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Anthrax, Matrix Biology, and Angiogenesis: Capillary Morphogenesis Gene 2 Mediates Activity and Uptake of Type IV Collagen-Derived Anti-Angiogenic Peptides

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ABSTRACT

Anthrax, Matrix Biology, and Angiogenesis: Capillary Morphogenesis Gene 2 Mediates Activity and Uptake of Type IV Collagen-Derived Anti-Angiogenic Peptides

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Capillary Morphogenesis Gene 2 (CMG2) is a type I transmembrane, integrin-like receptor. It was originally identified as one of several genes upregulated during capillary formation. It was subsequently identified as one of two physiological anthrax toxin receptors, where CMG2 serves as a cell-surface receptor for anthrax toxin and mediates entry of the toxin into cells via clathrin-dependent endocytosis. Additionally, loss-of-function mutations in CMG2 cause the genetic disorder hyaline fibromatosis syndrome (HFS), where the core symptom is dysregulation of extracellular matrix homeostasis (ECM), including excessive accumulation of proteinaceous hyaline material; HFS clearly indicates that CMG2 plays an essential function in ECM homeostasis and repair. Most often, these situational roles have been evaluated as separate intellectual and experimental entities; consequently, whereas details have emerged for each respective situational role, there has been little attempt to synthesize knowledge from each situational role in order to model a holistic map of CMG2 function and mechanism of action in normal physiology.

The work presented in this thesis is an example of such a synthesis. Interactions between CMG2 and type IV collagen (Col IV) were evaluated, to better understand this putative interaction and its effect on CMG2 function in angiogenesis. Using an overlapping library peptide array of the Col IV α1 and α2 chains, it was found that CMG2-binding peptides were enriched within the NC1 domains. This finding was corroborated via another epitope mapping peptide array, where we found a major epitope for CMG2-binding within the α2 NC1 domain (canstatin). Identification of CMG2 interactions with Col IV NC1 domains (including canstatin) was both surprising and intriguing, as these domains are potent endogenous inhibitors of angiogenesis. To further evaluate the physiological relevance of interactions with Col IV NC1 domains, a canstatin-derived peptide from the original array was synthesized and used for further studies. This peptide (here known as S16) binds with high affinity (K_D = 440 ± 160 nM) to the extracellular, ligand-binding CMG2 vWA domain; specificity was confirmed through competition studies with anthrax toxin PA, and through demonstration of divalent cation-dependent binding. CMG2 was found to be the relevant endothelial receptor for S16. CMG2 in fact mediates endocytic uptake of peptide S16, as demonstrated by flow cytometry, and colocalization studies. S16 further inhibits migration of endothelial cells. These findings demonstrate that CMG2 is a functional receptor for Col IV NC1 domain fragments. CMG2 may exert a pro-angiogenic effect through endocytosis and clearance of anti-angiogenic NC1 domain fragments. Additionally, this is the first demonstration of CMG2-mediated uptake of an endogenous matrix fragment, and suggests a mechanism by which CMG2 regulates ECM and basement membrane homeostasis, thereby establishing a functional connection between the receptor's role in matrix biology and angiogenesis.

Keywords: angiogenesis, CMG2/ANTXR2, matrix biology, peptide array, type IV collagen

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1.1. Introduction

Capillary Morphogenesis Gene 2 (CMG2) is an integrin-like type I transmembrane receptor. As the name denotes, this receptor was originally identified as one of several genes upregulated in endothelial cells undergoing capillary-like tubule formation[1], suggesting a role in vascular formation and angiogenesis. CMG2 was later described as one of two physiological anthrax toxin receptors[2], earning it the additional title of anthrax toxin receptor 2 (ANTXR2). The other anthrax toxin receptor, known as ANTXR1 or tumor endothelial marker 8 (TEM8)[3], shares an overall 40% amino acid identity with CMG2; these two proteins are each other's closest homologs throughout the genome. Notably, TEM8 was also originally implicated as having a role vascular formation, specifically tumor vascularization[4]. Considering the historical frame during which TEM8 and CMG2 were discovered as anthrax toxin receptors—2001[3] and 2003[2], respectively—their function in anthrax intoxication took center stage. This led to the rapid elucidation of the cellular mechanisms by which anthrax toxin infects cells; but it also resulted in a temporary overshadowing of the apparent roles of CMG2 and TEM8 in normal physiology, including angiogenesis.

CMG2 is also the culprit behind another human disease, known as hyaline fibromatosis syndrome (HFS). Unlike anthrax, this is not an infectious disease, but rather a rare genetic disorder. In 2003, two independent groups reported that putative loss-of-function mutations in CMG2 were the causative agent behind both infantile systemic hyalinosis (ISH) and juvenile hyaline fibromatosis (JHF)[5-6], and it was later realized that both diseases represented different points along the disease spectrum denoted HFS[7]. In addition to HFS, recent work has identified mutations in CMG2 as associated with the arthritic disease known as ankylosing spondylitis[8-10]. These genetic disorders suggest that CMG2 plays an essential function in extracellular matrix (ECM) homeostasis, and provides clues as to what this role may be; however, the exact nature of this essential function remains to be determined.

Regarding the role of CMG2 in angiogenesis, we have fortunately not been left without progress. Additional studies have validated a role of CMG2 in endothelial cell function and angiogenesis[11-12]. Others have clearly demonstrated the efficacy of CMG2-targeting towards an inhibition of pathologic angiogenesis[12-16]. These findings suggest that pharmacological inhibition of CMG2 function would be a novel and effective anti-angiogenic therapy, applicable for the treatment of cancer and eye disease, among others. Despite this new insight, the mechanism(s) by which CMG2 acts as an angiogenic regulator remains elusive.

In this review, we summarize the current state of research and understanding of CMG2 biological function, in both health and disease. Specifically, we attempt to derive insight into the "normal" biological role of CMG2 in matrix homeostasis and angiogenesis, using observations of CMG2 function (or malfunction) in disease. There have been limited attempts to reconcile the disparate roles of CMG2, in health and disease; but disease, after all, nearly always represents a

hijacking, or adulteration, of existing biological mechanisms; thus, the function of CMG2 in disease will shed light on the function of CMG2 in health.

1.2. CMG2 architecture and structure

The *CMG2* gene yields 4 distinct protein isoforms, generated through alternative splicing. These are the full length $CMG2^{489}$; $CMG2^{488}$, which is identical to $CMG2^{489}$ excepting the c-terminal 13 residues; $CMG2^{386}$, which lacks the extracellular Ig-like domain; and $CMG2^{322}$, which lacks both transmembrane and cytosolic domains, and thus is predicted to be secreted[2]. The role that each of these distinct isoforms play is not known in detail.

A comprehensive analysis of CMG2 protein structure is outside the scope of this review; nonetheless, for the sake of clarity, we will present an overview (for a detailed description of CMG2 structure, see Lacy et al.[17] and Deuquet et al.[18]). As most studies have utilized the full-length variant, $CMG2^{489}$, we will briefly discuss the structure and domain topology of this isoform. Overall, CMG2 is a type I membrane glycoprotein. At the N-terminus lies a signal peptide, which targets CMG2 to the endoplasmic reticulum during synthesis. The signal peptide is followed by a von Willebrand factor type A domain (vWA domain), then an extracellular Ig-like domain. CMG2 then traverses the membrane via a single transmembrane helix, and terminates with a 148-residue cytosolic tail. CMG2 is a highly-conserved protein, sharing 84% sequence homology from human to mouse. CMG2 also shares significant homology (overall 40%) with TEM8, particularly within the vWA (60%) and cytosolic domains.

The vWA domain mediates binding to extracellular ligands. As with vWA domains from integrins and other matrix-binding molecules, the CMG2 vWA domain contains a metal-ion dependent adhesion site (MIDAS), where the receptor coordinates a divalent cation (likely Ca^{2+}

or Mg^{2+}). The metal-bound receptor is then ready to engage ligands, which can interact with high affinity by completing the metal coordination with an electron-rich side chain (such as aspartate or glutamate). Several mutations within the vWA domain lead to CMG2 loss of function (these will be discussed alongside HFS). The CMG2 vWA structure (including the MIDAS) has been solved, both on its own[17] and in complex with the receptor-binding subunit of the anthrax toxin, protective antigen (PA)[19]. Whereas the interaction between CMG2 and PA has been thoroughly characterized[20] (as will be discussed below), less is known of interactions between the CMG2 vWA domain and ECM proteins. Bell et al. showed binding between the CMG2 vWA domain and type IV collagen, laminin-111, and fibronectin[1]; but details and physiological relevance of these interactions remain the subject of active research.

The precise function of the Ig-like domain of CMG2 remains a mystery. A few mutations within the Ig-like domain result in HFS through CMG2 loss-of-function[18], suggesting that the integrity of the domain is essential for proper protein function. This domain contains two disulfide bridges, both of which are required for proper folding of CMG2, and effective trafficking of the protein from the ER to the plasma membrane—HFS mutations within the Ig-like domain disrupt proper disulfide formation, and lead to ER retention and degradation via ERAD[21]. $CMG2^{386}$ lacks the Ig-like domain, and is sequestered within the endoplasmic reticulum, the function of which has not been characterized. Taken together, these findings suggest the Ig-like domain is essential for effective targeting of CMG2 to the cell surface. CMG2 is endogenously glycosylated[22], and the only potential glycosylation sites are within the Ig-like domain; though the function of this glycosylation remains unclear.

CMG2 contains a single membrane-spanning helix of 23 residues. The TM helix is suspected to mediate receptor dimerization (or possibly higher order oligomers) for both CMG2

and TEM8[21, 23]. This oligomerization is required for entry of the anthrax toxin into the cell[24-25], but whether CMG2 oligomerization occurs in normal biological function is unknown. An HFS causing mutation (L329R) is found in the center of the TM helix. This could result in a loss-of-function because of insertion of a positive charge within a membrane that would likely impact protein stability and proper localization. Alternatively, it could affect CMG2 oligomerization, which may impact proper CMG2 function. It should also be noted that the stability of CMG2 within the membrane is further facilitated by two cytosolic, juxtamembrane cysteines, which are both sites of palmitoylation[26].

The cytosolic domain of CMG2 appears to serve in various capacities. This domain is predicted to be intrinsically disordered (does not undergo a traditional hydrophobic collapse)[27], owing in part to its large proportion (40%) of charged residues. This likely makes it amenable to various post-translational modifications, and subsequent interaction with different partners. Little is sure at this point, but the cytosolic domain does interact with various components of the endocytic machinery required for anthrax uptake; it also contains a putative actin-binding region, which may directly or indirectly associate with the actin cytoskeleton. Both features will be discussed below.

CMG2 expression in humans is fairly ubiquitous, and has been detected in human heart, skeletal muscle, small and large intestine, spleen, kidney, liver, placenta, lung, and peripheral blood, with no expression observed in the brain and thymus[2]. Expression of CMG2 has additionally been found within both normal and tumor-stromal vasculature, with no observed expression within the tumor itself[11]. CMG2 and TEM8 both show temporally and spatially regulated expression during chick development[28], suggesting that the receptors may play an important role in developmental processes.

1.3. CMG2 function in anthrax intoxication

Anthrax infection is caused by a tripartite toxin produced by *bacillus anthracis*. The toxin consists of two enzymatic components: edema factor (EF), an adenylate cyclase that impairs phagocytosis in neutrophils[29-30]; and lethal factor (LF), a zinc-dependent protease that cleaves MEK (mitogen-activated protein kinase kinase, MAPKK), leading to lysis of macrophages[31-33].

The third component of the anthrax toxin is anthrax protective antigen (PA). PA is an 83 kDa protein; it is non-enzymatic, but is the receptor-binding subunit, and escorts LF and EF from the extracellular space to the cytoplasm[25]. PA can bind to both CMG2 and TEM8[2-3], through their respective vWA domain. PA binds with high affinity to the anthrax toxin receptors (with highest affinity for CMG2, $K_D \approx 170$ pM[34]) by engaging the MIDAS, and completing the metal coordination by donating an aspartate side chain, with additional contacts occur within the vWA domain but outside the MIDAS[19]. Upon binding to the host receptor, full length PA (PA-83) is cleaved by furin, leaving PA-63 bound to the cell[35]. Receptor-bound PA-63 is then able to oligomerize, forming a heptamer or octamer of PA-CMG2/TEM8 complexes[19, 24]. The removal of PA-20 further exposes a large hydrophobic surface area, which serves as binding sites for LF and EF on the PA-63 heptamer[36].

This CMG2/TEM8-PA-LF/EF complex will then be taken up through clathrin-dependent endocytosis[25]. This process involves several coordinated steps. First, binding and heptamerization of the PA/CMG2 complex activates src-like kinase, triggering phosphorylation of cytosolic tyrosine residues on CMG2[37]. Second, a ubiquitination complex (including ß-arrestin and the E3 ubiquitin ligase Cbl) recognizes phosphorylated CMG2 and ubiquitinates lysine residues on CMG2[26]. This ubiquitination leads to recruitment of the endocytic complex, including clathrin, actin and the adaptor protein AP-1[38]. This triggers uptake of the receptor/toxin complex into

endosomes. Upon maturation and acidification of the endosomes, the PA heptamer rearranges and forms a pore through the endosomal membrane[39]. The acidification also causes unfolding of LF and EF, which are then able to traverse the PA-pore and enter the cytosol where they can act on their enzymatic substrates.

Despite the earlier discovery of TEM8 as an anthrax toxin receptor[3], multiple studies have suggested that, at least in mice, CMG2 may be the primary anthrax toxin receptor. Using mice that were either deficient in CMG2 or TEM8, it was demonstrated that anthrax lethality in mice is mostly mediated by CMG2[40]. This could be because PA shows at least one order of magnitude higher affinity for CMG2 than TEM8, both in cells[40], and using recombinant purified protein[34]. This being said, the observation of anthrax resistance in $CMG2^{-/-}$ mice should be interpreted with prudence, as the CMG2-null mouse model from this study does not represent a complete knockout: the transmembrane domain was deleted, suggesting that the CMG2 (same as for $TEM8^{-/-}$) will be secreted into the extracellular milieu. This secreted domain could still be capable of interacting with ligands, including anthrax toxin, potentially preventing ligands from interacting with cellular receptors.

The work summarized above has enabled us to understand the critical and essential role of CMG2 in anthrax infection. This understanding has informed work towards development of new vaccines and treatment for anthrax. Additionally, and importantly, the high-affinity interaction between non-toxic PA and CMG2 has also provided a safe and accessible means to investigate the normal physiological role of CMG2, including in angiogenesis; the insight gained from this approach will be summarized in a later section.

1.4. Insight into CMG2 function from HFS and Ankylosing Spondylitis

1.4.1. CMG2 in Hyaline Fibromatosis Syndrome

The first apparent case report of what is now recognized as hyaline fibromatosis syndrome was in 1873[41-42]. Since that time, there have been roughly 150 described cases of HFS in the medical literature, showing no ethnic or geographic predisposition[18]. It is largely a disease of the connective tissue. HFS was previously described as two separate disorders, infantile systemic hyalinosis (ISH) and juvenile hyaline fibromatosis (HFS). The distinction between the two classifications is that ISH represents the more severe disease phenotype (with fatality occurring often during infancy), and is accompanied by recurrent respiratory infections and diarrhea[7]. The cause remained unknown for some time, until the advent of genome sequencing technologies; in 2003, genome sequencing from both ISH and JHF patients revealed putative loss-of-function mutations in CMG2[5-6]. Since then, loss-of-function mutations in CMG2 have been identified in every case of HFS[18, 43], precluding the possibility of genetic heterogeneity for the disease. These mutations have been found to span the CMG2 gene, with most occurring within exons.

Of the mutations that map to exons, Deuquet et al. defined 4 classes[18]. Class I HFS-inducing mutations occur within the extracellular ligand-binding vWA domain, including mutations that disrupt the metal chelation ability of the CMG2 MIDAS (such as T118K, where insertion of a lysine in the MIDAS coulombically opposes metal chelation). Primary fibroblasts from two HFS patients with vWA-domain mutations were unable to adhere to a laminin matrix[5], suggesting that the binding capacity of CMG2 to laminin is essential for adhesion of certain cell types. And we have shown that two ISH-causing MIDAS mutations (D50N and T118K) result in a 1000-fold reduced affinity for PA (T118K) or completely abolish the interaction (D50N, unpublished data). And of the seven identified HFS mutations that result in amino acid

substitutions within the vWA-domain, six of these result in the more severe ISH[18]. Taken together, these data suggest that interactions between CMG2 and its ligands (ECM components) are critical for appropriate CMG2 function.

Other mutations, denoted Class II, within the extracellular Ig-like domain decrease the folding stability of CMG2[21-22]. Several of these disrupt disulfide bond formation within the vWA and Ig-like domains. Due to poor folding, these are often retained in the ER and trafficked through the ERAD pathway for degradation. Excitingly, it was demonstrated using patient primary fibroblasts that proteasome inhibitors could restore CMG2 cell-surface expression and alleviate the phenotype, suggesting that proteasome inhibition may be a feasible treatment strategy for this rare but personally devastating disease.[21-22] This defective folding class also includes a mutation within the transmembrane helix (L329R)[5]; insertion of a charged residue within the membrane is expected to prevent appropriate plasma membrane targeting, and possibly interfere with CMG2 oligomerization.

Two other general classes of mutations remain. Class III consists of frameshift mutations that result in premature stop codons. Several of these mutations have been shown to lead to unstable mRNAs, that are rapidly degraded via the nonsense-mediated mRNA decay pathway[21, 44]. Class IV mutations map to the cytosolic tail of CMG2[6, 45-46]. These mutations do not affect membrane targeting or protein stability—the short cytosolic tail is predicted to be intrinsically disordered; rather it is presumed that they affect intracellular interactions and signaling. Interestingly, one of the mutations (Y381C, leading to JHF) occurs in a tyrosine shown to be 1 of 4 phosphorylated during anthrax intoxication, and that this phosphorylation was required for CMG2-mediated endocytosis of the toxin[6, 37]. This suggests that CMG2 phosphorylation may play

a role in endogenous function, potentially by signaling activation or endocytosis of CMG2 and endogenous ligands, similar to anthrax intoxication.

HFS has several hallmark symptoms, all of which suggest that CMG2 plays a vital role in homeostasis and repair of the ECM. For those with ISH, life expectancy does not span past childhood; for JHF, seldom do patients exceed early adulthood. Throughout the course of the disease, patients are intellectually normal. But, newborns and children begin to show thickening of the dermis. They develop subdermal tissue nodules. These appear to develop preferentially over sights of frequent mechanical stress or pressure (microtrauma), including the knees, fingertips, and perioral, perianal, and perinasal locations[42], suggesting a role for CMG2 in ECM repair after microtrauma. These nodules begin with a cellular composition of fibroblast-like cells within an eosinophilic matrix. But as the nodules age, they have been observed to shift to a more proteinaceous, acellular composition[47]. The exact composition of these nodules has not been definitively determined, but findings suggest that they consist largely of collagens, including type I and type VI[47-48]. It is likely that other basement membrane and ECM components (including glycoproteins and glycosaminoglycans) exist within these plaques. And considering that CMG2 is an ECM receptor[1, 5], the build-up of extracellular matrix components upon CMG2 loss-of-function suggests that CMG2 may be involved in cellular uptake, clearance and lysosomal degradation of ECM fragments. This remains, however, a question yet to be investigated.

1.4.2. CMG2 in ankylosing spondylitis

Ankylosing spondylitis (AS) is an auto-inflammatory arthritic disease, affecting roughly 1 out of 200 in the Caucasian population[49]. AS mostly affects the spine, where in severe cases new bone formation (ankylosis) can occur leading to fusion of the vertebra; but the arthritis can affect

other joints, including the sacroiliac joint, and the shoulders. Inflammation can also extend to the heart, lungs and kidneys[8]. The disease is strongly associated with MHC class I molecule, HLA-B27 (in the UK, HLA-B27 is present in 90-95% of cases of ankylosing spondylitis)[50]. Yet, less than 5% of individuals positive for HLA-B27 develop AS, suggesting that the disease pathogenesis is more complex, and likely involves other genes. This realization has triggered a search for non-MHC genes with strong associations for AS. And several have been identified, including IL23R and ERAP1 (endoplasmic reticulum aminopeptidase 1)[51], and recently, CMG2.

CMG2 linkage to AS was first reported in a genome-wide association study (GWAS)[8]; specifically, this report identified a SNP (re4333130) within a non-coding region of CMG2 as associated with the disease. Subsequently, several other GWAS confirmed the disease association with this SNP, and identified novel SNPs within CMG2[9-10]. Together, these association studies confidently identify a link between CMG2 SNPs and AS. Interestingly, all identified SNPs lie within putative gene regulatory regions, rather than coding regions, suggesting that defective CMG2 expression may be the contribution of these SNPs to AS pathology.

These association studies provide no functional insight to the role of CMG2 in AS; for this, the genetic analysis must be succeeded by molecular and cell biology. One study suggested a role of microRNA in the regulation of CMG2 in AS[52]. The authors sought to characterize the role of miR-124 in AS, and discovered that miR-124 possessed sequence complementarity for CMG2. With a cohort of AS patients, they observed that AS patients had higher levels of miR-124, and lower levels of CMG2 in peripheral blood (compared to healthy controls), suggesting that down-regulation of CMG2 may contribute to spondylitis pathology. Further, when overexpressed in Jurkat cells, miR-124 was indeed capable of silencing CMG2 expression. Findings from another study suggest that CMG2 mRNA is upregulated, and protein is down-regulated in patients with

AS[53]. This seemingly counter-intuitive finding could indicate that, in AS, translation of the CMG2 mRNA cannot proceed efficiently. This could cause a build-up of CMG2 mRNA, as the cell continues to signal for increased CMG2 expression, to no avail. However, the findings within that study are tenuous, and were not sufficiently validated to afford a reliable interpretation, as CMG2 protein levels were not directly measured, but indirectly using a lipopolysaccharide (LPS) stimulation assay.

Together, these findings suggest that a down-regulation of CMG2 contributes to AS pathology; and while more work must be done to understand this role, previous discoveries regarding CMG2 can shed light on this interesting question. Low-density lipoprotein receptor-related protein 6 (LRP6) was identified by Wei et al. as a requisite co-receptor for anthrax toxin internalization[54]. This suggests an interaction between LRP6 and CMG2 (or TEM8). And as LRP6 is also a receptor within the Wnt/β-catenin pathway affecting, among other things, bone formation, this interaction may indicate that CMG2 plays some unknown role in bone morphogenesis. This interpretation, however, must be taken cautiously, as the putative interaction between LRP6 and the anthrax toxin receptors is controversial; Young et al. published work where they found that LRP5/6 were not required for anthrax toxin internalization[55]. Then, a subsequent report found that, while not being required for anthrax intoxication, LRP6 does functionally interact with both CMG2 and TEM8[56].

There are also potential functional connections to be made between HFS and AS. In AS, impaired bone homeostasis, specifically excessive bone deposition, is the disease hallmark; patients also experience osteoporosis (severe low bone mineral density) and an increased risk of fracture[57]. In HFS, patients suffer from osteopenia and osteoporosis (moderate to severe low bone mineral density) with an increased susceptibility to fracture[47], indicating that loss of CMG2 affects

bone homeostasis in AS and HFS, possibly through interaction with LRP5/6, as noted above. In both diseases, patients suffer from painful and abnormal joint contractures, and an arthritic decrease in joint space[5, 57]. In both cases, patients may suffer from inflammatory bowel syndrome[6, 57]. The rationale behind these phenotypic connections remains to be investigated. One potential explanation centers on type I collagen homeostasis. It has been discussed previously that compositional studies of HFS patient nodules demonstrate abnormal type I collagen deposition[47-48]. As bone is composed predominantly of minerals (such as hydroxyapatite) and type I collagen, abnormal collagen deposition within bone could lead to abnormal bone formations, with low bone mineral density (if collagen deposition were to outpace hydroxyapatite formation), in agreement with the ankyloses and osteoporosis in AS. In relation to the observed inflammatory bowel syndrome, abnormal matrix and basement membrane homeostasis could result in decreased integrity of the gut wall, increasing the permeability and exacerbating intestinal inflammation. This has been observed as protein-losing enteropathy in HFS patients[6], and a similar hypothesis has been proposed for AS patients and their families, where increased intestinal permeability is observed relative to healthy controls, independent of HLA-B27[58]. If both diseases experience diminished CMG2 activity (definite in HFS[5] and suggested in AS[52]), one could envision a model wherein decreased CMG2 expression or function leads to dysregulation of collagen homeostasis, either through increased deposition of collagen, or decreased degradation and clearance. In addition to type I collagen, other matrix and ECM components are likely also affected.

1.5. CMG2 as a regulator of angiogenesis

Angiogenesis is the process of sprouting new blood vessels from existing vasculature; as opposed to vasculogenesis, which is the developmental process of forming *de novo* vessels from

endothelial progenitor cells. Whilst angiogenesis is critical for certain healthy-state functions—including wound healing—pathological angiogenesis is essential to the development and progression of many significant diseases. Aberrant blood vessel formation is the leading cause of blindness in the developed world[59-60]. Angiogenesis is also required for exponential tumor growth and metastasis[61-62]. Effective inhibition of angiogenesis would not only starve a tumor of needed nutrients and deny it a highway for metastasis; it would do so by targeting non-cancerous endothelial cells, potentially side-stepping the classic issue of drug resistance acquired through genomic instability within the transformed cells. Considering the severe consequences—and potential benefits from effective inhibition—there is an urgent need to understand the cellular and molecular processes underlying this vessel growth, so as to identify potential therapeutic targets. VEGF and VEGFR-2 are among the most common targets for anti-angiogenic therapies, and have been for several decades; but the efficacy of these treatments is underwhelming in most cases[63]. Although targeting the VEGF axis may halt certain angiogenic pathways, others seem to arise rapidly to compensate; in some cases, the cancer cells and vasculature will develop "resistance" by drastically upregulating VEGF and VEGFR-2 expression. This is to say that, while inhibition of angiogenesis is still seen as a promising strategy for treating cancer and eye disease, the eventual success of that strategy relies on the identification of novel anti-angiogenic targets.

The anthrax toxin receptors have emerged as potential targets for anti-angiogenic therapies in both eye disease and cancer[16], with recent evidence suggesting that targeting of CMG2 may be more efficacious than TEM8[64] (also, Rogers et al. unpublished). In 2007, Rogers and Christensen et al. demonstrated for the first time that targeting of the anthrax toxin receptors was a viable strategy for angiogenesis inhibition[12]. In that study, the authors used an anthrax toxin PA mutant known as PA-SSSR, which possesses a mutant furin-cleavage site, rendering it unable to undergo

proteolytic processing and endocytosis, thus leaving it bound to the CMG2/TEM8 at the cell surface. PA-SSSR drastically inhibited vessel formation, up to 58% of control, in a mouse corneal neovascularization assay[65]. This effect was dependent on the capacity of PA-SSSR to bind CMG2/TEM8, as PA-SSSR mutations within the receptor binding site abolished the inhibitory capacity of PA. PA-SSSR treatment further resulted in reduced tumor volume. This effect was observed to be the result of reduced endothelial cell migration, rather than proliferation. It is suspected that this phenotype is the result of PA-SSSR competitively inhibiting the interaction of CMG2/TEM8 with basement membrane and ECM proteins. Despite its efficacy, PA is seen by most as an impractical anti-angiogenic therapeutic, owing in large part to its immunogenicity—in fact, it is actively being investigated as an antigen in vaccines against anthrax[66-67]. Nonetheless, these findings served as rationale for further studies to understand the mechanism by which CMG2 and TEM8 regulate angiogenesis, and to identify means to therapeutically target these receptors.

In 2010, Reeves et al. made the notable discovery that CMG2 is expressed in both tumor and normal vasculature within the breast, and that CMG2 expression colocalizes with that of type IV collagen, a major component of vascular basement membrane and putative CMG2 ligand[1, 11]. The authors observed that targeted silencing of CMG2 resulted in impaired endothelial proliferation and capillary morphogenesis, but observed no effect on cellular migration. This finding appears at odds with those of Rogers et al.,[12] where migration, but not proliferation was affected by PA. There are a few potential explanations for this discrepancy. First, targeted silencing, and downregulation of CMG2 expression may affect endothelial cell function differently than competitive inhibition of CMG2 binding to extracellular ligands. Second, Rogers et al. used human microvascular endothelial cells (HMVECs), whereas Reeves et al. used human umbilical vein endothelial cells (HUVECs); differences in phenotype could arise from cell-type differences.

Third, the migration assays used in each paper (transwell migration and scratch-healing assay) are different, and suffer from their respective drawbacks, sharing those of reproducibility and sensitivity; thus, assay differences could have influenced the different conclusions. It is worth noting that subsequent studies demonstrated that different CMG2-binding pharmaceutical agents were capable of inhibiting either endothelial migration or proliferation, and that generally, inhibition of either of these functions results in inhibition of capillary network formation[13, 15, 68]; and one of these small molecules was even capable of modest inhibition of angiogenesis in the mouse cornea[15].

Other CMG2-targeting anti-angiogenic strategies do not aim to inhibit CMG2, but rather to use it as a cellular Trojan horse, effectively infecting tumor and/or stromal cells with anthrax toxin (or a more potent/selective analogue). By mutating the furin recognition sequence to that of a tumor-associated protease (such as urokinase activator or matrix metalloproteinase), several PA constructs have been engineered that exhibit high selectivity for tumor cells[69-71]. Once bound to CMG2 and cleaved at the cell surface, these PA mutants function as they normally would, binding to lethal factor and translocating this toxic enzyme to the cytosol of tumor cells. Liu et al.[64] recently showed that the ability of these tumor selective PA-LF complexes to treat solid tumors was not so much an effect on the primary cancer cells directly, but rather the result of CMG2-dependent "infection" of the endothelial stroma, and subsequent decrease in endothelial proliferation, resulting in destabilization and degradation of tumor vasculature. This then lead to a significant decrease in tumor volume. Systemic administration of several doses of PA-LF was enabled using an immunosuppressive regimen (as discussed earlier, PA is a highly immunogenic molecule).

It has become clear that targeting of CMG2 is an effective strategy for anti-angiogenic therapy, both in corneal and tumor models. This efficacy appears to arise from reduced endothelial cell proliferation and/or migration. But what extra- and intra-cellular molecules interact with CMG2 to effect this role in angiogenesis has remained unclear. Recent work from our group (Tsang et al., data unpublished) is using Bio-ID[72] to characterize intracellular interacting partners with CMG2. Work presented in this thesis has identified CMG2 as a receptor for known anti-angiogenic fragments of the type IV collagen NC1 domains. We have shown that CMG2 can mediate the anti-angiogenic effect of these extracellular molecules, as well as their endocytosis and clearance. These findings together represent a start to mapping the physiological CMG2 "interactome" and understanding the biological relevance of those interactions, particularly to angiogenesis.

1.6. Synthesizing disparate roles towards a unified understanding of CMG2 function

Whereas CMG2 was originally identified as a player in capillary morphogenesis[1], the limelight quickly shifted towards its role in disease: predominantly in anthrax intoxication[2], and also in hyaline fibromatosis syndrome[5-6]. Today, anthrax intoxication via CMG2 and TEM8 is among the most well characterized infectious pathways, with nearly every step of infection defined on a molecular level[73-76].

These three primary situational roles for CMG2—as a 1) anthrax receptor, 2) culprit gene in HFS, and 3) involved in regulating angiogenesis—have traditionally been evaluated as separate intellectual and experimental entities. There have been some connections established—notably including the use of anthrax toxin PA to validate/exploit CMG2 as an anti-angiogenic target[12, 16]. But, to the authors' knowledge, there has been little hypothesis-driven experimentation to unify

all three of these disparate situational roles. Such an approach would drastically improve our basic understanding of CMG2 biology, and would provide novel insight and potential therapeutic strategies for the treatment of HFS, AS, anthrax intoxication and for the inhibition of angiogenesis.

Based on current data, we herein propose three plausible functional—rather than situational—models for CMG2: 1) as a mediator of endocytosis and clearance of degraded/damaged matrix fragments; 2) as a linkage between the extracellular matrix and the intracellular actin cytoskeleton; and 3) as a component of complexes that signal to the nucleus. While these propositions represent some degree of speculation, they are based on existing knowledge and nonetheless provide an intellectual framework from which to devise testable hypotheses. It is critical to note that these models are not proposed as mutually exclusive; rather, it is our opinion, based on current data for CMG2 and analogy to TEM8, that CMG2 function is best defined as a synthesis of the three above models. Below, we will elaborate on each of these functions, and justify them from the position of anthrax intoxication, hyalinosis and ankylosing spondylitis, angiogenesis, and other observed physiologic functions of CMG2.

1.6.1. CMG2 as a mediator of ECM homeostasis and remodeling, through endocytic uptake and clearance of ECM fragments

This model casts CMG2 in a more "mechanical role", as a mediator for uptake and clearance of degraded or damaged fragments of ECM components. It is apparent from HFS that human CMG2 is *biologically essential* for extracellular matrix homeostasis. The logic goes that when CMG2 loss-of-function mutations occur (as in HFS), the receptor can no longer facilitate clearance of ECM fragments, leading to their systemic deposition and build-up in nodules, as experienced by HFS patients. Relatively little is known regarding cellular uptake and lysosomal

degradation of ECM components (as opposed to the broad knowledge available regarding extracellular processing by MMPs and other proteinases). A few pathways have been identified, involving binding and subsequent cellular uptake of ECM through different receptors, including: heterodimeric complexes of integrin-ß1 (phagocytic, ESCRT- or caveolin-1-mediated), Endo-180 (clathrin-mediated) and dystroglycan receptor[77]; but these do not yet fully explain the complete picture of cellular processing of ECM. There are several lines of evidence which suggest that this may be an endogenous function of CMG2. Notably, build-up of aberrant extracellular matrix components at sites of microtrauma (such as around the joints) in HFS patients suggests that CMG2 plays a critical role in clearance of damaged matrix fragments[78]. And perhaps most obviously, CMG2 mediates endocytic uptake of its best characterized ligand, anthrax toxin PA[79]. Endogenous ligands of CMG2 include ECM proteins. As pathogens almost always exploit existing biological mechanisms, it follows that CMG2 likely mediates endocytic uptake of endogenous ligands, including ECM components and fragments thereof. Further TEM8 (the closest homolog of CMG2) has been show to recycle back and forth from endosomes to the cell surface[80], indicating that receptor endocytosis occurs in normal biology; the same may be expected of CMG2. Yang et al. also demonstrated that anti-TEM8 antibodies were internalized via TEM8[81].

Additional support for this model can be drawn from phenotypic observations in CMG2$^{-/-}$ mice. In two different knockout strains, the only major observed phenotype was an inability of pregnant CMG2$^{-/-}$ females to progress through parturition[82-83]. It was determined, independently in both studies, that this defect in parturition was the result of extensive collagen and other matrix deposition and decellularization of the myometrium. While interesting that CMG2-null mice did not systemically phenocopy human HFS, the uterine phenotype of massive hyaline deposition closely resembles patient tissues in HFS. The difference between human and murine phenotypes

could be explained temporally. HFS is characterized by a *progressive* deposition of hyaline material. HFS symptoms do not manifest until several months to a few years after birth. It could be that this is the time required for sufficient hyaline deposition to occur. In mice, only tissues that undergo rapid and regular remodeling (such as the uterus) would be predicted to be affected in their short lifetime. Regardless, the parturition-defective phenotype with collagen deposition within the uterus supports a role for CMG2 in ECM remodeling and homeostasis, potentially through endocytic uptake and degradation of ECM components[82].

Reeves et al. suggested that the observed uterine fibrotic phenotype could be explained through CMG2-dependent MMP regulation[83]: that loss of CMG2 led to a concomitant decrease in MMP activity, resulting in build-up of extracellular hyaline material. They observed an apparent increase in MT1-MMP (MMP14) function upon interaction with CMG2; however, this increase in activity was modest, and has not been thoroughly reproduced[84]. By coimmunoprecipitation, they observed an interaction between CMG2 and MT1-MMP; this occurred, however, only when both proteins were overexpressed in 293T cells, raising questions as to if they interact at physiologically relevant protein concentrations. Thus, more work should be done to definitively identify any functional relationship between CMG2 and MMPs, though such a connection is plausible.

This model of matrix homeostasis and remodeling draws a clear connection between hyalinosis and anthrax (through endocytosis), but how might it influence the role of CMG2 in angiogenesis? An early step in the angiogenic process is vascular basement membrane remodeling, where MMP and other proteinase activity leads to degradation of the surrounding ECM (type I collagen and fibronectin) and the basement membrane (type IV collagen and laminin). These fragments must then be cleared by the surrounding cells, allowing endothelial migration and capillary formation[85]. In this thesis, the first evidence is presented of CMG2 mediating endocytic

uptake of an endogenous matrix fragment. Intriguingly, this fragment is derived from type IV collagen NC1 domains; these domains are well characterized as potent, endogenous inhibitors of angiogenesis[85-86]. These NC1 fragments are produced during the above described process of proteolytic basement membrane remodeling during angiogenesis. They can then signal through cellular receptors, as a sort of negative-feedback loop for angiogenesis. As CMG2 endocytoses and clears these anti-angiogenic peptides, it removes them from the extracellular space; this not only removes an anti-angiogenic molecule, it also physically clears the way for endothelial motility. Thus, CMG2 activity results in a pro-angiogenic phenotype. The anti-angiogenic effect of PA-SSSR treatment agrees with this model[12]. If PA-SSSR were to competitively engage and sequester CMG2, the receptor would no longer mediate uptake of the anti-angiogenic fragments, and thus the peptides would remain in the extracellular space and continue to inhibit angiogenesis, both through sterics and signaling.

Several questions need to be addressed to evaluate and expand upon this model. For example, during anthrax intoxication, PA must oligomerize prior to endocytic uptake[24, 35]. This oligomerization of PA is accompanied by oligomerization of CMG2 at the cell surface, demonstrating that receptor oligomerization is required for efficient uptake of the anthrax toxin. Is oligomerization also required for endocytic uptake of endogenous proteins? Additionally, much of the cellular machinery required for anthrax intoxication has been delineated[26, 37-38]; does this same machinery facilitate post-translational modification of CMG2 and subsequent endocytosis of ECM components and fragments?

Of note, an isoform of CMG2 ($CMG2^{386}$) was found to localize to the endoplasmic reticulum of endothelial cells, colocalizing with Hsp47, a collagen-specific chaperone[1, 87]. This could suggest that, in addition to collagen degradation, CMG2 may also be involved in the proper

folding/assembly of collagen. Improper folding assembly could also contribute to aberrant collagen deposition in HFS. This is an intriguing hypothesis that remains to be further investigated.

1.6.2. CMG2 as a linker between ECM and the actin cytoskeleton, regulating cell adhesion and migration

The original identification of CMG2 observed that the extracellular vWA domain of CMG2 interacted with ECM proteins; but they also identified block homology of the cytosolic CMG2 tail with the WH1 domain of WASP, a protein family fundamental for actin reorganization[1]. Physical linkage between the ECM and the cytoskeleton could mechanistically explain the role of CMG2 in cell migration and adhesion. Both CMG2 and TEM8 are clearly characterized as ECM interactors[1, 88]. And both have been identified as interacting with actin. For TEM8, that data is robust. It has been clearly established that TEM8 couples with the actin cytoskeleton[89-91], and current evidence suggests that the interaction can be either direct between TEM8 and actin[81, 91-92], or involve binding to other adaptors in the actin cytoskeleton[81]. For CMG2, the role of binding with actin has been less studied, but it has been demonstrated that actin dynamics are essential for TEM8- and CMG2-dependent anthrax toxin uptake[38, 91]. Intriguingly, binding to actin has been shown to regulate inside-out TEM8 signaling, as actin binding to the TEM8 cytosolic tail influences the extracellular conformation and subsequent ligand binding, specifically actin binding decreased the affinity of TEM8 for PA[81, 90-91]. This inside-out signaling is also a hallmark of integrin regulation[93]; but whether actin binding influences the affinity of CMG2 for extracellular ligands remains unknown.

Endothelial cell migration is a key step of vessel formation, and requires coupling of the cytoskeleton with the ECM[94]. Several publications have shown a role of CMG2 in regulating cell

migration. Most have focused on endothelial cells, where targeting of CMG2 with either PA[12] or CMG2-binding small molecules[13, 15, 68] inhibited endothelial migration. Unpublished data from our lab using CMG2-binding small molecules (that have been screened to select against TEM8-binding), provide additional support that CMG2 targeting is sufficient to inhibit endothelial migration. And as will be presented in this thesis, anti-angiogenic type IV collagen NC1 fragments inhibit endothelial migration through binding to the vWA domain of CMG2. A recent study demonstrated that CMG2 knockdown inhibits migration of human uterine smooth muscle cells (HUSMCs)[84], suggesting that the role of CMG2 in cell migration extends beyond endothelial cells (this observation suggests an additional mechanism for parturition defects in CMG2-null mice[82-83]). TEM8 has also been identified as a positive regulator of endothelial migration on type I collagen[88]. This could represent functional redundancy between CMG2 and TEM8; alternatively, the two receptors could respectively mediate migration on differing substrates (for example, type IV collagen for CMG2 and type I collagen for TEM8).

Another important cellular function is adhesion and spreading on ECM substrates, which also require linkage between the ECM and the actin cytoskeleton. Several studies have demonstrated a clear positive role for TEM8 in both cellular adhesion and spreading[88], and have shown that this process is dependent on TEM8 endosomal recycling[80] and on interactions with actin[89]. Again, for CMG2, less has been studied here. But recent work from our lab (Tsang et al., unpublished) demonstrates that CMG2 overexpression increases HEK293 cell adhesion to several substrates, including PA, type IV collagen, fibronectin, and laminin, and that this increased adhesion is inhibited by PA treatment, indicating that the adhesion is the result of interactions between the ECM substrates and CMG2 vWA domain. Connected to this, we are also currently investigating the role of CMG2 in regulating actin cytoskeletal dynamics, a hypothesis supported

by analogy with TEM8[92], and the observation that CMG2 is required for actin-mediated contractility of HUSMCs[84].

1.6.3. CMG2 as a component of signaling complexes regulating gene transcription

Unfortunately, much less is known on the role of CMG2 in regulating gene transcription, as compared to the receptor's function in endocytosis and cellular adhesion and migration. That being said, we can draw early insights (leading to testable hypotheses) using a few studies that have linked Wnt signaling with the anthrax toxin receptors, and other studies that have shown clear signaling functions of TEM8.

The interplay between Wnt signaling and anthrax toxin receptors began as controversial. Wei et al. originally reported that LRP6 (a Wnt co-receptor) was necessary for anthrax intoxication[54]. Despite being received with excitement, attempts to reproduce the requirement of LRP6 in anthrax intoxication demonstrated that LRP6 (and homolog LRP5) were *not* required for anthrax lethality[55, 95]. A subsequent study by Abrami et al. helped to clarify this contradiction: both CMG2 and TEM8 directly associate with LRP6, and this has a positive effect on Wnt signaling, but this interaction is not required for, though does accelerate anthrax intoxication[56]. RNAi against either CMG2 or TEM8 led to reduced levels of LRP6 (owing to increased proteasomal degradation), and this resulted in a destabilization of ß-catenin during Wnt signaling. Intriguingly overexpression of either CMG2 or TEM8 also led to reduced LRP6 levels, suggesting a "Goldilocks" effect in their regulation of LRP6. In 2011, a study by Verma et al. confirmed a role of TEM8 in regulating canonical Wnt signaling[28]. Using the embryonic chicken chorioallantoic membrane (CAM), which does not express CMG2, but expresses TEM8 in a temporally regulated fashion, the authors demonstrate that TEM8 expression amplifies Wnt signaling through

stabilizing ß-catenin and leads to increased expression of Wnt-induced reporter genes. Additionally, as this assay was examining vessel development within the CAM, these findings implicate TEM8 as an important regulator of Wnt-dependent developmental angiogenesis. An interesting question is raised by this apparently positive relationship between CMG2/TEM8 and LRP5/6, as loss-of-function mutations in CMG2 (HFS), TEM8 (GAPO, discussed below), and LRP5[96] all lead to disorders displaying osteopenia (low bone mineral density); could CMG2 and TEM8 be important co-receptors in Wnt-dependent bone formation[96]?

A role for TEM8 in developmental angiogenesis is further supported by the discovery of TEM8 mutations associated with infantile hemangioma[97]. A mutation in TEM8 was found to enhance the interaction of a novel signaling complex composed of ß1 integrin, TEM8, VEGFR2 and NFAT, and this enhanced interaction decreased ß1 integrin-dependent NFAT activation and gene regulation; this provides an additional example of TEM8 functioning in gene regulation. The result of this decreased signaling was an increase in VEGFR2 activation and signaling, resulting in, among other things, rapid proliferation of hemangioma endothelium as compared to control[97].

Recently, loss-of-function mutations in TEM8 have been identified as the causative agent of GAPO syndrome[98], which name is an acronym for the hallmark symptoms: growth retardation, alopecia, pseudoanodontia (teeth develop but fail to erupt), and in most but not all cases, progressive optic atrophy[99]. The disease results in significantly reduced lifespan, with death generally occurring within an individual's third or fourth decade. Identification of TEM8 loss-of-function mutations was the first demonstration of the underlying genetic causes of GAPO syndrome, and has since been validated in other case studies[100]. Remarkably, the underlying cause of GAPO syndrome symptoms is dysregulation of extracellular matrix homeostasis[98], demonstrating that TEM8 deficiency, like that of CMG2 in HFS, results in impaired ECM

homeostasis. This further suggests an apparent functional redundancy of CMG2 and TEM8. The distinction (and relationship) between the two anthrax toxin receptors is, at the moment, poorly understood. Importantly though, GAPO and HFS have consistent differences. From close inspection of the literature, GAPO appears to directly affect developmental processes, whereas HFS seems to regulate repair processes (with ECM-composed matrix nodules most often appearing at sites of microtrauma). The precise rationale for this distinction is unknown; but it may suggest that CMG2 and TEM8 perform similar functions, in different situations, with TEM8 controlling development and CMG2 influencing repair after injury, and the idea remains largely speculative. Indeed, many cell types have been identified as expressing both CMG2 and TEM8, and how these two receptors might function together must further be investigated. For example, Abrami et al. observed that RNAi against TEM8 had no effect on CMG2 mRNA levels, but led to a drastic decrease in CMG2 protein levels, suggesting some functional cooperation between the two receptors[56].

A novel TEM8-null mouse model[101] has enabled many exciting discoveries of TEM8 function regarding GAPO and infantile hemangioma and can inform the development of critical questions in understanding CMG2 and TEM8 biology. The model was generated by inducing a frameshift deletion of TEM8 exons 2-8, and replacing exon 1 with a TM-LacZ reporter under the endogenous TEM8 promoter, allowing the visualization of TEM8 expression patterns[101]. These mice recapitulated much of the GAPO phenotype, including growth retardation, vascular defects, and overall excessive deposition of fibrillary ECM components.

In agreement with infantile hemangioma, the authors reported increased levels of VEGF-A and VEGFR2 signaling. TEM8-null mice also displayed decreased expression of VEGFR1 and ß1 integrin, and increased activity of HIF-1alpha and TGF-ß. And, strikingly, they observed that

TEM8 deletion results in increased synthesis of the fibrillar ECM components type I and type VI collagen and fibronectin, and decreased synthesis of vascular basement membrane components type IV and XVIII collagen and laminin alpha-5 (changes seen both on the mRNA and protein levels); this finding suggests that TEM8 negatively regulates production of fibrillar matrix, but positively regulates formation of vascular basement membrane. A subsequent report clearly showed that, whereas some of the increased type I collagen and fibronectin synthesis resulted from the increased VEGF-A signaling[102-103] observed upon TEM8 knockdown, overexpression of the predominant TEM8 isoform in mutant fibroblasts was sufficient to reduce type I collagen and fibronectin transcripts, without any effect on VEGF-A expression[104]; the observed VEGF-A-independent regulation of matrix synthesis may occur through interaction between TEM8 and connective tissue growth factor (CTGF).

This is the first observation that an anthrax toxin receptor can regulate synthesis of matrix proteins, and could be a critical point in understanding both GAPO and HFS. A role of CMG2 in regulating matrix synthesis has not been demonstrated, but would be supported by observations that certain CMG2 isoforms localize to the ER and may participate in collagen folding and assembly[1] (as discussed above), and that CMG2 colocalizes *in vivo* with type IV collagen[11]. It was also observed that TEM8 knockout prevents proper ECM degradation, through a loss of MMP2 activity[101] resulting from impaired endothelial-fibroblast paracrine communication. Intriguingly, this finding appears to parallel the CMG2-dependent activity of MMP2, as observed by Reeves et al.[83]. Finally, it will be interesting to investigate any cross-talk occurring between CMG2/TEM8-depedent activation of Wnt signaling and the pathways identified by Besschetnova et al.[101, 104]; for example, activation of Wnt signaling is required for TGF-ß-mediated fibrosis[105], and TGF-ß is upregulated upon TEM8 loss.

As described above, there is sufficient evidence to comfortably suspect an involvement of CMG2 in various gene regulatory pathways, including the Wnt/ß-catenin pathway and others identified in TEM8-null mice. Addressing this question promises to yield exciting results and increased understanding of the physiological roles of CMG2.

1.7. Final comments

Compared to its role in disease, much less is known of the originally identified function of CMG2 as a player in angiogenesis. The function of CMG2 and interacting cellular machinery in mediating anthrax intoxication is thoroughly characterized. From HFS (and AS to a lesser degree), it is known that CMG2 is essential from proper regulation of ECM; apparently the same is true of TEM8, as learned from GAPO syndrome. Several studies have implicated CMG2 as an important regulator of pathological angiogenesis. But, in the 16 years of study, little has been identified of the molecular mechanisms whereby CMG2 regulates these important physiological processes (with the notable exception of anthrax intoxication). To improve understanding of the biological roles of CMG2, we proposed herein novel functional models for CMG2, as inspired by evidence of the situational roles of CMG2, and by analogy to the more thoroughly studied TEM8. These models are not proposed as an attempt to establish scientific dogma; but rather, it is our goal that they will serve as a catalyst for designing and conducting hypothesis-driven work that will accelerate our understanding of CMG2 biology, and improve capabilities to therapeutically target this receptor in cancer, eye disease, anthrax, hyalinosis, and other diseases where it may be involved.

2. Capillary Morphogenesis Gene 2 mediates anti-angiogenic function and endocytic uptake of type IV collagen NC1 domain peptide fragments

2.1. Abstract

Capillary Morphogenesis Gene 2 (CMG2) is a type I transmembrane, integrin-like receptor. In addition to its function as an anthrax toxin receptor (where its role is well characterized), reports have repeatedly demonstrated that CMG2 plays a role in the regulation of angiogenesis. But the mechanism by which CMG2 regulates angiogenesis remains elusive. Previous studies demonstrate that CMG2 interacts with type IV collagen (Col IV), a key component of vascular basement membrane; but the role of this interaction in vessel formation has not been investigated. To study this interaction, we designed a peptide array representing the Col IV α1 and α2 chains. From this array, we report here the novel observation that CMG2 is a receptor for peptide fragments of the Col IV non-collagenous domain-1 (NC1). These C-terminal NC1 domains (arresten for Col IVα1 and canstatin for Col IVα2) are well characterized as endogenous anti-angiogenic molecules, but their mechanism of action is not fully defined. This observation was validated by a second peptide array that was used to map a binding epitope. We selected top hits from the initial array, and subsequently identified a canstatin-derived peptide that binds to the CMG2 ligand-binding (vWA) domain with high affinity (peptide S16, K_D = 440 ± 160 nM), and found this interaction to be

competitive with anthrax toxin PA. CMG2 was validated as the relevant endothelial receptor for S16. We demonstrate that CMG2 mediates endocytic uptake of S16. Peptide S16 inhibits endothelial migration in two orthogonal assays, whereas S16 had no effect on endothelial cell proliferation. This report represents the first identification of CMG2 as a functional receptor for Col IV NC1 domains and provides important mechanistic insight regarding angiogenic regulation by CMG2. Additionally, considering the essential role of CMG2 in extracellular matrix (ECM) homeostasis, the observation that CMG2 mediates endocytic uptake of an ECM-derived peptide suggests a mechanism by which CMG2 can control ECM degradation and cellular clearance.

2.2. Introduction

CMG2 (also known as anthrax toxin receptor 2, ANTXR2) is an integrin-like single-pass transmembrane receptor that was originally identified because it is upregulated in endothelial cells forming capillary-like tubes *in vitro*[1]. Like integrins, CMG2 contains an extracellular von Willebrand factor type A (vWA) domain that chelates a divalent metal ion in a metal ion dependent adhesion site (MIDAS) and binds various extracellular matrix proteins. The initial report qualitatively showed CMG2 binding to type IV collagen, fibronectin, and laminin[1]; however, affinity, specific binding sites, and cellular relevance of these extracellular interactions have not been extensively examined.

CMG2, along with its close homolog TEM8, is best known for its role as an anthrax toxin receptor. These two cellular receptors bind the anthrax toxin subunit protective antigen (PA, itself non-toxic), and mediate entry of toxin enzymatic subunits, lethal factor and edema factor, into the cell[2, 19]. Engineered mice lacking full-length CMG2 or TEM8 challenged with *B. anthracis* spores indicate that CMG2 is the major receptor of anthrax toxin[40]; this observation is corroborated by

affinity studies, which demonstrate that CMG2 has higher affinity for PA than does TEM8[17, 106]. Fortuitously, interaction of CMG2 with PA has been used to confirm the role of CMG2 in angiogenesis. PA inhibits angiogenesis and reduces tumor volume *in vivo* and blocks endothelial cell migration *ex vivo*[12]. Further, knockdown of CMG2 in endothelial cells inhibits angiogenic phenotypes, including proliferation and tubule formation[11]. Several additional studies have demonstrated the relevance of CMG2-targeting to the inhibition of angiogenesis[13, 15-16, 68].

Despite its role in angiogenesis, little is known of the physiologic function of CMG2. Intriguingly, loss-of-function mutations in CMG2 cause a severe disorder known as hyaline fibromatosis syndrome (HFS; a disease spectrum that includes infantile systemic hyalinosis-ISH- and juvenile hyaline fibromatosis-JHF)[7, 18, 21-22], characterized by aberrant accumulation of hyaline material under skin and in other organs. These disorders generally lead to death in infancy (ISH) or during early adulthood (JHF). From HFS, it is clear that CMG2 plays an essential role in maintenance of ECM, possibly through endocytosis and clearance of matrix degradation products[77]. There has previously been no functional connection between the role of CMG2 in ECM homeostasis and the role of CMG2 in regulating angiogenesis. However, vascular basement membrane (VBM, composed predominantly of type IV collagen and laminins, as well as other glyco-components) remodeling is an essential step in angiogenesis[85]. It may be that CMG2 regulation of ECM/VBM degradation is intimately connected to angiogenic regulation by CMG2; indeed, here we present the first evidence that such is the case.

VBM remodeling generates fragments of matrix proteins, many of which exhibit angiogenic activity[85]. One class of VBM fragments, namely the type IV collagen (Col IV) C-terminal non-collagenous (NC1) domains, has been well characterized as potently anti-angiogenic[86, 107-108]. They appear to act as a form of negative feedback for angiogenesis: as

angiogenesis proceeds, the VBM is remodeled to allow vessel outgrowth and formation; but this remodeling process generates Col IV NC1 fragments that act through cell surface receptors to suppress further angiogenesis. In addition to the anti-angiogenic function of these NC1 domains, they are essential for proper Col IV assembly (where NC1 trimer formation drives triple helix formation) and extracellular network formation (where adjacent NC1 trimers can associate to form a hexamer)[109]. While there are 6 distinct Col IV chains that can trimerize in 3 different combinations, the most abundant Col IV isoform (and the isoform found within the VBM) is composed of two α1 chains and an α2 chain. The NC1 domains of the α1 and α2 chains are arresten and canstatin, respectively; both potently inhibit angiogenesis[107-108].

Since their initial discovery, there have been strides to elucidate the mechanism by which canstatin and arresten inhibit angiogenesis, including identification of specific integrin receptors and downstream signaling pathways[110-112]. But still, questions remain regarding the end fate of these NC1 domains, including potential receptor-mediated endocytic and degradation pathways. In addition to biological questions, significant issues remain regarding NC1 pharmacological utility. Initial excitement of their promise as therapeutic candidates has been diminished by several key issues, including difficulty of expression, low stability and propensity for aggregation[113].

Here, we report characterization of a novel interaction between CMG2 and fragments of canstatin and arresten. This unexpected finding was initially demonstrated via overlapping-library peptide arrays. Using a high-resolution overlapping peptide array, we report a binding epitope of CMG2 to Col IV NC1 domains. Top array hits were then synthesized for further binding analysis and characterization of anti-angiogenic effect. Of the fragments analyzed, only a canstatin-derived 15-mer peptide (denoted as S16) exhibited high affinity for CMG2 and potent anti-angiogenic activity, suggesting that this small peptide can mimic the anti-angiogenic behavior of full-length

NC1 domains. In addition, we find that CMG2 mediates endocytosis and lysosomal delivery of this peptide fragment. Together, these findings demonstrate that CMG2 is an important component in mediating the anti-angiogenic function of Col IV NC1 fragments. Additionally, CMG2-mediated endocytosis and degradation of ECM and BM fragments has been long suspected; but, to the authors' knowledge, this report represents the first demonstration of CMG2-mediated uptake of an endogenous matrix fragment. And uptake and degradation of anti-angiogenic matrix fragments provides a potential functional explanation for the pro-angiogenic behavior of CMG2.

2.3. Materials and Methods

2.3.1. Proteins, antibodies, and other reagents

CMG2-avi and PA were expressed and purified as previously described [34]. To a pGEX 4T1 (Amersham Biosciences) derived plasmid encoding CMG2 vWA residues 40-217, with R40C and C175A mutations, an avitag sequence was added (GLNDIFEAQKIEWHE) for *in vivo* biotinylation. This construct was then transformed into BL21 T7 Express *E. coli* (New England Biolabs) and CMG2 was expressed as a GST-fusion protein via fermentation in a 5L bioreactor. Upon induction with 0.5mM IPTG, D-biotin (Amresco) was added to 50 uM to facilitate biotinylation. We found that BirA overexpression was not necessary to achieve near stoichiometric biotinylation. Cells were lysed via French press and sonication, and CMG2-avi was purified with Glutathione Superflow Agarose (Pierce). Purity was confirmed as >85% by SDS-PAGE and colloidal Coomassie staining [114]. Glutathione was removed and CMG2-avi exchanged into HBS-T with 50% glycerol using Sephadex G50 (Amersham Biosciences) on an Äkta Start chromatography system. Purified protein was stored at -80 °C. CMG2-GST (without avitag) was expressed and purified in a similar manner.

Recombinant PA was expressed from pET-22b (Novagen) into the periplasm and purified from the periplasmic lysate via anion exchange chromatography (Q-sepharose, Amersham Biosciences), using 20 mM Tris-HCl pH 8.0 with 20 mM NaCl (Buffer A) and Buffer A + 1 M NaCl. >85% purity was confirmed by SDS-PAGE; PA was stored in TBS with 50% glycerol at -80°C. Endotoxin was removed from PA samples prior to cell-based assays by passing twice through poly-lysine coated cellulose beads (ThermoFisher), per manufacturer protocols. PA was verified as endotoxin free by the limulus amebocyte lysate coagulation test, per manufacturer protocols. Two constructs were expressed: PA-SSSR (contains mutations within the furin cleavage sequence that prevents processing and cellular uptake; remains bound to the cell surface) and PA-E733C (for fluorescent conjugation), as PA lacks endogenous cysteine residues.

CMG2-GST (AlexaFluor™ 488, DyLight™ 800) and PA-E733C (Alexa Fluor™ 546) were conjugated via maleimide chemistry. Maleimide-dyes were purchased from ThermoFisher. Protein was reduced by reacting with 5 mM TCEP (GoldBio) at room temperature for 30 minutes. Maleimide-dyes were then added (dye:protein ratio, 5:1), and incubated for 2 hours at room temperature, or overnight at 4C. Conjugated protein was then purified from free dye by size exclusion (Sephadex G50). Unfortunately, we found that the presence of TCEP in the labeling reaction limited the degree of labeling to about 50%. Nonetheless, this labeling was sufficient for the assays described herein. Conjugated proteins were stored in HBST, 50% glycerol at -80C.

Peptide S16 was synthesized by and purchased from GenScript and Biomatik. Peptide EPG was synthesized by GenScript. S16 conjugated at the N-terminus to HiLite™-488 was synthesized by and purchased from Anaspec. Transferrin-AF633 was purchased from ThermoFisher. Dextran-CascadeBlue (10,000 MW) was purchased from ThermoFisher.

2.3.2. Membrane-based peptide array

An overlapping peptide array (15 residue peptides, with 5 overlapping residues) composed of the Col IV α1 (P02462) and α2 (P08572), fibronectin (P02751), and anthrax toxin PA-63 (P13423) sequences was created by direct synthesis of peptides onto a nitrocellulose membrane. The membrane was first blocked in TBS-T with 1% milk. It was then probed by incubation with 250 nM CMG2-biotin (without GST) in blocking buffer with Mg^{2+} and Ca^{2+}. Bound CMG2-biotin was detected by incubation with avidin-biotin-HRP and subsequently developed by ECL on film. Spot intensity was qualitatively and blindly scored by visual image inspection.

2.3.3. PEPperPRINT overlapping peptide micro-array and epitope analysis

A peptide array containing the Col IV α1 and α2 NC1 domains as 15-mer peptides, with a 13-residue overlap, was printed by PEPperPRINT. Incubation of this array was performed according to manufacturer protocol. Assay buffer was 50 mM HEPES, 150 mM NaCl, pH 7.2, 0.05% Tween-20, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. All incubations were performed on an orbital shaker at 140 RPM. Briefly, the array was blocked by incubation in assay buffer with 1% milk. It was then stained with an anti-HA-680 antibody, as a 1:1000 dilution in assay buffer with 0.1% milk. The array was washed by three 30s incubations in assay buffer, dipped in salt-free Tris buffer, dried in a gentle air stream, and then imaged using a LI-COR Odyssey CLx. This pre-staining enabled visualization of the HA-control border, and demonstrated that the anti-HA antibody did not bind to any of the array peptides. The array was then re-equilibrated in assay buffer, and incubated with 2 uM CMG2-GST-800 in assay buffer with 0.1% milk. The array was washed by three 20s washes in assay buffer, dipped, dried, and imaged as above.

PepSlide® Analyzer (Sicasys) was used to analyze the raw TIF image of the probed array. Spot-by-spot quantification was performed with local background subtraction. This quantification data was used for further analysis.

CMG2-binding epitopes were identified using the quantification data and visual inspection of the array image. To be scored as an epitope, a peptide hit must be flanked by adjacent hits. The strongest intensity hit within an epitope was selected as the best representation of that epitope. False epitopes (identified as irregularly shaped noise or background) were recognized upon visual array inspection, and removed from further analysis. Epitope mapping molecular graphics were then generated using the UCSF Chimera[115-116] package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081).

GibbsCluster-2.0[117-118] was used to perform clustering analysis on hits from the PEPperPRINT array. Briefly, hits with intensity above one-third of maximum were selected. These hits were verified by visual image inspection to remove any "false" hits. True hits from the entire array (65 in total) were submitted for clustering analysis. As no consensus sequence for CMG2 binding has been identified, parameters were systematically varied to evaluate the effect of motif length and insertions and deletions on the quality of the alignment. Seq2Logo[119] was used to generate a visual representation of the clustering analysis.

2.3.4. CMG2-PA FRET assay

Top hits from the above peptide array screen (including S16) were synthesized and validated for CMG2-specific binding using a FRET assay to detect competition with PA, as described previously[68]. Briefly, 10 nM each of CMG2-488 and PA-546 were incubated in the

presence of peptide of varying concentration. With no competing ligand, CMG2 and PA remain bound and energy is transferred from the excited CMG2-488 (donor quenching) to PA-546 (acceptor stimulation). As increasing concentrations of competing ligand are added, the ratio of energy transfer decreases. All samples were allowed to incubate for 4 hours at room temperature to approximate equilibrium. This method provided an IC_{50} for S16 binding to CMG2.

2.3.5. Bio-layer interferometry

An Octet RED96 Bio-layer interferometer (BLI) was used to characterize the interaction of peptide S16 with CMG2. Assay buffer was 50 mM HEPES, 150 mM NaCl, pH 7.2, 0.1% Tween-20, 1 mg/mL BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3. First, streptavidin biosensors (ForteBio) were coated with 5-10 ug/mL CMG2-avi overnight at 4C. 8 sensors were loaded and run in parallel (6 sensors for S16 binding, 2 for reference control). Binding assays were performed the following day using the Octet 8.2 Data Acquisition software. Assays were performed at 30°C and a 1000 rpm plate shake speed. The CMG2-avi loaded sensors were equilibrated in assay buffer (1200 sec) followed by an association step with a serial dilution (1-90 uM) of peptide (300-1200 sec), and a dissociation in assay buffer (600-1800 sec). Binding data for S16 concentrations below 1 uM were not obtainable, due to the small peptide size and the corresponding low signal/noise ratio at sub-micromolar concentrations. Following acquisition, data was processed and analyzed in the Octet Data Analysis 8.2 software. Processing involved subtraction of reference controls, y-axis alignment and inter-step correction. A global fit to a 1:1 binding model was performed on the processed data, to obtain a single K_D (and kinetic parameters) across all concentrations. Data presented here represents the average and standard deviation of calculated K_D from three independent experiments.

2.3.6. Cell lines and culturing technique

To investigate processes relevant to angiogenesis, two endothelial cell lines were used for this report. EOMA (CRL-2586™) are a murine endothelial cell line from hemangioma[120]. EA.hy926 (CRL-2922™) are an immortalized vascular endothelial cell, generated from the fusion of human umbilical vein cells with lung carcinoma cells[121]. Both cell lines are routinely used to study vascular endothelial cell biology. Both were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and sodium pyruvate and 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified environment with 5% CO_2. Cells were subcultured at 80-95% confluency by tryspinization (0.25% trypsin, 1 mM EDTA)

CHO-K1 (generously provided by Dr. Scott Weber, WT CHO express neither CMG2 nor TEM8) and derivative CHO-CMG2 and CHO-TEM8 (generously provided by Dr. Michael Rogers) cell lines were cultured in Minimum Essential Medium (MEM)α, with 5% FBS, 10 mM HEPES and 50 ug/mL gentamycin to prevent microbial contamination. Stably transfected CHO-CMG2 and CHO-TEM8 were kept under selection with 500 ug/mL Hygromycin B. Cells were grown at 37°C in a humidified environment with 5% CO_2. As above, cells were subcultured at 80-95% confluency.

2.3.7. Endothelial cell binding assays by flow cytometry

Cells were seeded into 12- or 24-well plates and assays were conducted at 30-70% confluency. Cells were conditioned in low-serum media (appropriate medium, with 1% FBS, and 200 nM endotoxin-free PA-SSSR for certain conditions) to synchronize receptor recycling. S16-488 (2 uM) and transferrin-633 (10 ug/mL) were added to appropriate wells, with or without 200 nM endotoxin-free PA-SSSR. Cells were incubated at 37°C with staining solution for indicated

times to allow for ligand binding/uptake. For cold treatment assays, well plate with staining solution was left on ice for indicated time. After incubation, cells were washed three times with serum-free media to remove unbound S16-488 and Tf-633. Cells were then trypsinized for 5-10 minutes at 37C, and mixed to form a single-cell suspension. Cells were then spun down for 5 minutes at 300 xg, to remove trypsin and wash into cytometry buffer (PBS, 1% BSA, 5 mM glucose). Cell suspensions were transferred to a 96-well plate, and analysis was performed using a 6-color Attune Acoustic Focusing Cytometer, equipped with 488nm and 633nm lasers. Controls for each experiment included unstained, single stains, DMSO (S16 vehicle), and endotoxin-free PA-SSSR alone. Cytometry data was analyzed in FlowJo®.

2.3.8. Confocal microscopy to track ligand endocytosis

Cell preparation and staining for confocal microscopy was as described above for flow cytometry, except that cells were seeded in appropriate optical plates. Staining with S16-488 (2 uM) and transferrin-633 (10 ug/mL), and dextran-CascadeBlue (250 ug/mL) was allowed to proceed at 37°C for 1-3 hrs. After staining, cells were washed three times with serum-free media. Imaging media (SDM79, 7.5 mM glucose, ProLong™ live cell antifade reagent) was added to dishes, and incubated at 37°C for at least 1 hour. Live-cell images were then acquired on a Leica DMi8 confocal microscope, in resonant scanning mode, using 405nm, 488nm and 633nm lasers. Images were acquired with the Leica Application Suite X (LAS X), deconvoluted in Huygens Essential, and further analyzed in LAS X to examine S16-488 and Tf-633 intracellular colocalization.

2.3.9. Wound-scratch migration assay

EOMA cells were seeded in 96-well plate and cultured in DMEM supplemented with 10% FBS. Cells were incubated at 37ºC in a humidified environment with 5% CO_2 until completely confluent. A 200uL pipette tip was used to draw a vertical line on the confluent cell layer to create a wound area, followed by three washes with PBS to remove dead cells and debris. Cells were then treated under different conditions (i.e. Complete medium, serum free medium, and complete medium with 1uM peptide). Pictures were taken every four hours, and the change in wound area was quantified using ImageJ.

2.3.10. Cell proliferation assay

15,000 EOMA cells were seeded into each well in a 96-well plate and incubated 1 hour to ensure cells attached to the well. Treatment with different peptides at a final concentration 1uM was prepared in DMEM with 10% FBS. After cells were completely seeded, untreated medium was replaced by the treatment media. Negative controls were generated by adding 80% ethanol into the well and incubating at room temperature for 5 minutes. Ethanol was than replaced by normal DMEM with 10% FBS. Cells were then incubated at 37°C with 5% CO_2 for 24 hours, followed by addition of 20uL CellTiter-Blue Reagent (Promega #G8080) into each well and incubation for 4 hours. Fluorescence signal (Ex: 560nm / Em: 59nm) was obtained from a BioTek Synergy H2 plate reader. All readings were normalized to the non-treated, full serum control to obtain percent proliferation. ANOVA analysis was used to determine statistical difference between treatments and control.

2.3.11. CellAsic endothelial migration assay

The gradient migration assay protocol followed the description in the *CellASIC® ONIX M04G-02 Microfluidic Gradient Plate User Guide*. To prime the gradient plate, PBS from well 6 and well 8 (including liquid inside the PTFE ring) was removed. PBS from well 1 and 7 (leave liquid inside the PTFE ring) was also removed. 10 uL of complete medium supplemented with 25mM HEPES was added into the PTFE ring of well 6 then the plate was sealed onto the F84 manifold. The default priming program for M04G plate (0.25psi for 2 minutes on well 6) on the CellASIC ONIX Microfluidic FG software was run to flow solution into the cell culture chamber. Once the plate was primed, it was detached from the manifold and kept at 37°C until cell loading. All media used in the plate (excepting the cell suspension) was filtered through a 0.2 um syringe filter to prevent clogging of system and gradient-perfusion barriers.

A suspension of 3E6 cells/mL was prepared freshly in complete medium supplemented with 25mM HEPES. Solution in the PTFE ring from well 1 and 6 were removed. 50uL of completed medium with HEPES were added into both well 7 and 8 to prevent cell loading prior sealing onto the CellASIC manifold.

Following cell loading and seeding, a stable gradient of 0 – 10% FBS was generated by perfusing DMEM with either 0% or 10% FBS on either side of the culture chamber. Depending on condition, inhibitory compounds were added to both perfusion media in order to have uniform concentration of inhibitor throughout cell chamber. Migration of individual cells was monitored by taking images every 10 minutes, over a 12-hour period.

For data analysis, the manual tracking plugin within Image J was used to track individual cells. Fifty cells were manually tracked and data was transferred into the Chemotaxis and Migration Tools 2.0, available from the ibidi website. The values for endpoint-x displacement (no

chemoattractant gradient), endpoint-y displacement (chemoattractant gradient), accumulated and Euclidean displacement were obtained. The average displacement was calculated for each variable that was measured, along with the standard error of the mean (n=50). ANOVA was used to determine statistical significance between the 10% to 10% and 10% to 0% assays as well as the 20uM S16 treatment, and PA-SSSR treatment.

2.3.12. Statistical analysis and data visualization

Unless otherwise indicated, data was analyzed and visualized using KaleidaGraph 4.1 (Synergy Software). ANOVA was conducted to determine statistical significance.

2.4. Results

2.4.1. Peptide array demonstrates that CMG2 binds preferentially to the Col IV NC1 domains

We sought to investigate the putative interaction of the CMG2 vWA domain with the VBM component type IV collagen, to understand the relevance of this interaction to the role of CMG2 in angiogenesis. First, we designed a peptide array, where peptides were directly synthesized onto a nitrocellulose membrane. These array peptides represented the linear sequences of human Col IV α1 and α2 chains, divided into 15-mer peptides in a 10-residue sliding window. This array was probed using CMG2-biotin and read out with avidin-HRP, and spots were qualitatively scored with an intensity 0(min) to 5(max). Analysis of the binding data demonstrated, clearly though unexpectedly, that CMG2-binding to Col IV occurs predominantly within the anti-angiogenic Col IV NC1 domains (Fig 1A-B). When considering only hits with intensity of 2 or above to avoid weak or non-specific signal, the array shows only 1.4% of synthesized peptides within the

collagenous domain of Col IV α1 bind to CMG2, whereas 50% of peptides within arresten (NC1 domain) bound (Fig 1A). Similarly, we see for Col IV α2 that only 8.9% of collagenous peptides bound, whereas 48% of peptides within the canstatin NC1 domain exhibited affinity for CMG2 (Fig 1B). Noting that, in this array, the entire peptide library was generated in a 10-residue sliding window, the prevalence of CMG2-binding peptides within the NC1 domain strongly suggests that CMG2 may be a novel receptor for the Col IV NC1 domains.

Considering the established role of CMG2 in angiogenesis[1, 11, 16], the observation of CMG2 interacting with peptides derived from the anti-angiogenic NC1 domains was too intriguing to ignore. To validate this finding and further characterize the interaction between CMG2 and arresten/canstatin, we synthesized a higher resolution peptide array, with the NC1 domains arrayed as 15-mer peptides in a 2-residue sliding window. The array was probed using CMG2-GST-800 and anti-HA-680 (for control border peptides), and imaged on a LI-COR Odyssey CLx (Fig 1C). Image data was quantified using PepSlide Analyzer; this image and quantification were used or downstream analyses.

With the high-resolution overlapping library, we sought to map an epitope for CMG2 binding to Col IV NC1 domains. Binding epitopes were identified from quantitation and visual inspection as a string of peptides exhibiting binding to CMG2. For these strings, the highest binding peptide was selected, and the 15-mer sequence of this peptide was called the epitope. False epitopes (noise and array aberrations) were identified by visual array inspection as irregular, and removed from analysis. Using UCSF Chimera[115], we mapped the binding epitopes onto a NC1 hexamer structure (PDB 1LI1) solved by Than et al.[122]. The hexamer structure shown consists of two interacting NC1 heterotrimers, each composed 2 α1 and 1 α2 chain. This is the physiologically relevant NC1 hexamer formation. The generated epitope map (Fig 1D) shows a large contiguous

epitope of CMG2 for canstatin (Fig 1D, left), but a relatively sparse epitope for CMG2 binding to arresten (Fig 1D, right). This epitope model suggests that, in addition to binding synthesized fragments of arresten/canstatin, CMG2 likely interacts with intact Col IV through the NC1 domains, particularly the α2 NC1 domain (canstatin). Identification of this interaction led us to ask what affect CMG2 binding to canstatin-derived peptides might have on endothelial cell behavior, and how that might influence angiogenic phenotypes.

We further used the PEPperPRINT array quantification to identify highest binding peptides, and these, after removal of false hits, were subjected to clustering by GibbsCluster-2.0, with the goal of uncovering a CMG2-binding motif. In contrast to many integrins, where the RGD-tripeptide motif is well established amongst ligands[123], no such motif has yet been reported for CMG2 ligands. Our relatively small peptide sampling (< 70 peptides) surprisingly produced a consistent motif element (Fig 1E). The most noticeable element from these consensus sequence analyses is a hydrophobic/aliphatic residue followed two residues by an acidic enrichment (either aspartate or glutamate). This pattern was observed consistently within simulations of varying motif length and allowed insertions and deletions (Fig 1E). This acidic enrichment is not unexpected for CMG2-ligands, and supports the relevance and accuracy of this peptide array, as most high-affinity CMG2 ligands would be expected to coordinate a divalent cation within the CMG2 MIDAS.

2.4.2. Canstatin-derived peptide S16 binds with high affinity to CMG2, via the MIDAS

Being one of the highest intensity hits from the original peptide array, peptide S16 was synthesized and binding to CMG2 was analyzed by bio-layer interferometry (BLI) using CMG2-avi loaded streptavidin biosensors (Fig 2A). Analysis of data from BLI revealed a K_D of 440 ±

170 nM (mean ± SD, n = 3 independent experiments). This interaction was characterized by a slow on-rate (≈ 2×10^2 $M^{-1}s^{-1}$) and slow off-rate (≈ 1×10^{-4} s^{-1}).

Interestingly, the observed association was inverted (association resulted in negative signal, and dissociation gave a positive signal). While this does not impact the analysis, it is necessary to consider why this inverted binding signal might occur. In BLI, the readout (via white-light interferometry) is directly related to the change in effective size or thickness of the biosensor tip. Generally, as molecules bind to the sensor tip, this increases the size of the sensor, and results in a shifting interference pattern that yields a positive binding signal. However, if a relatively small molecule is binding to a surface, and causes a large conformational change that results in reduced hydrodynamic radius of a larger, loaded ligand, this binding event would be recognized as a negative signal, since upon binding the effective size of the sensor actually decreased. In the case of S16, this inverted signal could result from an "induced-fit" type interaction between CMG2 and S16, where binding induces a significant conformational change in CMG2. While possible, no other evidence exists to support a conformational change within the CMG2 vWA domain upon ligand binding. Alternatively, binding of a large enough particle could shift the interference pattern in the opposite direction as expected, and thus another explanation (and more accessible to evaluate) is that large S16 aggregates are binding to CMG2. Indeed, both dynamic light scattering of S16 and fluorescence anisotropy using S16-488 demonstrated that S16 does form large aggregates at concentrations > 10 uM (data not shown), which is within the range for our BLI assays. Further, when S16 dilutions are prepared under conditions that encourage monomerization, binding is not observed on BLI (data not shown). However, this does not necessarily indicate that CMG2 binds only to S16 aggregates. Our BLI assay may not have sufficient sensitivity to detect the binding of the small S16 monomer (1600

Da). In support of this hypothesis, data below demonstrates that S16-488 prepared to minimize peptide aggregation is capable of binding to the surface of endothelial cells in a CMG2-dependent manner (Fig 3A).

Specificity of the interaction between CMG2 and S16 was further confirmed using a PA/CMG2 competition FRET-based assay[68] (Fig 2B). This assay demonstrates that S16 interacts with CMG2-488 in competition with PA-546, as increasing S16 concentrations reduced the FRET between CMG2-488 and PA-546 (IC_{50} = 1.9 uM, 95% CI: 1.3 – 2.6 uM). This value is in close agreement with the BLI-observed K_D. As one assay is a detection of direct binding, and the other involves competition with a high affinity interaction, we do not expect the values to be in exact agreement. The ability of S16 to bind to CMG2 in competition with PA demonstrates that S16 interacts with the known ligand binding surface of the CMG2 vWA domain. As further support of specificity, EDTA inhibits the interaction of S16 and CMG2, as determined by BLI (Fig 2C), indicating that the interaction requires a Mg^{2+} or Ca^{2+} to be present in the MIDAS of CMG2. Indeed, S16 contains an aspartate (that is preceded by several aliphatic residues) that could participate in metal coordination. In addition to the FRET assay, BLI was used to verify that S16 binds in competition with PA; PA-saturated CMG2-avi loaded biosensors demonstrate significantly reduced binding to S16 (Fig 2D).

2.4.3. CMG2 is the relevant endothelial surface receptor for S16, and mediates endocytosis of S16

Having determined that S16 bound to CMG2 with high specificity and affinity (Fig 2), we then sought to verify that full length CMG2, on the surface of endothelial cells, bound to S16. HUVEC-derived EA.hy926 cells were co-incubated with an S16 conjugate (S16-HiLyte488, at 2

uM) and transferrin-AlexaFluor633 for various durations. Knowing that S16 binds to CMG2 in competition with PA (Fig 1C, E), we postulated that, if cell surface binding to S16 occurred through interaction with CMG2, we should see less S16-488 signal when co-incubated with high concentrations of PA-SSSR (non-endocytosable PA construct). Following incubation, cells were washed and harvested by trypsinization. Without PA-SSSR, EA.hy926 cells showed a time-dependent increase in S16-488 signal, as analyzed by flow cytometry (Fig 3A). When co-incubated with 200 nM PA-SSSR, a positive slop was observed, but there was a drastic and statistically significant decrease compared with cells without PA (60% decrease at 10 hrs., Fig 3A). To verify that the effect of PA-SSSR was a result of specific receptor binding, and not off-target effects, we included Tf-633 in all experiments (positive control for both cell-binding and receptor mediated endocytosis, the relevance of which will be discussed below). In contrast to S16-488, Tf-633 signal did not change when cells were treated with PA-SSSR (Fig 3B), confirming that the decrease in S16-488 binding upon PA-SSSR treatment was the result of a receptor-specific effect, with PA inhibiting the interaction between S16-488 and CMG2. It is necessary to note that, as EA.hy926 cells are expected to express both CMG2 and TEM8, we cannot exclude, with current data, the possibility that cell binding is mediated in part by TEM8, though there is clearly a high-affinity interaction between S16 and CMG2 (Fig 2A).

Knowing that CMG2 is essential for proper regulation and homeostasis of the extracellular matrix, and that loss-of-function mutations in CMG2 lead to excessive accumulation of collagenous hyaline material[5-6, 18], it has been hypothesized that CMG2 mediates endocytic uptake of extracellular matrix fragments, and that these fragments may then be trafficked for lysosomal degradation. This hypothesis is strengthened by the well documented and elucidated role of CMG2 in mediating endocytic uptake of the anthrax toxin[2, 26, 37-38], considering that pathogens most often

are opportunistic organisms which subvert pre-existing biological mechanisms. To the authors' knowledge, there has been no investigation of CMG2-mediated endocytic uptake of ECM or VBM fragments. With this in mind, we sought to identify whether S16 (a fragment of Col IV α2 NC1 domain) was endocytosed through CMG2. This question was further spurred when considering that cellular S16 binding (Fig 3) was investigated immediately following trypsinization for cell harvesting; as trypsinization will cleave off cell surface receptors and any bound ligands, we posited that the observed S16-488 signal was accounted for by internalized S16-488. To validate this, additional assays were conducted to specifically track cellular uptake. First, we compared S16-488 and Tf-633 binding signal to EA.hy926 when treated at 37°C, or on ice for the same time. Cells kept on ice are unable to undergo endocytosis[124]. Following incubation, both treatments were harvested identically via washing and trypsinization. And, indeed, we see that cold treatment nearly completely abolishes the observed signal for both S16-488 (Fig 4A) and Tf-633 (Fig 4B), indicating that the previously observed signal arises from internalized S16-488 and Tf-633.

Internalization of S16-488 could occur through receptor-mediated endocytosis following CMG2 binding, or by non-specific fluid-phase pinocytosis. While we considered the latter to be unlikely, considering the relatively low concentrations of S16-488 used (2 uM, 4 ug/mL), we turned to confocal microscopy to further interrogate the mechanism of S16 internalization. As transferrin is internalized via transferrin-receptor-mediated endocytosis, co-localization of S16-488 with Tf-633 demonstrates that both are within endosomes (Fig 4C). In addition to the punctate endosomal colocalization (white arrows), strong colocalization is seen within apparent perinuclear lysosomes. The figure shown is representative of n = 20 cells imaged from two independent experiments. Compared to S16-488 and Tf-633, significantly less uptake was observed after incubation with a 10,000 MW dextran-CascadeBlue (ThermoFisher), indicating that the process

of pinocytosis is slow within EA.hy926 cells (data not shown). Considering the weak signal from 250 ug/mL dextran-CascadeBlue, which will only be taken in by fluid-phase uptake, the strong signal observed for S16-488 at 4 ug/mL rules out the possibility of fluid-phase S16 uptake.

Together, flow cytometry and confocal microscopy clearly demonstrate that CMG2 is the relevant endothelial cell receptor for S16-488, and that CMG2 in fact mediates endocytic uptake and apparent lysosomal delivery of this canstatin-derived peptide.

2.4.4. Peptide S16 inhibits endothelial cell migration, but not proliferation

Chemotactic endothelial cell migration is an essential step in the process of angiogenesis that requires coordination of growth-factor mediated signaling pathways with functional connections between the actin cytoskeleton and underlying basement membrane and ECM[94]. Previous work clearly shows that binding of certain angioinhibitors to CMG2—including PA and other small molecules—inhibits endothelial cell migration, but not proliferation in microvascular endothelial cells[12, 15]. We sought to determine whether peptide S16 was functionally active, specifically, whether it affected angiogenic phenotypes in cell culture, including migration and proliferation. S16 treatment results in drastic inhibition of EOMA cell migration, as seen by two orthogonal assays. In the traditional wound scratch assay, S16 exhibited strong inhibition of endothelial cell migration over the untreated, full-serum control (Fig 3A). Impressively, there was no statistical difference in migration between the S16-treated and serum-free control, indicating that S16 completely blocked endothelial migration. Of note, fibronectin-derived peptide EPG (identified from original peptide array, binds to CMG2, $K_D \approx 2$ uM, data not shown) did not inhibit migration in the wound scratch assay (Fig 5A), suggesting that the two may interact with CMG2 by different modes. Indeed, EPG-CMG2 binding was also found to be insensitive to EDTA (data

not shown), suggesting that the interaction occurs away from the MIDAS, in contrast to S16-CMG2 binding. Neither S16 nor EPG, showed inhibition of endothelial cell proliferation (Fig 5B).

The wound scratch assay is standard in assessing cell migration, but is limited in relevance, owing to several issues, including reproducibility and the lack of a chemoattractant gradient. To further assess the effect of S16 on endothelial chemotactic migration, an orthogonal migration assay was used. Microfluidic migration platforms provide greater control, and allow for more insight into cellular processes, by tracking more parameters of cell motility. The CellAsic Onix microfluidic migration platform allows migration to be assayed within a stable, unidimensional and fairly linear chemoattractant gradient. In this assay, S16 significantly inhibits migration of endothelial cells towards a serum gradient (Fig 5C). Surprisingly, and in keeping with results from the wound scratch assay, the observed inhibition by S16 is nearly complete, and is as strong as migratory inhibition by PA-SSSR (Fig 5D). Together, both migration assays demonstrate that S16 potently inhibits anti-angiogenic phenotypes.

2.5. Discussion

CMG2 has recently been shown to be an important regulator of angiogenesis[11-12, 16], but there has been little mechanistic insight to explain how this regulation occurs. Here, we made the novel identification of an interaction between the extracellular ligand-binding CMG2 vWA domain and Col IV NC1 domains (Fig 1). As these NC1 domains are themselves potent anti-angiogenic molecules, binding to CMG2—an emerging regulator of angiogenesis—is particularly interesting, and likely relevant to the role of each in angiogenesis. In order to better understand the functional connection of CMG2-NC1 interactions and their relevance to the regulation of angiogenesis, we conducted further experiments using a canstatin-derived 15-

residue peptide, denoted S16. This peptide binds with high affinity to CMG2 vWA domain, at the MIDAS in a manner competitive with anthrax toxin PA. CMG2 (and possibly TEM8) is indeed the relevant cell surface receptor for S16 binding to endothelial cells, as shown by PA-SSSR competition flow cytometry assays, where a 60% inhibition in S16-488 fluorescence signal was observed upon co-incubation with PA-SSSR (Fig 3A). While this decrease is notable, one might expect that co-incubation with a competitive ligand such as PA-SSSR, which binds to CMG2 with high affinity ($K_D \approx 170$ pM) should result in complete loss of S16 binding. There could be several reasons for the deviation of our results from the expected outcome. First, S16 may be taken up slowly by EA.hy926 cells via pinocytosis; though, evidence from confocal microscopy suggests that any S16-488 uptake by pinocytosis would be slow, and should be a minimal contribution to the total signal observed. Second, while PA does interact with CMG2 with much higher affinity than does S16, the assay concentration of S16 (2 uM) were 10-fold higher than those of PA-SSSR (200 nM). The higher S16 concentration could result in increased binding of S16. Finally, the incomplete reduction by PA-SSSR treatment may indicate that S16 binds also to other cell surface receptors. Regardless, it is clear that much of the interaction of S16 with endothelial cells is mediated by CMG2.

In addition to binding S16, CMG2 mediates the endocytic uptake of this Col IV-derived peptide fragment. This is the first observation of CMG2-mediated uptake of an endogenously relevant matrix fragment; but it does echo evidence from HFS that CMG2 is essential for ECM homeostasis and repair[5-6, 18], and established knowledge of the role of CMG2 in mediating clathrin-dependent endocytosis of the anthrax toxin[2, 38] A role for CMG2 in mediating uptake and cellular degradation of ECM/VBM fragments[77] could explain the excessive hyaline build-up arising from CMG2 loss-of-function mutations in HFS. This finding is, however, preliminary.

More work must be done to understand the scope of this role for CMG2, and whether this receptor is capable of mediating endocytic uptake of any matrix fragment to which it binds, or if there is some additional specificity. Further, assays should be designed that examine the ability of CMG2 to mediate uptake of proteolytically generated matrix fragments, rather than synthesized peptides. It also remains to be seen whether endocytic uptake of matrix fragments requires similar steps as endocytosis of anthrax toxin, namely: ligand binding and receptor clustering, phosphorylation of intracellular tyrosine residues on CMG2 by *src*-like kinases, binding to ß-arrestin, ubiquitination by Cbl, and subsequent recruitment of AP-1, clathrin and the actin cytoskeleton[18, 25-26, 37-38].

As S16 is an apparently angiogenically active peptide (derived from the anti-angiogenic canstatin), endocytosis and clearance by CMG2 suggests a partial mechanism whereby CMG2 may exert its pro-angiogenic phenotype. For example, if CMG2 binds to, endocytoses, and traffics to lysosome anti-angiogenic peptides from the extracellular space, these will no longer be able to exert their anti-angiogenic effect through interactions with integrins and other functional receptors. Thus, one role of CMG2 may be to remove anti-angiogenic molecules from the extracellular space, and removal of a negative stimulus results in a positive response (pro-angiogenic). With that said, CMG2 also likely regulates angiogenesis through controlling endothelial cell migration, as discussed below.

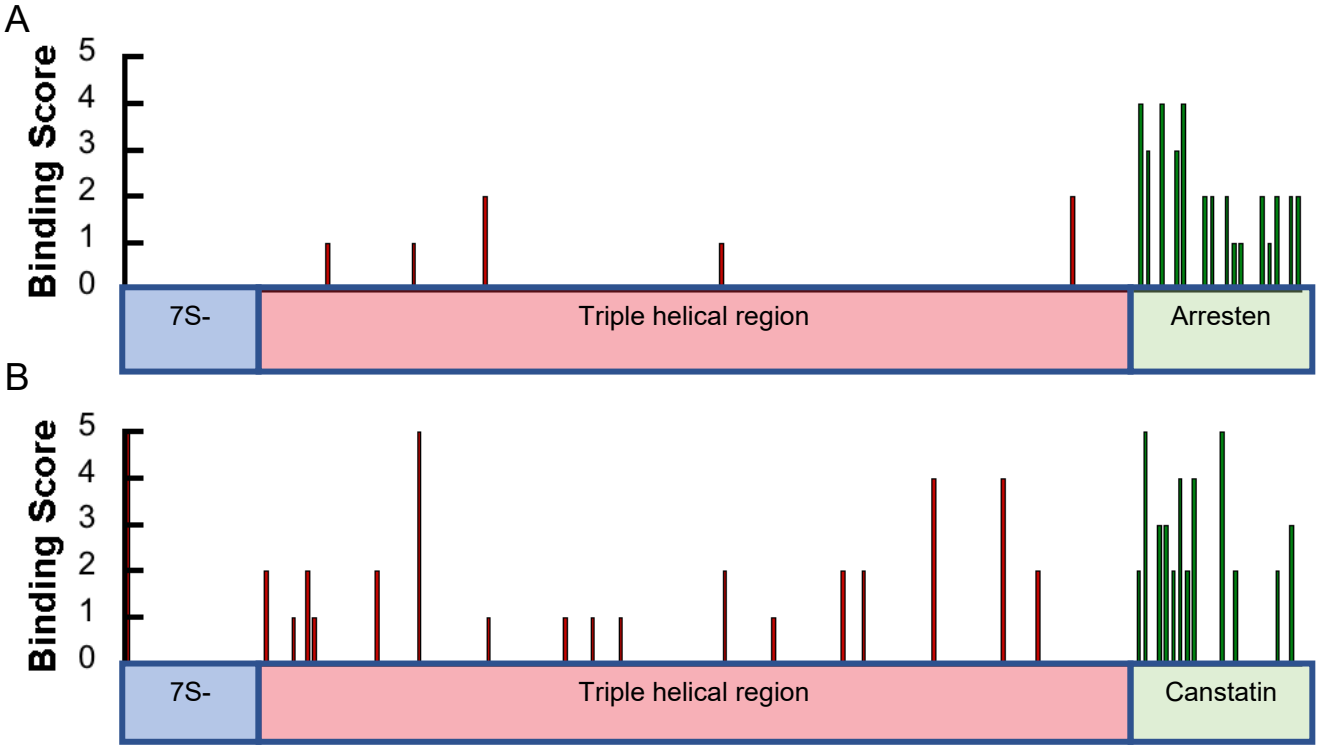
Peptide S16 potently inhibits vascular endothelial cell migration, but does not affect cell proliferation. This finding agrees with previous work showing that various exogenous ligands of the CMG2 vWA domain inhibit endothelial cell migration, without affecting proliferation[12-13, 15]. However, these findings stand in apparent conflict with work by Reeves et al., where shRNA-mediated knockdown of CMG2 resulted in a decrease in HUVEC proliferation, with no

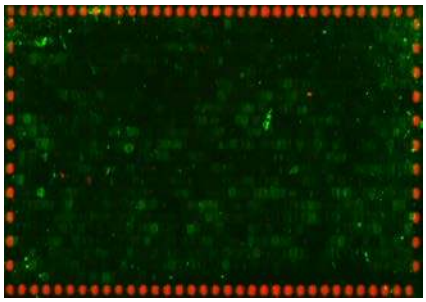
discernible effect on migration[11]. But, what appears to be conflict may be representations of the different functional roles of CMG2 in vascular biology. It is plausible to imagine that CMG2-dependent regulation of endothelial cell proliferation requires the involvement of CMG2 in various signaling pathways and that this requires the presence and post-translational modification of the CMG2 cytosolic tail. As knockdown will reduce total CMG2 expression, it may infringe on this proliferative ability. Interaction with exogenous ligands, such as PA and S16, is not expected to affect CMG2 expression, and thus may not be expected to inhibit endothelial proliferation. These ligands do, however, competitively inhibit binding of the extracellular CMG2 vWA domain to endogenous ligands (components of the ECM/VBM). This competitive inhibition of ECM/VBM binding could lead to decoupling of a CMG2-mediated ECM-cytoskeleton linkage. Such linkages are an essential element of vascular endothelial cell migration[94], and decoupling of these linkages inhibits chemotactic migration. While interactions between CMG2 and the actin cytoskeleton have not yet been thoroughly investigated, there are several reasons they are likely to exist. CMG2 contains a putative actin-interacting peptide within its cytosolic tail[1]. Indeed, anthrax intoxication was shown to be dependent on the actin cytoskeleton[38]. Further, TEM8 (the closest structural and functional homolog of CMG2) interacts both directly and indirectly with the actin cytoskeleton[89, 92], and these interactions lead to an ECM-actin coupling that enables endothelial cell adhesion and migration[88]. This rationale will support further work to understand the role that CMG2 plays in coupling the ECM/VBM with the actin cytoskeleton, and the influence of this coupling on endothelial cell migration.

Inspired by the findings discussed above, novel anti-angiogenic strategies have emerged that target CMG2[13, 15-16, 68]. One such strategy that we have developed and pursued in this report involves the identification of CMG2 ligand-derived small peptides that are capable of binding

the receptor with high affinity and exerting anti-angiogenic behavior. In addition to the information regarding basic CMG2 biology, this report also demonstrates the feasibility of this small-peptide approach to developing CMG2-targeted anti-angiogenic therapeutics. Future work should strive to identify and affinity-mature tighter binding peptides, that can be investigated as they were here, and subsequently evaluated for *in vivo* efficacy in eye disease and tumor angiogenesis models. Additionally, as peptide S16 was derived from canstatin, this reductionist approach of finding a bio-active, minimally-sized peptide may yield potent anti-angiogenic peptides with pharmacological advantages over full length collagen NC1 domains.

2.6. Figures





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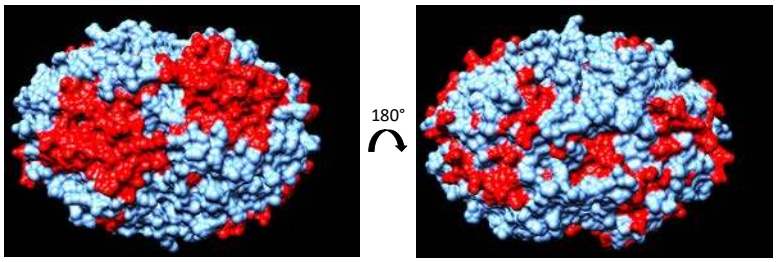

D

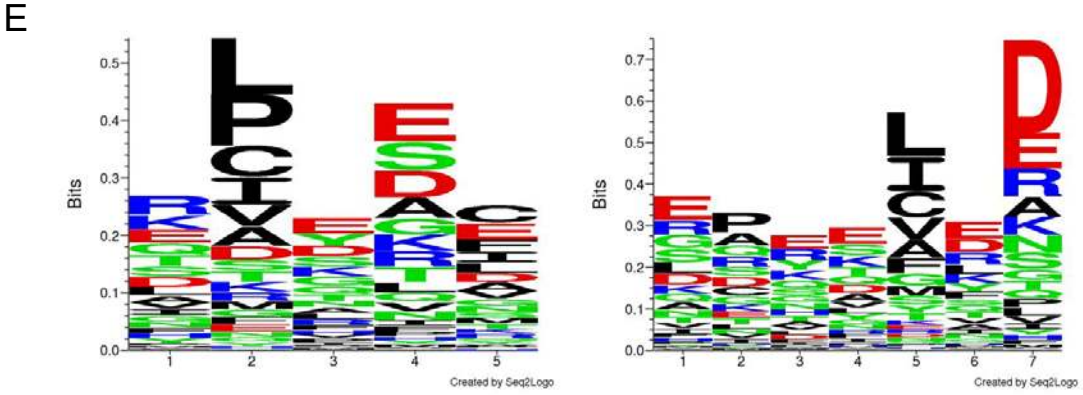

Figure 2-1: CMG2-binding peptides are enriched within the anti-angiogenic Col4 NC1 domains

A peptide array was constructed by synthesizing 15-mer peptides with a 10-residue sliding window, covering the sequences of Col IV-A1 (A) and Col IV-A2 (B). This array was probed with 250 nM CMG2-biotin and read out using avidin-HRP. After imaging the membrane, the spots were scored from 0 (no observed binding) to 5 (maximal binding). Domain topology is shown below. There is a greater proportion of CMG2-binding peptides within the c-terminal NC1 domains than the collagenous and 7S domains. (C-E) PEPperPRINT peptide array validates and clarifies the interaction of CMG2 with NC1-domain derived peptides, and identifies a putative CMG2-binding motif. (C) Image of array, showing binding of anti-HA-680 (red) to HA border control peptides, and binding of CMG2-800 (green) to peptides within the array. Array, as seen, is composed of Col IV α1 and α2 chains, laminin-111 and PA-63. (D) Molecular representation of CMG2-Col IVNC1 epitope. Binding data, quantified by PepSlide Analyzer was used to identify CMG2 binding epitopes within Col IV NC1 domains. False epitopes, as identified by visual array inspection, were removed from analysis. Graphics generated in UCSF Chimera. Protein in blue, epitope sequences are shown in red. Left shows canstatin-exposed face of NC1 hexamer (PDB 1LI1), demonstrating a large, uniform epitope for CMG2 to canstatin. Right displays face with arresten exposed. Epitopes are present, but are more sparse. These data may suggest that the predominant Col IV NC1 ligand for CMG2 is cansatin. (E) Identification of a potential CMG2-binding motif. Top hits (65 peptides, after removing false hits) from entire array were pooled and subjected to clustering analysis with GibbsCluster-2.0. Left, 5-residue motif, with no insertions or deletions allowed. Right, 7-residue motif with up to 3 insertions and deletions allowed. Consistent motif consists of an enriched acidic position, preceded two positions by a hydrophobic/aliphatic residue.

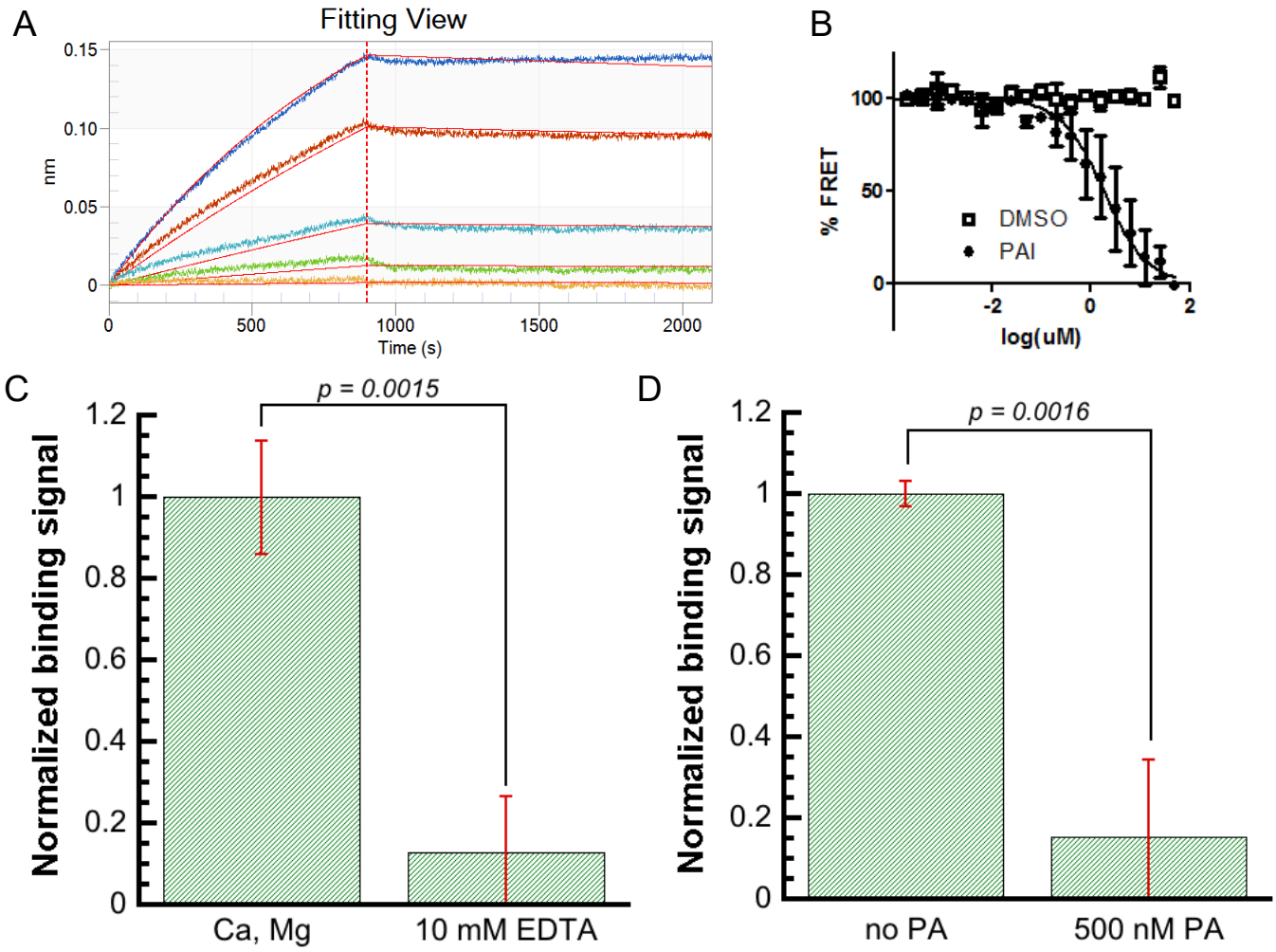

Figure 2-2: In vitro characterization of CMG2 interaction with peptide S16

(A) Representative trace showing association and subsequent dissociation of S16 binding to CMG2-GST-biotin-coated streptavidin biosensors. K_D = 440 ± 170 nM (average of 3 independent experiments). (B) FRET inhibition assay confirms the specificity of S16 (PAI) binding to CMG2. PAI disrupts the interaction of CMG2-488 and anthrax toxin PA-546, as demonstrated by FRET, with an IC50 of 1.9 uM. (C-D) Peptide S16 interacts with the MIDAS domain of CMG2. (C) Mg and/or Ca are required for CMG2 binding to S16. CMG2-GST-biotin coated streptavidin sensors were equilibrated either in buffer with 2 mM $CaCl_2$ and 1 mM $MgCl_2$, or 10 mM EDTA, and then interaction with S16 was probed by BLI (n=3). (D) CMG2-GST-biotin coated streptavidin biosensors were allowed to equilibrate with 500 nM PA until saturation, and then binding to S16 was probed by BLI. In agreement with the FRET assay, S16 and PA bind competitively to CMG2, further confirming S16 binding to the MIDAS domain.

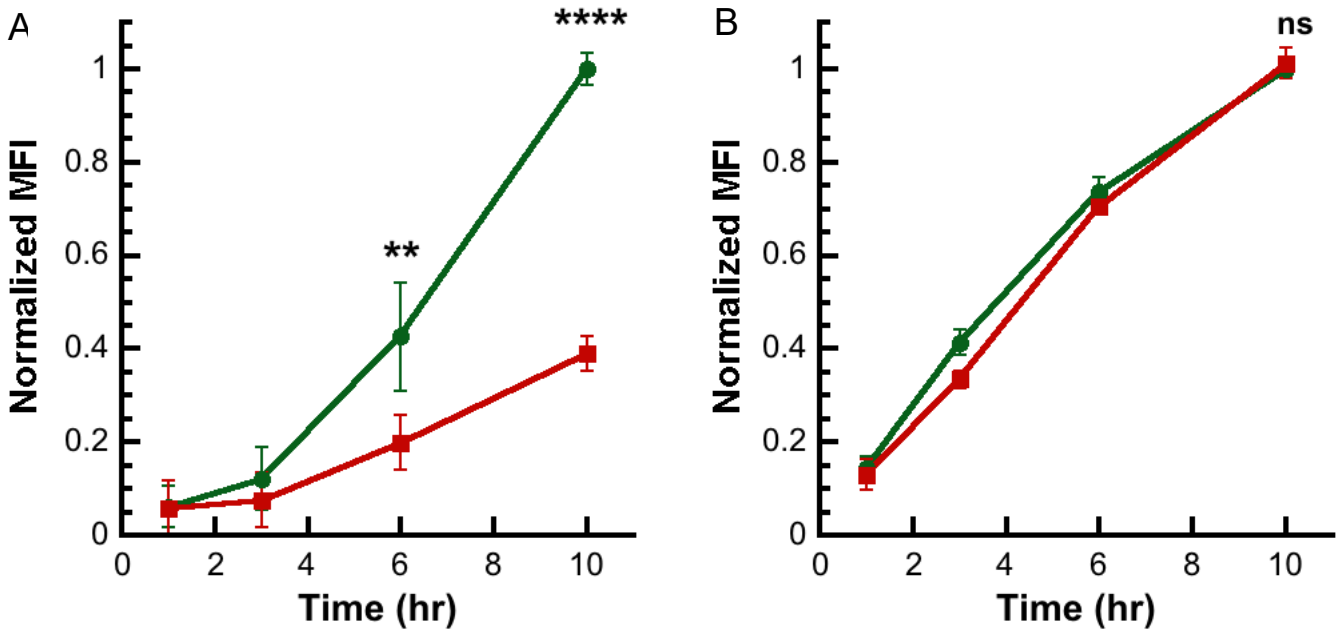

Figure 2-3: CMG2 is the relevant cell surface receptor for S16

(A-B) Conditioned eaHY926 cells were treated with both S16-488 (A) and transferrin-633 (B), with 200 nM PA-SSSR (red squares) or without (green circles), for 1, 3, 6, or 10 hrs. After incubation, the cells were washed in serum-free DMEM and harvested by trypsinization for 5 minutes at 37C. Cells were rinsed into flow buffer (PBS, 5 mM glucose, 1% BSA), and run on an Attune Acoustic Focusing Cytometer. Treatment with PA-SSSR specifically reduces binding of S16-488 to endothelial cells, but does not reduce the binding of transferrin-633, demonstrating that at least 60% of S16 cell binding occurs through the anthrax toxin receptors. MFI, mean fluorescence intensity. N=6 from two independent experiments. **, $p < 0.01$; ****, $p < 0.0001$; ns, not significant.

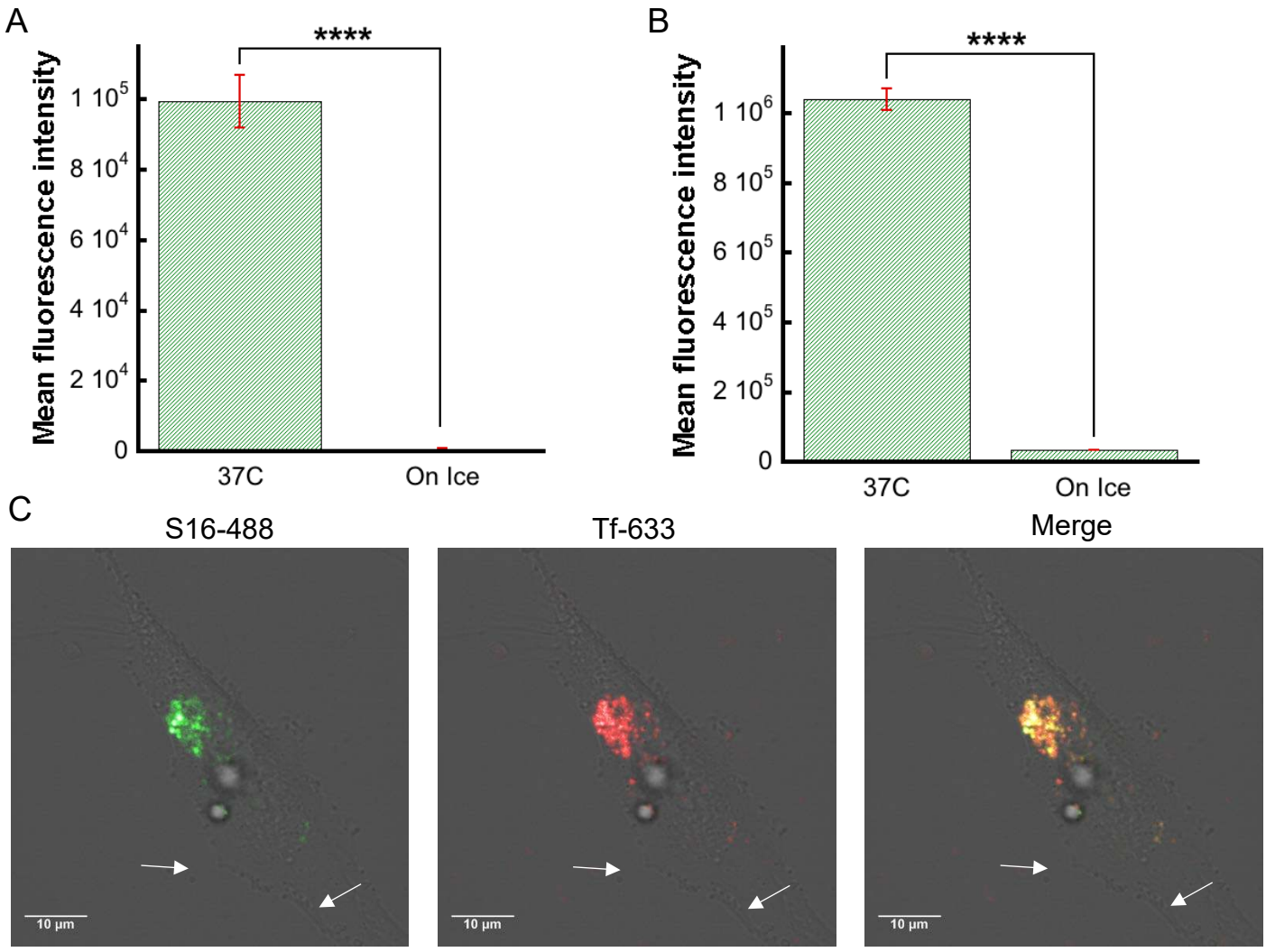

Figure 2-4: CMG2 mediates endocytosis of S16

(A-B) Cold treatment abolishes the signal of both S16-488 (A) and transferrin-633 (B), indicating that, as transferrin, S16 is being endocytosed. Conditioned eaHY926 cells were incubated with 2 uM S16-488 and 10 ug/mL transferrin-633 in DMEM (with 20 mM HEPES and 1% FBS) for 6 hours, either at 37C or on ice. After incubation, cells were washed with serum-free DMEM and harvested by trypsinization. Cells were washed into flow buffer, and analyzed using an Attune Acoustic Focusing Cytometer. Plot shows mean fluorescence intensity of three replicates. ****, $p < 0.0001$. (C) To confirm endocytic uptake of S16-488, we incubated conditioned eaHY926 with 2 uM S16-488 and 10 ug/mL Tf-633 at 37C for 3 hours at 37C, then washed with serum-free DMEM and incubated in imaging buffer (SDM79 with 7.5 mM glucose and ProLong™ antifade) for 1 hour at 37C. Cells were then imaged within an incubation chamber on a Leica DMi8. Representative image is shown. We observed consistent colocalization between S16 and Tf in endosomes (white arrows) and in apparent perinuclear lysosomes. These findings were consistent across n=20 cells from 2 independent experiments; in every cell, we observed endosomal and lysosomal colocalization.

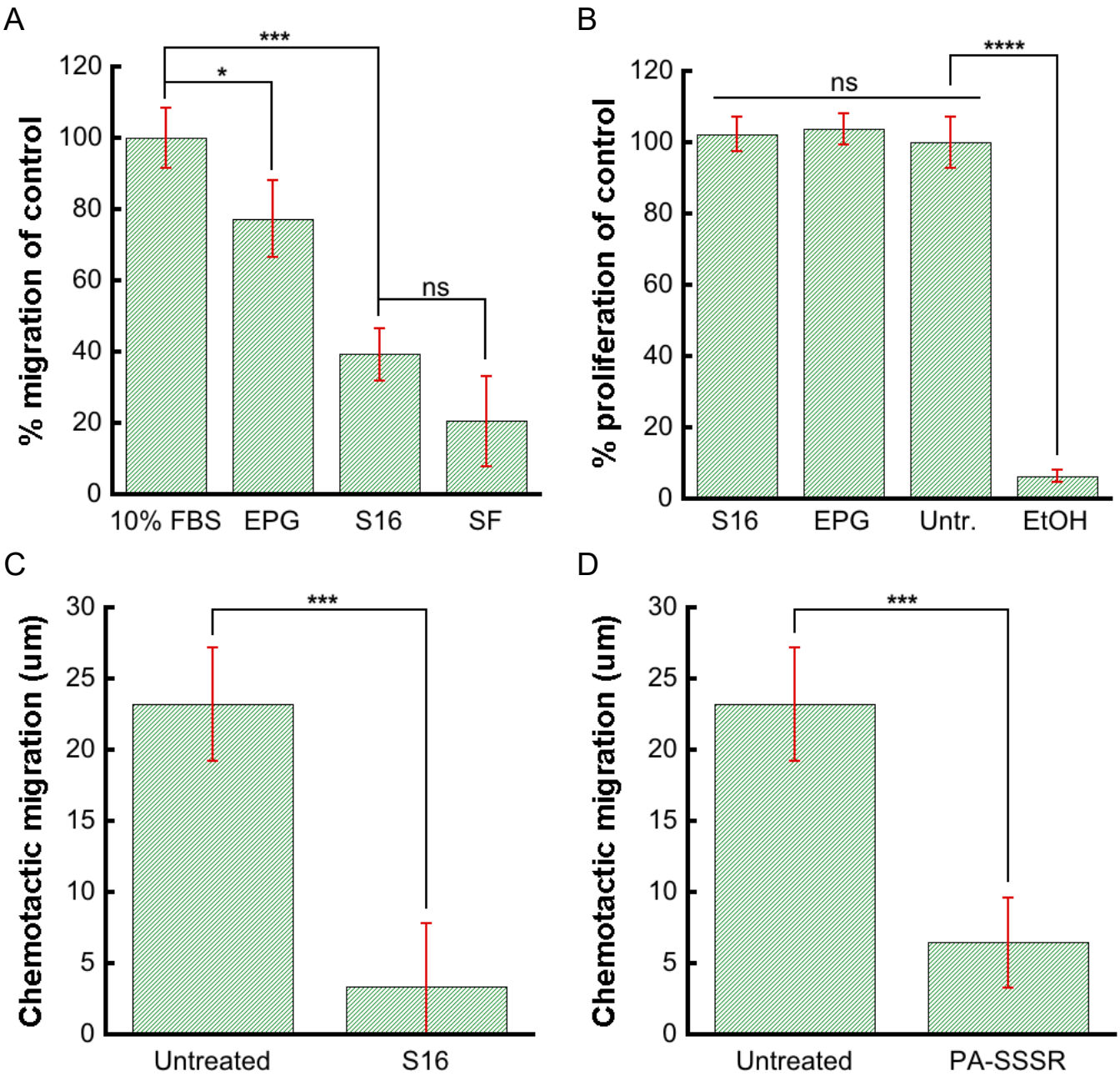
A
% migration of control
120
100
80
60
40
20
0
***
*
ns
10% FBS
EPG
S16
SF
B
% proliferation of control
120
100
80
60
40
20
0
ns
****
S16
EPG
Untr.
EtOH
C
Chemotactic migration (um)
30
25
20
15
10
5
0
***
Untreated
S16
D
Chemotactic migration (um)
30
25
20
15
10
5
0
***
Untreated
PA-SSSR

Figure 2-5: Peptide S16 inhibits migration of endothelial cells through binding CMG2, with no effect on proliferation

(A) Wound scratch assay. EOMA cells were seeded at high confluency, and then a wound was created by scratch. This wound was imaged, then the cells were incubated in DMEM with 10% FBS for 4 hours, after which the wounds were again imaged. The degree of wound closure represents the degree of cell migration. Samples were treated either with canstatin-derived S16, fibronectin-derived EPG, or no serum. Peptide EPG has only a marginal effect on migration. S16 results in 60% inhibition of migration, and is not statistically different from the serum-free (negative) control. Error bars are SD of n=3 replicates. (B) Proliferation of EOMA cells was monitored. Treatment with S16 or EPG resulted in no change in proliferation relative to the untreated control. In contrast, cells fixed in ethanol showed a complete loss of proliferation. Error bars are SD of n=8 replicates from two independent experiments. (C-D) To validate the effect of S16 on endothelial migration, assays were performed with the CellASIC Onix microfluidic platform, using plates that enable monitoring of chemotaxis through the generation of a stable serum gradient in the imaging chamber. A serum gradient was generated (0-10% FBS), and chemotactic migration (in the y-direction) was monitored for 4 hrs. The gradient was reversed, and cells S16 (C) or PA-SSSR (D) was flowed at a uniform concentration to the cell chamber. Migration was monitored in the presence of these compounds for 4 hrs. Chemotactic migration under the different conditions were then compared. Error bars are SEM of n=50 cells for each condition. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

3. Conclusion

Capillary Morphogenesis Gene 2 (CMG2) has diverse situational roles: it has been well characterized as the major anthrax toxin receptor[2, 40]; it is apparently essential for proper homeostasis and repair of the extracellular matrix[5-6, 18]; and CMG2 is emerging as an important regulator in pathological angiogenesis[1, 11-12, 16]. While progress has been made on each of these fronts separately, most studies to date focus on one, sometime two, of these aspects, and the broader question of the functional role of CMG2 has remained elusive.

Careful analysis of the existing literature reveals interesting overlap between these disparate situational roles. For example, CMG2 is essential for proper ECM homeostasis and repair, and ECM remodeling is a key step in angiogenesis. It is possible that CMG2 regulation of angiogenesis is mediated, in part, by its role in ECM homeostasis. Indeed, data presented herein supports this hypothesis. A novel type IV collagen NC1 domain-derived peptide (S16) was identified that binds with high affinity to the CMG2 vWA domain ($K_D \approx 400$ nM). This peptide interacts with human immortalized endothelial cells through binding to CMG2, and inhibits endothelial cell migration, a critical process for angiogenesis[94]. Additionally, CMG2 mediates endocytic uptake of peptide S16, as identified by flow cytometry and confocal microscopy. This observation of S16 endocytosis presents an interesting model whereby CMG2 may regulate angiogenesis through endocytic clearance of angiogenically active matrix fragments. As it has here, hypothesis-driven synthesis of the disparate biological roles of this should continue to accelerate and improve understanding of the physiological function and relevance of capillary morphogenesis gene 2.

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