
may therefore be advantageous to have a molecule that can aggregate, and then therefore activate, the Tie2 receptor without binding at the ang1/ang2 binding region. Additionally COMP ang1 is still a large, relatively complex, protein weighing approximately 38kDa, despite the loss of the N-terminal residues. Modifications which might later become desirable to tailor the function of the molecule would be more difficult than with a smaller ligand, such as a peptide ligand. Furthermore COMPang1 not only stabilises the vasculature, but there are also multiple studies documenting a very potent proangiogenic effect noted less with ang1 (and in the latter case only when high expression was used). Whilst this might be advantageous in some circumstances it would be disastrous in others, as discussed below.

There appears to be a contrast between the roles of ang1 in some of the above studies, where its predominant effects are quiescence, and those of others where ang1 or ang1 variants were able to produce angiogenesis. This may be explained by the observation that in many of the latter experiments ang1 was present in high concentrations, and it is likely that such levels do not replicate the ‘normal’ role of ang1 in vivo. Nevertheless one has to consider that, whilst attempting to harness the effects of vascular protection from the use of ang1, one might inadvertently induce deleterious effects, such as unhelpful neovascularisation (Takagi, 2003). For example COMPang1 showed a protective effect in a mouse radiation-induced enteritis model (Cho, 2004b). However if it also a potent inducer of angiogenesis (Cho, 2006; Cho, 2005) it could in theory promote angiogenesis at the time when least desired, such as during cancer radiotherapy. Such effects could confound attempts to reduce disease in diabetic retinopathy simply by the encouragement of neovascularisation. Ideally one would seek to be able to modulate only the desired protective effects in some circumstances, such as those discussed in
section 1.8. However in others one might actually desire angiogenesis, for example short term, to promote healing of a wound

**1.11. Conclusions and Aims of Project**

The mechanism of Tie2 activation is only partially known. In particular, there has not yet been resolution of debate about why ang1 and ang2, with similar receptor-binding moieties, have marked differences in abilities to activate Tie2. The most likely explanations may relate to the dimeric nature of ang2 not enabling sufficient clustering of receptors to elicit a strong intracellular response, or that there is an inhibitory coreceptor which may associate preferentially with ang2. The answer may be a combination of both features, as discussed in section 1.4.3.

There is abundant, promising, evidence in vitro and in preclinical models of potentially beneficial vascular protective effects of angiopoietin-1 in several pathologies, particularly models of sepsis. However, in all pathologies there is still a paucity of human studies. There are also many conditions in which vascular inflammation and leakage play a role, such as burns, acute pancreatitis, neovascularisation and the instability of atherosclerotic plaques, where there is little or no literature to address the potential involvement of ang1.

Most models to assess the efficacy of angiopoietin-1 have been hindered by the problematic pharmacokinetics of administration. This may be a result of the tendency to form higher order multimers leading to aggregation. Attempts to circumvent this problem have led to the generation of a variety of recombinant alternatives, as discussed in section 1.10. These use, principally, the requirement for a minimally tetrameric version of the Tie2 receptor-binding domain together with a
surrogate multimerisation motif. The best characterised is COMPangiopoietin-1, which seems to replicate the effects of ang1 in vivo more efficiently than ang1 itself. However, COMPang1 is still a relatively large protein. Since it binds to the site on Tie2 to which native ang1 and ang2 bind it would, therefore, also be susceptible to competitive antagonism by ang2. This could lead to an increase in the dosing requirement for any therapeutic application, which might in turn lead to enhanced side-effects. Furthermore, COMPang1 has been described to have a particularly potent proangiogenic effect not observed to the same extent with its native counterpart. Unwanted angiogenesis may be a barrier to use for conditions requiring longer term administration because of the potential for induction of tumour growth.

Another option for the induction of Tie2 signalling is to test the ability of receptor-binding moieties other than the FRD domain to activate Tie2. Since the requirement is for a tetramer of ang1 FRDs in order to induce substantial signalling, it is theorised that such a moiety would need to be able to form a minimally tetrameric conformation. Thus, such a receptor-binding portion would need to be attached to a surrogate multimerisation domain. Since it is attractive to create a small molecule, the large size imposed by the presence of the FRD could be reduced by the use of small receptor-binding peptides, clustered into a minimally tetrameric conformation. Indeed such a method has been used for the FGF receptor (Ballinger, 1999), where dimerisation of peptides selected for specific binding resulted in a small recombinant agonist with substantial ability to activate the receptor itself, in addition to downstream pathways known to be activated by FGF itself. Using a related approach for Tie2 could lead to the generation of putative small molecule peptide agonists. Additionally, if some of these peptides bind to areas on Tie2 other than the ang1/ang2 binding site, theoretically they may also avoid competitive antagonism by ang2.
This project aims to take this approach, using two different Tie2-binding peptides, clustered using a surrogate multimerisation domain, to create recombinant proteins which are minimally tetrameric. These will then be tested for their ability to bind and activate the Tie2 receptor. The project hypothesis is, ‘Synthetic ligands can be constructed in which a heptameric Tie2-binding peptide, when presented in a minimally tetrameric, oligomeric format, will activate receptor signalling’. Such ligands, or their derivatives, might then ultimately be applicable in therapeutic systems.
2: Materials and Methods
2.1. General Solutions

All aqueous solutions were prepared with double-deionised water. pH measurement was performed with the Mettler-Toledo Seven Easy S20 model pH meter (Fisher Scientific, Loughborough, UK).

*Phosphate-Buffered Saline (PBS)*

140mM NaCl, 2.7mM KCl, 10mM NaPO$_4$, 1.8mM KH$_2$PO$_4$, pH 7.4

*Tris-Buffered Saline (TBS)*

25mM tris, 144mM NaCl, pH 7.4

*Tris-Acetate (TAE)*

40mM tris, 40mM glacial ethanoic acid, 1mM EDTA, pH 8.0

2.2. Gene Construction and Cloning

2.2.1. General Solutions

*DNA Loading Buffer for Agarose Gels*

65% sucrose (w/v), 10mM tris, 10mM EDTA, 0.05% bromophenol blue (w/v), pH 7.5

*LB broth*

Tryptone 10gL$^{-1}$, yeast extract 5gL$^{-1}$, 10gL$^{-1}$ NaCl. Solubilised in deionised water, pH corrected to 7.4 and autoclaved. Stored at rtp. For transformant selections ampicillin (Boehringer, Mannheim) or kanamycin (Sigma) were added at 100μgml$^{-1}$ (1μL of a 100mgml$^{-1}$ stock solution per ml broth) or 50μgml$^{-1}$ (1μL of a 50mgml$^{-1}$ stock solution per ml broth) respectively.

*LB agar plates*

Tryptone 10gL$^{-1}$, yeast extract 5gL$^{-1}$, 10gL$^{-1}$ NaCl, agar 15gL$^{-1}$, pH corrected to 7.4 and autoclaved. Stored at rtp. For plates agar heated gently until melted, allowed to cool to $<40^\circ$C before immediate addition and mixing of selective antibiotic
(ampicillin at 100 μg/ml or kanamycin at 50 μg/ml). Approximately 25ml agar was poured into each 100mm petri dish (Bibby Sterilin) in close proximity to a blue flame and allowed to set at rtp. Plates were then either used immediately or stored < 7 days at 4°C.

2.2.2. Primers

All oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Primers used were:

*Sense COMP*
5’-
GACTACAAGGACGACGACGACAGACAAGGGCAACCAGC GGCGACCTGGCCC CCCAGATGCTGCGC GAGCTGCAGGAGACCAACGCCGCCCTGCAGGACGTGCGAGAGCTGCTGA GACAAACAA-3’

*Asense COMP*
5’-
GCTGTGGCTGTAGAAGGGGCCGGCCTGCGCCGCCGAGGC TCGACTCCA TCACGGGTGTCTTTC AGGAAGGTTGATCTCCTTTCACTTTGTGTCAGCAGCTCTCGCACG-3’

*COMP5PCR*
5’-GACTACAAGGACGACGACGA-3’

*COMP3PCR*
5’-CTATGCACTTCCGCCGCC-3’

*Pep1.1.4.*
5’-
CTATGCACTTCCGCCGCCCA CGTTGCCGCGGCTGGTCACG CTGTGGCTGTAGAAGGGGC 171’

*NLLM*
5’-
TCAGGGCGCTGCAGCGCGCCGACTGCTGCTGGCTGTGA AGAAGGGGC-3’
AJM5
5'-TACTTCCAATCCATGCTTGACTACAAAGGACGACG-3'

AJM3
5'-TATCCACCTTTACTGTCAAGCTC GGATCAAAGCCTCGCCCT-3'

NLMContDownstream
5'-
TATCCACCTTTACTGTCACTAGGCGCTGGC CGCCGCAAGTCGTGGCTGTC AGGGCCAGGCTG GCCTGCC-3'

RP Downstream
5'-
TATCCACCTTTACTGTCACTAGGCGCTGCCGCCGCCGCTGGCGGCC-3'

Extender
5'-
GCTGTGGCTGTAGAAGGGGCCACCACCGCGGCCCACCTGAACC CGCCGCCTGCCGCCCGCC-3'

P1.1.14.
5'-
TATCCACCTTTACTGTCACTATGCACTTCCCCCGGAGCGCGCTGTGGCTGTAGAAGGGGC

NLMLei
5'-
TATCCACCTTTACTGTCACTAGGCGCTGC CGCCGCCGCGTGGCGGCCATCG AGGGCCAGGCTG GCCTGCC-3'

2.2.3. Polymerase Chain Reaction

Unless otherwise stated all PCR reagents were purchased from Bioline (London). PCR reactions were prepared in an RNA free area of the laboratory, with a bench washed with RNAaseZAP (Ambion, Texas), and using RNA-free
microcentrifuge tubes (Ambion). Unless specifically stated otherwise in the relevant later chapter, the PCR conditions were as detailed below.

For PCR of templates using Biotaq DNA polymerase (Bioline, London), in order to leave ‘A overhangs’ suitable for TA cloning, PCR mixtures of 25 or 50 μL were prepared. For a 25 μL reaction the following were mixed: 2.5 μL of 10xNH₄ buffer; 2 μL of 50mM MgCl₂; 5 μL of 10mM dNTP mix; 10ng of template DNA; 0.5 μL of relevant 5’-primer (from stock concentration 100pmolμL⁻¹); 0.5 μL of relevant 3’-primer (from stock concentration 100pmolμL⁻¹); added deionised, RNAase- and DNAase-free water (Ambion, USA) to make the reaction up to 24 μL; and delayed addition of 0.5μL Biotaq polymerase (from stock concentration 5UμL⁻¹) after the initial hot start. Each reaction was covered with a single drop of mineral oil (Sigma, London). Thus the final concentrations were: 1x NH₄ Buffer (16mM (NH₄)₂SO₄, 67mM tris-HCl pH 8.8); 4mM MgCl₂; 2mM dNTPs; 0.4ngμL⁻¹ DNA template; 2μM 5’- and 3’-primers; and 0.2 UμL⁻¹ Biotaq.

As a result of a tendency for errors for Biotaq in later experiments, PCR of templates using Accuzyme DNA polymerase was adopted. For a 25 μL PCR reaction the following were mixed: 2.5 μL of 10x AccuBuffer; 2 μL of 50mM MgCl₂; 5 μL of 10mM dNTP mix; 10ng of template DNA; 0.5 μL of relevant 5’-primer (from stock concentration 100pmolμL⁻¹); 0.5 μL of relevant 3’-primer (from stock concentration 100pmolμL⁻¹); added deionised, RNAase- and DNAase-free water (Ambion, USA) to make the reaction up to 24 μL; and delayed addition of 0.5 μL Accuzyme DNA polymerase (concentration 2.5 UμL⁻¹) after a hot start. Again, each reaction was covered with one drop of mineral oil. Thus the final concentrations were: 1x Accubuffer (60mM tris-HCl, 6mM (NH₄)₂SO₄, 10mM KCl, 2mM MgSO₄ pH 8.3);
4 mM MgCl₂; 2 mM dNTPs; 0.4 ngµL⁻¹ DNA template; 2 µM 5’- and 3’-primers; and 0.1 UµL⁻¹ Accuzyme.

The PCR cycling reaction, unless stated otherwise in the results, was carried out as described below, using the Perkin Elmer (Massachusetts, US) thermal cycler. An initial hot start of 94°C for 1 minute was followed by 30 cycles of: 94°C for 30 seconds (denaturation); 65°C for 30 seconds (annealing); followed by 72°C for 1 minute (elongation). Products were analysed on 1% agarose gels and purified as detailed below.

For the addition of 3’ adenine overhangs to blunt-ended PCR products to allow for TOPO-II cloning, 1 U Biotaq was added to PCR product on ice. The mixture was incubated for 8 minutes on a preheated block at 72°C, followed by replacement on ice. Products were then analysed and purified as detailed below.

2.2.4. Agarose Gel Electrophoresis

DNA was run on 1% agarose gels for visualisation, as well as subsequent purification in some experiments. For a 10 well agarose gel, 0.5 g agarose was measured and heated in 50 ml TAE buffer until solubilised completely. Subsequently, using a fume cupboard, ethidium bromide (Sigma) was added to a concentration of 50 ng ml⁻¹ and mixed. The gel was poured into a gel tray and a 9 well 1 mm comb inserted to give well volumes of approximately 25 µL per well. For larger gels twice the amount of agarose was used in 100 ml of TAE, with an 18 well gel comb, and a larger accommodating gel plate.

After the gel had set, DNA samples were loaded, after mixing with DNA loading buffer in a 5:1 ratio of DNA:buffer. A marker lane consisting of 2 µL of Trackit 1 kb plus DNA ladder (Invitrogen) was run with each set. Gels were run at
120V in TAE buffer until desired fragment separation was judged to have occurred. DNA was visualised on an ultraviolet light transilluminator and images were acquired using the MultiImage Light Cabinet (Flowgen).

### 2.2.5. Gel Purification of DNA

For the purification of desired DNA fragments from PCR reactions, run out on a 1% agarose gel, the Qiaquick Gel Extraction Kit (Qiagen, Crawley, UK) was used. The desired gel fragment was visualised using UV transillumination. Immediately the fragment was cut out of the gel with a clean, sharp scalpel, ensuring the minimal amount of surrounding nonspecific agarose was taken. The gel slice was weighed. Three volumes of solubilisation and binding buffer QG were added to one volume of gel (100mg = 100µL) immediately and the sample was incubated at 50°C, with frequent vortexing, until the gel slice had dissolved. 10 µL 3M sodium acetate was added to the sample, followed by mixing, and addition of one gel volume of isopropanol, with a further mix.

For each sample a Qiaquick spin column was inserted into a provided 2ml collection tube, and the sample loaded onto the column. Centrifugation at 13000g was carried out for one minute to bind DNA to the column. Flow-through was discarded. 0.5ml buffer QG was applied to the column and centrifuged for one minute at 13000g to remove any traces of agarose. Flow-through was discarded. 0.75ml wash buffer PE was applied to the column, spun for one minute at 13000g and flow-through discarded. A further one minute spin at 13000g was performed to remove residual ethanol from the wash buffer. The column was placed into a clean 1.5ml collection Eppendorff tube and DNA eluted by applying 30µL of buffer EB (1mM tris pH 8.4). The column was left to stand for one minute and then centrifuged at 13000g for a
further minute, to maximise DNA elution. The purified product was used in cloning or PCR amplification and was stored at – 20°C.

2.2.6. Cloning into a Mammalian Expression Vector and E.Coli Transformation

For protein expression in mammalian cells the relevant PCR products were cloned into the pSecTag/FRT/V5-His TOPO II expression vector (Invitrogen, LA) using the TOPO TA cloning kit (Invitrogen), with subsequent transformation of TOP10 chemically competent E coli (Invitrogen). A prokaryotic ampicillin resistance DNA element is conferred by this plasmid. Proteins are conferred with a secretory tag and thus extracellular protein can be generated. In order for cloning to occur a 

*Vaccinia* virus topoisomerase I enzyme is supplied already bound to the vector. This enzyme catalyses efficiently the cloning into a specified site on the vector of PCR products with 3’ A (adenine) overhangs. *Taq* polymerase adds 3’ A overhangs to all PCR products and therefore no further manipulation of such PCR products is necessary for efficient cloning. When Accuzyme was used addition of 3’ A overhangs was performed as described in section 2.2.3, where products were required to be inserted into the pSecTag/FRT/V5-His TOPO-II expression vector. The genomic map of the pSecTag/FRT/V5-His TOPO-II vector is detailed in Appendix A.

In all cases 2-4μL purified PCR product was added to 1 μL of salt solution (stock concentration 1.2M NaCl, 60mM MgCl₂) in a 0.5ml Eppendorff. The mix was made up with addition of sterile water to a final volume of 5μL. Then 1μL of the vector was added. The mix was allowed to stand at rtp for 5 minutes before the addition of 2μL to a thawed TOP10 cell vial on ice. After gentle mixing, reactions were left on ice for 30 minutes (to maximise colony numbers) before a 30 second heat shock, at 42°C, without shaking. Thereafter the vials were replaced on ice
for two minutes. Then 250μL SOC medium, pre-warmed to 37°C, was added to the vial. The vial was placed in an incubator at 37°C for one hour, with shaking at 250rpm. After 1 hour both 20μL and 200μL of the total vial were pipetted onto separate ampicillin-impregnated agar. Plates were placed in an incubator at 37°C for 15-18 hours, inspected, and suitable colonies chosen for plasmid amplification and extraction.

2.2.7. Amplification and Extraction of Plasmids

For the extraction of high quantities of plasmid for subsequent mammalian transfections or for protein expression in E. coli the Qiaprep Miniprep kit (Qiagen, US) was used. Selected agar colonies were incubated into 8mls per sample of LB broth with ampicillin at 100μgml⁻¹ or kanamycin at 50μgml⁻¹ for 15 hours, at 37°C, shaking at 250rpm. Then samples were centrifuged at 1000g for 10 minutes and supernatant disposed of. All subsequent steps were carried out at rtp. Pellets were resuspended in 250μL resuspension buffer P1 (50mM tris pH 8.0, 10mM EDTA, 100μgml RNase A) and transferred to a 1.5ml microcentrifuge tube. 250μL lysis buffer P2 (200mM NaOH, 1% w/v SDS) was added to samples with immediate gentle mixing by inverting contents six times. Exactly 5 minutes later 350μL neutralisation buffer N3 was added to each sample, followed by six tube inversions to mix. Immediately samples were centrifuged at 13000g for 13 minutes. Supernatant was removed carefully and applied to a Qiaprep spin column. Samples were centrifuged for 60 seconds and flow-through discarded. 0.5ml PB was applied to each column, to remove trace nuclease activity, and flow-through discarded after a further 60 second centrifugation. The column was washed by the application of 0.75ml buffer PE, centrifugation for 60 seconds with disposal of flow-through being followed by a
further 60 second centrifugation step in order to remove residual ethanol from buffer PE. Plasmid was eluted by placing the Qiaprep column in a standard, clean, 1.5 ml microcentrifuge tube. 30µL buffer EB (10mM tris-Cl pH 8.5) was added to each column and allowed to stand for 5 minutes. Then centrifugation was carried out for 60 seconds and the eluted plasmid DNA collected.

2.2.8. Quantitation of Plasmid DNA

DNA concentration and purity was estimated using UV spectrophotometry (UV-1601 spectrophotometer, Shimadzu, Japan). 5µL DNA in solution was diluted into 500µL sterile water. Concentration was measured using absorbance at 260nm. Calculation of DNA concentration assumes that 50µgml⁻¹ of DNA measured in a cuvette with a 1cm path length has an absorbance of 1. Purity was estimated using the ratio of absorbance at 260nm (abs₂₆₀) divided by absorbance at 280nm (abs₂₈₀). For pure DNA abs₂₆₀/abs₂₈₀ is 1.8-2.0.

2.2.9. Cloning into a Prokaryotic Expression Vector and E.Coli Transformation

For protein expression in prokaryotes the pLEIC-03 plasmid vector was used. This plasmid carries a prokaryotic kanamycin resistance element and an upstream hexahistidine tag, and is transcribed by the T7 RNA polymerase of DE3 BL21 E.Coli. Cloning was carried out by the Protein Expression Laboratory; Department of Biochemistry; University of Leicester, since this group supplied the vector. Cloning was performed directionally, using specific upstream (TACTTCCAATCCATG) and downstream (TATCCACCTTTACTGTCA) homology sequences on the vector. A genomic map of pLEIC-03 is illustrated in Appendix B.
The PCR method described previously was used to incorporate the relevant peptide nucleotide sequences onto the colony 3 template, as well as the 5’ and 3’ extensions required for directional cloning at the 5’ and 3’ ends of the amplified PCR product. This was achieved by using the primer AJM 5, together with a variable 3’ primer, and the PCR conditions already described. Products were purified after running on a 1% agarose gel and the appropriately-sized extracted PCR product was then cloned by the Protein Expression Laboratory. If storage was required this was for 48 hours maximum at -20°C. The Protein Expression Laboratory extracted plasmid from incorporating clones screened by PCR which was confirmed by sequencing.

For protein expression in bacteria BL21-Gold (DE3) competent cells (Stratagene, UK) were transformed. This cell type uses T7 polymerase expression system on a lacUV5 promoter. Expression is therefore inducible with Isopropyl-1-thio-β-D-galactopyranoside (IPTG). Additionally these bacteria lack a number of proteases which can degrade recombinant proteins, for example, Lon protease.

Transformations were carried out within 14ml BD Falcon Polypropylene Round Bottom tubes. 50μL competent cells were thawed on ice. 15ng plasmid DNA containing the required protein nucleotide sequence and a resistance element for kanamycin was added. Gentle mixing was followed by incubation on ice for 30 minutes. Cells were exposed to a 30 second heat shock at 42°C. Cells were placed on ice for 2 minutes immediately, after which 0.45ml SOC medium was added to the aliquot. Then cells were incubated at 37°C for 1 hour, shaking at 250rpm. 20μL and 200μL were placed on two separate kanamycin-impregnated agar plates and incubated for 15-18 hours at 37°C. Colonies were inspected for selected transformants on each plate. A control transformation was carried out using the same conditions and plasmid quantity with the pUC18 plasmid, supplied with cells.
2.3. Mammalian Protein Expression

Several mammalian cell lines were transfected with a TOPO-II plasmid encoding secreted expression of the recombinant protein of interest. Cells were then grown in both serum-free media to assay expression of recombinant proteins. Multiwell plates were obtained from Nunc International (Thermofisher, Denmark).

2.3.1. Cell Culture

2.3.1.1. Chinese Hamster Ovary Cell (CHO) Lines

CHO-K1 cell lines were obtained from the European Collection of Cell Cultures (ECACC). Cells were used in passage numbers p29-p37 for transfections. Cells were maintained in αMEM (Biowhitakker), plus 2.5mM L-glutamine (Lonza, Walkersville), with 10% fetal calf serum (FCS). Cells were grown at 37°C, 5% CO2. For seeding cells for transfection T80 flasks of confluent CHOs were washed in 3ml PBS twice. 1ml 0.1% trypsin in PBS was applied for just long enough for cells to dissociate from the flasks (approximately 1-2 minutes). 5mls fresh complete medium was added, mixed to neutralise trypsin, and the suspension was centrifuged for 6 minutes at 300g. Pellets were resuspended in a suitable volume of αMEM and seeded for desired confluence.

2.3.1.2. HEK-293 Cell Lines

HEK-293 cell lines were obtained from the ECACC. Cells were used between passages p5 and p20. Cells were maintained in αMEM plus 2.5mM L-glutamine with 10% FCS, and incubated at 37°C, 5% CO2. When passaging cells, complete medium was changed, after two washes in PBS. Gentle agitation was used to disrupt the
association of cells with T80 flasks. Cells were then collected in the fresh complete medium and mixed to homogeneity before seeding.

2.3.2. Transient CHO Transfections

Transient transfection of CHO lines for expression of recombinant protein was carried out in 6-well plates. Transfection was carried out using engineered pSecTag/FRT/V5-His TOPO-II plasmid containing an encoding sequence for the recombinant ligand of interest, purified as detailed in section 2.2.7. Experiments were carried out by the method of lipofection, using Superfect (Qiagen, Crawley), Polyfect (Qiagen) and Lipofectamine 2000 (Invitrogen, Paisley). Efficiency of transfection was estimated using a plasmid containing GFP as a positive control. For each transfection a negative control was also included. This was a pSecTag/FRT/V5-His TOPO-II plasmid from the original cloning procedure which had the sequence of a recombinant COMP-peptide ligand, but in the reverse orientation with respect to transcription. The control plasmid was named, ‘7D’. Fifteen hours after transfection complete medium was removed from CHOs and replaced with 1ml per well (6-well plate) of serum-free αMEM. Cells were incubated under standard conditions for 48 hours before medium was removed for analysis.

2.3.2.1. Transfection using Superfect

For transfection of CHOs in 6-well plates, 4.75x10^5 CHOs were seeded into each well, in 2mls per well fresh complete medium. After an overnight recovery of 15 hours cells were always noted to be at approximately 80% confluence. For each sample, and for controls, 2µg plasmid DNA was mixed with 10µL Superfect in 150µL serum-free αMEM. The mixture was allowed to stand at rtp for 10 minutes for
transfection complexes to form. During this period CHOs were removed from the incubator. Cells were washed with 2mls PBS per well and 600µL of fresh complete medium per well was applied. Transfection complexes were then added to the CHOs and cells were incubated for 3 hours at 37°C, 5% CO₂. Subsequently all medium was removed and replaced with fresh complete medium for 15 hours. Transfection efficiency was assessed using the GFP well. Also, medium was changed, cells were washed with 2mls PBS per well, and 1ml serum-free αMEM was placed in each well. This was collected after 48 hours for protein estimation.

2.3.2.2. Transfection Using Polyfect

For CHO cell transfection using Polyfect, cells were seeded at 4x10⁵ per well of a 6-well plate. After a 15 hour recovery cells were washed with 2mls per well PBS. 1.5 ml per well complete medium was then added to cells. Transfection complexes were prepared for samples and controls by adding 1.5µg of the plasmid DNA of interest to 100µL of serum free medium, at rtp. After gentle mixing, 10µL of Polyfect was then added to the DNA in solution, and further gentle mixing was performed. The mixture was left at rtp for 10 minutes to allow the formation of complexes. Then 600µL complete medium was added to complexes, the solution was pipette up and down twice, and added to the CHOs in wells. After 15 hours of incubation at 37°C, 5% CO₂ medium was changed to 1ml per well serum free medium. After 48 hours medium was collected for protein estimation.

2.3.2.3. Transfection Using Lipofectamine 2000

For CHO cell line transfection using lipofectamine 2000 in 6-well plates, cells were seeded at 5-6x10⁵ in order to observe that after 15 hours cells had reached at
least 90% confluence. For each sample and controls, two separate samples were prepared in sterile Eppendorffs. To one tube was added 4μg plasmid DNA in 250μL Optimem medium (Invitrogen). To the other was added 10μL lipofectamine 2000 reagent in 250μL Optimem. After 5 minutes incubation both samples were combined and complexes were allowed to form by incubating for 15 minutes rtp. During this period medium was removed from previously seeded CHOs, each well was washed with 2ml PBS, and replenished with 2ml per well fresh complete medium. Complexes were then added to cells and incubation at 37°C, 5% CO₂ was performed. After 6 hours medium was replaced with 2ml per well fresh complete medium. After a further 15 hours medium was replaced with 1ml per well serum free medium. At 48 hours medium was retrieved for protein estimation.

2.3.3. Transient 293-HEK Cell Transfections

For transient 293-HEK transfections in 6-well plates, Superfect, Polyfect and Lipofectamine 2000 were used. The transfection protocols for Superfect and Lipofectamine 2000 were identical to those in CHOs, described above. For the use of Polyfect, again the protocol was similar, the only differences being that slightly more plasmid DNA was used per well (2μg) and more Polyfect was added per well (20μL). For seeding 293-HEK cells in wells of a 6-well plate, pre-coating of each well with 0.5ml sterile 0.01% poly-L-lysine solution (Sigma) was performed. Plates were incubated for 10 minutes at rtp, rocking at 35rpm. Then coating solution was removed and discarded and plates were incubated at 37°C for 2 hours to dry before cell seeding. 293-HEK were seeded in higher numbers per well (7x10⁵) for 90% confluency. After 15 hours for recovery transfections were performed.
2.3.4. Estimating Efficiency of Mammalian Transfections

Transfection efficiencies were estimated by using a GFP-containing plasmid. Conditions used for the transient transfection were performed with the GFP expression plasmid. A total of 48h after transfection, efficiency of transfection was estimated by counting fluorescent cells in 5 high power fields (x40 magnification), using light and fluorescence microscopy, and deriving the mean percentage of fluorescent viable cells versus non-fluorescent viable cells.

Quantification of protein derived after transfection of mammalian cells was also an indirect measure of transfection efficiency. This involved harvesting serum-free medium, 1ml per well, placed on the cells 15h after transfection. Medium was harvested after 48h incubation and centrifuged at 13000g for 10 minutes to remove debris. Quantification was then performed as described in section 2.5.3.

2.3.5. Concentrating Mammalian Ligands for Cellular Stimulation Experiments

Low yields from mammalian synthetic ligand expression experiments required protein to be concentrated in order to be useful for subsequent cellular stimulation experiments. To do this mammalian cellular transfections using the pSecTag/FRT/V5-His TOPO II containing the COMP-peptide of interest, or the negative control vector described in section 2.3.2, were performed in 6-well plates of HEKs. After transfection, at 48h approximately 6 mls serum-free medium was removed from wells and concentrated using the YM-10 Macrocon (Amicon), which has a 10kDa lower cut-off, to 0.5ml. Briefly, medium was applied to the concentrator column and centrifuged at 1000g, 4°C until the reservoir volume was approximately 0.5ml. Then medium was concentrated to approximately 30μL by the use of the YM-10 Microcon (Millipore), which additionally has a 10kDa lower cut-off. Medium from the
Macrocon was placed in the microcon and centrifuged at 13000g until approximately 30μL remained in the reservoir. Medium from the reservoir was quantified as described in section 2.5.3.

2.4. Bacterial Protein Expression

2.4.1. General Solutions

**BL21 Resuspension Buffer**

50mM tris pH 8, 150mM NaCl, 10mM imidazole, 5% glycerol, 0.5mM DTT, 1x protease inhibitors (143μLml⁻¹ of 7x stock solution, from 1 Complete, Mini, EDTA-free tablet [Roche] in 1.5ml deionised water).

**Wash Buffer I**

50mM tris pH 8, 150mM NaCl, 5% glycerol, 10mM imidazole

**Wash Buffer II**

50mM tris pH 8, 150mM NaCl, 5% glycerol, 30mM imidazole

**Elution Buffer**

1x TBS pH 7.4, 5% glycerol, 1M imidazole

**Dialysis Buffer**

1x TBS pH 7.4

2.4.2. Test Expression of Bacterial Proteins

Transformation of BL21-Gold (DE3) competent cells was performed with pLEIC-03 plasmid encoding the nucleotide sequence for each recombinant construct, as described in section 2.2.9. Transformed agar plates were incubated overnight at 37°C, in CO₂ free conditions, for 12-15 hours, in the presence of 50μgml⁻¹ kanamycin.
For test expression, selected colonies were placed into 1ml LB broth with kanamycin at 50µgml⁻¹. Colonies were incubated for 12-15 hours in a shaker at 250rpm, 37°C. Then 100µL from each culture was placed in 900µL fresh LB broth without antibiotics. Samples were incubated for 2h at 250rpm, 37°C. Subsequently 100µL of each of the cultures was placed on ice, whilst to the remaining 900µL was added 1mM IPTG. Samples were then incubated for a further 2h at 250rpm, 37°C. At this time, for each of the three uninduced and the induced samples, 80µL was removed for further analysis. 40µL was added to 20µL 3xSB and the remaining 40µL was added to 20µL 3xSB/DTT. Samples were sonicated using the MSE Sanyo Soniprep 150 (Gemini BV, Netherlands). Each sample received six 10 second pulses of 3microns. Samples were boiled at 95°C for 6 minutes, centrifuged for 1 minute at 13000g, and 50µL of each of the six samples was loaded onto a 20% SDS-PAGE gel. The gel was run until maximal separation was achieved. The gel was then transferred into Coomassie blue, stained overnight at rtp, rocking, and destained to assess evidence of production of the recombinant proteins. On a fresh 20% gel, in parallel, 10µL of each of the three cultures, both uninduced and induced, was solubilised in 5µL of either 3xSB or 3xSB/DTT, sonicated as described, boiled, centrifuged and loaded. After optimal marker separation, the gel was transferred overnight onto Hybond ECL membrane and probed for the FLAG epitope as described (section 2.10).

2.4.3. Large-Scale Bacterial Protein Expression

The later mainstay of recombinant protein generation used prokaryotes. As a result, larger quantities of functional protein were produced with significantly more ease than with the previously employed mammalian methods.
The adopted bacterial expression host was the BL21-Gold(DE3) competent cell, already described (section 2.2.9.). A colony was selected from the transformant plates (section 2.2.9.) and inoculated into 25 ml LB broth containing 50μg/ml kanamycin. Culture at 37°C, 250 rpm was performed for 15 hours. 15 ml of the culture was then inoculated into 350ml fresh LB broth without antibiotics (which had already been warmed to 37°C). Culture was performed at 250rpm, 37°C until the OD$_{600}$ of the broth reached 1.2, after which protein expression was induced by the addition of 1mM IPTG. Culture was then continued under the same conditions for a further 4 hours. Culture was then centrifuged at 4°C, 18000g for 10 minutes using the Sorvall RC-5B Superspeed Centrifuge (Dupont, Block Scientific, NY). Supernatant was removed and discarded. The BL21 cell pellet was resuspended in 2ml BL21 Resuspension Buffer. Cells were stored overnight at -20°C.

Next, cells were thawed on ice and immediately snap frozen in liquid nitrogen. Again pellets were thawed on ice. To extract the soluble cellular fraction, suspension was sonicated at 10 microns for 15 seconds, on ice. The cycle was repeated a further 9 times, with 30 second intervals between cycles. The suspension was placed in ice for 30 minutes, then centrifuged at 13000g, 4°C for 30 minutes. Supernatant was removed and the pellet discarded. Supernatant was again centrifuged identically for 30 minutes to remove any residual debris. Supernatant was collected.

In order to extract protein from the soluble fraction, firstly, into a Falcon 14 ml conical tube was placed 500μL of Proaffinity IMAC Ni$^{2+}$-charged resin (Biorad). Resin was washed by the addition of 12mls cold Wash Buffer I, and subsequent centrifugation at 2000g for 10 seconds, 4°C. Supernatant was removed and the resin resuspended in the sonicated BL21 soluble fraction. The resuspended mixture was transferred to two 1.5ml Eppendorffs and binding allowed to occur for 1.5 hours at
4°C on a roller. The mixture was then transferred to a fresh Falcon tube, centrifuged at 2000g for 10 seconds, 4°C, and supernatant was removed. To the pellet was added 12ml Wash Buffer I. The pellet was resuspended gently by several tube inversions and then centrifuged at 2000g for 10 seconds, 4°C. This was repeated twice. Then, using 12ml per wash of Wash Buffer II, the wash cycle was repeated a further three times. The remaining pellet was resuspended in 0.5ml Elution Buffer, contents were transferred to a 1.5ml Eppendorff, and elution was allowed to proceed for one hour at 4°C, rolling. Centrifugation at 2000g was then performed for 10 seconds, 4°C, and eluted protein solution removed as supernatant.

Using the Slyde-a-Lyzer (Pierce), with a 2kDa molecular weight lower cut off, protein solution was applied to a cassette and dialysed for 22 hours. Initially, the cassette was suspended in 350ml Dialysis Buffer. After 2 hours this was changed for fresh buffer. After a further 2 hours buffer was again changed and left for the final dialysis for a further 18 hours. Recombinant protein was then removed from the cassette and quantified.

2.5. Analysis of Protein Expression

2.5.1. General Solutions

**Coomassie Blue Stain**

10% ethanoic acid, 40% ethanol, 0.1% Coomassie Blue Stain R-250, in deionised water

**Destain Solution**

10% ethanoic acid, 40% ethanol in deionised water
2.5.2. Characterisation of Recombinant Proteins

Recombinant proteins from the mammalian and bacterial expression systems were analysed by electrophoretic mobility in 10%, 12% or 20% SDS-PAGE gels under reducing and nonreducing conditions. The preparation of SDS-PAGE gels is described in section 2.7.4. Proteins were loaded and resolved as described in section 2.7.4. Proteins were then analysed by staining gels for 24 hours in Coomassie Blue, followed by destaining and visualisation. Proteins were also visualised by Western Blotting, using transfer of SDS-PAGE gels to nitrocellulose membranes and subsequent probing using an anti-FLAG antibody with high specific affinity for the N-terminal FLAG epitope engineered into all recombinant proteins (section 2.10.).

2.5.3. Quantification of Expressed Protein

Quantification of protein was achieved by the use of the FLAG epitope attached to the protein, along with knowledge of the molecular weight of the protein. Dilutions of purified protein in TBS pH 7.45 were dotted in minimum triplicate onto a nitrocellulose Hybond ECL strip, each dot representing 1μL of the diluted COMP-peptide. Dilutions of a stock of COMPangiopoietin-1 of known concentration were also dotted in minimum triplicate on the same strip, at varying concentrations, with each dot representing 1μL of the diluted protein. Dots were allowed to dry for 10 minutes at room temperature. Strips were then probed for the FLAG epitope using identical methods to those described in section 2.6.2 and 2.10, with anti-FLAG M2 antibody. Once developed dot densities were measured by scanning densitometry. Comparing densitometry plots from dilutions of COMP-peptides with dilutions of known quantities of COMPangiopoietin-1 allowed derivation of an estimation of the concentration of the COMP-peptide solution.
2.6. Protein Binding Assays

2.6.1. General Solutions

*Target Dilution Buffer*

0.1M NaHCO₃ pH 8.4

*ELISA Block Buffer (EBB)*

1x TBS pH 7.4, 1% (w/v) BSA, 0.1% (v/v) Tween-20 (polyoxyethylene sorbitan monolaurate detergent) made up in deionised water

*TBSTX-100*

1xTBS pH 7.4, 0.1% Triton X-100 (Sigma), dH₂O

2.6.2. Dot Binding Assay

This assay was used to detect binding of recombinant proteins to Tie2-Fc (R & D systems) adsorbed onto nitrocellulose, or of binding of Tie2-Fc in solution to adsorbed recombinant proteins on nitrocellulose. The assay was conducted by two methods. In the first, recombinant Tie2-Fc chimera was dotted in 50ng, 100ng and 200ng triplicates onto a long rectangular piece of Hybond ECL and allowed to adsorb for 15 minutes at rtp. After drying, the strip was blocked in blocking buffer (TBSTX-100/5% (w/v) milk pH 7.4) for one hour rtp, rocking. Subsequently the longer strip was cut into individual triplicates, each small enough to fit into one well of a 6-well plate. Ministrips were exposed to the protein of interest, at 5µgml⁻¹ (experimental test proteins) or 1µgml⁻¹ (positive control COMPangiopoietin-1), in a total volume of 1ml TBSTX-100 for one hour at rtp, rocking, in separate wells of a six-well plate. Five washes of 5mls TBSTX-100 per well were then performed at rtp, rocking. Thereafter ministrips were incubated together in 1µgml⁻¹ anti-FLAG M2 antibody in TBSTX-100/5% milk for 30 minutes, washed five times together in 40mls per wash TBSTX-
Ministrips were then incubated together for 45 minutes with anti-mouse secondary antibody at 1\,\mu\text{g}\,\text{ml}^{-1} in TBSTX-100/5\% milk at rtp, rocking, washed a further five times and developed and exposed in the same cassette at the same intervals with ECL (see section 2.10.3.). Relative binding of recombinant proteins to Tie2-Fc was quantified by comparative dot densitometry.

In the second method, using adsorbed recombinant protein, a coin-shaped nitrocellulose membrane was fashioned, of a size rendering it sufficiently small to fit comfortably into a single well of a 6-well cell culture plate. 100ng of each recombinant protein of interest was diluted into 4\,\mu\text{L} of 1\times\text{TBS pH 7.4. For each protein 100ng was applied, 1\,\mu\text{L} at a time, to a pre-determined area on the membrane. 100ng COMPangiopoietin-1 was used as a positive control. In addition to the recombinant COMP-negative control protein 100ng recombinant IGF was adsorbed to the membrane as an additional negative control. After all proteins had been added drying was allowed. The membrane was blocked in TBSTX-100/5\% milk for 45 minutes and, after a 2 minute wash in TBSTX-100, incubated with 0.5\text{ml} 20\text{nM Tie2-Fc chimera in TBSTX-100 for one hour. After one hour Tie2-Fc solution was removed and the membrane washed in 6\text{ml} TBSTX-100 twice, for 5 minutes per wash (rtp, rocking 35rpm). Then the membrane was incubated for one hour in 1\text{ml} 1/1000 \textbf{Protein A solution in TBSTX-100/5\% milk. Subsequently unbound protein A was removed. Three washes in 6\text{ml} per well TBSTX-100 were performed, of length 5 minutes per wash (rocking 30rpm, rtp). The membrane was then developed using a chemiluminescent detection system (see section 2.10.3.).}
2.6.3. ELISA Binding Assays

ELISA binding assays were used to assay binding of recombinant proteins to an immobilised Tie2-Fc chimera (R & D systems, Abingdon) on a microtitre plate.

Plates were coated with Tie2-Fc chimera at 10µg/ml in target dilution buffer, 50µL per well, for 12 hours, rocking gently at 4°C. Wells were washed subsequently with 100µL per well of EBB for 5 minutes and then blocked for 45 minutes in 150µL per well of EBB. Recombinant protein ligand was then added, diluted to the required concentration in EBB, and allowed to bind for 60 minutes. Wells were then washed three times with 150µL of EBB, for 5 minutes each rocking. 100µL of detecting Anti-FLAG M2 monoclonal (Sigma) antibody was then added at 1µg/ml in EBB for 30 minutes. After removal of primary antibody 5 washes were carried out, with 150µL per wash of EBB. Each wash was again 5 minutes, rocking at rt. Then secondary anti-mouse HRP-linked antibody (Amersham) was added at 1µg/ml in EBB, 100µL per well, for 60 minutes. Then secondary antibody was removed and 5 further washes, 150µL per wash, preceded the application of 200µL per well OPD solution (SIGMA FAST OPD tablets, Sigma), containing o-phenylenediamine dihydrochloride substrate. After 30 minutes readings were taken at 450nm using the ELx808iu Ultra Microplate Reader (Biotex Instruments, INC) to determine optical density.
2.7. Cellular Stimulation Assays

2.7.1. General Solutions

Cellular Lysis Buffer
50mM tris pH 7.4, 50mM NaCl, 1mM NaF, 1mM EGTA, 1mM sodium
orthovanadate, 1% Triton X-100 (v/v), complete protease inhibitor mixture (Roche)

Phosphate-Buffered Saline (PBS)
140mM NaCl, 10mM NaPO$_4$, 1.8mM KH$_2$PO$_4$

Protein Electrophoretic Running Buffer
25mM tris, 250mM glycine, 0.1% (w/v) SDS

Protein Transfer Buffer
25mM tris, 150mM glycine, 20% methanol (v/v)

3 x Sample Buffer (3xSB)
150mM tris pH 6.8, 6% SDS (v/v), 30% glycerol (v/v), 15mM EDTA pH 6.8, 0.3%
bromophenol blue (w/v)

3x Sample Buffer + DTT (3xSB/DTT)
0.9 ml 3xSB, 45mg DTT

2.7.2. Cell Lines

2.7.2.1. Eahy926 Cells Lines (Eahys)
Eahys are an immortalised human umbilical endothelial cell line derived from fusion
of human umbilical vein endothelial cells with the human lung adenocarcinoma cell
line A549 (Edgell 1983). Cells were a gift from CJ Edgell (North Carolina). Cells
used were between passages 29 and 38. Eahys were grown in DMEM (Biowhitakker)
plus 10% FCS and 2.5mM L-glutamine at 37°C, 5% CO$_2$ For stimulation in various
experiments cells were used in 6-well, 12-well, 24-well, 48-well (2x wells in a 96-well plate) and 96-well plates. Respective seeding numbers were: 8; 4; 2; 1; and 0.5x10^5 cells per well for 75% confluence. To split T80s, medium was removed and cells were washed with 4ml PBS per wash, twice. 0.25% trypsin in PBS, 1ml per flask was applied for 2minutes at 37°C 5% CO2 and cells were then resuspended thoroughly in complete DMEM. Cells were then seeded as required.

2.7.2.2. Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were purchased from Promocell (Heidelberg, Germany). Cell lines were maintained in Medium 199 (Promocell) containing 20% FCS, 5 units/ml heparin and 50μg/ml endothelial cell growth supplement (Promocell) at 37°C, 5% CO2. For stimulation experiments cells in T80s were split into 6-well plates for 70-80% confluence. To split cells, medium was removed and cells in T80s were washed twice with 4mls per wash PBS. 0.25% trypsin in PBS, 1ml per flask, was applied for 1 minute at room temperature. Cells were then resuspended thoroughly in complete Medium 199. Subsequently, to remove residual trypsin, HUVECs were centrifuged for 4 minutes at 150g. After removal of medium cells were resuspended in fresh medium and seeded as required.

2.7.3. Phosphorylation Assays

Assays of the abilities of synthetic COMP-peptides to activate endothelial Tie2 and its downstream signalling mediators Akt were conducted in Eahy926 and HUVECs. The use of HUVECs also allowed assessment of a further downstream signalling mediator, ERK. When assessing the activities of prokaryotic-derived COMP-peptides, cells were stimulated in 6-well plates. However, when assessments
were conducted using mammalian derived COMP-peptides, availability of only smaller amounts of ligand required the development of a smaller-scale assay in 96-well plates.

2.7.4. HUVEC Cellular Stimulation Assays

HUVECs in T80 flasks, which had had a fresh medium replacement 12 hours previously, were trypsinised and split into 6-well plates to achieve 75-80% confluence. After a 24h recovery, medium was removed from each well and wells were washed twice in 1ml of serum-free growth medium at 37°C. Cells were placed in 1ml/well fresh serum-free medium and serum-starvation of wells was performed for 30 minutes at 37°C, 5% CO₂. Cells were removed from the incubator for no more than 1 minute whilst ligands were added to relevant wells. Orthovanadate was added at a total concentration of 1mM for each well to reduce de-phosphorylation of Tie2 (Jones, 1998). Negative controls were represented by either 1ml/well serum-free medium or by the presence of the COMP-RP-Long negative control protein at 1µg/ml⁻¹. 1mM orthovanadate was included with negative controls, in addition to test specimens. Cells were then stimulated at 37°C, 5% CO₂ for 30 minutes and then lysed as described in section 2.7.8.

2.7.5. Eahy Cellular Stimulation Assays in 6-well Plates

Eahys were trypsinised and split into 6-well plates, as described in section 2.7.2.1, to achieve approximately 75-80% confluence at stimulation. After a 24h recovery in complete medium, cells washed twice with 1ml per well serum-free medium. Cells were then placed in 1ml per well fresh serum-free medium and serum-starved at 37°C, 5% CO₂ for 4.5h. The period of serum-starvation of 4.5h was derived
by test experiments showing that, in negative controls, levels of Akt phosphorylation were sufficiently suppressed at this time to allow obvious distinction from negative control.

After serum-starvation ligands were added at the required concentration. Serum-free medium alone was used as negative control. Just after ligands were added, orthovanadate was added to all wells to achieve 1mM final concentration, in order to minimise de-phosphorylation of Tie2. After 30 minutes stimulation at 37°C, 5% CO₂ cells were lysed as described in section 2.7.8.

Note that there was no period of serum-starvation observed in which negative control levels of phospho-ERK could be suppressed to any significant degree in Eahy926. Therefore Eahy926 were not considered suitable for the observation of the effects of COMP-peptides on downstream ERK modulation.

### 2.7.6. Eahy Cellular Stimulation Assays in 96-well Plates

For assessing the effects of mammalian-derived COMP-peptides on Eahy926 Tie2 phosphorylation, small yields of ligand required smaller volumes to attain effective concentrations for stimulation. Development and verification of the principle is described in section 4.2.

Cells were prepared as already described (section 2.7.2.1.) with 0.5x10⁵ cells seeded per well of a 96-well plate. After a 24h recovery in complete medium cells were washed twice with 0.1ml per well serum-free medium. Cells were then placed in 0.1ml per well serum-free medium to serum-starve for 4.5h at 37°C, 5% CO₂. Then serum-free medium was removed and replaced with 25μL concentrated mammalian conditioned medium containing quantified COMP-peptide ligand. Stimulation and lysis was then carried out as described elsewhere in this section. Concentration of
mammalian cell-derived conditioned medium was performed as described in section 2.3.5.

<table>
<thead>
<tr>
<th>Well Size</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding Number Eahy/×10⁶</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Serum-starvation Volume/ml</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.25</td>
<td>0.1</td>
</tr>
<tr>
<td>Volume lysis Buffer/μL</td>
<td>50</td>
<td>40</td>
<td>40</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 2.1. Preparation of Eahy926 for Cellular Stimulation.** This table illustrates the initial seeding numbers of Eahy for cellular stimulation experiments in 6-, 12-, 24-, 48-, and 96-well plate experiments. Also shown are the subsequent volumes in which cells were placed for serum-starvation, in addition to the volumes of lysis buffer added to cells.

### 2.7.7. Cellular Kinetics Assays

In order to assess the kinetics of Tie2 activation in endothelial cells, Eahy926 and HUVECs were exposed to synthetic COMP-peptide ligands in 6-well plates. The principles are similar to those described in sections 2.7.4. and 2.7.5. However there are slight differences in cell treatments.

For Eahy926, cells were split into 6-well plates for approximately 75-80% confluence. Each well was serum-starved for 4.5h. Subsequently, cells were exposed for the allocated period to the ligand under test. Orthovanadate was not used in these experiments, in order to avoid a variable period of exposure of different time courses to vanadate. Negative controls were serum-starved for 4.5h before the application of serum-free medium alone for 30 minutes. After lysis, whole cell lysates were loaded onto 7.5%, 10% or 12% SDS-PAGE gels, run, transferred to Hybond and probed for
specific phospho-Tie2 antibody (Phosphodetect), phospho-akt (serine 473), and subsequently total Tie2 and total akt.

HUVECs were treated similarly. However the period of serum-starvation was 30 minutes only. Orthovanadate was not applied to cells at any point during the assay. Again, a negative control was provided by treating serum-starved cells with serum-free medium alone for 30 minutes. After loading and transferring whole cell lysates to Hybond ECL, membranes were probed for phospho-Tie2, phospho-ERK p44/p42 and phospho-akt. Membranes were then stripped and reprobed for total Tie2, total akt and total ERK p44/p42.

2.7.8. Mammalian Cellular Lysis

After stimulation cells were placed immediately on ice and transferred to a 4°C environment to minimise protein de-phosphorylation. Wells were washed with ice-cold PBS. Ice-cold, freshly prepared, lysis buffer was added to each well. Volumes of lysis buffer depended on well size and are indicated in table 2.1. Lysis buffer was mixed evenly across each well by gentle agitation. Immediately, wells were scraped using a cell scraper and lysates were collected in 1.5ml Eppendorffs. After centrifugation at 13000g for 15 minutes, supernatant was collected and debris discarded. Supernatant volumes were mixed with a 50% proportional volume of 3xSB/DTT, boiled at 95°C for 6 minutes using the Perkin Elmer Thermal Cycler, and either stored at -20°C or sampled for SDS-PAGE gel electrophoresis.
2.8. Protein Separation by SDS-PAGE

Cellular proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Resolving gels used for cell lysates were 7.5%, 10% and 12% for cell stimulation assays. For protein analysis, 20% gels were used predominantly. The stacking gel was a 5% gel. Gels were prepared using the Mini-Protean Gel Kit (Biorad), to create 10-well gels, of 1mm or 1.5mm depth, depending on sample loading volume required. Two gels per time were created using a double-gel casting stand. Long and short gel plates were loaded, filled with deionised water, and checked to observe that no appreciable leaks had occurred. Resolving and stacking gels were prepared according to the composition illustrated in table 2.2. Briefly, for a resolving gel, filtered acrylamide (Protogene) was added to a 0.37M tris-HCl (pH 8.8) solution made up with deionised water. After mixing, 0.1% SDS (v/v) was added, followed by a further mix. Ammonium persulphate was made fresh as a 10% solution (w/v). This was then added to resolving gel mix to a final concentration of 0.067% (v/v), followed by a further mix. To induce polymerisation tetramethylethlenediamine (TEMED) 0.7μL per ml of gel was added, mixed, followed by rapid application between gel plates. A layer of approximately 5ml deionized water was pipetted, extremely slowly, to cover the setting resolving gel. For a stacking gel polyacrylamide was added to a 0.125M tris pH 6.8 solution, made up with deionised water. After mixing 0.1% SDS and 0.1% ammonium persulphate were added sequentially, followed by mixing. Finally, 1μL per ml TEMED was added, mixed, and the gel gently laid on top of the resolving gel. Either a 1mm or 1.5mm, 10-well comb was inserted immediately. Once set, gels were applied to a casting frame in a gel tank, filled with running buffer, and loaded. If not used immediately, gels were stored in clingfilm at 4°C for a maximum of three days.
Between 30 and 60µL samples were loaded into each gel lane using a 100µL pipette. Protein separation was estimated by comparison with a lane, run concomitantly, in which 10µL protein molecular weight markers (Biorad) had been loaded. Gels were run at 120V until desired resolution was attained.

<table>
<thead>
<tr>
<th></th>
<th>20%</th>
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<th>10%</th>
<th>7.5%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/</td>
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<td>8.0</td>
<td>6.7</td>
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</tr>
<tr>
<td>0.8% bisacrylamide</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mL)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Tris pH 8.8 (mL)</td>
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<td>3.7</td>
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<td>Tris pH 6.8 (mL)</td>
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<tr>
<td>dH2O (mL)</td>
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<td>9.6</td>
<td>10.9</td>
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<tr>
<td>TEMED (µL)</td>
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<td>14</td>
<td>14</td>
<td>14</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.2: Composition of SDS-PAGE gels

2.9. Western Blot Analysis of Proteins

For probing, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech). Transfer buffer was poured into a tray. A transfer cassette was opened and a buffer-soaked gel-sized piece of sponge-paper, with a soaked piece of filter paper, was used to form one half of the transfer sandwich. The gel was gently removed from between the glass plates, underwater, and applied to one half of the sandwich. An appropriately-sized piece of Hybond ECL membrane was then applied directly over the gel, so that the latter would lie on the anode side of
the gel in the cassette. The sandwich was completed with a further piece of filter paper and a transfer sponge. To minimise air bubbles the whole process was carried out immersed in transfer buffer. The sandwich was closed gently and inserted onto a Mini-Trans Blot (Biorad), in a tank which had already been filled with transfer buffer. Transfer was carried out for a minimum of 12 hours at 0.15A. After completion transfer membranes were probed or stored at 4°C, wrapped in cling film.

2.10. Probing Nitrocellulose Membranes

2.10.1. General Solutions

TBSTX-100

1xTBS pH 7.4, 0.1% Triton X-100, dH2O

Developer Solution

1M tris pH 8.8

2.10.2. Probing Method

Membranes were probed for the proteins of interest using antibodies. Immediately after transfer, membranes were washed in 30-50 ml TBSTX-100 for 5 minutes. Then membranes were blocked for one hour at rtp rocking, using 20mls TBSTX-100 with either 5% (w/v) milk or 5% (w/v) BSA, depending on the antibody. After a 2 minute rinse in TBSTX-100 antibody was applied in TBSTX-100 alone, or containing 5% BSA or 5% milk, and incubated in a heat-sealed plastic wallet for the period required. Incubation temperature and times depended on antibody (see section 2.10.5.). Unbound primary antibody was washed off by the use of a minimum of three washes in TBSTX-100, lengths and volumes varying significantly depending on primary antibody type (see section 2.10.3.). Secondary antibody was then added at a
concentration of 1μgml⁻¹ in TBSTX-100 containing 5% milk for 45-80 minutes, depending on the primary antibody used. Again antibody and membrane were heat-sealed in a plastic wallet and incubation was carried out at rtp, rocking at 30rpm. After removal of secondary antibody three further washes were carried out in TBSTX-100, rocking at rtp at 30rpm, to remove unbound secondary antibody. The volumes and lengths of washes are again dependent on the primary antibody used (see 2.10.5).

2.10.3. Chemiluminescent Detection of Proteins

An enhanced chemiluminescent detection system was used to detect bound secondary antibodies, which were conjugated with Horseradish peroxidise (HRP). 1ml developer solution was diluted into 10mls dH₂O, resulting in an 0.1M tris solution. To this 22μL solution A (90mM p-coumaric acid in DMSO) and 50μL solution B (250mM luminol [=5-amino-2,3-dihydro-1,4-pthalazinedione in DMSO]) were added. After mixing, 3μL hydrogen peroxide was added and the solution was mixed again. The probe was then soaked in this ECL solution for one minute, immediately after the last wash-off of secondary antibody. After wrapping in clingfilm, and placing in a developer cassette, photosensitive film was then exposed to the probe.

2.10.4. Reprobing Nitrocellulose Membranes

The membrane was washed in TBSTX-100 for 5 minutes at rtp, rocking at 30rpm. Subsequently the membrane was placed in 1x Reblot Plus Mild (Chemicon International: 1ml of 10x stock solution plus 9mls dH₂O). Incubation was performed for 6 minutes at rtp, rocking at 30rpm. Then two 5 minute washes with 40-50mls TBSTX-100 were carried out, after which fresh blocking buffer was applied.
2.10.5. Antibodies used for Probing

*PhosphoDetect Phospho-Tie2 (Ab-1)(pTyr\(^{1102/1108}\))(Calbiochem)*

This rabbit antibody was used to detect activated Tie2 at tyrosine residues 1102 and 1108 in whole cell lysates. After blocking in TBSTX-100/5% BSA, antibody was applied at 1 in 4000 dilution from the manufacturer's aliquots, in TBSTX-100/5% BSA, for 12 hours. After removal of primary antibody, three washes of at least 50mls per wash, and each wash for 20 minutes were used. Secondary anti-rabbit HRP-linked Fab antibody was used in a 1 in 2000 dilution in TBSTX-100/5% milk. Following this, three 30-minute washes, at least 50mls per wash, were performed.

*PY99 (Santa Cruz Biotechnology)*

Mouse antibody, used to detect phospho-tyrosine residues in activated Tie2. Blocking was performed in TBSTX-100/5% BSA for one hour. Primary was applied at 0.2μg/ml for 12 hours at 4\(^{\circ}\)C. Washes for primary were 3 washes, at least 40mls per wash, and for 15 minutes each. Secondary anti-mouse was applied for 80 minutes at 1/2000 in TBSTX-100/5% milk, followed by three washes of at least 40mls per wash. The first two washes were 20 minutes each, followed by a final wash of at least 50mls for 30 minutes.

*Anti-Tie2 Antibody (R&D Systems)*

This goat antibody was applied at 0.5μg/ml\(^{-1}\) in TBSTX-100/5% milk for 12 hours at 4\(^{\circ}\)C, after one hour blocking in TBSTX-100/5% milk solution. After primary removal, three washes of 20mls each, for 10 minutes each, were performed. Secondary anti-goat HRP-linked antibody was applied subsequently in TBSTX-
100/5% milk for 60 minutes. This was followed by three washes, each of 20mls, for 10 minutes each.

Anti-Phospho-Akt (serine 473) Antibody (Cell Signalling)

A mouse monoclonal antibody was used for detecting activated Akt in whole cell lysates. Blocking was in TBSTX-100/5% milk for one hour, followed by three washes, for 5 minutes each, in 15mls per wash of TBSTX-100. Primary antibody was then applied at 2μgml⁻¹ for 12 hours at 4°C. Subsequently three washes of 15mls per wash, for 5 minutes each, were carried out at rtp. Secondary HRP-linked anti-mouse antibody was then incubated for 80 minutes. Then three further washes, of 15mls per wash, for 5 minutes each, preceded probe development.

Anti-Akt Antibody (Cell Signalling)

A rabbit polyclonal antibody was used for the estimation of total Akt in whole cell lysates. After blocking in TBSTX-100/5% milk for 45 minutes, primary antibody was applied at 1μgml⁻¹ in TBSTX-100/5% milk for 30 minutes. Then three washes were performed, using at least 50mls per wash, for 10 minutes each. This was followed by incubation with secondary anti-rabbit HRP-linked antibody for 45 minutes. Then three washes of at least 50ml per wash for 15 minutes each, were performed prior to developing.

Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) Antibody (Cell Signalling)

A mouse monoclonal antibody used to detect activated ERK, membranes were probed in the exact fashion described for the phospho-Akt (serine 473) antibody. Primary antibody was applied at 2μgml⁻¹
**P44/42 MAPK (Erk1/2) Antibody** (Cell Signalling)

A rabbit polyclonal antibody used to detect total ERK, membranes being probed in the same fashion as for total Akt. Primary antibody was applied at 1μgml⁻¹.

**Anti-FLAG M2 Antibody** (Sigma)

A mouse monoclonal antibody, used to detect the FLAG epitope fused to the N-terminal aspect of COMP-peptides. Membranes were blocked for 30 minutes in TBSTX-100/5% milk. Primary antibody was applied at 1μgml⁻¹ in TBSTX-100 for 30 minutes at room temperature, before six washes of two minutes each in TBSTX-100. Secondary anti-mouse was incubated for 30 minutes in TBSTX-100/5% milk, followed by three 15 minute washes and probe development.

### 2.11. Statistical Analysis

Probes were analysed by densitometry, using ImageJ (available online at: http://rsbweb.nih.gov/ij/). Where at least three experiments were performed, statistical significance was determined by the Student’s t-test. During ELISA binding assays using the synthetic ligands, best fit concentration response curves and EC50 concentration approximations were performed using Prism (available at: http://www.graphpad.com/prism/ ).
Results
3: Generation and Expression of Recombinant COMP-peptides
Introduction

Biochemical and structural studies conducted by a number of authors have demonstrated that induction of tetramer formation of the receptor-binding domain of angiopoietin-1 is necessary and sufficient for the activation of its receptor, Tie2 (Kim, 2005; Davis, 2003). Several authors have also shown that the ang1 receptor-binding domain can activate Tie2 as a minimally tetrameric construct in a variety of conformations (Cho, 2005; Davis, 2003; Maisonpierre, 1997).

It was hypothesised, therefore, that synthetic, small molecular mass proteins capable of binding Tie2 might be used as synthetic agonists for the receptor. The small mass proteins would require to be presented in a minimally tetrameric format. In order to test this hypothesis it was necessary to design such molecules comprising of receptor-binding proteins presented in a multimeric scaffold. To minimise size, and therefore simplify production and reduce complicating structural interactions, chosen receptor-binding domains were heptameric peptides which are known to bind Tie2 specifically.

3.1. Receptor-Binding Domains

Peptides chosen were isolated by phage display from large random libraries of heptapeptides fused to the M13 bacteriophage pIII coat protein. These peptides have been shown to bind specifically to the Tie2 receptor. The first was isolated using the Ph.D.™-7-Phage Display Peptide Library (New England Biolabs) and shown to bind specifically to Tie2, but not to VEGFR2 (Kang M, unpublished data). The amino acid sequence of this peptide is VTSRGNV, which does not bear any homology with the fibrinogen-related domain of ang1 in BLAST homology searches (Kang M, unpublished data). The second peptide, with the amino acid sequence NLLMAAS,
was also derived using the Ph.D.™-7-Phage Display Peptide Library as described by Tournaire, et al (2004). NLLMAAS was able to antagonise the binding and actions of angiopoietin-1 at the Tie2 receptor in a concentration dependent fashion, both in vitro and in vivo. This suggests that NLLMAAS binds within the angiopoietin-1 binding site on Tie2.

Figure 3.1: Schematic representation of design of multimeric peptide construct. A: An N-terminal FLAG epitope is attached to the coiled-coil domain of rat cartilage oligomeric matrix protein (COMP) via a short linker (GSGG). Between COMP and the heptameric peptide presented to Tie2 is a further linker, GSGGPFYSHS, containing an upstream flanking sequence (PFYSHS) from M13 bacteriophage pIII minor coat protein. The peptide is then followed by a short C-terminal flanking segment, GGGSA, also presented downstream of the peptide in the original pIII coat protein. B: The construct is hypothesised to form a disulphide-linked multimer, predominantly pentameric.
3.2. Peptide-Presentation Scaffold

As discussed above and in section 1.4.3, angiopoietin-1 requires presentation in a minimally tetrameric oligomerisation state for significant activation of Tie2. Therefore, to present Tie2 receptor-binding peptides designed to oligomerise to induce Tie2 activation, a multimerisation motif with a minimally tetrameric conformation is required. Additionally this should be a stable protein, preferably an endogenous protein. Recent approaches by Cho and colleagues (Cho, 2004a) have resulted in the design of the recombinant protein, COMP-angiopoietin-1. This is a fusion construct of the C-terminal Tie2-binding domain of native human ang1 with the N-terminal coiled-coil domain of rat cartilage oligomeric matrix protein (COMP, amino acid sequence DLAPQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMEDACG, residues 28-77). The resulting protein forms stable, soluble tetrameric and pentameric oligomeric conformations of the ang1 fibrinogen-related domain.

It was therefore hypothesised that small peptides may be presented in a stable multimeric format to the Tie2 receptor using COMP as a multimerisation motif. The design for the construct is illustrated in figure 3.1. The construct consists of an N-terminal FLAG epitope, DYKDDDDK, fused to a short linker region, GTSGG. The short linker is fused directly to the presented heptameric peptide sequence. Immediately downstream of the peptide is a C-terminal sequence, GGGSA. The two flanking sequences, upstream PFYSHS and downstream GGGSA, were flanking sequences present in the M13 bacteriophage pIII coat protein of the phage display library. It was therefore decided to incorporate these flanking segments into
the final construct because this context of presentation of the peptide might be important.
Figure 3.2. Schematic Diagram of Generation of cDNA Encoding COMP-peptides.
A: Two overlapping oligonucleotides were used initially. One encoded FLAG at the N-terminus attached to Linker 1 and the N-terminal segment of the nucleotide sequence for cartilage oligomeric matrix protein. The other encoded the downstream C-terminal sequence of COMP attached to Linker 2. There was a 10 nucleotide sequence complementary overlap. The two oligonucleotides correspond to ‘SENSE COMP’ and ‘ASENSE COMP’ respectively, the sequences of which are illustrated in section 2.2.2. PCR was carried out as described in sections 2.2.3. and 3.3. B: The resulting FLAG-Linker1-COMP-Linker2 oligonucleotide was used as a template for further PCR using upstream primer ‘COMP5PCR’ and either downstream primer ‘Pep1.1.4.’ or ‘NLLM’ (section 2.2.2.). The two downstream primers contain sequences for VTSRGNV and NLLMAAS respectively, together with a short overlapping complementarity to Linker2. Thus PCR with these primers, as described in sections 2.2.3. and 3.4, enabled cDNA for the full length COMP-VTSRGNV or COMP-NLLMAAS to be generated (C). Colour code: FLAG, green; Linker1, black; COMP, yellow; Linker2, red; C-terminal heptameric peptide, blue.
3.3. Engineering The FLAG-COMP-Linker Construct

The strategy used to generate the construct illustrated in figure 3.1 is as follows. Overlapping oligonucleotides were used in overlap PCR to create the full-length cDNA encoding FLAG-Linker1-COMP-Linker2. The resulting cDNA was used as a ‘scaffold’, since different peptides could subsequently be added onto the C-terminus of the scaffold to potentially alter the final properties of the complete FLAG-Linker1-COMP-Linker2-Peptide construct. Different peptides were added to the scaffold by overlap incorporation PCR. The process is illustrated in figure 3.2.

In order to construct the COMP encoding sequence, with an N-terminal FLAG epitope encoding sequence and a C-terminal 9 amino acid linker, overlapping oligonucleotides SENSE COMP and ASENSE COMP (section 2.2.2.) were used in PCR, using standard conditions with Biotaq, as described in section 2.2.3. PCR product was run on a 1% agarose gel (section 2.2.4.) and visualised under UV light. The expected band was observed at approximately 200 bp (expected size 204 bp), as demonstrated in fig 3.3. Remaining PCR product was cloned into the pSECTagFRT/V5/His-TOPO-2 vector, amplified and colonies plated onto selective agar, as described in section 2.2.5.

Figure 3.3: Overlap PCR of SENSE COMP and ASENSE COMP. PCR was performed using the overlapping oligonucleotides SENSE COMP and ASENSE COMP, under standard PCR conditions described in section 2.2.3. PCR product was analysed by separation on a 1% agarose gel and visualised using UV light. The expected product from overlap and amplification is 204bp, representing the cDNA comprising of the FLAG-Linker1-COMP-Linker2 (see also figure 3.2A).
From the resulting agar colonies, six were used in a colony screen to select appropriate positive colonies for sequencing. Primers used were \textit{COMP5PCR} (forward) and \textit{Pep1.1.4.} (reverse) under standard PCR conditions with Biotaq. The expected band in the resulting agarose gel for the test was 243bp. This was observed in 4 of the 6 tested colonies (figure 3.4). cDNAs from four colonies were sequenced, confirming the desired FLAG-Linker1-COMP-Linker2 product. This scaffold could subsequently be used to generate full length nucleotide sequences for the complete constructs.

\textbf{Figure 3.4: Colony Screen PCR for FLAG-COMP-Linker Oligonucleotide.} To detect colonies transformed with the pSECTagFRT/V5/His-TOPO-2 vector containing the FLAG-Linker1-COMP-Linker2 scaffold cDNA, small amounts from 6 individual colonies on agar were used as templates for PCR amplification, under standard conditions. Forward primer (COMP5PCR) and reverse primer (\textit{Pep1.1.4.}) were included. From each colony PCR, product was run on a 1% agarose gel and visualised under UV light. The expected product from positive colonies, representing the FLAG-Linker1-COMP-Linker2-VTSRGNV cDNA, was observed in colonies 1, 3, 5 and 6 at approx 243bp (black arrow).
3.4. Engineering Genetic COMP-peptide Constructs

From colony DNA containing the FLAG-Linker1-COMP-Linker2 sequence, DNA was used as a template for an incorporative PCR reaction, under standard conditions with Biotaq, using upstream primer COMP5PCR and downstream primer Pep1.1.4. This is illustrated schematically in figure 3.2B. PCR product was analysed by running on a 1% agarose gel as described. Visualisation under UV light confirmed the expected band at approximately 250bp (figure 3.5: actual size 243bp) in the test specimen but not in the control. Immediately, the band was purified as described in section 2.2.5. A sample of purified PCR product was cloned into the pSECtagFRT/V5/His-TOPO-2 vector, using conditions described in section 2.2.6, and used to transform competent E coli. After plasmid amplification positive colonies were selected by PCR screening.

![Figure 3.5: Overlap PCR of FLAG-Linker1-COMP-Linker2 cDNA With Pep1.1.4. Downstream Primer.](image)

Colony DNA containing the cDNA for FLAG-Linker1-COMP-Linker2 was used as a template with upstream primer COMP5PCR and downstream primer Pep1.1.4. under standard PCR conditions. PCR product was analysed by running on a 1% agarose gel, as described previously, and visualising under UV light. The expected oligonucleotide band is observed (arrow).
As illustrated in figure 3.6, three of the six colonies chosen appeared to contain the expected nucleotide segments representing the cDNA for FLAG-Linker1-COMP-Linker2-VTSRGNV (colonies 7C, 7D and 7F). This was confirmed by sequencing (see Appendix C).

COMP-NLLMAAS was engineered using the same principles. Again colony DNA containing the FLAG-Linker1-COMP-Linker2 sequence was used as a template for an incorporative PCR reaction, under standard conditions with Biotaq, using upstream primer COMP5PCR and downstream primer NLLM. Subsequent sequencing of the product confirmed the desired cDNA (see Appendix C).
3.5. Mammalian Protein Expression of COMP-Peptides

In order to use the mammalian recombinant COMP-peptides it was necessary to produce functional, stable, quantifiable protein. CHO-K1 cell lines were transfected transiently as described in section 2.3.2. The transfecting plasmid contained the FLAG-Linker1-COMP-Linker2-Peptide construct. The peptide was either VTSRGNV or NLLMAAS. Additionally, a control plasmid was used. This contained the construct encoding VTSRGNV but in the reverse reading frame, ie not encoding for protein. This was named, ‘7D’ (see also section 2.3.2.).

After 48 hours incubation in serum-free medium, removed medium was centrifuged. Samples of supernatant were then solubilised under reducing or nonreducing conditions, heated to 95°C and run on 20% SDS-PAGE gels. Proteins were transferred to Hybond ECL nitrocellulose membrane by Western blotting. Subsequently, probing with anti-FLAG M2 antibody was used to detect proteins versus control (48 hour medium from CHO cells transfected with 7D plasmid). Both COMP-VTSRGNV and COMP-NLLMAAS showed the expected multimerisation under nonreducing conditions (fig 3.7), with protein existing mainly as pentamers at approximately 60-65kDa. To a lesser extent, tetramers, trimers, dimers, and a very small proportion of monomers (12kDa) were also observed under these conditions. Under reducing conditions, however, the expected stronger monomer band was not seen. CHO cell lysates were also prepared for protein detection. Each well of a 6-well plate was lysed directly using reducing or nonreducing conditions. Cells were scraped and lysates were collected, sonicated, centrifuged and samples loaded onto a 20% gel. Transfers were probed for FLAG epitope, as with medium. In CHO lysates under
reducing conditions the monomer was visible easily at approximately 12 kDa (fig 3.7).

Figure 3.7: Mammalian recombinant COMP-peptide proteins in reducing and nonreducing conditions. A: Reducing conditions from CHO cell lysates: 40μL of lysate from a well transfection of CHOs from a 6-well plate, from either wells transfected with COMP-VTSRGNV or control (7D) plasmid, solubilised and boiled with 20μL 3xSB/DTT. Samples were run on a 20% SDS-PAGE gel, transferred to Hybond ECL and probed using anti-FLAG M2 antibody. B: Nonreducing conditions from CHO cell medium: 40μL supernatant from extracellular medium from CHO transfections was solubilised in 20μL 3xSB and run on a 20% SDS-PAGE gel. The gel was transferred to Hybond ECL and probed with anti-FLAG M2 antibody as described previously.
3.6. Time Course of Accumulation of Secreted Recombinant Protein

In order to estimate the most efficient time to harvest medium from CHO transfections, the time course of accumulation of COMP-VTSRGNV was observed after CHO cell transient transfections. CHO cells were seeded into 8 wells on two six-well plates, transfected using Superfect and 7C plasmid, with 7D as control transfection. Fifteen hours after transfection, cells were placed in 1ml per well serum-free medium. After a further 24, 48, 72 and 96 hours medium was removed from separate wells. 40µL medium from each time interval was analysed via SDS-PAGE gel electrophoresis under nonreducing conditions, as described in sections 2.5. and 3.5. Dot quantification of protein, in triplicate, was performed in parallel. The results are represented in figure 3.8. Though a single experiment, this analysis suggested that a maximal yield of protein was attainable by harvesting conditioned media from CHO cells at 48 hours. After this time the results suggest that COMP-VTSRGNV content is reduced in media. Absolute maximum under these conditions was apparent at 48h, at approximately 11.3ng protein per well.
Figure 3.8: Accumulation of COMP-VTSRGNV in CHO cell conditioned medium. CHOs were transfected transiently using the method described for Superfect in 6-well plates (section 2.3.2.), using the plasmid pSECtagFRT/V5/His-TOPO-II vector containing either VTSRGNV or negative control (7D) nucleotide sequence. Fifteen hours post transfection medium was replaced with 1ml per well serum-free CHO medium (α-MEM). Medium was removed at 24, 48, 72 or 96 hours, centrifuged to remove debris, analysed by SDS-PAGE gel electrophoresis with probing for the FLAG epitope, and assayed by dot quantification. A: 20% SDS-PAGE gel of protein accumulation versus control. B, Dot quantification blot of protein in medium at alternative times of harvest. C: Graphical representation of calculated protein in test conditioned CHO medium. Data bars represent means of triplicate dot quantification from a single experiment.
3.7. Optimisation of Protein Expression

In the previous section, the estimated generation of COMP-VTSRGNV per transfection of a well of CHOs was approximately 11.3ng. Generation of large quantities of COMP-peptides in this fashion would be inefficient and costly.

To attempt to increase the efficiency of mammalian protein expression, experiments to assess the effects of varying different transient transfection parameters were performed. The experiments apply to production of COMP-VTSRGNV. The conditions tested were: effects of optimising transfections using Superfect; effects of varying initial seeding densities of CHOs using Superfect; effects of using alternative cellular media during transfection with Superfect; effects of using alternative lipofection reagents to Superfect; the effects of using alternative cell lines for transfection; and the effects of incubation of cells in serum-free versus complete medium after transfection.

Transfection efficiencies were estimated using GFP as a marker of transfected cells, as described in detail in section 2.3.4. Additionally, generation of COMP-VTSRGNV was estimated directly. This involved harvesting serum-free medium, 1ml per well, placed on the cells 15h after transfection. Medium was harvested after 48h incubation and centrifuged at 13000g for 10 minutes to remove debris. Dot quantification was then performed, as described in section 2.5.3.

3.7.1. Effects of Optimising Superfect

The effects on transient transfection of varying the DNA:Superfect ratio from 1:0.5 to 1:10 was investigated by keeping total amount of plasmid DNA constant, at 2µg per well of a 6-well plate, whilst increasing the relative quantity of
Superfect, from 1μL up to 20μL. Transfections were otherwise performed in the fashion described (section 2.3.2.1.). Relative efficiency was estimated by comparing proportions of GFP-transfected CHOś post-transfection in high power fields by light microscopy. As suggested in figure 3.9A, the manufacturer’s initial specifications of 2μg DNA and 10μL Superfect appeared to be the most efficient protocol.
Figure 3.9. Optimisation of transient transfections using Superfect in CHO cells. A: Effect of altering Superfect: DNA ratio. CHO cells were seeded into 6-well plates and transfected as described for Superfect (section 2.3.2.1.) except for variation of the Superfect:DNA ratio in the transfection complexes from 0.5 to 10. This was achieved by keeping the total DNA constant at 2μg per reaction whilst varying volume of Superfect from 1μL to 20μL. GFP plasmid was used to transfect CHO cells. In control CHO cells no GFP plasmid was added. 48h after transfection efficiency was estimated by counting green fluorescent cells in five high power (x40) fields. Bars are representative of mean counts from 5 high-power fields, with error bars representing standard error, from a single experiment.

B: Effect of altering initial seeding number in wells of a 6-well plate. Transfections using the GFP plasmid were carried out as described in section 2.3.2.1, whilst varying initial seeding number of CHO at: 1.25; 3.5; 4.75 and 7 x10⁵ per well. In control CHO cell wells, no GFP plasmid was added. 48h post transfection counts of GFP expressing cells were used to estimate efficiencies of transfection. Bars represent mean and standard errors of counts from 5 high power (x40) fields by fluorescence microscopy, from a single experiment.
Figure 3.10 . Effects of Alternative Transfection Media on CHO Cell Transfections: To estimate the effects of using different media, CHOs were transfected using the GFP plasmid under the conditions described in section 2.3.2.1. However DMEM and Ham’s F12 were used as alternatives to α-MEM. In control CHO cells no GFP plasmid was added during the transfection. 48h post transfection efficiencies were estimated by counting green fluorescent cells under fluorescence microscopy. Bars represent means and standard errors of counts from 5 high power (x40) fields, from a single experiment.
3.7.2. Effects of Optimising CHO Cell Seeding Density

Variation of seeding numbers of cells could alter confluency during transfection. To estimate the effects of altering initial 6-well plate seeding number, CHO cells were seeded at alternative seeding densities of 1.25, 3.5, 4.75 and \(7 \times 10^5\) cells per well of a six well plate. Transfection was then continued in a standard fashion as described in section 2.3.2.1, using GFP as the transfecting plasmid. 48h after transfection, efficiency was estimated by comparing mean counts of fluorescent cells in five high power fields (x40 magnification) per variable. Results are shown in figure 3.9B and suggest \(4.75 \times 10^5\) cells per well seem to lead to maximise efficiency of transfection. On this basis further transfections using CHO cells and Superfect were carried out using initial seeding numbers of \(4.75 \times 10^5\) cells per well.

3.7.3. Effects of Using Alternative Media During Transfection

An experiment was conducted to determine the effects of the use of alternative media during transient CHO transfection using Superfect. Standard \(\alpha\)-MEM, with 2.5mM L-glutamine, with or without 10% FCS was compared with DMEM (Biowhitaker, Lonza) plus 2.5mM L-glutamine +/- 10%FCS, or Ham’s F12 (Invitrogen) plus L-glutamine +/-10% FCS. Other than using alternative media, transfections were conducted in an identical fashion, as described in section 2.3.2.1. GFP plasmid was used as the transfecting DNA, and efficiencies were again estimated by comparative GFP expression at 48 hours after the first medium change. Results are represented in figure 3.10. This experiment did not suggest there would be any benefit to changing the incubating medium used for transfection, since there seemed no obvious improvement using any of the alternatives to \(\alpha\)-MEM.
3.7.4. Effects of Alternative Transfection Reagents in CHO Cells

Cells were transfected as described in section 2.3.2. Alternative reagents used for lipofection were Polyfect, in which the manufacturer’s protocol (specifically pre-optimised for CHO cells) was used, and lipofectamine 2000, which required optimisation. In order to optimise lipofectamine 2000 optimisation, experiments were performed in which DNA: lipofectamine ratios from 1:0.5 to 1:5 were achieved during transfection. DNA was fixed at 4μg per reaction whilst Lipofectamine was applied at 2,5,10,15 or 20μL. A negative control was achieved by transfecting in the absence of a lipofection reagent. Proportional GFP expression results initially suggest that 15mL Lipofectamine 2000 is perhaps most efficient (fig 3.11). However at least 50% of viable cells had been lost at 48h, compared to the use of 10mL. Therefore the latter was used in subsequent experiments.

Subsequently, in experiments comparing the three reagents in CHO cells, transfection was estimated using the GFP plasmid. Additionally, protein yield was also estimated by using plasmid containing COMP-VTSRGNV, and estimating protein yield as described (section 2.5.3.). Figure 3.12 shows the results of the use of alternative lipofection reagents. Both the GFP expression experiment (fig 3.12A) and COMP-VTSRGNV dot quantification experiment (fig 3.12B), though single experiments, suggest that lipofectamine 2000 is the most effective reagent for the transient transfection of CHO cells under these circumstances.
Figure 3.11: Optimisation of Lipofectamine 2000 in CHO Cells. A: Optimisation of lipofectamine2000 transfections in CHOs. Cells in 6-well plates were transfected as described in section 2.3.2. During cellular transfections, all other parameters were kept constant, whilst volume of lipofectamine 2000 was varied from 2µL to 20µL per transfection. GFP expression was quantified at 48h post transfection, bars representing mean and standard error of counts from 5 high power fields, from a single experiment.
Figure 3.12: Effects of alternative reagents on transfection in CHO cells. A: CHO cells in 6-well plates were transfected with three alternative transfection reagents, as described in section 2.3.2., using the manufacturer’s specifications and GFP expression plasmid. The figure demonstrates mean proportion of GFP-transfected cells at 48 hours in cells transfected with Superfect, as compared with Lipofectamine2000, Polyfect, and cells not transfected with the GFP plasmid (Control). Bars represent means of 5 high power field counts for fluorescent CHO cells from a single transfection. Error bars represent standard error of the mean. B: Similar experimental transfection performed as in A, but using the pSECtagFRT/V5/His-TOPO-II vector containing either VTSG or 7D (Control). Medium was replaced with 1ml/well serum-free medium 15h post-transfection. Recombinant protein was quantified at 48h by dot blotting. Each bar represents a mean of triplicate dot quantification from a single experiment.
3.7.5. Effects of Using an Alternative Cell Line for Transfection

A 293-HEK cell line was also used comparatively to determine whether protein yield could be improved. An initial experiment was conducted to estimate the comparative effects of Superfect, Lipofectamine2000 and Polyfect on transfection of the GFP plasmid. HEKs were seeded and transfected as described in detail in section 2.3.3. Fifteen hours later medium was changed, and GFP transfection proportions were observed in an identical fashion to CHO cells (section 2.3.4.). The results, shown in figure 3.13A, suggested lipofectamine 2000 to be the most efficient reagent.

For comparison between the effects of lipofectamine 2000 on transient protein expression in CHO and 293-HEK cells, an experiment was conducted comparing one well of each cell type transfected with the plasmid for COMP-VTSRGNV. The optimal transfection conditions established were used for lipofectamine 2000. Medium was collected and centrifuged at 48 hours. Dot quantification in triplicate was performed as described (section 2.5.3). A negative control involved transfection of CHO cells with the same quantity of the 7D negative control plasmid. The results are shown in figure 3.13B. Though 293-HEK appeared more efficient than CHO cells in this single experimental comparison, the yield for the 293-HEK cells in this experiment is quite similar to that of CHO cells for COMP-VTSRGNV in figure 3.12B. This suggests that there is likely to be little real difference between the two cell types using lipofectamine 2000. Since 293-HEK were in abundance at this time they were used for future experiments.
Figure 3.13: Transfection using 293HEK cell lines. A: HEK-293 were seeded at $7 \times 10^5$ per well in 6-well plates and transfected in the manner described previously (section 2.3.4.) with GFP plasmid, using either Superfect, Polyfect or lipofectamine 2000. A negative control well was included in which no GFP plasmid was present in the transfection reaction. 48h post-transfection mean proportional GFP expression was quantified as described previously. Bars represent means with error bars representing standard error of the mean of five counts at high power magnification fluorescence microscopy, from a single experiment. B: Direct comparison of efficiency of protein production in a single well of HEK-293 versus a single well of CHO-K1 with pSECtagFRT/V5/His-TOPO-II vector encoding COMP-VTSRGNV. HEK-293 and CHO cells were seeded and transfected as described in section 2.3, using lipofectamine 2000. As a negative control CHOs were transfected as described with lipofectamine but using the TOPO-II plasmid 7D (negative control plasmid). After 15h recovery in complete medium cells were placed in serum-free medium. The latter was harvested 48h later and assayed by the methods described for secreted COMP-VTSRGNV. Bars represent a mean derived concentration of COMP-VTSRGNV in medium from dot densitometry.
3.7.6. Effect of Serum on Protein Expression

The presence of serum in the cellular medium might exert an effect on protein production. To test this HEK-293 were seeded into two wells of a 6-well plate for transfection with pSECtagFRT/V5/His-TOPO-II vector encoding COMP-VTSRGNV. Transfection was performed in a standard fashion (see section 2.3.3.). After 15h medium was changed for either serum-free medium or complete medium (1ml/well). Medium was harvested at 48h and expression of COMP-VTSRGNV quantified by dot blotting. A single experiment was performed.

As shown in figure 3.14 there is no clear advantage suggested from the use of either serum or serum-free medium.

![Figure 3.14](image)

**Figure 3.14. Effects of Serum on Protein Expression in 293-HEK Cells.**
HEKs were plated into wells of a 6-well plate and transfected using pSECtagFRT/V5/His-TOPO-II vector encoding COMP-VTSRGNV, as described in detail in section 2.3.4. Fifteen hours post transfection medium was changed for 1ml per well serum-free medium or complete medium. After a further 48h medium was harvested and quantified by dot blotting. A control was included by tranfecting a well of HEKs with 7D plasmid. Bars represent mean protein expression from triplicate dot quantification from a single experiment.
3.8. Comparison of Optimised Protein Generation Versus Pre-Optimised Conditions

After establishing an optimal set of transfection conditions, both COMP-VTSRGNV and COMP-NLLMAAS were generated using these conditions. Mean yield of COMP-VTSRGNV was investigated both pre- and post-optimisation, by comparing yields from three transient transfections using CHO-s transfected with Superfect in the standard method (sections 2.3.2.) versus yields from four transient transfections where HEK-293 were transfected using the optimised protocol with lipofectamine 2000 (sections 2.3.3. and 3.7.4.). As illustrated in figure 3.15A there was an observed difference between pre-optimised yield and post-optimised yield. Mean yield was 28ngml\(^{-1}\) post-optimisation versus a mean yield of 7.8ngml\(^{-1}\) pre-optimisation. However, the apparent improvement was not statistically significant (p=0.138: see figure 3.15A). Optimised conditions were also used for the expression of COMP-NLLMAAS. Mean yield for the latter was lower, at approximately 7.7ng, but not significantly lower when compared directly with yields for COMP-VTSRGNV over three independent experiments (p=0.23: see figure 3.15B).

Subsequently protein was used from transfections of 293HEK in 6-well plates. Expressed medium was concentrated as described (section 2.3.6.) This smaller volume of conditioned medium with a higher concentration of protein was used for cellular stimulation assays in 96-well plates (section 4.1).

In addition to transient transfections, stable expression of ligands was also achieved by using antibiotic selection following co-transfection with test plasmid and an antibiotic resistance plasmid. However, protein yield for stable transfectants was consistently lower than transient transfectants (data not shown). Therefore, work with these stable transfectants was not continued.
Figure 3.15: Yield of recombinant protein from transient transfection systems. A: Effect of optimisation of transient transfections on yield of recombinant protein. Four transient transfections using optimised conditions were compared with three transient transfections before optimisation. 48h post-transfection COMP-VTSRGNV was collected in serum-free medium and quantified as described previously. Bars represent mean concentration of COMP-VTSRGNV in ng/ml⁻¹. Error bars represent standard error of the mean of concentration. B: Comparison of Yield of COMP-VTSRGNV and COMP-NLLMAAS using Optimised Conditions. 293-HEKs were transfected in 6-well plates using the conditions optimised for Lipofectamine 2000. Methods were as described in section 2.3.4. After 48h serum-free medium was collected and quantified as previously described. Bars represent mean concentration, error bars representing standard error of the mean from three independent experiments.
3.9. Expression of COMP-peptides in a Prokaryotic System

Using the mammalian systems described above, convincing generation of sufficient quantities of COMP-peptides for subsequent purification and cellular assays had not been achieved, despite attempts to optimise the yield of transient systems and the creation of a stable CHO cell line. Therefore, the feasibility of expression of COMP-peptides in bacterial cells was examined.

3.9.1. Generation of COMP-peptide Oligonucleotide Constructs

The first step was to use PCR to extract the oligonucleotide sequences of COMP-VTSRGNV and COMP-NLLMAAS from their mammalian plasmids, with the addition of 5’ and 3’ regions which would allow subsequent insertion of the PCR products into the prokaryotic vector, pLEIC-03 (see figure 3.16). As a result of both COMP-peptides possessing common 5’ and 3’ ends common primers were able to be used for the extraction of both sequences from their mammalian expression vectors.

Mammalian expression plasmids pSecTag/FRT/V5-His TOPO II containing COMP-VTSRGNV or COMP-NLLMAAS which had already been created (section 3.4) were used to extract the nucleotide sequences of COMP-VTSRGNV and COMP-NLLMAAS. Purified plasmid DNA was used in PCR reactions with the two plasmids using the common primers AJM5 and AJM3. The PCR conditions were standard, as described in section 2.2.3. The primers include sequences for the generation of the 5’ and 3’ overhangs required for subsequent directional cloning into the pLEIC-03 expression vector (see section 2.2.9.). After completion of PCR, products were run out on a 1% agarose gel as described previously (section 2.2.5.), and visualised under UV light. The expected 276bp products were observed, as shown in figure 3.16. Bands were purified as described in section 2.2.6. Purified product was cloned into
the prokaryotic expression vector pLEIC-03 by the Protein Expression Laboratory (University of Leicester). Sequencing confirmed the correct sequences for COMP-VTSRGNV and COMP-NLLMAAS.

Figure 3.16: PCR of pSecTag/FRT/V5-His TOPO II using AJM5 and AJM3 primers. Mammalian pSecTag/FRT/V5-His TOPO II plasmids, containing the insert for either COMP-VTSRGNV or COMP-NLLMAAS, were used as templates with the primers AJM5 and AJM3, for PCR under standard conditions. PCR products were separated on a 1% agarose gel and visualised under UV light. The blue arrow indicates the observed bands.
3.9.2. Protein Expression of Prokaryotic COMP-peptides

Transformation of BL21-Gold (DE3) competent cells was performed with pLEIC-03 plasmid containing the oligonucleotide sequences for COMP-VTSRGNV or COMP-NLLMAAS (also section 2.2.9.). Subsequently a small scale test expression was conducted, to estimate the productivity and the feasibility of subsequent larger-scale expression of COMP-peptides by this method. Test expressions were carried out in small volumes of broth, and are described in detail in section 2.4.2. After expression small volumes of induced and non-induced samples were run in parallel on 12% SDS-PAGE gels. Gels were either stained for expressed protein using Coomassie blue, or transferred to Hybond ECL and probed for the FLAG protein tag on the ligands. The process is also described in sections 2.4.2. and 2.5.2.

Results of the test expression are shown in figure 3.17. Clear expression of both proteins was observed versus the negative control, particularly in the test sample for COMP-VTSRGNV. On the Coomassie stain (figure 3.17A) convincing bands for protein expression versus control are only observed between 10 and 15kDa, presumed to represent the monomer. The FLAG probes in figures 3.17B and 3.17C indicate that for both COMP-VTSRGNV and COMP-NLLMAAS, most protein is observed in the reduced form, regardless of the application of DTT. However small bands are seen suggesting multimerisation for both test proteins, particularly in the absence of DTT (figure 3.17B, C).

Figure 3.17 (p112): Test Expression of COMP-peptides in the BL21-Gold (DE3) Prokaryotic System. BL21 transformed with the pLEIC-03 plasmid containing oligonucleotides for expression of COMP-VTSRGNV or COMP-NLLMAAS, or a negative control plasmid, were grown in 1ml broth containing kanamycin 50µg/ml and induced to express protein using IPTG. For detailed method refer to section 2.4.2. After induction, samples of both induced and uninduced BL21 were solubilised, sonicated, and run on 12% SDS-PAGE gels, in the presence or absence of DTT, as indicated. Then gels were either stained using Coomassie blue (A) or transferred to Hybond ECL nitrocellulose and probed using anti-FLAG antibody (B). C: FLAG probe, darker exposure, to show protein bands of COMP-NLLMAAS. Arrows indicate position of monomeric (red) or polymeric (blue) proteins.
3.9.3. Extraction of Protein-Containing Cellular Fraction

Initially, the partitioning of expressed protein in BL21-Gold (DE3) cells was investigated. A test expression was performed in 1ml culture as described (section 2.4.2.), for each protein and negative control. Induction was performed, after which each 1ml culture was split into two equal parts. All samples were centrifuged at 13000g for 5 minutes. Supernatant was removed and the pellet solubilised in 300μL Wash Buffer I, either with or without 1% triton X-100. Three episodes of 10 seconds sonication were then performed for each sample. Centrifugation at 13000g was performed for 10 minutes to separate the soluble fraction from the insoluble pellet. Supernatant was removed and resolved by SDS-PAGE, followed by Coomassie staining or transfer to nitrocellulose membrane and probing for FLAG. Results are illustrated in figure 3.18A and B. Both recombinant proteins were present in both soluble and insoluble cellular fractions. The effect of adding Triton X-100 seems minimal. Furthermore, what is observed in the soluble fraction represents only approximately one tenth of the total from a test specimen, whereas that from the insoluble fraction represents almost one half of the test specimen. Thus it was decided to extract protein only from the soluble fraction, since it was expected that this would be simpler, and would avoid potentially denaturing protein in an attempt to remove it from inclusion bodies.
Figure 3.18. Investigation of BL21-Gold (DE3) Soluble and Insoluble Fractions for Test Expression of COMP-peptide Ligands. A test expression of BL21 containing either the pLEIC-03 plasmid for COMP-VTSRGNV, COMP-NLLMAAS, or the negative control, was induced then split into two equal parts. Cultures were then centrifuged, supernatant removed and solubilised in Wash Buffer I, with or without 1% triton X-100 to aid solubilisation. After sonication and further centrifugation, samples were resolved by SDS-PAGE. Gels were either stained for protein using Coomassie blue (A) or transferred to Hybond ECL and probed for with anti-FLAG antibody (B). Red arrows indicate proteins.
3.9.4. Extraction of COMP-peptides from the Soluble Cellular Fraction

Having established that the desired ligands were both produced clearly into the soluble cellular fraction under test conditions, protein generated subsequently was purified from the soluble fraction by binding with nickel beads, followed by elution using 1M imidazole. This is described in detail in section 2.4.3.

3.9.5. Large Scale Preparation of Prokaryotic COMP-Peptides

In order to generate larger amounts of COMP-VTSRGNV and COMP-NLLMAAS for experiments of its activity, the test expression and extraction system was scaled up to larger cultures. As described in sections 2.4.3 and 2.5.3., protein was produced and quantified by dot densitometry. Approximate yields were 0.14-0.21mg per litre of culture medium used.
3.10. Characterisation of Prokaryotic COMP-peptides

To assess whether structural organisation of prokaryotic COMP-peptides is similar to that of mammalian-derived ligand, one microgram of each purified recombinant protein was solubilised in either reducing or nonreducing conditions. Samples were resolved on a 20% SDS-PAGE gel and the gel stained using Coomassie Blue. Additionally, 50ng each protein was resolved on an additional 20% gel, under both reducing and nonreducing conditions. The gel was transferred to a Hybond ECL membrane and probed using anti-FLAG antibody. Results are shown in figure 3.19. Under reducing conditions one band at the expected molecular weight of approximately 12kDa is observed for both COMP-VTSRGNV and COMP-NLLMAAS. Under nonreducing conditions the protein is observed to oligomerise to a predominantly pentameric configuration. The Coomassie gels show only the bands observed on the FLAG probes, confirming that the purified protein is free from detectable protein contaminants.

Formal tests of stability were not performed for prokaryotic COMP-peptides. However, using dot quantification (section 2.5.3), it was estimated that after 2 weeks in storage at 4°C, both prokaryotic COMP-peptides had diminished at least 50% from the original quantity. Protein was still able to produce the appropriate multimers under nonreducing conditions on SDS-PAGE gel and FLAG probe analysis. After 3 months storage at -20°C prokaryotic COMP-peptides had lost <5% of original quantity, as observed on dot quantification. At this time such stored COMP-peptides were able to form the normal constellation of oligomers seen in figure 3.19.
Figure 3.19. Characteristics of prokaryotic COMP-peptides. COMP-VTSRGNV and COMP-NLLMAAS were prepared in BL21 expression systems and purified (see section 2.4.3.). For each ligand, 1 µg was run on a 20% SDS-PAGE gel under reducing and non-reducing conditions, and stained for protein using Coomassie blue. Also, 50 ng of each protein was also run under reducing and non-reducing conditions and transferred to Hybond ECL, with subsequent probing for the FLAG epitope. A: Coomassie Blue staining (left) versus transfer and FLAG probe (right) for COMP-VTSRGNV. B: Coomassie Blue staining (left) versus transfer and FLAG probe (right) for COMP-NLLMAAS. Red arrows indicate monomeric protein. Blue arrows indicate various oligomeric forms.
3.11. Generation of a Negative Control COMP-peptide

In chapters 4 and 5 COMP-peptides are tested for their ability to bind and activate endothelial Tie2. In some experiments COMP-peptides are tested against the ability of serum-free medium applied to cells alone to activate Tie2. However the ideal negative control is a COMP-peptide with the FLAG-Linker1-COMP-Linker2 scaffold attached to a peptide not selected for Tie2 binding, named COMP-NEGATIVE. The initial aim in creating COMP-NEGATIVE was the creation of a ligand similar to COMP-NLLMAAS by random shuffling of the heptapeptide to LALANSM. This peptide would not be expected to bind Tie2. Methods used for the generation of the ligand are similar to those described in section 3.2.6. PCR using template containing FLAG-Linker1-COMP-Linker2 with upstream primers AJM5’ and NLMContDownstream, under standard conditions, generated the presumed cDNA for FLAG-Linker1-COMP-Linker2-LALANSM, which was subsequently cloned into pLEIC-03 by the Protein Expression Laboratory. Sequencing did not confirm the expected product, COMP-LALANSM, but instead a longer oligonucleotide product with the same FLAG-Linker1-COMP-Linker2 backbone as COMP-peptides, but with an irrelevant 35 amino acid peptide on its C-terminus (see appendix C). The reason for this was a point mutation, most likely during PCR with Biotaq, leading to a frameshift. Protein was produced in prokaryotes, using the same fashion described in section 2.4.3. Yields of protein were lower, at 60-80μg per litre of culture medium.

Protein was characterised by SDS-PAGE gel electrophoresis and probing for anti-FLAG in the same fashion as for COMP-peptides, described in sections 2.5.2. and 3.10. Results are represented in figure 3.20. The COMP-NEGATIVE construct appears to form similar sized monomers and multimers to COMP-VTSRGNV. However, additionally, there is a longer heavier monomeric fragment in the reduced
species, which also appears to form different-sized multimers in the nonreduced species. Though this was not the intended negative control, it is a construct containing the FLAG-COMP-linker with a peptide on the C-terminus. For this reason, in addition to time constraints, further assays were conducted using this COMP-NEGATIVE construct. It is used principally as a negative control during binding assays of COMP-peptides to Tie2, described in section 4.2.1.
Figure 3.20: Structural Analysis of COMP-Negative. For Coomassie staining analysis (right panels), 1μg of purified COMP-NEGATIVE protein (labelled C-NEG) was solubilised in either 3xSB/DTT (A) or 3xSB (B) and loaded onto a 20% SDS-PAGE gel. For probing with anti-FLAG antibody (left panels), 20ng protein was loaded in a similar fashion, run, transferred to Hybond ECL and probed (also described in section 2.5.2.). In each case the same quantity of COMP-VTSRGNV was run in parallel for comparison (labelled C-VTSR). Red arrows indicate oligomers observed with both recombinant proteins. Blue arrows indicate oligomers observed only with COMP-NEGATIVE.
3.12. Generation of Extended Length COMP-peptides

It was of interest later in the project to create COMP-peptides with a longer Linker2 region, subsequently named COMP-Long peptides. The rationale is discussed in detail in section 5.2. The aim was the creation of COMP-peptides with a potentially greater armspan to cluster Tie2 receptors.

The plasmid containing the FLAG-Linker1-COMP-Linker2 cDNA was used as a template in standard PCR with upstream primer COMP5PCR and downstream primer Extender to insert a longer nucleotide segment, GGTTCAGGTGGTGGT, into the original sequence. This encoded an extra 9 amino acids, GSGGGSGGG, onto the original scaffold. The resulting product was isolated from a 1% agarose gel and product cloned into the pcDNA3.1/V5-His TOPO plasmid. Screening for appropriate colonies was performed and a number of positive colonies were sequenced, confirming the insert for the longer scaffold, FLAG-Linker1-COMP-Long Linker (see Appendix C). Purified plasmid was used in further PCR reactions, under standard conditions, using upstream primer AJM5’ and downstream primers, p1.1.14. or NLMLei (to incorporate peptide VTSRGNV or NLLMAAS, respectively). PCR product was cloned into pLEIC-03 by the Protein Expression Laboratory (University of Leicester) and sequencing confirmed the cDNAs of the desired COMP-Long Peptides, COMP-VTSRGNV-Long and COMP-NLLMAAS-Long (see Appendix C).

3.12.1. Protein Expression of COMP-Long-peptides

Sequenced pLEIC-03 plasmids containing the oligonucleotides encoding COMP-VTSRGNV-Long and COMP-NLLMAAS-Long were used to transform
BL21-(Gold) DE3 competent cells and generate protein in the exact manner and conditions described in section 2.4.3. Yields were similar to those for the shorter COMP-peptides, at 0.14-0.21mgL\(^{-1}\).

3.12.2. Biochemical Characterisation of COMP-Long-peptides

To characterise COMP-Long-peptides, 1\(\mu\)g of each of COMP-VTSRGNV, COMP-VTSRGNV-Long and COMP-NLLMAAS-Long were resolved on a 20\% SDS-PAGE gel under reducing or nonreducing conditions. In parallel, 50ng of each was also run on a 20\% gel and transferred to Hybond ECL. The membrane was then probed with anti-FLAG antibody. Detailed methods are described in section 2.5.3. Results are shown in figure 3.21. Under reducing conditions prokaryotic COMP-VTSRGNV-Long and COMP-NLLMAAS-Long have the predicted molecular mass at around 12.5kDa. Under nonreducing conditions all three proteins run very similarly as pentamers (figure 3.21B), with a smaller proportion of tetramers, trimers, dimers and monomers. There appears to be little difference between the pentamers of prokaryotic COMP-VTSRGNV and prokaryotic COMP-Long-peptides. This is expected, since the difference in predicted size between prokaryotic COMP-VTSRGNV pentamers and those of COMP-Long-peptides is only 3kDa (59.5kDa versus 62.5kDa, respectively), not visible on the resolution of the gel. Again, purity of the protein appears good.

Thus, biochemically, prokaryotic COMP-Long-peptides behave very similarly to their shorter counterparts.
Figure 3.21. Structural Characteristics of Prokaryotic COMP-Long-peptides. 1μg of each of COMP-VTSGN, COMP-VTSGN-Long, and COMP-NLLMAAAS-Long were solubilised in or 3xSB/DTT (A) or 3xSB (B), loaded onto a 20% SDS-PAGE gel, run and stained for protein with Coomassie blue (left panels). Additionally, 50ng of each protein was also solubilised in 3xSB or 3xSB/DTT, run on a 20% SDS-PAGE gel, transferred to Hybond ECL nitrocellulose and probed with anti-FLAG antibody (right panels). For each condition control lanes were run with 3xSB+/- DTT alone. Red arrows indicate monomeric protein, blue arrows show various oligomers.

As a control for the COMP-Long-peptides, COMP-TMYQLNF-Long (COMP-RP-Long) was created. This consists of the same FLAG-Linker1-COMP-LongLinker construct as COMP-VTSRGNV-Long and COMP-NLLMAAS-Long but with the randomly chosen peptide TMYQLNF in place of the specific peptides VTSRGNV or NLLMAAS. Such a peptide has not been selected for its ability to bind Tie2. It was intended to have an identical oligomerisation scaffold, weight and structural characteristics to COMP-VTSRGNV/NLLMAAS-Long, but to be unable to bind Tie2. To generate the oligonucleotide for COMP-RP-Long, plasmid containing the oligonucleotide for the FLAG-Linker1-COMP-LongLinker construct, described in this section, was used as template in standard PCR (see section 2.2.3. for further detail) using upstream primer AJM5 and downstream primer for peptide incorporation, RPDownstream. PCR product was purified and used in directional cloning into pLEIC-03 as previously described. Sequencing confirmed the correct oligonucleotide (see appendix C). Protein expression was carried out as for other prokaryotic COMP-peptides. This is described in detail in section 2.4.3. Yields were on average slightly lower than for COMP-VTSRGNV-Long and COMP-NLLMAAS-Long, at approximately 0.1mgL⁻¹.

Characterisation of COMP-RP-Long was performed in a similar fashion to other COMP-peptides, described in the preceding sections. Results are shown in figure 3.22. Under reducing conditions prokaryotic COMP-RP-Long appears to form a similar sized monomer as prokaryotic COMP-VTSRGNV-Long (figure 3.22A). Under nonreducing conditions both proteins form very similar pentamers, with lesser proportions of tetramers and some trimers (figure 3.22B). This suggests that, structurally, COMP-RP-Long, behaves similarly to COMP-VTSRGNV-Long. Purity
of protein from large scale expression can be observed to be good (Coomassie stains, figure 3.23).

Unfortunately, after storage at -20°C, COMP-RP-Long was observed to precipitate, with loss of >50% protein after a single storage and thaw. Under storage conditions at 4°C, stability was similar to COMP-VTSRGNV-Long, with dot quantification suggesting minimal protein loss at 1 week (<10%) and remaining protein able to form standard structural elements, but >50% loss after 2 weeks storage. Protein therefore had to be produced and used within several days.
Figure 3.22. Structural Characteristic of Prokaryotic COMP-RP-Long Negative Control Protein. 1 μg of each of COMP-VTSRGNV-Long and COMP-RP-Long were solubilised under reducing (A) or nonreducing (B) conditions, run on a 20% SDS-PAGE gel and stained using Coomassie blue (left panels). In parallel, 50ng of each protein were also solubilised under reducing or nonreducing conditions, run on a 20% SDS-PAGE gel, transferred to nitrocellulose and the membrane probed with anti-FLAG antibody (right panels). In each case a negative control consisting of solubilisation buffer alone was run in concert with proteins. Red arrows indicate protein monomers, whilst blue arrows indicate variable-sized oligomers.
**Discussion**

The experimental work in this chapter describes the construction of soluble, small, multimeric constructs of peptides, previously selected for their ability to interact specifically with the Tie2 receptor. The structure chosen as a multimerisation motif was rat cartilage oligomeric matrix protein (COMP). The reason for this was that COMP has been used successfully by Cho and colleagues (Cho, 2004a) to express a multimeric recombinant construct of the angiopoietin-1 FLD domain.

Initially, generation of the COMP-peptide constructs was performed in mammalian cell lines. Initial experiments to characterise the proteins suggested both mammalian COMP-VTSRGNV and COMP-NLLMAAS formed the expected pentameric structures at approximately 60kDa, with a lesser proportion of tetramers and lesser-order oligomers. Under reducing conditions both mammalian COMP-VTSRGNV and COMP-NLLMAAS were observed as approximately 12kDa monomers, as predicted. Despite attempts to optimise the system, principally using alternative transfection reagents and alternative cell lines, overall yields were too low to enable purification of large quantities of protein. Nevertheless, as is described in section 2.3.5, concentration of medium from transient transfections will enable some cellular assays to be performed using mammalian protein (see chapter 4).

The next system utilised in the production of recombinant proteins was the prokaryotic BL21-Gold (DE3) expression system. These cells have been used successfully for the expression of many proteins. One theoretical disadvantage was the lack of mammalian posttranslational modification of proteins. However, analysis of the literature concerning residues 28-77 of the coiled-coil domain of COMP, which was the part of COMP required, suggested that covalent bonding between interdomain cysteine residues is not responsible for its oligomerisation (Malashkevich, 1996).
Furthermore, no posttranslational glycosylation of COMP occurs in any of the residues from amino acids 28-77 (Zaia, 1997) Additionally, work by Belitskaya and colleagues suggested that a stable multimerising version of residues 28-73 of COMP could readily be produced as part of an unrelated fusion construct in BL21s (Belitskaya, 2004). This suggested that prokaryotic systems might be feasible for the generation of COMP-peptides. Indeed, cloning and expression of COMP-peptides in BL21 here proved to generate adequate yields of proteins for cellular assays. Structural characterisation suggested that these prokaryotic-derived COMP-peptides form the expected monomers under reducing conditions, whilst multimerising predictably into predominantly pentameric form under nonreducing conditions. Proteins were stable under freezing storage for several months at -20°C.

An additional type of ligand with extended linker was also constructed. Here, for each protein, the linker2 region between COMP and C-terminal peptide (see figure 3.1) was lengthened by 9 amino acids. The extra length consisted of serines and glycines only, with the emphasis on retaining flexibility. The resulting proteins were produced in the same way as for prokaryotic COMP-peptides. Structural analysis revealed them to behave physically in the same way as COMP-peptides and to be of similar stability. The further use of these molecules will be discussed in detail in chapter 5.

Two proteins were created and expressed whose role was to act as negative control proteins. The aim was to develop proteins with identical structures to COMP-peptides but with C-terminal heptameric peptides which are not selected to bind Tie2. This would enable them to be used as controls of COMP-peptides. The first, COMP-NEGATIVE (see section 3.11), was the culmination of attempts to create COMP-LALANSM, ie a FLAG-Linker1-COMP-Linker2 oligomer attached to a reshuffled
version of NLLMAAS as peptide. This should not be able to bind Tie2 and therefore function as an appropriate negative control. However, as a result of a point mutation during PCR, the resulting protein consisted of the FLAG-Linker1-COMP-Linker2 oligomer attached to a 35 amino acid C-terminal peptide. Structurally, this formed both similar oligomers to COMP-VTSRGNV, in addition to larger oligomers. The disadvantages of this are that COMP-NEGATIVE is not the ideal intended negative control structurally. However, the 35 amino acid on the C-terminus is not selected to bind Tie2, and indeed any potential nonspecific interaction between peptide and Tie2 would expect to be enhanced with a longer peptide. Therefore because of this, with the secondary consideration of additional time constraints, this protein was used as a negative control in chapter 4.

The second negative control created was designed to be an appropriate negative control for COMP-Long-peptides (section 3.12.3.). Therefore it was designed using the same oligonucleotide scaffold as these proteins, ie the FLAG-Linker1-COMP-Long-Linker oligonucleotide. However, a randomly chosen heptameric C-terminal peptide was utilised, namely, TMYQLNF. The resulting COMP-TMYQLNF-Long, or ‘COMP-RP-Long’, was shown structurally to form identical stable multimers to COMP-VTSRGNV-Long. This suggested it might be useful as a control for COMP-Long Peptides. Whilst being almost ideal as a negative control, this protein tended to precipitate out of solution on storage at -20°C. Since it was identical to COMP-VTSRGNV-Long and COMP-NLLMAAS-Long in every other way than the C-terminal peptide, this suggests that perhaps an alternative peptide would have more ideal in terms of practical storage. An additional alternative which was not explored would have been the possibility of using the FLAG-Linker1-COMP-Linker2 scaffold without any attached peptide. This may have been easier to
generate, in fact. However, it would not have been as close to the ideal control as a construct with a C-terminal peptide.
4: Interaction of COMP-peptides with the Tie2 Receptor
Introduction

In chapter 3 the rational design and protein expression of multimeric ligands expressing heptameric Tie2-binding peptides on an oligomerising COMP scaffold was described. In this chapter the aims are to assess the ability of COMP-VTSGNVR and COMP-NLMAAS to bind and induce phosphorylation of Tie2. Though mammalian expression of recombinant proteins was not achieved on a large scale, methods are demonstrated in the following sections by which these proteins could be used for cellular stimulation assays on vascular endothelial cells. However, the majority of the succeeding experiments are conducted using the prokaryotic proteins.

Results

4.1. Binding Characteristics of COMP-peptide Ligands to Tie2

To investigate whether the prokaryotic recombinant proteins can bind Tie2 two approaches were adopted. Assays were performed in which Tie2 ectodomain, or ligand, was immobilised onto a nitrocellulose surface. ELISA assays were also performed for soluble COMP-peptide ligand binding to adsorbed Tie2 ectodomain.

4.1.1. Binding of COMP-peptides to Tie2-Fc Under Conditions of Immobilisation

The binding of COMP-peptides to Tie2-Fc chimera was investigated for conditions in which one element was immobilised on nitrocellulose, whilst the other was incubated and allowed to bind. Bound element could then be detected by probing using antibody to detect an epitope on the surface of the soluble component. The methods adopted are described in detail in section 2.6.2.

In the first set of experiments Tie2-Fc was immobilised by adsorption onto nitrocellulose membrane. Then, by incubating with soluble synthetic COMP-peptides
followed by incubation with antibody specific to the N-terminal FLAG epitope on soluble ligands, binding could be assayed. The binding of a 5μgml⁻¹ solution of COMP-peptides was tested against 50ng, 100ng or 200ng of immobilised Tie2-Fc. Binding of the control construct, COMP-NEGATIVE (see section 3.11), was also tested. Binding of COMP-VTSRGNV, COMP-NLLMAAS and COMP-NEGATIVE to adsorbed Tie2-Fc was assessed as described in section 2.5.3. Briefly, quantities of Tie2 ectodomain were adsorbed onto nitrocellulose and incubated in solutions of COMP-peptides, allowing binding. Unbound ligand was then washed off before incubating the membranes with anti-FLAG M2 antibody to assay for FLAG (indicating bound ligand). Membranes were then developed simultaneously and dot size and densitometry used to estimate binding. Results are shown in figure 4.1, revealing that both ligands COMP-VTSRGNV and COMP-NLLMAAS exhibit concentration-dependent binding to adsorbed Tie2 ectodomain. However, COMP-NEGATIVE does not.

Binding was also assessed by immobilizing the ligand itself onto nitrocellulose and then incubating with soluble Tie2-Fc. Using protein A, bound Tie2-Fc could then be detected. Binding was tested against a further negative control, recombinant IGF-1, by adsorbing 100ng of each test protein to nitrocellulose in 4μL dots, allowing to dry, and subsequently exposing the nitrocellulose to soluble Tie2-Fc chimera at 20nM for one hour before probing using protein A. The method has been described in detail in section 2.6. As shown in figure 4.2 A and B, significant binding was observed of soluble Tie2-Fc to adsorbed COMP-VTSRGNV and to COMP-NLLMAAS but not to recombinant IGF-1. Also of note was that under these conditions there was a significant and sizeable difference apparent in the binding of soluble Tie2-Fc to 100ng of COMP-VTSRGNV as compared to that to 100ng COMP-NLLMAAS.
Figure 4.1. Binding of COMP-peptides to Immobilised Tie2. Tie2-Fc was dotted and allowed to adsorb at 50ng, 100ng and 200ng on nitrocellulose membrane. After blocking strips were exposed to COMP-VTSRGNV, COMP-NLLMAAS or COMP-NEGATIVE at 5µg/ml in TBSTX-100, or COMPang1 at 1µg/ml for one hour at rtp. Unbound ligand was washed off and strips were then probed together with anti-FLAG M2 antibody (see also section 2.6). A: The probe shows a representation of results from one experiment. B: Plot of mean proportional optical density for COMP-VTSRGNV versus COMP-NEGATIVE over three independent experiments. C: Plot for COMP-NLLMAAS versus COMP-NEGATIVE over three independent experiments. D: Comparative plot of COMP-VTSRGNV and COMP-NLLMAAS using the data in B and C. All data is presented as mean proportional optical density, with error bars representing standard error of the mean. * indicates p<0.05, ** indicates p<0.01 (Student’s t-test).
Figure 4.2: Binding of Soluble Tie2 to Immobilised COMP-peptides. 100ng of COMP-VTSRGNV, COMP-NLLMAAS, recombinant IGF-1 or COMPangiopoietin-1 was adsorbed onto nitrocellulose, blocked and exposed to soluble Tie2-Fc at 20nM for one hour. After washing membrane was probed with protein A and developed in ECL. Dot densitometry was then used to measure the degree of binding of soluble Tie2-Fc to immobilised ligands on nitrocellulose. A: Representation of a probe from one experiment. B: Mean proportional optical densities for COMP-peptides versus recombinant IGF-1 from thee independent repetitions of the experiment described in A. Bars represent mean dot densitometry from three experiments, with error bars representative of standard error of the mean. *** indicates p<0.001 versus recombinant IGF, ** indicates p<0.001 versus COMP-NLLMAAS (Student’s t-test).
4.1.2. ELISA Assays and Quantitative Analysis of Binding of COMP-peptides to Tie2-Fc

In section 4.1.1, results indicate that COMP-VTSRGNV and COMP-NLLMAAS bind specifically to Tie2-Fc chimera. Under certain conditions, COMP-NLLMAAS was observed not to be as efficacious a binder as COMP-VTSRGNV. The use of ELISA assays of binding of soluble ligand to Tie2-Fc adsorbed onto a plastic surface was adopted to investigate this relationship further. Assays were conducted according to the method described in section 2.5.4. COMP-VTSRGNV, COMP-NLLMAAS and COMP-NEGATIVE were tested in direct comparisons over three independent repetitions of the same experiment, involving exposure of Tie2-Fc coated wells to; 50ng, 100ng, 250ng, 500ng, 1µg and 5µgml⁻¹ soluble ligand. Results are demonstrated in figure 4.4.

Each of COMP-VTSRGNV and COMP-NLLMAAS demonstrated concentration-dependent binding to Tie2-Fc, whereas this did not occur with COMP-NEGATIVE (fig 4.3A, B). Direct comparison of Tie2-binding by COMP-VTSRGNV and COMP-NLLMAAS suggests that COMP-VTSRGNV has increased binding ability. However, this did not reach statistical significance (fig 4.3C).
A

![Graph A: Proportional Optical Density vs. COMP-peptide Concentration/µg ml⁻¹](image)

- **COMP-VTSRGNV**
- **COMP-NEGATIVE**

B

![Graph B: Proportional Optical Density vs. COMP-peptide Concentration/µg ml⁻¹](image)

- **COMP-NLMAAS**
- **COMP-NEGATIVE**

COMP-peptide Concentration/µg ml⁻¹:

- 0.05
- 0.1
- 0.25
- 0.5
- 1
- 5
Figure 4.3: ELISA Binding Assay Determination of Binding of COMP-peptides to Tie2-Fc. Plates were incubated with Tie2-Fc overnight at 10μgml⁻¹. Wells were blocked and incubated with COMP-VTSRGNV, COMP-NLLMAAS or COMP-NEGATIVE at concentrations 0.05, 0.1, 0.25, 0.5, 1 or 5μgml⁻¹ for one hour. After washing plates were then incubated with anti-FLAG M2 antibody, followed by secondary HRP-linked anti-mouse antibody. Plates were then developed using OPD substrate and ODs recorded. A (p137): Bar Plot of proportional OD for concentration range of COMP-VTSRGNV versus COMP-NEGATIVE B (p137); Bar Plot of proportional OD for concentration range of COMP-NLLMAAS versus COMP-NEGATIVE C: Bar plot comparing relative binding of COMP-VTSRGNV and COMP-NLLMAAS. For A-C data represents mean proportional OD for three independent experimental repetitions. Error bars represent standard error of the mean. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001 (Student’s t-test).
Plotting mean proportional optical densities versus logarithmic concentration for the three experiments enables the sigmoidal binding response of the two specific ligands to be observed, compared to COMP-NEGATIVE (figure 4.4). From these plots the maximal binding under these experimental conditions for COMP-VTSRGNV occurs at a ligand concentration of approximately 650ngml⁻¹, and for COMP-NLLMAAS occurs at approximately 490ngml⁻¹. EC₅₀ for COMP-VTSRGNV is approximately 136ngml⁻¹, whilst that for COMP-NLLMAAS is approximately 101ngml⁻¹. Interestingly, both peptides show a concentration-dependent increase in binding, before reaching a plateau, and subsequently declining.
Figure 4.4: Binding Curves for COMP-VTSRGNV (A) and COMP-NLLMAAS (B) versus COMP-NEGATIVE. Data was derived from ELISA assays in which binding of the three synthetic ligands to Tie2 adsorbed onto ELISA plates was measured. Plot of mean proportional ODs versus $\log_{10}$[concentration] of COMP-peptide enables observation of the ligand receptor binding relationship. Approximated binding curves are included.
4.2. Establishing a 96-well Plate Assay to Detect Endothelial Tie2 Activation

Yields of synthetic COMP-peptides from the mammalian expression experiments were low. In order to test the ability of these ligands to activate Tie2 in endothelial cells, it was first necessary to establish an assay in which low volumes of extracted mammalian culture medium could be used. This would allow significant concentration of synthetic ligands to be attainable for testing.

To establish the assay Eahy926 were seeded into 12-, 24-, 48-, and 96-well plates and stimulated with either COMPangioptien-1 at 360ngml\(^{-1}\) or serum-free medium as a negative control for 30 minutes, as described in sections 2.7.6. and 2.7.7. After lysis samples were loaded onto a 7.5% SDS-PAGE gel. The gel was run, transferred to Hybond ECL and probed with PY99 to detect activated phosphotyrosines on Tie2 (Santa Cruz mouse antibody), and then Tie2 (Santa Cruz rabbit antibody). Results are shown in figure 4.6. From the results of three experiments it is observed that there is a difference between test and control in 12-, 24-, 48- and 96-well plates. This is significant statistically for the 12-well and 96-well plate experiments (p= 0.028 and p<0.0001 respectively). Therefore this suggests that 96-well plates are a sensitive medium for the detection of Tie2 phosphorylation in Eahy926, allowing smaller volumes of concentrated ligand to be presented to endothelial cells.
**Figure 4.5: Scaling Down Eahy Stimulation Experiments.** Eahy926 were trypsinised and plated into 12-, 24-, 48-, or 96-well plates, serum-starved and stimulated with COMP-angiopoietin-1 or control (serum-free medium) as described in detail in section 2.7.6. After stimulation cells were lysed and total whole cell lysates from each well were loaded and run on a 7.5% SDS-PAGE gel. After transfer to Hybond ECL, membranes were probed for phosphorytyrosine (PY99), then stripped and re-probed for total Tie2. A: Representative comparative probe from confluent Eahy926 in 12-, 24-, 48- or 96-well plates, stimulated with COMP-angiopoietin-1 or control. B: Representation of mean phospho-Tie2/total Tie2 ratios observed over three independent experiments. Bars represent mean ratio of observed Tie2 phosphorylation by PY99 densitometry, divided by total Tie2 densitometry. Error bars are representative of standard error of the mean. * indicates p<0.05, ** indicates p<0.01, versus control (Student’s t-test).
4.3. Effects of Mammalian Cell-Derived COMP-peptides on Endothelial Tie2 Activation

Experiments were performed to assess whether recombinant COMP-peptides produced in transient mammalian transfections could activate Tie2 phosphorylation.

Mammalian protein was generated and experiments were conducted in which one complete 6-well plate of 293-HEKs per experiment was transfected transiently, as described in section 2.3.3, with pSecTag/FRT/V5-His TOPO II for COMP-VTSRGNV or COMP-NLLMAAS, and another 6-well plate with the 7D control vector. In the latter case no recombinant protein will be produced (see figure 3.7). 48h after transfection, conditioned medium containing synthetic COMP-peptides or control medium was collected from cells, concentrated (see section 2.3.5.), quantified (see section 2.5.3.) and applied to serum-starved Eahy926 in 96-well plates (see section 2.6.3.3. for further details). After cellular lysis and resolving of total whole-cell lysates on 7.5% SDS-PAGE gels, specimens were transferred to Hybond ECL. Probing was carried out for activated Tie-2 using PY99 and total Tie2. Densitometric analysis of probes was used to calculate relative ratios of P-Tie2/Tie2 for each sample relative to P-Tie2/Tie2 ratios in the presence of the corresponding control application (7D). There were a total of six applications of freshly concentrated COMP-peptides (0.16; 0.57; 2.7μg/ml COMP-VTSRGNV and: 0.08; 0.15 and 1μg/ml COMP-NLLMAAS), each versus a similar volume of concentrated conditioned medium derived from HEKs transfected with the 7D control vector.

Results are represented in figure 4.6 for COMP-VTSRGNV and 4.7 for COMP-NLLMAAS. Though there are only three data points for COMP-VTSRGNV versus control, a relationship between increasing concentration of COMP-VTSRGNV and increasing Tie2 phosphorylation is suggested. For COMP-NLLMAAS, Tie2...
activation at 1μgml⁻¹ seems to be more significant on Tie2 phosphorylation than the effect at the two smaller concentrations.

Figure 4.6. Effects of Mammalian Cell-Derived COMP-VTSRGNV on Endothelial Cell Tie2 Activation. Conditioned medium was harvested from 293-HEKs in 6-well plates transfected transiently with pSecTag/FRT/V5-His TOPO II plasmid encoding either the synthetic COMP-peptide COMP-VTSRGNV or the negative control plasmid 7D. Medium was concentrated into 30μL, quantified by dot blotting, and 25μL of this concentrated conditioned medium was applied each to a well of Eahy926 in a 96-well plate for 30 minutes. Lysates were loaded onto 7.5% SDS-PAGE gels, transferred to Hybond ECL, and probed for phosphotyrosine (PY99) indicating activated Tie2, then for total Tie2. Ratios of activated to total Tie2 were quantified by densitometry. A: Probes from each application of COMP-VTSRGNV versus its control. Concentrations of ligand are indicated. B: Plot of relative P-Tie2/Tie2 versus COMP-VTSRGNV concentration, from probes in A. Each data point represents a single experiment, with ratios relative to each negative control.
Figure 4.7. Effects of Mammalian Cell-Derived COMP-VTSRGNV on Endothelial Cell Tie2 Activation. Conditioned medium was harvested from 293-HEKs in 6-well plates transfected transiently with pSecTag/FRT/V5-His TOPO II plasmid encoding either the synthetic COMP-peptide COMP-NLLMAAS or the negative control plasmid 7D. Medium was concentrated into 30µL, quantified by dot blotting, and 25µL of this concentrated conditioned medium was applied each to a well of Eahy926 in a 96-well plate for 30 minutes. Lysates were loaded onto 7.5% SDS-PAGE gels, transferred to Hybond ECL, and probed for phospho-tyrosine (PY99) indicating activated Tie2, then for total Tie2. Ratios of activated to total Tie2 were quantified by densitometry. A: Probes from each application of COMP-NLLMAAS versus its control. Concentrations of ligand are indicated. B: Plot of relative P-Tie2/Tie2 versus COMP-NLLMAAS concentration, using probes in A. Each data point represents a single experiment, with ratios relative to each negative control.
4.4. Effects of Prokaryotic COMP-peptides on Tie2 Activation

In order to assess whether the specific binding of prokaryotic COMP-peptides to Tie2 observed in section 4.1. might lead to receptor phosphorylation, assays of cellular stimulation were conducted. EAhy926 were serum-starved for 4.5h and exposed for 30 minutes to COMP-VTSRGNV, COMP-NLLMAAS or serum-free medium alone (negative control) at 37°C. Ranges of COMP-peptides were as used in ELISA binding assays, ie 0.05, 0.1, 0.25, 0.5, 1 and 5μgml⁻¹. Whole cell lysates were solubilised in 3xSB/DTT and loaded onto 7.5% SDS-PAGE gels. After running and transferring, gels were probed for specific Tie2 activation (PY1102/1108) using Phosphodetect Phospho-Tie2 antibody, followed by reprobing for total Tie2. Methods are described in more detail in sections 2.7.5. and 2.10. There were a minimum of three independent experiments, with four independent experiments for 250ngml⁻¹, and five independent experiments at 1 and 5μgml⁻¹. Results are shown in figures 4.8 and 4.9.

Results for both COMP-peptides versus control show a concentration-dependent increase in specific Tie2 phosphorylation which reaches maximum towards 1μgml⁻¹ for each ligand. This appears to reflect to some degree the binding response observed in section 4.1.2. For both COMP-peptides maximal specific Tie2 phosphorylation is observed at a ratio of approximately 2.2-fold versus control.
Figure 4.8: Effects of COMP-VTSRGNV on Endothelial Tie2 Phosphorylation.

Whole cell lysates derived from Eahy926, stimulated for 30 minutes with COMP-VTSRGNV at 0.05, 0.1, 0.25, 0.5, 1 or 5 μg/ml or serum-free medium alone (control) were loaded onto 7.5% SDS-PAGE gels, run and transferred to Hybond ECL. Subsequently membranes were probed for activated Tie2 using a specific Phosphodetect Phospho-Tie2 antibody (PY1102/1108), then stripped and reprobed for total Tie2. A: Representative probe. B: Bar plot for mean proportional phospho-Tie2: total Tie2 versus control (measured by densitometry) for between three and five independent experiments over the concentration range tested. Error bars represent standard error of the mean. ** indicates p<0.01 (Student’s t-test).
Figure 4.9: Effects of COMP-LLMAAS on Specific Endothelial Tie2 Phosphorylation. Whole cell lysates derived from Eahy926, stimulated for 30 minutes with COMP-VTSRGNV at 0.05, 0.1, 0.25, 0.5, 1 or 5μgml⁻¹ or serum-free medium alone (control) were loaded onto 7.5% SDS-PAGE gels, run and transferred to Hybond ECL. Subsequently membranes were probed for activated Tie2 using a specific Phosphodetect Phospho-Tie2 antibody (PY1102/1108), then stripped and reprobed for total Tie2. A: Representative probe. B: Bar plot for mean specific Tie2 phosphorylation: Tie2 ratio versus control (measured by densitometry) for between three and five independent experiments over the concentration range tested. Error bars represent standard error of the mean. ** indicates p<0.01 (Student’s t-test).
4.5. Kinetics of COMP-peptide Effects on Tie2 Activation

In section 4.4, evidence is shown for specific phosphorylation of Tie2 induced by COMP-peptides, maximal at 1µgml⁻¹ for each ligand after 30 minutes of application to vascular endothelial cells. It was of interest to determine the kinetics of Tie2 activation. Therefore experimental time courses of ligand application were conducted on serum-starved Eahy926 in 6-well plates, as described in detail in section 2.7.8. COMP-VTSRGNV at 1µgml⁻¹ was tested for 5, 15, 30, 60, 120 and 240 minutes versus control. For COMP-NLLMAAS, 1µgml⁻¹ lig and was added for 10, 30, 60, 120 and 180 minutes. As a negative control for each ligand, one well of cells was serum-starved, followed by exposure to serum-free medium alone for 30 minutes. Whole cell lysates were run on SDS-PAGE gels, transferred to Hybond ECL and probed with specific anti-phospho-Tie2 antibody specific for tyrosine phosphorylation of Tie2 at residues 1102 and 1108. Membranes were then stripped and reprobed for total Tie2. One kinetics experiment was conducted for each of COMP-VTSRGNV and COMP-NLLMAAS using Eahy926. Results are shown in figures 4.10 and 4.11. COMP-peptides induced Tie2 phosphorylation from 30 minutes. This was sustained for between 120 and 180 minutes. However, caution should be exercised in interpreting these data, since only a single experiment was performed for each ligand.

Activation of Tie2 has been shown to be responsible for the induction of the downstream signalling mediator Akt1, or Akt (Kim, 2000b; Papetropoulos, 2000). It was thus of interest to assess whether evidence of activation of Akt could be observed in the kinetics experiments. From the above kinetics experiments, probes were stripped and reprobed for activated Akt, assessed by serine 473 phosphorylation (see section 2.10). Subsequently probes were stripped and reprobed for total Akt. Results
are shown in figures 4.12 and 4.13. Similar to their effects on Tie2 activation, both COMP-peptides increased \textit{akt} phosphorylation. The kinetics of \textit{akt} activation are similar to those of Tie2 activation. However, caution should be used in interpreting the results of a single experiment.
Figure 4.10. Kinetics of Endothelial Tie2 Phosphorylation Induced by COMP-VTSRGNV. Serum-starved Eahy926 in 6-well plates were exposed to COMP-VTSRGNV at 1μg/ml for 5, 15, 30, 60, 120 or 240 minutes versus a control (exposure to serum-free medium alone for 30 minutes). Whole cell lysates were loaded onto a 10% SDS-PAGE gel, run and transferred to Hybond ECL. Membranes were probed using specific phospho-Tie2 antibody for tyrosine phosphorylation of Tie2 at residues 1102 and 1108, followed by stripping and re-probing for total Tie2. A single experiment was performed. A: Experimental probe. B: Bar plot showing densitometric analysis of proportional phospho-Tie2/Total Tie2 for the specified times.
Figure 4.11. Kinetics of Endothelial Tie2 phosphorylation Induced by COMP-NLLMAAS. Serum-starved Eahy926 in 6-well plates were exposed to COMP-NLLMAAS at 1μg/ml for 10, 30, 60, 120 or 180 minutes versus a control (exposure to serum-free medium alone for 30 minutes). Whole cell lysates were loaded onto a 10% SDS-PAGE gel, run and transferred to Hybond ECL. Membranes were probed using specific phospho-Tie2 antibody for tyrosine phosphorylation of Tie2 at residues 1102 and 1108, followed by stripping and re-probing for total Tie2. A single experiment was performed. A: Experimental probe. B: Bar plot showing densitometric analysis of proportional phospho-Tie2/Total Tie2 at the specified times.
Figure 4.12. Kinetics of COMP-VTSRGNV Effects on akt Activation in Endothelial Cells. Serum-starved Eahy926 in 6-well plates were exposed to COMP-VTSRGNV at 1μg/ml for 5, 15, 30, 60, 120 or 240 minutes versus a control (exposure to serum-free medium alone for 30 minutes). Whole cell lysates were loaded onto a 10% SDS-PAGE gel, run and transferred to Hybond ECL. After probing for Tie2 (see figure 4.10) the membrane was stripped and reprobed with antibody specific for phospho-Akt (serine 473), followed by probing for total Akt. A: Actual probe. B: Densitometric analysis of proportional phospho-Akt/total Akt ratios.
Figure 4.13. Kinetics of COMP-NLMAAS Effects on akt Activation in Endothelial Cells. Serum-starved Eahy926 in 6-well plates were exposed to COMP-NLMAAS at 1μgml⁻¹ for 10, 30, 60, 120 or 180 minutes versus a control (exposure to serum-free medium alone for 30 minutes). Whole cell lysates were loaded onto a 10% SDS-PAGE gel, run and transferred to Hybond ECL. After probing for Tie2, membranes were stripped and reprobed for phospho-Akt (serine 473), then stripped and reprobed for total Akt. A: Actual probe. B: Densitometric analysis of proportional phospho-Akt/total Akt ratios.
Discussion

In this chapter the aims were to investigate whether multimeric versions, predominantly pentameric, of VTSRGNV and NLLMAAS could bind and activate the Tie2 receptor specifically in the context of vascular endothelial cells. In the case of angiopoietin-1 a minimal requirement for a tetrameric configuration of the receptor-binding domain has been demonstrated to be necessary for phosphorylation of Tie2, at least in the presence of a surrogate multimerisation domain (Davis, 2003). Additionally, Cho and colleagues have demonstrated that significant binding and activation of Tie2 can occur in vitro and in vivo when COMP is used to present the receptor binding domain of ang1 to Tie2 in a stable, soluble format (Cho, 2004a). Here, the effects of introducing the receptor-binding peptides into a related system were investigated.

In the mammalian expression system only low levels of expression of COMP-peptides had been achieved. However since there is a potential structural advantage in mammalian-cell derived protein, in terms of posttranslational modification, it was interesting to perform experiments using this protein. Since too little protein was produced to allow proper purification use was limited to concentration of fresh serum-free medium from transfectants. This was then applied immediately to serum-starved Eahys for 30 minutes periods, in 96-well plates. Results for both COMP-VTSRGNV and COMP-NLLMAAS suggested that higher concentrations of applied ligand led to increased Tie2 phosphorylation versus identically concentrated conditioned medium containing no ligand, from the control transfectants. However, the data here must be interpreted with caution, since only individual and single applications of medium were available at a given concentration.
In the prokaryotic system high levels of expression of COMP-VTSRGNV and COMP-NLLMAAS had been achieved. Furthermore, biochemical characterisation of the molecules in chapter 3 suggested that these constructs were able to multimerise under native conditions to the expected, predominantly pentameric, format. Assays in which different amounts of Tie2 were immobilised as small dots on nitrocellulose and subsequently exposed to soluble COMP-peptides at 5μgml⁻¹ provided evidence of specific binding of COMP-VTSRGNV and COMP-NLLMAAS to immobilised Tie2-Fc (section 4.1.1.). Additionally, a negative control protein, COMP-NEGATIVE, was fashioned (see also section 3.11) and included in these experiments. The aim was to generate COMP-LALANSM, identical to COMP-NLLMAAS in every other respect apart from scrambling of the heptameric peptide on the C-terminus. Unfortunately a point mutation, most likely during overlap PCR, led to a frame shift which resulted in a FLAG-COMP-linker scaffold but with a random peptide on the C-terminus. The peptide was not heptameric but five times longer than intended. Actually, the resulting structure did appear to form multimers comparable to COMP-VTSRGNV, in addition to a larger monomer and a more heterogeneous accompanying group of multimers. Though not an ideal control, taking into account additional time constraints, it was observed that this control forms stable multimers under nonreducing conditions. Additionally a peptide five times longer than that intended to be presented might have potential for a greater degree of nonspecific binding than COMP-LALANSM. Therefore COMP-NEGATIVE was included in the experiments involving Tie2-Fc immobilisation. As can be observed in figure 4.1, there was very little specific binding to Tie2, as expected, for COMP-NEGATIVE, regardless of the total amount of Tie2-Fc immobilised. At 100ng and 200ng Tie2-Fc this difference was very significant statistically for COMP-VTSRGNV. Also, for COMP-NLLMAAS the difference was
significant at 50ng and 200ng Tie2-Fc. However significant variability between responses in three experiments led to a nonsignificant difference at 100ng Tie2-Fc. No significant difference was observed between binding of the two COMP-peptides under these conditions. Additionally of note is that no plateau was observed in binding as Tie2-Fc was increased, implying room in further experiments for increasing the total amount of Tie2-Fc immobilised to ascertain the maximal binding attainable.

In order to compare the binding of Tie2-Fc to COMP-peptides versus an entirely different, unrelated protein which is known not to bind Tie2, experiments were also conducted in which 100ng COMP-VTSGN, COMP-NLLMAAS or recombinant IGF-1 were adsorbed onto nitrocellulose in dots of equal size. Subsequently dots were exposed together to soluble Tie2-Fc chimera at 20nM. The reason for the use of this particular concentration of Tie2-Fc was that it had been demonstrated to lead to sensitive detection of binding to 200ng adsorbed native ang1 in preceding work by Davis et al (1996). The results again indicated specific binding of both COMP-VTSGN and COMP-NLLMAAS to soluble Tie2-Fc which was not observed with recombinant IGF-1 (figure 4.2). Interestingly, a robust finding in three independent experiments was that, under these conditions, COMP-VTSGN bound more strongly to Tie2 for a given quantity of ligand than COMP-NLLMAAS. This differs from the observations in the case of immobilised Tie2-Fc. It is possible that elements of the two constructs were preferentially adsorbed so that the receptor-binding region of Tie2-Fc could not gain access as easily to NLLMAAS as the unknown site on Tie2-Fc responsible for binding VTSGN could gain access to the latter.
To gain further quantitative binding information, ELISA experiments were conducted in which Tie2 was immobilised on 96-well plates and subsequently exposed to differing concentrations of COMP-peptides (figure 4.3). Results support those from nitrocellulose immobilisation experiments. They suggest strongly that COMP-VTSRGNV and COMP-NLLMAAS, but not COMP-NEGATIVE, are able to bind specifically to Tie2-Fc in a concentration dependent fashion. Significantly greater binding was observed for COMP-VTSRGNV versus COMP-NEGATIVE from 100ngml\(^{-1}\) to 5μgml\(^{-1}\). Though COMP-NLLMAAS appeared also to produce a significant dose-binding response versus COMP-NEGATIVE over three independent experiments, this only attained statistical significance between 250 and 500ngml\(^{-1}\).

Plotting of proportionate ELISA optical densities for Tie2-binding versus concentrations on a logarithmic scale showed a binding response curve with a sigmoidal feature, as expected. Plotting this for each experiment allowed estimation of EC\(_{50}\). This was similar for both COMP-VTSRGNV and COMP-NLLMAAS. Indeed there was no significant difference between binding between the two at any given concentration of applied ligand (figure 4.3C).

Subsequently, cellular stimulation assays were used in which serum-starved Eahy926 were exposed to concentrations of COMP-peptides reflective of the observed ELISA binding results, for 30 minutes. Specific Tie2 phosphorylation versus control (no COMP-peptide applied) was observed to increase to a maximum at 1μgml\(^{-1}\) for each COMP-peptide (figures 4.8-4.9). For COMP-VTSRGNV, significant Tie2 phosphorylation was observed at ligand concentrations of 250ngml\(^{-1}\), 1μgml\(^{-1}\) and 5μgml\(^{-1}\). However in the case of COMP-NLLMAAS only 1μgml\(^{-1}\) demonstrated statistical significance. It can also be noted that even maximal Tie2 phosphorylation seemed modest, at 2.2-fold. For instance maximal Tie2 phosphorylation with native
ang1 is seen to be around 3-4 fold, whereas COMPang1 can achieve 15-fold specific phosphorylation (Cho, 2004a). Naturally, however, it is impossible to state precisely how COMP-peptides would compare with other Tie2 ligands without direct comparison.

Kinetics experiments suggested that both COMP-VTSGRGNV and COMP-NLLLMAAS may activate Tie2 from 30 minutes up to between 120 and 180 minutes. Activation of Tie2 has been shown to be responsible for the induction of the downstream signalling mediator, Akt (Kim, 2000b; Papetropoulos, 2000). Since Akt is known to be important in the protection of vascular endothelial cells from apoptosis in response to ang1 acting through Tie2, it was of interest to observe that there was evidence of Akt activation for both COMP-peptides which was similar to that of Tie2 on kinetics. However, these data are limited by the acquisition of single experiments. If time had allowed, further experiments would have been performed in this area.

In conclusion data in this chapter supports proof of concept, that oligomeric Tie2-binding peptides can activate Tie2 specifically. Though the activity appeared modest compared to other known ligands for Tie2, this was not tested directly. Such a proof of principle has also been demonstrated for the FGF receptor, where the ability of a dimeric ligand expressing a receptor-binding peptide appeared potent (Ballinger, 1999).
5: Modification of COMP-peptides and Downstream Signalling
**Introduction**

In chapter 4, COMP-VTSRGNV and COMP-NLLMAAS were shown to bind and activate the Tie2 receptor, in a concentration-dependent manner, in vascular endothelial cells. However it was noted that a relatively modest level of maximal phosphorylation was observed, being approximately 2.2-fold above controls.

One reason for the modest degree of Tie2 activation observed might be a lesser binding affinity of the heptameric peptides VTSRGNV and NLLMAAS to Tie2, in comparison with the FRD domain of ang1.

Another feature of COMP-peptides which may contribute to weaker activation of Tie2 might be the inability to recruit an as yet unidentified cofactor which strengthens the interaction, individually, or in groups of receptors. The related tyrosine kinase Tie1 is known to associate with Tie2 in the cell membrane (Marron, 2000), and indeed is able to modulate the activity of Tie2 (Marron, 2007). In fact Tie-1 may be involved in part in mediating differential responsiveness between alternative Tie2-binding ligands, particularly ang1 and ang2, in which the receptor-binding domains are structurally very similar (Hansen, 2010; Seegar, 2010).

An additional mechanism which might play a role in the modest observed Tie2 phosphorylation in endothelial cells may be the molecular constraints of a relatively small molecule, which may be insufficient to engage receptors adequately to enable sufficient clustering.

Since NLLMAAS is known to bind to the ang1 FRD binding site on Tie2, this information has been used in the first part of this chapter to investigate, as far as known research enables, the potential intermolecular dimensions involved in COMP-peptide binding to Tie2. Based on the theoretical and experimental findings, a modification of the COMP-peptides is undertaken, with the emphasis of this
modification being on creating a new COMP-peptide with a longer ‘reach’ to bridge binding sites on aggregated Tie2 receptors. The effects of this modification are explored. Following this some of the downstream signals initiated by COMP-peptides are explored, along with their kinetics.

5.1. Potential Constraints in Tie2 Aggregation Induced by COMP-peptides

Most of the evidence for the shape and conformation of the interaction of angiopoietins with Tie2 derives from the studies of Barton and colleagues on the crystallographic structure of the ang2 receptor binding domain (Barton, 2005), and the complex of this domain interacting with Tie2 itself (Barton, 2006). Using the actual crystal structure of the ang2 FRD complexed with Tie2, visualised in the the JMol programme (Java), allows the estimation of the potential distances between ang2 binding sites in Tie2 receptors as they might approach and interact with one another. Since ang1 is thought to bind at a similar site to ang2 this also allows derivative estimations with regard to the shortest potential distances between ang1 binding sites on associated Tie2 receptors. Since NLLMAAS also binds to the ang1/ang2 binding site of Tie2 (Tournaire, 2004), this therefore also allows estimations of potential distances between NLLMAAS binding sites on associated Tie2 receptors.

Since the crystallographic structure of the coiled-coil domain in cartilage oligomeric matrix protein has been described (Malashkevich, 1996) representation in JMol allows estimation of the width of the pentameric coiled-coil of COMP which suspends the Linker2 structures with C-terminal peptides in COMP-NLLMAAS (see also figure 3.1).

Figure 5.1A shows a bird’s eye representation of the Tie2 receptor, with the proposed ang2/ang1/NLLMAAS binding site highlighted in red. In figure 5.1B the diagram on the left illustrates again the bird’s eye view of the Tie2 receptor, with the
receptor-binding domain of ang2 in blue. However, in this diagram J Mol is used to illustrate the approximate dimensions of the receptor, as ‘observed’ from above. In figure 5.1B the right-hand diagram is an illustration of these dimensions and how they are used to create a trapezium with dimensions representative of Tie2 in a bird’s eye view. Also, using J Mol, the outline of the ang2-binding site is outlined in red dash. In figure 5.1C the left-hand panel shows a cross-sectional view of the cartilage oligomeric matrix protein coiled-coil domain, as derived from J Mol, featuring the molecular dimensions plotted from the actual crystal structure of rat COMP (Malashkevich, 1996). This is the domain of which residues 28-77 are used as oligomerisation construct in COMP-peptides (also see figure 3.1). The right-hand panel shows that, in cross-section, the coiled-coil domain of rat COMP can be viewed as a pentagon with sides of approximately 12 Angstroms.
B

Ang2/ang1
Binding Region

Derived trapezium
showing estimated
region of ligand
binding site

C

Dimensions of COMP
oligomer (from Jmol)

Derived Pentagon
with sides \( \sim 12 \text{Å} \)

12 Å
Figure 5.1: Structural Aspects of Tie2 Aggregation. A (p163): The left-most diagram illustrates the Tie2 ectodomain in a complex with the ang2 receptor-binding domain, as observed from the plane of the cell membrane. The ligand-binding site is illustrated in red. The illustration is removed for copyright reasons. A 90° rotation about the x-axis illustrates a bird’s eye view of the interacting structures. B (p164): Left panel shows bird’s eye view of Tie2 ectodomain, in particular, the position of the interaction with the ang2 FRD, i.e., the ligand-binding site. Right panel shows approximate trapezium, with ligand binding site dimensions in red. C (p164): Cross-section through the coiled-coil oligomerisation domain of rat COMP represented in Jmol. D (p165): Two possible tetrameric aggregates of Tie2. On the left, Tie2 is aggregated in a homo-tetrameric fashion, with all ligand-binding regions ‘pointing’ in the same direction. In the right-hand diagram Tie2 are aggregated into an alternative tetrameric. Lengths indicate distances between adjacent ligand binding regions. E (p165): Diagrams to illustrate a pentameric aggregate of Tie2 receptors. Blue arrows indicate shortest and longest distances between ligand binding regions and central COMP. F: Sagittal view of a schematic representation of COMP- NLLMAAS, to show actual bridging distance of Linker2, described by 35cosα.
Using the data derived from figure 5.1A-C allows the creation of scaled diagrams to allow representation of Tie2, as viewed from above in the plane of the cell membrane, in different states of aggregation. A scaled representation of the COMP pentagon can subsequently be superimposed upon these aggregates to give an approximation of the distances Linker2 may need to bridge in order for the C-terminal peptide to bind Tie2 appropriately. Since the peptide NLLMAAS is known to bind somewhere in the ligand-binding site of Tie2, whereas it is not at all known where the peptide VTSGRNV binds, the bridging distances are estimated assuming the peptide in question is NLLMAAS.

As has been discussed previously, it is known that a minimum requirement for the ang1 receptor-binding domain to induce strong activation of Tie2 is as a tetramer. Assuming a stoichiometry of 1:1 between FRD and receptor, an assumption supported by Barton’s crystallographic studies, the minimum aggregation of Tie2 receptors for strong phosphorylation is expected to be four. In figure 5.1D two alternative representations are given for bird’s eye views of tetrameric aggregates of Tie2 receptors in the cellular membrane, with the ang1-binding region highlighted in red. Onto each is superimposed the COMP pentagon in cross-section. In the left-most representation ligand-binding domains of Tie2 are all in the same orientation. However, the distance which must be bridged by Linker2 to enable NLLMAAS binding to similar points on each ligand-binding domain are at least 30-35 Angstroms. In the right-most diagram Tie2 receptors are aggregated as tetramers in a different fashion, with the distance which must be bridged by Linker2 being only 15-20 Angstroms. However, in this case 50% of the Tie2 ligand binding domains are orientated in reverse fashion to the remaining 50%. This might be feasible, but might
provide steric hindrance for the Linker2-NLLMAAS component of COMP-peptides, unless the peptide were able to rotate.

It was noted in chapter 3 that COMP-peptides under native conditions, like the related COMP-angiopoietin-1, form mainly pentameric oligomers. This is hypothesised as one possible reason for the enhanced potency of COMP-angiopoietin-1 over native ang1, in that it can aggregate more Tie2 receptors. In fact if a scaled representation of a pentameric aggregation of Tie2 receptors is drawn, and the pentagonal COMP superimposed, it can be observed that COMP-peptides would be able to bind all five receptors with equal bridging for each Linker2. The minimum bridging distance required could be as little as 20 Angstroms, but might be as great as 35 Angstroms (figure 5.1E).

The actual linker length can be calculated for Linker2 of COMP-peptide constructs. Using previously calculated lengths (derived from structures of the relevant amino acids, to be found at: www.3dchem.com) of the specific amino acids in Linker2, GSGGPFYSHS, as well as including the lengths of peptide bonds between them, enables an estimate of the maximal length of Linker2 of 35 Angstroms. Therefore COMP-peptides might have difficulty binding tetrameric aggregates of Tie receptors if they were presented as in the left-hand fashion in figure 5.1D. They should be able to bind if the receptors were presented as in the right-hand diagram in figure 5.1D. However, again, this is presuming the peptides are flexible enough to rotate. In figure 5.1E the minimum distance between ligand-binding sites on a pentameric aggregate of Tie2 receptors looks favourable for COMP-peptides. However the furthest distance COMP-NLLMAAS might be required to bind (35 Angstroms) is the maximum possible distance Linker2 could span, assuming full extension and minimum flexibility (see figure 5.1F).
This suggests there might be limitations in the ability of the Linker2 to bridge Tie2 receptors in practice. Therefore variants of the ligands with altered Linker2 length were produced and tested for their ability to activate Tie2.

5.2. Design and Construction of Extended Length COMP-peptides

Ideally, the assessment of optimal length for Linker2 could be achieved by testing a range of lengths. However, practically this was not feasible in the time limits of the project. Instead the impact of increasing the length of Linker2 to 60-70 Angstroms was tested, based on the conclusions from section 5.1. The composition of the new length of linker would be required to be flexible, soluble, relatively resistant to proteolytic degradation, and with minimal secondary structure. Therefore G3S repeats, which fulfill these criteria (Robinson, 1998; Huston, 1988) were substituted between the first four amino acids of Linker2, ie GSGG, and the last six amino acids, namely PFYSHS. Thus the original Linker2, GSGGPYSHS, becomes the ‘LongLinker’, GSGGGSGGGSGGGPFYSHS (see also Appendix D).

The generation of extended length COMP-peptides, or COMP-Long-peptides, according to the design discussed here, is described in detail in section 3.12. As discussed in section 3.12, examination of the characteristics of COMP-Long-VTSRGNV and COMP-Long-NLLMAAS reveal they behave very similarly to COMP-peptides, forming predominantly stable pentamers under nonreducing conditions.

5.3. Effects of COMP-Long-peptides on Tie2 Activation in Eahy926

The degree of Tie2 phosphorylation induced by COMP-Long peptides in Eahy926 vascular endothelial cells was examined initially. Eahy926 were prepared in
6-well plates and serum-starved as described previously in detail (section 2.7.5.). Cells were exposed to COMP-VTSRGNV-Long at 0.1, 1, 5, 10 or 30μgml\(^{-1}\), or serum-free medium alone (control), or COMPangiopoietin-1 at 0.36μgml\(^{-1}\) for 30 minutes. In separate experiments cells under the same conditions, and using the same controls, were exposed to COMP-NLLMAAS-Long at 0.1, 1, 2 or 5μgml\(^{-1}\) for 30 minutes. Cells were lysed and whole cell lysate samples run on 12% SDS-PAGE gel electrophoresis, followed by transfer to Hybond ECL membranes. Membranes were probed initially for phosphorylated Tie2 using Phosphodetect rabbit Phospho-Tie2 Antibody (Calbiochem), and then stripped and reprobed for total Tie2 using goat anti-Tie2 antibody (R&D Systems). Results are illustrated in figure 5.4 for COMP-VTSRGNV-Long and figure 5.5 for COMP-NLLMAAS-Long.

Significant specific phosphorylation of Tie2 was observed at concentrations of COMP-VTSRGNV-Long of 1 and 5μgml\(^{-1}\), with a maximal increase of 2.6-fold versus control (fig 5.4). The peak phosphorylation of Tie2 attained with COMP-VTSRGNV was 2.2-fold in Eahy926 (figure 4.8), suggesting that increasing Linker2 length for this ligand did not markedly affect its ability to activate Tie2 in these cells.

COMP-NLLMAAS-Long induced significant phosphorylation of Tie2 at 0.5, 1 and 2μgml\(^{-1}\). The maximal increase in specific Tie2 phosphorylation versus control is approximately 3.2-fold at 1μgml\(^{-1}\) (figure 5.5). Compared with COMP-NLLMAAS (figure 4.9), where maximal observed Tie2 phosphorylation was approximately 2.2-fold versus control, this suggests lengthening Linker2 might enhance the activity of this ligand.
Figure 5.4: COMP-VTSRGNV-Long Phosphorylates Tie2 in Eahys. Eahys in 6-well plates were exposed to COMP-VTSRGNV-Long at 0.1, 1, 5, 10 or 30μgml⁻¹, or serum-free medium alone, for 30 minutes. Whole cell lysates were run 12% SDS-PAGE gels and resulting transfers probed for phosphorylated Tie2 (PY1102/1108), followed by reprobing for total Tie2. A: Representative blot. B: Plot of phospho-Tie2/total Tie2 ratios, normalised to peak activation. The number of independent experiments at 0.1, 1 and 5μgml⁻¹ was five. The number of independent experiments at 30μgml⁻¹ was three, whilst at 10μgml⁻¹ two experiments were performed. * indicates p<0.05, *** indicates p<0.001 versus control. Error bars indicate standard error of the mean.
Figure 5.5: Effects of COMP-NLLMAAS-Long on Specific Phosphorylation of Tie2 in Eahy926. Eahys in 6-well plates were exposed to COMP-NLLMAAS-Long at 0.1, 1, 2 or 5 µg/ml, or serum-free medium alone (control), for 30 minutes. Samples of whole cell lysates were run 12% SDS-PAGE gels and resulting transfers probed for phosphorylated Tie2 (PY1102/1108), followed by reprobing for total Tie2. 

A: Representative probe. B: Plot of proportional phospho-Tie2/total Tie2 ratios over three independent experiments. Bars represent mean phospho-Tie2/total Tie2, normalised to peak activation. Error bars represent standard error. * indicates p<0.05, ** indicates p<0.01 versus control (Student’s t-test).
5.4. Effects of COMP-Long Peptides on Tie2 Activation in Human Umbilical Vein Endothelial Cells

Eahy926 are an immortalised endothelial cell line derived from fusion of human umbilical vein endothelial cells with the human lung adenocarcinoma cell line A549 (Edgell, 1983). Therefore such cells, though able to express Tie2 and respond to angiopoietins in a defined manner, are not purely physiological, though they are more robust. It was thus of interest to investigate whether the Tie2 phosphorylation observed in Eahy926 could be observed in HUVECs.

Since HUVECs would subsequently be used for the investigation of downstream signalling pathways if evidence of signalling via Tie2 were observed, it was necessary to determine the serum-starvation period of the assay for the minimal time required to suppress basal phosphorylation of the downstream signalling mediators Akt and ERK, without compromising the healthy state of the cells. To investigate serum-starvation conditions required, semi-confluent HUVECs were prepared in a six-well plate and serum-starved for 0.5, 2, 5 or 12h before a 30 minute exposure to either COMPangiopoietin-1 at 0.36µgml⁻¹ or serum-free medium alone as a control. Samples of whole cell lysates were loaded onto a 12% gel, run, transferred to Hybond ECL, and probed for phospho-Akt (serine 473 activation) using the mouse antibody described in section 2.10. Subsequently the membrane was stripped and reprobed for total Akt. The membrane was then probed for phospho-ERK, followed by total ERK. Results are illustrated in figure 5.6.

From figure 5.6, 0.5h seems sufficient for clear suppression of phosphorylation of Akt at serine 473, and ERK. In the experiments which follow 0.5h is thus taken as the time for total serum deprivation preceding the addition of ligand.
Figure 5.6: Testing Appropriate Serum-Starvation Periods in HUVECs. A six well plate of subconfluent HUVECs was serum-starved for 0.5, 2, 5 or 12 hours. Cells were then stimulated for 30 minutes with either COMPangiopoietin-1 at 0.36 μg/ml or serum-free medium alone as negative control. Samples of whole cell lysates were run on a 12% gel, transferred to Hybond ECL and probed for phospho-Akt (s473) and total Akt (A), phospho-ERK and total ERK (B). Results represent a single experiment.
For assessment of the effects of the synthetic ligands on Tie2 phosphorylation, sub-confluent six-well plates of HUVECs were serum-starved for 0.5h and then exposed to COMP-VTSRGNV-Long, COMP-NLLMAAS-Long, or to serum-free medium alone (control), for 30 minutes. Cells were lysed and samples of whole cell lysate samples run on 12% gels, as described previously (section 2.7-2.8). After transfer to Hybond ECL, membranes were probed for phospho-Tie2 using the specific Phosphodetect Phospho-Tie2 antibody, then stripped and reprobed for total Tie2. Results are shown in figures 5.7. and 5.8.

In HUVECs maximal activation of Tie2 is observed at 1µgml⁻¹ for both COMP-VTSRGNV-Long and COMP-NLLMAAS-Long, and in each case is significantly greater than control (p=0.0001 and p=0.001, respectively). At higher concentrations of 5µgml⁻¹ Tie2 activation still reaches statistical significance for COMP-VTSRGNV-Long (p=0.001). This parallels its activity in Eahys (figure 5.4). However for COMP-NLLMAAS-Long in HUVECs, in comparison to Eahys, significant specific Tie2 phosphorylation seems to reduce markedly at concentrations above 1µgml⁻¹ (see figure 5.8). Maximal Tie2 phosphorylation in applications of COMP-VTSRGNV-Long to HUVECs is approximately 2.8-fold greater than control (fig 5.7). For COMP-NLLMAAS-Long this maximum is 2.1-fold (fig 5.8). These data are similar to experiments in Eahy926 (section 5.5.).
Figure 5.7: Effects of COMP-VTSRGNV-Long on Phosphorylation of Tie2 in Human Umbilical Vein Endothelial Cells. Subconfluent, serum-starved HUVECs in 6-well plates were stimulated for 30 minutes with COMP-VTSRGNV-Long at 0.1, 1, 5 or 30µgml\(^{-1}\) versus control (serum-free medium alone for 30 minutes). Samples of whole cell lysates were run on a 12% SDS-PAGE gel, transferred to ECL Hybond and probed for phosphorylated Tie2 followed by total Tie2. A: Representative blot. B: Cumulative results of three independent repetitions of the same experimental protocol. Bars represent plots of mean Tie2 phosphorylation normalised to peak activation. Error bars represent standard error of the mean. ** indicates p<0.01, *** indicates p<0.001 versus control (Student’s t-test).
Figure 5.8. Effects of COMP-NLLMAAS-Long on Phosphorylation of Tie2 in Human Umbilical Vein Endothelial Cells. Subconfluent, serum-starved HUVECs in 6-well plates were stimulated for 30 minutes with COMP-NLLMAAS-Long at 0.1, 1, 2 or 5 μg/ml, or serum-free medium alone (control). Samples of whole cell lysates were run on a 12% SDS-PAGE gel, transferred to ECL Hybond and probed for phosphorylated Tie2 followed by total Tie2. A: Representative probe. B: Plot of mean Tie2 phosphorylation/ total Tie2 for three independent experimental repetitions. Bars represent mean phospho-Tie2/total Tie2, normalised to peak activation. Error bars represent standard error of the mean. ** indicates p<0.01 versus control (Student’s t-test).
5.5. Direct Comparison of COMP-Long-Peptides with COMP-peptides

In order to determine whether increasing the length of Linker2 affects the ability of the synthetic ligands to activate Tie2, experiments were performed in which COMP-VTSRGNV, COMP-VTSRGNV-Long, COMP-NLLMAAS and COMP-NLLMAAS-Long were tested directly together. Since the maximal observed Tie2 phosphorylation for each ligand was at approximately 1µg/ml\(^1\) (see sections 4.4. and 5.4.) , all four ligands were applied simultaneously at this concentration for 30 minutes to serum-starved HUVECs in 6-well plates, as described in section 2.7.4. Two controls were used in this direct comparison. The first was serum-free medium alone, as used previously. In addition, a negative control protein was produced as described in section 3.12.3. This protein consists of the FLAG-Linker1-COMP-Long Linker2 construct, similarly to the COMP-Long peptides, but with the random peptide, TMYQLNF. The construct was named COMP-RP-Long. Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels and analysed as described, for assessment of Tie2 phosphorylation. Four independent experimental repetitions were performed. Results are shown in figure 5.9.

Over four independent experiments application to HUVECs of each of the four experimental constructs, COMP-VTSRGNV; COMP-NLLMAAS; COMP-VTSRGNV-Long, and COMP-NLLMAAS-Long, at 1µg/ml\(^1\) induced greater Tie2 phosphorylation versus COMP-RP-Long or serum-free medium alone. Tie2 activation by the shorter versions of the COMP-peptide ligands failed to reach statistical significance compared with either negative control. Both COMP-VTSRGNV-Long and COMP-NLLMAAS-Long induced significant activation of Tie2 phosphorylation, as observed previously (fig 5.7 and 5.8). Interestingly, COMP-NLLMAAS-Long
induced a significantly greater activation of Tie2 phosphorylation than COMP-NLLMAAS (fig 5.9).
Figure 5.9. Direct Comparison of Tie2 Phosphorylation Induced by Alternative COMP-peptides. Serum-starved HUVECs were exposed to COMP-VTSRGNV, COMP-VTSRGNV-Long, COMP-NLLMAAS, COMP-NLLMAAS-Long or COMP-RP-Long at 1 μg/ml for 30 minutes as described elsewhere. Control consisted of exposure to serum-free medium alone for 30 minutes. Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed for specific Tie2 phosphorylation followed by total Tie2. A: Representative blot from one experiment. B: Summary of four independent experimental repetitions. Bars represent mean Tie2 phosphorylation/total Tie2 ratios, normalised to peak activation. Error bars represent standard error. * indicates p<0.05 versus medium control (Student’s t-test), Ψ indicates p<0.05 versus COMP-RP-Long control construct (Student’s t-test), ΨΨ indicates p<0.01 versus COMP-RP-Long construct (Student’s t-test), ζ indicates p<0.05 versus COMP-NLLMAAS (Student’s t-test).
5.6. Effects of COMP-Long-Peptides on Induction of Downstream Akt in Vascular Endothelial Cells

The downstream signalling mediator Akt1, or \( Akt \), has a number of roles in vascular endothelial cells. Certainly this mediator is known to be of critical importance in protection of vascular endothelial cells from apoptosis in response to ang1 acting through Tie2 (Harfouche, 2002; Papetropoulos, 2000; Kim 2000b), in a dose-dependent fashion.

5.6.1. Eahy926

In order to assay the potential effects of COMP-Long-Peptides on the activation of \( Akt \) in Eahy926, cells were prepared in 6-well plates and serum-starved for 4.5h, as described in section 2.7.5. Subsequently COMP-Long-peptides or serum-free medium alone (control) were added to cells for 30 minutes. After cold lysis, samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed initially for mouse phospho-Akt serine 473. Membranes were then stripped and reprobed for total \( Akt \) using a rabbit antibody. Results are shown in figures 5.10 and 5.11.

The pattern of activation of \( Akt \) in Eahy926 when stimulated with COMP-VTSRGNV-Long is similar to that of specific Tie2 activation. There is significant serine 473 phosphorylation of \( Akt \), maximal at 1\( \mu \)gml\(^{-1} \) of applied ligand (\( p=0.0005 \)). Here an approximate 3.8-fold increased serine phosphorylation is observed versus control (see figure 5.10). Additionally, 5\( \mu \)gml\(^{-1} \) applied ligand is observed to lead to a significant increase in \( akt \) phosphorylation versus control (\( p=0.021 \)), with an approximate 2.3-fold increase. At lower and higher concentrations of ligand, phosphorylation declines.
For COMP-NLLMAAS-Long at 1μgml\(^{-1}\), serine phosphorylation of Akt is also observed to be significantly greater than control (p=0.003). At this concentration an approximately 6.2-fold increase versus control is observed. Akt is also phosphorylated significantly at 2μgml\(^{-1}\) (p=0.046), with an approximate 4.8-fold rise above control. Though the trend at 0.5μgml\(^{-1}\) is also reflective of observed Tie2 phosphorylation (figure 5.5), this does not reach statistical significance. Again, at 5μgml\(^{-1}\) significant phosphorylation of Akt is not observed, comparable to Tie2 activation (figure 5.5).
Figure 5.10. Effects of COMP-VTSRGNV-Long on Activation of Akt in Eh926.
Subconfluent Eh926 in 6-well plates were exposed to COMP-VTSRGNV-Long at 0.1, 1, 5, 10 or 30μg/ml, or serum-free medium alone (control), for 30 minutes. Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed for phospho-Akt (s473), followed by total Akt. A: Representative probe. B: Summary of three independent experiments. Bars represent mean phospho-Akt/total Akt ratios, normalised to peak activation. Error bars represent standard error. Note that in a single experiment the concentration 30μg/ml was substituted for 10μg/ml. * indicates p<0.05, *** indicates p<0.001 versus control (Student’s t-test).
Figure 5.11. Effects of COMP-NLLMAAS-Long on Activation of Akt in Eahy926. Eahy926 in 6-well plates were exposed to COMP-NLLMAAS-Long at 0.1, 0.5, 1, 2 or 5 µg/ml, or serum-free medium alone (control), for 30 minutes. Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed for phospho-Akt (s473), followed by total Akt. A: Representative probe. B: Summary of three independent experiments. Bars represent mean phospho-Akt/total Akt normalised to peak activation. Error bars represent standard error. * indicates p<0.05, ** indicates p<0.01 versus control (Student’s t-test).
5.6.2. Human Umbilical Vein Endothelial Cells

As HUVECs are an unmodified vascular endothelial cell, reflective closely of a physiological phenotype, it was of interest to investigate specific serine 473 phosphorylation of Akt in these cells in response to COMP-Long-Peptides. Cellular stimulation assays were performed as described, using a serum-starvation period of 30 minutes prior to stimulation with each ligand (see figure 5.6). Cells were stimulated with COMP-VTSGRNV-Long, COMP-NLLMAAS-Long, or control (serum-free medium alone) for 30 minutes. Results are shown in figures 5.12 and 5.13.

COMP-VTSGRNV-Long induced a rise in phosphorylation of Akt in HUVECs which was significant at 1 and 5 μgml⁻¹ (p=0.0011 and p=0.012, respectively), being maximal at 1 μgml⁻¹ (figure 5.12). This is similar to the activation of Akt observed in Eahy926 (figure 5.10). Additionally, the pattern of Akt activation also reflects the activation of Tie2 phosphorylation in HUVECs induced by COMP-VTSGRNV-Long (figure 5.7). COMP-NLLMAAS-Long induced significant activation of Akt at 1 and 2 μgml⁻¹ (p<0.0001 and p=0.029, respectively), stimulation again being maximal at 1 μgml⁻¹ (figure 5.13). This is similar to the pattern observed in Eahy926 (see figure 5.11). Additionally, maximal Akt induction is also observed, as with COMP-VTSGRNV-Long, at the same concentration as maximal activation of Tie2 phosphorylation (figure 5.8).
Figure 5.12. Effects of COMP-VTSRGNV-Long on Serine Phosphorylation of Akt in Human Umbilical Vein Endothelial Cells. Subconfluent HUVECs in 6-well plates were exposed to 0.1, 1, 5, 20, or 30μgml⁻¹ COMP-VTSRGNV-Long for 30 minutes, or serum-free medium alone for 30 minutes (control). Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed for phospho-Akt (s473), followed by total Akt. A: representative probe. B: Plot showing five independent experiments at 0, 0.1, 1 and 5μgml⁻¹, three experiments at 20μgml⁻¹, and two experiments at 30μgml⁻¹. Bars represent mean phospho-Akt/total Akt ratios, normalised to peak activation. Error bars represent standard error. * indicates p<0.05, ** indicates p<0.01 versus control (Student’s t-test).
Figure 5.13. Effects of COMP-NLLMAAS-Long on Serine Phosphorylation of Akt in Human Umbilical Vein Endothelial Cells. Subconfluent HUVECs in 6-well plates were exposed to 0.1, 1, 2 or 5µg/ml COMP-VTSRGNV-Long for 30 minutes, or serum-free medium alone for 30 minutes (control). Samples of whole cell lysates were loaded onto 12% SDS-PAGE gels, run, transferred to Hybond ECL and probed for phospho-Akt (s473), followed by total Akt. A: Representative probe. B: Summary plot of three independent experimental repetitions. Bars represent mean phospho-Akt/total Akt ratios, normalised to peak activation. Error bars represent standard error. * indicates p<0.05, *** indicates p<0.001 versus control (Student’s t-test).
5.6.3. Comparison With Shorter COMP-peptides

In order to compare directly the effects of long and short synthetic COMP-peptides on the activation of Akt, experiments were performed with COMP-VTSRGNV and COMP-VTSRGNV-Long, as well as COMP-NLLMAAS and COMP-NLLMAAS-Long. Western blots for the four experiments represented in figure 5.9 were stripped and reprobed phospho-Akt (serine 473), then stripped and reprobed for total Akt. Results are shown in figure 5.14.

Both short and long versions of COMP-peptide ligands induced significant activation of Akt phosphorylation (fig5.14). There was no difference in the ability of the short versus long ligands to stimulate Akt phosphorylation. This contrasts with the differing effects of COMP-NLLMAAS and COMP-NLLMAAS-Long on Tie2 phosphorylation (see figure 5.9).
Figure 5.14: Direct Comparison of Serine 473 Phosphorylation of Akt Induced by Alternative COMP-peptides. HUVECs in 6-well plates were exposed to COMP-VTSRGNV, COMP-VTSRGNV-Long, COMP-NLLMAAS or COMP-NLLMAAS-Long at 1µg/ml for 30 minutes as described previously. Control wells containing the same confluence of HUVECs were exposed to either COMP-RP-Long at 1µg/ml, or serum-free medium for 30 minutes, under the same conditions. Whole cell lysates were analysed for specific Tie2 phosphorylation (see figure 5.9). Subsequently blots were stripped and reprobed for phospho-Akt (s473), followed by total Akt.

A: Representative blot. B: Summary of mean proportional Akt phosphorylation from four independent repetitions of the experiment described. Bars represent mean serine 473 phosphorylation of Akt, normalised to peak activation. Error bars represent standard error. * indicates p<0.05 versus serum-free medium, ** indicates p<0.01 versus serum-free medium, *** indicates p<0.001 versus serum-free medium, ψ indicates p<0.05 versus COMP-RP-Long control, ψψ indicates p<0.01 versus COMP-RP-Long control, ψψψ indicates p<0.001 versus COMP-RP-Long control (Student’s t-test).
5.7. Effects of a 30-Minute Exposure to COMP-Long-peptides on ERK Activation in Human Umbilical Vein Endothelial Cells

ERK-1/2 (ERK p44/p42) is known to be activated by angiopoietin-1 in endothelial cells (Harfouche, 2003). It was therefore of interest to investigate whether activation of the ERK pathway could be observed in the presence of Tie2 and Akt phosphorylation for the assays carried out in sections 5.5. and 5.6, on HUVECs treated for 30 minutes with COMP-long-peptides. To examine this, blots from the experiments described were stripped and reprobed for phospho-ERK followed by total ERK (see also section 2.10). Results are shown in figures 5.15 and 5.16.

Surprisingly, COMP-VTSRGNV-Long did not appear to activate ERK-1/2 (see figure 5.15 for phospho-ERK p42; data for phospho-ERK p44 not shown). This is in contrast to the significant activation of Akt which was observed on the same probes in response to COMP-VTSRGNV-Long. For COMP-NLLMAAS-Long induction of phospho-ERK p42 (see figure 5.16) is at least partially reflective of the induction of phospho-Akt on the same probes (figure 5.14). As COMP-NLLMAAS-Long concentration increases there was a progressive increase in phospho-ERK p42, peaking at 1μgml⁻¹ (p=0.0002) and reducing but still significant at 2μgml⁻¹ (p=0.047) versus control. No induction of phospho-ERK p44 versus control was observed for the application of COMP-NLLMAAS-Long. These data demonstrate a difference between the signalling induced by COMP-VTSRGNV-Long and COMP-NLLMAAS-Long, under these conditions.
Figure 5.15. Effects of a 30 Minutes Exposure to COMP-VTSRGNV-Long on Induction of phospho-ERK in Human Umbilical Vein Endothelial Cells. For the experiment illustrated in figure 5.12, probes run to detect phospho-Akt and Akt in HUVECs after a 30 minute exposure to COMP-VTSRGNV-Long were stripped and reprobed for phospho-ERK then total ERK as described (section 2.10). A: Representative probe. B: Summary of at least three independent experiments. Bars are representative of mean phospho-ERK p42/total ERK p42, normalised to peak activation. Error bars represent standard error. Numbers of repetitions are indicated above bars.
Figure 5.16. Effects of a 30 Minutes Exposure to COMP-NLLMAAS-Long on Induction of phospho-ERK in Human Umbilical Vein Endothelial Cells. For the experiment illustrated in figure 5.13, probes run to detect phospho-Akt and Akt in HUVECs after a 30 minute exposure to COMP-NLLMAAS-Long were stripped and reprobed for phospho-ERK then total ERK as described (section 2.10). A: Representative probe. B: Summary of three independent experiments. Bars are representative of mean phospho-ERK p42/total ERK p42, normalised to peak activation. Error bars represent standard error. * indicates p<0.05, *** indicates p<0.001 versus control (Student’s t-test).
5.7.1. Comparison with Shorter COMP-peptides

In order to compare directly the effects of long and short synthetic COMP-peptides on the activation of ERK, experiments were performed with COMP-VTSRGNV and COMP-VTSRGNV-Long, as well as COMP-NLLMAAS and COMP-NLLMAAS-Long. Western blots for the four experiments represented in figure 5.9 were stripped and reprobed for phospho-ERK and then total ERK. Results are shown in figure 5.15.

Compared to controls, COMP-NLLMAAS and COMP-NLLMAAS-Long at 1µg/ml induced significant activation ERK p42, with no difference between short and long ligands. Interestingly, though COMP-VTSRGNV-Long was unable to induce statistically significant activation of ERK p42 versus controls, COMP-VTSRGNV did appear to induce ERK. However, there was no significant difference between activation of ERK p42 for COMP-VTSRGNV versus COMP-VTSRGNV-Long directly. None of the peptides were able to induce ERK p44 under these conditions.
Figure 5.19: Direct Comparison of Ability of Alternative COMP-peptides to Induce Phosphorylation of the ERK Signalling Pathway. Subconfluent HUVECs in 6-well plates were serum-starved as described and exposed to COMP-VTSRGNV, COMP-VSTRGNV-Long, COMP-NLLMAAS or COMP-NLLMAAS-Long at 1µgml⁻¹ for 30 minutes. As negative controls identically treated HUVECs were exposed to COMP-RP-Long at 1µgml⁻¹ or serum-free medium (‘Control’) for 30 minutes. Samples of whole cell lysates were analysed by SDS-PAGE gel electrophoresis and Western blotting. After probing for P-Tie2/Tie2, and subsequently P-Akt (s473)/Akt (see figures 5.9 and 5.14), blots were reprobed for phospho-ERK and total ERK. A: Representative probe. B: Summary from four independent experiments. Solid bars represent mean phosphorylation of ERK-p42/total ERK-p42, normalised to peak activation, with error bars representing standard error. * indicates p<0.05 versus serum-free medium; Ψ indicates p<0.05 versus COMP-RP-Long control protein (Student’s t-test)
5.8. Kinetics of COMP-Long-peptides in Human Umbilical Vein Endothelial Cells

It was of interest to examine the kinetics of the effects of COMP-Long peptides on signalling in endothelial cells. HUVECs in 6-well plates were serum-starved and exposed for various periods to COMP-VTSRGNV-Long or COMP-NLLMAAS-Long at 1μg/ml. The detailed methods are described in section 2.7.8. After lysis, samples of whole cell lysates were run on 12% SDS-PAGE gels. After transfer to nitrocellulose, membranes were probed for phospho-Tie2 (PY1102/1108), phospho-Akt (serine 473), phospho-ERK p44/p42, total Tie2, total Akt and total ERK p44/p42. Results of the analysis of Tie2 phosphorylation kinetics in response to exposure to COMP-Long-peptides are illustrated in figures 5.20 and 5.21.

COMP-VTSRGNV-Long induced peak activation of Tie2 phosphorylation at 60 minutes exposure. Tie2 phosphorylation was decreased at 180 minutes (fig 5.20). For COMP-NLLMAAS-Long, only two experimental repetitions were performed as a result of time constraints (figure 5.21). Serum-starved HUVECs were exposed to 1μg/ml COMP-NLLMAAS-Long for 15, 30, 60, 120 or 180 minutes, or to control (serum-free medium alone). The results suggest, surprisingly, that little increase in Tie2 phosphorylation is observed versus control at 15 or 30 minutes of exposure. However, peak phosphorylation of Tie2 appears to occur towards 60 minutes of exposure.

As shown in figure 5.22, COMP-VTSRGNV-Long induced significant activation of Akt in HUVECs from as early as 15 minutes, peaking at 180 minutes. For COMP-NLLMAAS-Long, again, peak activation occurred after 180 minutes (figure 5.23). Interestingly, for both ligands, peak activation of Akt appeared to occur
at a much later time point than peak activation of Tie2 (compare figures 5.20 and 5.22; and figures 5.21 and 5.23).

COMP-VTSRGNV-Long induced rapid stimulation of ERK (figure 5.24). Maximal activation was observed after 15 minutes, with an apparent decrease towards 60 minutes, followed by a rise again which culminated in a second peak of activation at 180 minutes. For COMP-NLLMAAS-Long, over two experiments, activation of ERK required up to 120 minutes exposure to the ligand (figure 5.25). Interestingly, though activation of ERK p44 was not observed using COMP-VTSRGNV-Long, COMP-NLLMAAS-Long seemed to be able to recruit ERK p44 in addition to ERK p42 (figure 5.25).
Figure 5.20: Kinetics of Tie2 Phosphorylation in HUVECs Induced by COMP-VTSRGNV-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-VTSRGNV-Long at 1µg/ml for 15, 60, 180, 300 or 420 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose and probed for phospho-Tie2 (PY1102/1108) followed by total Tie2. A: Representative probe B: Summary of up to four independent experiments (three repetitions at 180 minutes; two repetitions at 30, 120, 300 and 420 minutes). Bars represent mean P-Tie2/total Tie2, normalised to peak activation, for each exposure. Error bars represent standard error. * indicates p<0.05 versus control, *** indicates p<0.001 versus control (Student’s t-test).
Figure 5.21: Kinetics of Tie2 Phosphorylation in HUVECs Induced by COMP-NLLMAAS-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-NLLMAAS-Long at 1μg/ml for 15, 30, 60, 120 or 180 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose and probed for phospho-Tie2 (PY1102/1108) followed by total Tie2. A: Representative probe. B: Summary of two independent experiments. Bars represent mean P-Tie2/total Tie2, normalised to peak activation. Error bars represent standard error.
Figure 5.22: Kinetics of Serine 473 Phosphorylation of Akt in HUVECs Induced by COMP-VTSRGNV-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-VTSRGNV-Long at 1μgml⁻¹ for 15, 30, 60, 120, 180, 300 or 420 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose. After probing for P-Tie2/Tie2, membranes were stripped and probed for phospho-Akt followed by total Akt. A: Representative probe. B: Summary for up to four independent experiments for 15, and 60 minutes (three repetitions at 180 minutes; two repetitions at 30, 120, 300 and 420 minutes). Bars represent mean phospho-Akt/total Akt ratio, normalised to peak activation, with error bars representative of standard error. ** indicates p<0.01, *** indicates p<0.001 versus control (Student’s t-test).
Figure 5.23: Kinetics of Serine 473 Phosphorylation of Akt in HUVECs Induced by COMP-NLLMAAS-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-NLLMAAS-Long at 1 μg/ml for 15, 30, 60, 120 or 180 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose. After probing for P-Tie2/Tie2, membranes were stripped and reprobed for phospho-akt then total akt. A: Representative probe. B: Summary of two independent experimental repetitions. Bars represent mean P Akt/total Akt ratio, with error bars representative of standard error.
Figure 5.24 (p202): Kinetics of Phospho-ERK Induction HUVECs Induced by COMP-VTSRGNV-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-VTSRGNV-Long at 1µgml⁻¹ for 15, 60, 180, 300 or 420 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose. After probing for P-Tie2/Tie2, membranes were stripped and probed for phospho-ERK p44/p42 and then total ERK p44/p42. A: Representative probe for kinetics. B: Summary of up to four independent experiments (three repetitions at 180 minutes; two repetitions at 30, 120, 300 and 420 minutes). Bars represent mean phospho-ERK/total ERK ratio, normalised to peak activation, with error bars representative of standard error. C: Second representative probe from a shorter kinetics experiment. * indicates p<0.05 versus control.
Figure 5.25: Kinetics of Phospho-ERK Induction HUVECs Induced by COMP-NLLMAAS-Long. Serum-starved HUVECs in 6-well plates were exposed to COMP-NLLMAAS-Long at 1μg/ml for 15, 30, 60, 120 or 180 minutes, or to control (serum-free medium for 60 minutes). After cell lysis samples were loaded onto 12% SDS-PAGE gels, run, transferred to nitrocellulose. After probing for P-Tie2/Tie2, membranes were stripped and reprobed for phospho-ERK p44/p42 followed by total ERK p44/p42. A: Representative probe. B: Summary of two independent repetitions of the experiment. Bars represent mean phospho-ERK p44/p42/total ERK p44/p42 ratios, normalised to peak activation. Error bars represent standard error of the mean.
**Discussion**

The experiments described in this chapter compare the abilities of synthetic ligands with two different Linker2 lengths to activate Tie2 and its downstream *Akt* and *ERK* pathways. A theoretical analysis of the structure of the ang2/Tie2 complex was used to estimate the molecular distances which COMP-NLLMAAS might require to bridge between adjacent Tie2 receptors. It was concluded that such bridging might in theory be difficult with the length of Linker2 originally designed. Thus, it was decided to construct COMP-NLLMAAS with a longer Linker2, leading to COMP-NLLMAAS-Long. COMP-VTSRGNV-Long was also created, though the binding site for VTSRGNV on Tie2 was entirely unknown. Although a range of lengths for Linker2 would have been ideal, in order to compare them directly, time constraints allowed only for construction and analysis of one length. This was based on the maximum length of the armspan of COMP-angiopoietin-1, derived from measurements of electron micrographs from the authors’ paper (Cho, 2004a). Also, other authors had suggested that linker lengths exceeding 19 amino acids might be less stable (Robinson, 1998). Therefore Linker2 was increased from 10 to 19 amino acids.

Testing the effects of COMP-Long-peptides in Eahy926 indicated the ligands with increased Linker2 length activate Tie2 phosphorylation over a 30 minute period, in addition to that of *Akt*. Similarly in HUVECs both COMP-VTSRGNV-Long and COMP-NLLMAAS-Long were able to induce significant activation of Tie2 and *Akt* versus a serum-free medium control. Interestingly, only COMP-NLLMAAS-Long, over a 30 minute period of stimulation, appeared capable of inducing significant activation of *ERK*. The reason why COMP-VTSRGNV-Long Tie2 activation leads to *Akt*, but not *ERK*, induction at 30 minutes is unclear. However it might relate to
differential access or orientation of tyrosines mediating the Akt versus the ERK pathway for this particular ligand.

Direct comparison of COMP-VTSRGNV and COMP-VTSRGNV-Long revealed no significant increase in signalling activity associated with increased Linker2 length. However, COMP-NLLMAAS-Long had a significantly increased ability to induce Tie2 activation versus COMP-NLLMAAS, though enhanced Akt or ERK activity were not observed. This does, to some extent, support the hypothesis that increased Linker2 length allows increased bridging and therefore better recruitment of Tie2 receptors. This may not have been observed for peptide VTSRGNV because, as alluded to in section 5.1, it is not at all known where VTSRGNV binds on Tie2. Linker2 lengthening may not have been sufficient, therefore, to observe any effect for VTSRGNV.

The effects of COMP-VTSRGNV-Long and COMP-NLLMAAS-Long were also compared directly with a negative control, COMP-RP-Long. This construct was designed to present an identical FLAG-Linker1-COMP-LongLinker2 structure to Tie2, but containing the random C-terminal heptameric peptide, TMYQLNF, which was not expected to bind Tie2. Indeed, whilst COMP-VTSRGNV-Long and COMP-NLLMAAS-Long were able to activate Tie2 at 1μg/ml, COMP-RP-Long was not (figure 5.9). This demonstrated that the activation of Tie2 observed by the test constructs was specific.

Examination of the kinetics of Tie2, Akt and ERK activity for COMP-VTSRGNV-Long in HUVECs showed maximal Tie2 activation at 60 minutes of exposure. However, this was not mirrored by rises at similar times in either Akt or ERK. In fact Akt activity was maximal at 180 minutes, whilst ERK activation appeared to be maximal after only 15 minutes, with a possible biphasic activity, peaking again
at 180 minutes post exposure to ligand. The activity of Akt reflects that observed in 30 minute exposures to COMP-VTSRGNV-Long in HUVECs (see figure 5.12). For ERK activity, low levels of Tie2 activation by COMP-VTSRGNV-Long might be sufficient to recruit ERK, which may be short-lived compared to Tie2 and Akt. This has been observed, in fact, for both ang1 and COMPang1 (Cho, 2004a; Harfouche, 2003). However, the second peak may relate to another unknown mechanism of activation, including the possibility that it is independent of a ligand effect on Tie2.

For COMP-NLLMAAS-Long there are only two experiments, so statistics cannot be performed. However, there was a trend for Tie2 activation to peak at 60 minutes, with Akt and ERK both peaking at 180 minutes. Here, however, there is a delay in Akt activity, when compared to 30-minute exposures in HUVECs (see figure 5.13). Additionally, COMP-NLLMAAS-Long had induced ERK activity in 30-minute exposures previously (figure 5.16). This is difficult to account for since assays were conducted in a very similar fashion. Though vanadate was not added during kinetics assays, if this were to exert any contributory effect to the apparent discrepancy it would be expected also to be observed for COMP-VTSRGNV-Long. Another possibility may be that this is the result of analysing only two experiments, though the standard error of the two experiments was not large for 30 and 60 minutes exposures. Another observation is that, in contrast to COMP-VTSRGNV-Long, there appears to be only one peak of ERK activation. Furthermore, it appears that both ERK p42 and ERK p44 are activated by COMP-NLLMAAS-Long, whereas no activity of ERK p44 was detected for COMP-VTSRGNV-Long.

Thus, the kinetics of Tie2 phosphorylation appear similar for both COMP-VTSRGNV-Long and COMP-NLLMAAS-Long. The kinetics of Akt activation for COMP-VTSRGNV-Long show a gradual activation, reaching a peak later than Tie2
activation. This is also observed for COMP-NLLMAAS-Long, though the degree of activation of Akt at earlier time points appears less than that of COMP-VTSRGNV-Long. The kinetics of ERK activation appear very different. One possible explanation for this is that the peptides are unlikely to bind in the same place on Tie2. Therefore they might recruit certain downstream signalling mediators in different ways. Additionally, it is possible that one or both of COMP-VTSRGNV-Long and COMP-NLLMAAS-Long are demonstrating effects on downstream signalling mediators which are independent of Tie2. If more time were available Tie2-knockout HUVECs would be invaluable in answering this question.
6: Discussion
Tie2 is an endothelial receptor which has been shown to be important for maintaining quiescence in the adult mammalian vascular endothelium. The protective effects of ang1 signalling through Tie2, namely reducing endothelial apoptosis, in addition to suppression of inflammation in, and breakdown of, the vascular endothelial barrier, have demonstrated beneficial effects in a number of preclinical models (see section 1.8). This suggests early potential for future therapy based on inducing signalling through Tie2. However, the native ang1 has encountered difficulties with use, principally difficulties with aggregation which contributes to unreliable pharmacokinetics. A number of ang1 variants have therefore been generated, including ang1* and COMPang1. As described in sections 1.8-1.10, these variants have been used in several models with success. However, there are still problems with these variants. Firstly, they are still large, relatively complex, molecules. This renders manipulation potentially more difficult than it is likely to be for a smaller peptide ligand. Such manipulation might be sought in order to alter a variety of features, such as stability, additional binding elements, and the nature of the receptor-binding element itself. The latter may be altered to alter receptor binding affinity or even to attempt to alter the profile of receptor signalling (Moss, 2009). Secondly, though the mechanism is unclear, COMPang1 is a potent inducer of angiogenesis. There are some therapeutic situations, such as wound healing, where this would likely be a desirable secondary effect. However, in some situations this would clearly be deleterious, for example, in diabetic retinopathy. Thus it might be advantageous to have a smaller protein, preferable exhibiting the protective effects of ang1 only. Thirdly, current ang1 variants retain the FRD Tie2-binding domain, rendering their interaction with Tie2 susceptible to antagonism by ang2. This could theoretically increase the concentrations required to achieve effects in vivo. Fourthly,
a small, easily modifiable agonist might be useful to examine Tie2 receptor function itself, by allowing particular receptor conformations or investigating the effects of binding by a peptide at a particular point on Tie2.

Therefore the aims of this project were to use small heptameric receptor-binding peptides in place of the ang1 FRD, together with a small multimerisation motif. Two peptides known to bind Tie2 were used, namely VTSRGNV and NLLMAAS. The former had already been shown to bind Tie2 specifically in previous work (Kang M, unpublished data). The latter had been shown to bind at the Tie2 ligand-binding domain (Tournaire 2004). Since the binding site of NLLMAAS is known, this might also be useful in modelling any further modifications to the recombinant protein which could be required. The multimerisation motif chosen for these peptides was the N-terminal segment, residues 28-77 of the superclustering domain of rat cartilage oligomeric matrix protein (COMP). This molecule had been used successfully to express a stable, pentameric version of the ang1 FRD (Cho 2004). Additional, ‘Linker2’ regions span the distance between the COMP motif and the attached peptides. These were designed to act as a flexible bridge for the aggregation of Tie2 receptors during binding and activation.

The recombinant multimeric COMP-peptide ligands, COMP-VTSRGNV and COMP-NLLMAAS, were synthesised in mammalian cells and subsequently, to improve yield, BL21-DE3(Gold) prokaryotic expression systems. A satisfactory yield of protein was produced. Biochemical analysis showed the proteins to behave as expected, forming approximately 11kDa monomers under reducing conditions, and predominantly 55kDa pentamers under nonreducing conditions. Both proteins showed a similar affinity for Tie2 binding in vitro. In assays of cellular stimulation of Eahy926 endothelial cells, both COMP-peptides showed a similar ability to activate
Tie2 compared with controls. Mean resulting activation of Tie2 was approximately 2-fold, maximal at 1μgml$^{-1}$ for each ligand.

It was noted that Tie2 activation appeared modest. Therefore, prior to further work, an attempt was made to modify the structure of the COMP-peptides in order to enhance Tie2 activity. It was theorised, from known structural data on the interaction of ang2 with Tie2, that the bridging Linker2 section may not be sufficient for efficient aggregation of Tie2 receptors. It was reasoned that a range of Linker2 lengths should be used, and their effects on Tie2 activity noted, since the structural data available on COMP-peptides was very limited. However, time constraints for this project did not allow this. Therefore, based on structural estimations about the width of COMPang1 in electron micrographs, and known information about the lengths of amino acids, Linker2 was lengthened by 9 amino acids, to a total of 19 amino acids. Testing the resulting COMP-Long-peptides against their shorter versions led to mixed results. For VTSRGNV, lengthening Linker2 did not lead to any enhancement of ability to activate Tie2. However, for COMP-NLLMAAS-Long, this seemed to lead to a small, but significant, enhancement in its ability to activate Tie2 versus the shorter COMP-NLLMAAS. Thus lengthening may have had a marginal effect for NLLMAAS. In theory, this may not have worked for VTSRGNV because of a binding site further away on Tie2.

Two downstream signalling pathways known to be activated by ang1 signalling through Tie2, and contributing to the protective effects of ang1, were also investigated. These were the Akt and ERK pathways. At 30 minutes both COMP-Long-peptide ligands seemed to lead to a significant induction of Akt versus controls. However, although COMP-NLLMAAS-Long appeared able to induce ERK, COMP-VTSRGNV-Long did not seem to do so at this time point. There was no significant
difference in the ability of short versus long COMP-peptides to activate downstream Akt in HUVECs. In comparative 30 minute stimulations both COMP-NLLMAAS and COMP-NLLMAAS-Long appeared capable of activating ERK in HUVECs, with no significant difference between their ability to do so. COMP-VTSRGNV-Long did not appear able to activate ERK at 30 minutes in HUVECs, whereas there was some evidence that COMP-VTSRGNV could activate ERK p42. However, there was no significant difference between them.

The interactions of COMP-peptides with Tie2 were also compared with specifically designed controls. The principal aim was to exclude the possibility that the actions of the synthetic ligands might be to some extent mediated by the FLAG-Linker1-COMP-Linker2 motif. The first control was designed for comparison with the shorter COMP-peptides, namely COMP-NEGATIVE. This consisted of the FLAG-Linker1-COMP-Linker2 motif attached to an irrelevant peptide at the C-terminus. Unfortunately, the peptide was not the desired peptide but a much longer one, probably a result of a mutation during PCR. However, it was reasoned that this peptide should still not be able to bind Tie2, and the overall COMP-NEGATIVE construct was able to form stable multimers. For this reason, in addition to time constraints, this ligand was used in competitive binding ELISA assays versus COMP-VTSRGNV and COMP-NLLMAAS. Whilst the latter ligands were able to induce dose-dependent binding to Tie2, COMP-NEGATIVE was not, confirming that the FLAG-Linker1-COMP-Linker2 motif was not involved in binding Tie2. Later a second control with the FLAG-Linker1-COMP-Linker2 motif was designed attached to a random peptide, TMYQLNF, which was tested against COMP-VTSRGNV-Long and COMP-NLLMAAS-Long. Whilst the motifs containing the two Tie2-specific peptides induced significant activation of Tie2 and its downstream signalling
mediators, Akt or ERK, after a 30 minute exposure, COMP-RP-Long did not. This suggested that both binding and activation of Tie2 were specifically induced by COMP-VTSRGNV-Long and COMP-NLLMAAS-Long.

Kinetics in HUVECs were analysed using COMP-VTSRGNV-Long and COMP-NLLMAAS-Long. Kinetics of Tie2 activation appeared similar for both peptides, being maximally activated after 60 minutes, though there were only two experiments with COMP-NLLMAAS-Long. For COMP-VTSRGNV-Long, the kinetics of Akt activation showed an early increase in phosphorylation, from 15 minutes onwards. For COMP-NLLMAAS-Long, Akt activation was much more modest early on, though still appearing to peak at 180 minutes. This suggested a discrepancy between kinetics and earlier 30 minute stimulations in HUVECs (section 5.6.2.). One possible explanation is that because only two experiments were performed for the kinetics of COMP-NLLMAAS-Long, these were not fully reflective.

The kinetics of ERK activation appeared quite different between the two peptides, with COMP-VTSRGNV-Long appearing to induce an early peak at 15 minutes, with a possible second peak at 60 minutes. In contrast, only one peak seemed to be observed with COMP-NLLMAAS-Long, as with Akt much later than in the 30-minutes HUVEC stimulations, at 180 minutes. Again here there is a discrepancy between the kinetics of COMP-NLLMAAS-Long and the earlier 30-minutes stimulations (section 5.7), which might occur for the same reasons as with the kinetics of Akt. It is possible that, since NLLMAAS binds to the ang1/ang2 binding site on Tie2, but VTSRGNV binds at an unknown (and possibly distant) site, the recruitment of some secondary signals might be different as a result. However, it must also be considered that at least some of the observed intracellular signalling might not occur
secondary to Tie2 activation, but via an independent pathway. The use of HUVECs deficient in Tie2 would be an invaluable way of answering this question.

In conclusion, the initial hypothesis of whether small heptameric peptides which bind Tie2 specifically can activate the receptor has been accepted. This is therefore proof of a principle. This demonstrates that ligands other than the ang1 FRD can be multimerised to activate Tie2, and in addition, its signalling pathways. Moreover, the ligands created in this project were very small, easily generated, and potentially simple to modify. The use of peptides on oligomeric scaffolds to cluster tyrosine kinase receptors has been demonstrated also for the FGF receptor (Ballinger, 1999). However, at the start of this project there was no literature assessing whether similar approaches could be applied to Tie2. This work shows that similar approaches can indeed be applied to Tie2. Moreover, towards the conclusion of experimental work in this project, van Slyke and colleagues (2009) confirmed our findings by using avidin to form tetrameric multimers of a Tie2-binding peptide. This construct, termed ‘Vasculotide’, was also able to activate Tie2 and its downstream signalling mediators, Akt and ERK. The authors also demonstrated anti-apoptotic and pro-angiogenic effects of this ligand in vitro and in vivo. However, several points are of note. Firstly, their work on Tie2 activation and signalling was represented by single experiments in their paper. Secondly, concentrations of ligand which were able to induce significant phosphorylation of Akt were orders of magnitude different to those which provided most anti-apoptotic effects in vitro. Thirdly, vasculotide promoted additional effects which were different from those expected from ang1, promoting angiogenesis potently in vitro and in vivo. Given that the Tie2-binding peptide used by the authors, like VTSRGNV, has an unknown binding site on Tie2, these differing effects from what we would expect might be a further manifestation of an alternative clustering of
Tie2 by this ligand, with slightly differing recruitment of downstream effects. To some extent this supports some of the conclusions in the preceding paragraphs with regard to the potential for ligands with alternative binding sites on Tie2 perhaps functioning in slightly differing ways to recruit downstream signalling mediators. Another potential problem with proangiogenic effects is that, as discussed with reference to COMPang1 (section 1.9), such effects might not always be desirable in therapeutic situations.
**Further Work**

In this project the hypothesis of whether small heptameric Tie2-binding peptides can be presented in a multimeric fashion to activate Tie2 has been accepted. The work also suggests that two pathways known to be activated by ang1 signalling through Tie2, which have a role in its vascular protective actions, can be activated by these recombinant constructs. Also, there is evidence that the different peptides presented might work in slightly different ways in recruiting intracellular signalling pathways. There are, however, additional points which need to be addressed.

Activation of Tie2 by COMP-peptide ligands appeared modest. A hypothesis that Linker2 lengthening might improve molecular reach and therefore Tie2 activation characteristics was tested. Linker2 was therefore lengthened from 10 to 19 amino acids. This did not improve the activation of Tie2, Akt or ERK observed for peptide VTSRGNV. For NLLMAAS, there was a significantly greater induction of Tie2 over four independent experiments with the longer Linker2. However, a greater induction of Akt or ERK was not also observed. Thus, lengthening Linker2 may have increased the activity of COMP-NLLMAAS-Long to activate Tie2 over COMP-NLLMAAS. However this did not do so for VTSRGNV. This might perhaps have been to do with the unknown site where VTSRGNV binds on Tie2. Thus, it is impossible to exclude that COMP-VTSRGNV simply was not lengthened enough, and the hypothesis was indeed correct. However, against the short initial length of Linker2 being the only factor in the modest Tie2 activation observed are two observations. Firstly, the increase of COMP-NLLMAAS-Long over COMP-NLLMAAS to induce Tie2 phosphorylation was small. Secondly, the observed increase in Tie2 activity was not translated into downstream activation of Akt or ERK. Actually, another explanation
for this would be that *Akt* and *ERK* as observed in these experiments might have been independent of Tie2 signalling itself.

It does seem, however, that there are other reasons for the modest activation observed. It may be that the peptides chosen do not bind strongly to their sites on Tie2. Indeed, the peptides are selected by four rounds of phage display. However, of over a billion peptides in the library, this might not select the strongest binders. In fact, in Tournaire’s study (2004), peptide ‘H7’ appeared to be stronger individual Tie2 binder in ELISA assays. Many more rounds of phage display could be performed with the same libraries in an attempt to find stronger binders. These could then be inserted into the COMP-peptide structure. Alternatively, substituting H7 into the COMP-peptide structure, and comparing the resulting ligand with COMP-VTSRGNV or COMP-NLLMAAS in some of the assays performed in this work might help answer this question. Interestingly, as discussed in the previous section, van Slyke and colleagues (2009) had also created a tetramer of Tie2-binding peptides in parallel with this work. In fact they used the peptide H7 as the Tie2-binding peptide. A view of their kinetics data (figure 1 from their paper) suggests that quite a significant activation of Tie2, *Akt* and *ERK* was observed in HUVECs in response to vasculotide. In fact this appeared at least as great as with ang1 itself, supporting the concept that heptameric peptides may be able to influence Tie2 to the same degree as the ang1 FRD.

Alternatively, some other structural feature might limit the ability of the construct to bind multiple Tie2 receptors, for example, steric factors in the Linker2. This should not be the case as the Linker2 consists mainly of glycines and serines. However, the segment, ‘PFYSHS’, close to the C-terminal peptides might not be as mobile. This might restrict such a small peptide from rotating to be in its preferred
orientation. This segment was derived from the original bacteriophage in which phage display was carried out to isolate Tie2-binding peptides. Therefore it was kept in the FLAG-Linker1-COMP-Linker2 structure immediately upstream of the Tie2-binding peptide, as it had been in the bacteriophage, in case the context of presentation of the peptide adjacent to this segment was important. Naturally, this context could actually change when transferring the PFYSHS segment to an entirely new structure. There are several ways in which the effect of this C-terminal segment could be investigated. One method would be to substitute PFYSHS for a segment very likely to be flexible, for example, SGGGSG. Another would be to investigate the structure of the COMP-peptides and their interaction with Tie2 to observe binding interactions and orientation directly. However, this is the focus of a project in itself.

Another point for consideration is that the degree of activation of Tie2 visible in assays is not necessarily reflective of downstream functional changes. Small changes in cellular receptor activity might lead to significant functional changes in the cell. This has been noted by authors working in other receptor systems (Chen 2009). Such functional effects, such as apoptosis, prevention of leakage of endothelial monolayers, and suppression of an inflammatory endothelial phenotype, all require investigation. Another research project in the laboratory has started this investigation.

COMP-peptides were observed to activate downstream signalling pathways known to be associated with ang1 signalling through Tie2, suggesting that they can recruit at least two of these pathways. However, there is more to learn about their induction of these pathways. Whilst Akt induction did not appear vastly different between the two peptides, VTSRGNV and NLLMAAS, there appeared to be a difference in the induction of ERK between peptides. This is interesting since it is the first suggestion that the ligands might work slightly differently. The most obvious
reason might be related to their presumed different binding sites on Tie2. This information would be very useful since it might tell us something about differential signalling in Tie2. Therefore, gathering further structural information about peptide binding sites for VTSRGNV particularly, for instance from NMR studies, will be of paramount importance. Also of importance will be the substitution of other heptameric Tie2-binding peptides into the FLAG-Linker1-COMP-Linker2 structure. During the work of preceding laboratory colleagues, other peptides were also found to bind Tie2 specifically. Additionally, peptide H7 which is derived from the study of Tournaire and colleagues (2004), and which has been used in vasculotide, is another specific Tie2-binding peptide. Comparisons of the abilities of these peptides both to activate Tie2 and induce downstream signalling mediators, combined with structural studies aimed at observing where such peptides interact with Tie2, could actually provide valuable information about the receptor itself, optimum clustering proximities of receptors, and also whether peptides binding in different regions of the receptor can alter recruitment of downstream mediators. In turn such information would ultimately be valuable in attempting to modify Tie2 function to achieve specific effects which could be applied to therapy.

Another possibility accounting for some of the kinetics results might be that one or both of the inductions of Akt and ERK are not dependent on Tie2, but occur through other receptors or pathways. To answer this question, testing the ligands in HUVECs where Tie2 has been inactivated by siRNA would be invaluable. This work is in progress currently. Also, of interest may be the observations that VTSRGNV has some ability to bind the related Tie1 (Kang M, unpublished data). Since Tie1 has more recently been shown to have an inhibitory role in Tie2 signalling, it may be that COMP-VTSRGNV also binds Tie1 to some extent, and this might dampen activation
of Tie2. Whether NLLMAAS binds Tie1 is not known. Testing the ability of COMP-peptides to bind Tie1 would be useful in this regard, as would testing their ability to activate Tie2 in HUVECs lacking functioning Tie1.

It is also of interest to assess whether COMP-VTSRGNV or COMP-NLLMAAS could be antagonised by ang2. It would be expected that COMP-NLLMAAS may be subject to antagonism whereas COMP-VTSRGNV would not. This could in turn affect the concentration requirements for ligands in vivo. This is part of future work in this area. Naturally, given that the observed signals induced by the two synthetic ligands are relatively modest, this is perhaps not as pertinent at present.

The point alluded to in the previous paragraph allows reflection on another important potential problem. Though the COMP-peptides generated appear to multimerise into pentameric format, their actions through Tie2 are relatively modest. In vivo such effects could, arguably, potentially antagonise the effects of endogenous ang1. This might be expected more for COMP-NLLMAAS, since it would compete with ang1 for binding sites on Tie2. In vivo this could potentially have deleterious effects in which COMP-peptides could behave not dissimilarly from ang2. Indeed, ang2 exerts a measurable effect through Tie2 to activate the receptor and its downstream Akt, and is thought of more as a partial agonist of signalling through Tie2 (Yuan, 2009), acting as an antagonist principally in the presence of ang1. Simple experiments could be used to assess whether COMP-NLLMAAS, or indeed even COMP-VTSRGNV, might be able to exert such effects.

Although the synthetic COMP-peptides have been demonstrated to activate Tie2 and some of its downstream messengers, as discussed earlier, the final functional effects of this are not yet known and will be investigated in future ongoing work. It
has been noted that, although ang1 at relative physiological concentrations is not thought to have significant proangiogenic effects, some in vivo studies using higher expressed levels of ang1 have noted such effects (see section 1.9). This has also been noted for COMPang1 as well as for vasculotide. Such effects would doubtless be invaluable in the context of such processes as wound healing, and perhaps also in stroke. However, in the context of diseases which might require longer term administration of an ang1 mimetic, such as diabetic retinopathy, such effects would be unwanted and even deleterious. Furthermore, some authors have implicated ang1 in promoting pathology in certain circumstances. These include Tie2 dependent effects on synovium in rheumatoid disease (Hashiromoto, 2007), as well as apparently paradoxical effects to promote inflammation in some circumstances (Long, 2008).

Additionally, though studies of ang1 involvement in tumour growth have yielded divergent results, there is evidence that it is context-dependent, and that ang1 is able to promote tumour growth at least in some models (Huang, 2009; Holopainen, 2009; Shim, 2002). Such potential problems associated with the use of ang1 and analogues make full exploration of functional effects important when considering their value in therapies. This also highlights the value of exploring further the effects of alternative ligands on Tie2 signalling, in attempting to tailor specific desirable effects through the receptor whilst attempting to avoid those unwanted effects. The use of small Tie2-binding peptides may be an invaluable way to be able to do this.
Appendices
Appendix A:

Genomic Map of pSecTag/FRT/V5-His-TOPO II Expression Vector
Map of pSecTag/FRT/V5-His-TOPO-II expression vector.
Appendix B:

Genomic Map of pLEIC-03
Expression Vector
Appendix C:

Nucleotide Sequences of Ligands

FLAG-Linker1-COMP-Linker2
TTGACTACAAGGACGACGACGACAAGGGCACCAGCGGCGGCGACCTGGCCCC
CCAGATGCTGCGCGAGCTGACAGGAGACCAACGCCGCCCTGCAGGACGTGCGAGAGCTGCTGAGACAACAAGTGAAGGAGATCACCTTCCTGAAGAACACCGTGAT
GGAGTGCGACGCGCTGCGGCGGCAGCGGCGGCCCTTCTACAGCCACAGC

FLAG-Linker1-COMP-LongLinker2

TTGACTACAAGGACGACGACGACAAGGGCACCAGCGGCGGCGACCTGGCCCC
CCAGATGCTGCGCGAGCTGACAGGAGACCAACGCCGCCCTGCAGGACGTGCGAGAGCTGCTGAGACAACAAGTGAAGGAGATCACCTTCCTGAAGAACACCGTGAT
GGAGTGCGACGCGCTGCGGCGGCAGCGGCGGCCCTTCTACAGCCACAGC

Mammalian COMP-VTSRGNV

ACACTCCTGCTATGGGTACTGC
TGCTCTGGGTTCCAGGTTCCACTGGTGACGC
GGCCCAGC
CGGCCAGGCGCGCGCGCCGTACGAAGCTCGCCCTTGACTACAAGGACGACGACGACAAG
GCACCAGCGCGCGCGCGAAGCTGCTGAGAGCTGCTGAGACAACAAGTGAAGGAGATCACCTTCCTGAAGAACACCGTGAT
GGAGTGCGACGCGCTGCGGCGGCAGCGGCGGCCCTTCTACAGCCACAGC

Mammalian COMP-NLLMAAS
CACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGACG
CGGCCAAGCCGCCAGGGCGCGCGCGCGCTACGAAGCTCGCCCTTGACTACA
GGACGACGACGACAAGGGCACCAGCGGCGGCGACCTGGCCCCCCAGATGCT
GAGACAAACAGTGAAAGGAGATACACCTCTTCTGAAGAAACACCAGTGATGGAAGTGC
ACGCTCTGCCGGCAGCAGCGGGGCCCCTTCTCTACGCAACAGCAACTCTGCTGAT
GGCCGCCAGCAGGCGGCGGCGGAGCGCTGAAA

Prokaryotic COMP-VTSRGNY

AAGAAGGAGATATACATATGCAACCATCATCATCATCATTTCTTGGTGATATCT
GGTGACCCGAGACCTGTACTTCCAATCCATGCTTGACTACAAGGAGGAGACGC
ACAAGGGCACCAGCGGCGGACCTGGCCCCCCCCAGATGCTGCGAGCTGC
AGGAGACCAACGCCGCCCTGCAAGGACGTCGAGGCTGCTCGAGACAAACAGT
GAAGGAGATCACCTCTCTGGAAGAAACACCAGTGATGGAGTGCAGCAGCTGC
GGCGAGCGCGCCGTTCCAGGGTGATGGTGATTGGGTGTTGGGGCCCTTTCTACGC
ACAGCGTGACCAGCAGGCGAAGTGTCGATA GTG

Prokaryotic COMP-NLLMAAS
GAAGGAGATATACATATGCACCATCATCATCATCATCATCTTTCTGGTGTAGATCTGG
GTACCGAGAACCTGTACTTCAATCCATGCTTTGACTACAAGGACCGAGACGACA
AGGGCAACCAGCGGCAGCGACCTGGCCCCAGATGCTGCGCGAGCTGCAGG
AGACCAACGCGCCCTGCAGGACGTGCGAGAGCTGCTGAGACAACAAGTGAA
GGAGATCACCTTCTCCTGAAGAACACCGTGATGGAGTGGACTGAGAA
GAGGATCAACCCCTTCAGGATCGGATGAGGGGAGTGGCAGCGGCAGGCGC
AGCGGCGCGGCTCAGGCTGCTGAGTGGTGGTGGGCCCCCTTCCTACAGCCACA
GCAACCTGCTGATGGCCCGCAGCGGCGGCGAGCGGCACGCGCCT

**COMP-NEGATIVE**

GACTACAAGGACCGACGACCAAGGGCACCAGCGGCGGCGACCTGGCCCCC
CAGATGCTGCGCGAGCTGCAAGGAGACACCCGCGCCTGCAGGACGAGTGC
AGCTGCTGAGAAACTGAGTAGCAGCGGATCGGCTGAGATC
GAGTGCAGAGCCTGCGGAGCAGCGGCGGCGCCCTCCCTACAGCGGACAGGTG
CCTGCGGCGGCGGCGGCGCCCTCCCTACAGCGGACAGGTG
GGATCCGAACATTGACCTCCTGAGGCGCGCCACGCGCGCCACCTCGAGGCCTTAAT

**Long Linker2**

GGTTCAGGTGGTGGTGTAGGTGGTGGTGGC

**COMP-VTSRGNV-Long**
GAAGGAGATATACATATGCACCATCATCATCATCATATTCTTCTGGGTAGATCTTG
GTACCGAGAACCTGTACTTCCAATCCATGCTTGACTACAAGGACGCAGACGACGAC
AGGGCACCAGCGGCGGCGGACCTGCGCCCCCAGATGCGCGAGCTGCAGGAC
AGACCAACGCGCCCTGAGGACGTGCGAGAGCTGAGACAAACACGTGAGGAGCGCGGC
AGCAGCGCCGCTTCAGGTGGTTAGGGTGTGGTGGTTGGCGCCCTTCTACACGCAAC
GCGTGACCAGCGCCGCAACGTGGGCGCGGAGAAGTGATGAC

**COMP-NLLMAAS-Long**

AAGAAGGAGATATACATATGCACCATCATCATCATCATATTCTTCTGGGTAGATCTT
GGTACCGGAAACCTGTACTTCCAATCCATGCTTGACTACAAGGACGCAGACGACGAC
ACAGGAGGCAACCAGCGGCGGACCTGCGCCCCCAGATGCGCGAGCTGCAGGAC
AGCAGCGCCGCTTCAGGTGGTTAGGGTGTGGTGGTTGGCGCCCTTCTACACGCAAC
GCGTGACCAGCGCCGCAACGTGGGCGCGGAGAAGTGATGAC

**COMP-RP-Long**
AAGAAAGGAGATATACATATGCACCATCATCATCATCATCATATTCTTCTGTTGTAGATCT
GGGTACCGAGAACCTGTACTTCCAATCCATGCTTGACTACAAGGACGACGACG
ACAAGGGCACCAGCGGCGGCGACCTGCGCCCCAGATGCTGCGAGCAGCTGC
AGGAGACCAACGCGCGGCTGCAGGACGTGCGAGAGCTGCATGAGACAACAAAGT
GAAGGAGATCACTTTCTGAAAGAAACACCGATGATGGGAGATGCGACCGCTGCGGC
GGCAGCGCGCGGCTTCAGGGTGTGATGTTGGGTCAGGGTCGGGGCGCTTCTACAGCC
ACAGCACCATGTACCAGCTGAAGTCTGCAGCGGCGGAGCGCGCTTAG
Appendix D:

Amino Acid Sequences of Ligands

FLAG-Linker1-COMP-Linker2
DYKDDDDKGTSGGDLAPQMetLRELQETNAALQDVRELLRQQVKEITFLKNTVMet
ECDACGGSGGP FYSHS

**FLAG-Linker1-COMP-LongLinker2**

DYKDDDDKGTSGGDLAPQMetLRELQETNAALQDVRELLRQQVKEITFLKNTVMetECDACGGSGGP FYSHS

**Mammalian COMP-VTSRGNV**

TLLLWVLVLLWVGSTGDAAQPPARRARRTKLALDYKDDDDKGTSGGDLA
PQMetLRELQETNAALQDVRELLRQQVKEITFLKNTVMetECDACGGSGGPFYSHSVTSGNVGGS

**Mammalian COMP-NLLMAAS**

TLLLWVLVLLWVGSTGDAAQPPARRARRTKLALDYKDDDDKGTSGGDLA
PQMetLRELQETNAALQDVRELLRQQVKEITFLKNTVMetECDACGGSGGPFYSHSNLLMetAASGGGSA

**Prokaryotic COMP-VTSRGNV**
EGDIH Met HHHHHHSSGVDLGTENLYFQSMet LDYKDDDD
KGTSGGDLPQ Met LRELQETNAALQDVRELLRQQVK EIT
FLKNTV Met ECDACGGSGGPFSYSHSVTSRGNVGGSA

Prokaryotic COMP-NLLMAAS

EGDIH Met HHHHHHSSGVDLGTENLYFQSMet LDYKDDDD
KGTSGGDLPQ Met LRELQETNAALQDVRELLRQQVK EIT
FLKNTV Met ECDACGGSGGPFSYSHSNLL Met AASGGGSA

COMP-NEGATIVE

DYKDDDDGTKSGDLPQ Met LRELQETNAALQDVRELLRQQVK EIT FLKNTV Met
ECDACGGSGGPFSYSHSLPWPTAWARQRLTVKVDTPNSSSVDKLAAALEAStop

Amino Acid Sequence for Longer Linker2

GSSGGGSSGGG

Amino Acid Sequence for COMP-VTSRGNV-Long
E G D I H Met H H H H H S S G V D L G T E N L Y F Q S Met L D Y K D D D D
K G T S G G D L A P Q Met L R E L Q E T N A A L Q D V R E L L R Q Q V K E I T
V G G G S A

**Amino Acid Sequence For COMP-NLLMAAS-Long**

E G D I H Met H H H H H S S G V D L G T E N L Y F Q S Met L D Y K D D D D
K G T S G G D L A P Q Met L R E L Q E T N A A L Q D V R E L L R Q Q V K E I T
A S G G G S A

**Amino Acid Sequence for COMP-RP-Long**

E G D I H Met H H H H H S S G V D L G T E N L Y F Q S Met L D Y K D D D D
K G T S G G D L A P Q Met L R E L Q E T N A A L Q D V R E L L R Q Q V K E I T
N F G G G S A
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