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VEGF-A splicing:

the key to anti-angiogenic therapeutics?

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Abstract

The physiology of microvessels limits the growth and development of tumours. Tumours gain nutrients and excrete waste through growth-associated microvessels. New anticancer therapies target this microvasculature by inhibiting vascular endothelial growth factor A (VEGF-A) splice isoforms that promote microvessel growth. However, certain VEGF-A splice isoforms in normal tissues inhibit growth of microvessels. Thus, it is the VEGF-A isoform balance, which is controlled by mRNA splicing, that orchestrates angiogenesis. Here, we highlight the functional differences between the pro-angiogenic and the anti-angiogenic VEGF-A isoform families and the potential to harness the synthetic capacity of cancer cells to produce factors that inhibit, rather than aid, cancer growth.

The growth and progression of tumours, in line with that of all expanding cellular structures such as the placenta and the developing embryo, depends on a proliferating vasculature ensuring adequate supply of nutrients and efficient removal of waste products. The advent of anti-angiogenic therapies such as sorafenib1, sunitinib2 and bevacizumab3,4 stems from a huge leap in our mechanistic understanding of the initiation, development, refinement and maintenance of new vessels and microvessels. This in turn originates from the discovery in the 1980s by Ferrara5, Senger6 and Keck7 of the principal player in angiogenesis, vascular endothelial growth factor A (VEGF-A, also referred to as VEGF). VEGF-A exists in multiple isoforms of variable exon content and strikingly contrasting properties and expression patterns. This range of products from the 8-exon *VEGF-A* gene on chromosome 6 renders VEGF-A biology complex (FIG. 1), and alterations in isoform expression in cancer may be instructive for other genes involved in malignant change in general8 and in the pro-angiogenic cascade in particular. Indeed, the products of *VEGF-A*, rather than just being targets for inhibition, may hold the key to impeding tumour growth and act as a model for controlling the qualitative expression of other malignancy-associated genes.

In tumours, and most other angiogenic situations, new vessel development is primarily dependent on this 46 kDa glycoprotein acting on its endothelial cell receptors VEGF receptor 1 (VEGFR1), VEGFR2 and the co-receptor neuropilin 1. This view is supported by the finding that even heterozygous *Vegfa* knockouts are embryonically lethal9. The first VEGF-A isoform described, VEGF-A_{165} (REF. 5), has been extensively investigated for its function, signalling, expression and roles in cancer10. Other isoforms including VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{183}, VEGF-A_{189} and VEGF-A_{206}, identified

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between 1989 and 2003, are generated by alternative splicing of exons 6 and 7, which code for motifs that bind to the highly negatively charged glycosaminoglycan carbohydrate heparin and similar molecules. In 2002, an additional isoform was identified11: VEGF-A_{165}b, which is generated by exon 8 distal splice site (DSS) selection. This DSS choice can also occur in conjunction with exon 6 and 7 inclusion or exclusion. It therefore became apparent that *VEGF-A* mRNA splicing generates two families of proteins that differ by their C' terminal six amino acids (FIG. 1), and these are termed VEGF-A_{xxx} (pro-angiogenic) and VEGF-A_{xxx}b (anti-angiogenic)12, xxx denoting the amino acid number of the mature protein.

Details of the molecular control of C' terminal splice site choice (and the pro-angiogenic-anti-angiogenic balance) are emerging13 (FIG. 2). Upstream factors governing VEGF-A expression include hypoxia, cytokines, sex hormones, chemokines and growth factors (reviewed in REFS 10,14), although most studies have assessed VEGF-A expression using agents that would not distinguish between the two VEGF-A families. Subsequent downstream VEGF-A signalling of the conventional pro-angiogenic VEGF-A_{xxx} isoforms has been identified (reviewed in REFS 15,16) (FIG. 3a). Alterations in these pathways have not been identified in as much detail for the VEGF-A_{xxx}b family (FIG. 3b).

In this article we consider the significant functional differences between the isoform families and the progress made in determining the mechanistic differences between them.

Expression of VEGF-A_{xxx}b isoforms

Increased expression of VEGF-A appears to be a characteristic in several pathologies, including cancer, arthritis and cardiovascular disease, but it is upregulated from a basal level in normal tissues. The development of antibodies and probes that specifically detect VEGF-A_{xxx}b isoforms by enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, western blotting and quantitative PCR has revealed that basal expression is dominated by VEGF-A_{xxx}b isoforms in many tissues17. In human vitreous fluid, circulating plasma, urine, renal cortex, colonic epithelium, bladder smooth muscle, lung and pancreatic islets, VEGF-A_{xxx}b isoforms constitute more than or close to half of the total VEGF-A expressed12,17,18. To date, the placenta, in which angiogenesis is known to occur, is the only normal tissue identified to have VEGF-A_{xxx}b constituting significantly less than half its total VEGF-A17.

In primary cultured cells, such as differentiated visceral glomerular epithelial cells (podocytes), retinal pigmented epithelial cells and colonic epithelial cells, VEGF-A_{xxx}b isoforms predominate13,19,20. However, in melanoma, colorectal carcinoma and bladder cancer cells as well as proliferating dedifferentiated podocytes, VEGF-A_{xxx} isoforms comprise the majority of VEGF-A19-21.

VEGF-A_{xxx}b structure and properties

Receptor binding, downstream signalling and pharmacology

VEGF-A_{165}b differs from VEGF-A_{165} only in the carboxy-terminal six amino acids, a change from CDKPRR to SLTRKD11. The unique C'-terminal six amino acids encoded by exon 8b endow VEGF-A_{165}b (and other VEGF-A_{xxx}b isoforms) with radically different properties to those of VEGF-A_{165}. The key residue alterations are the loss of the cysteine and the replacement of the highly positively charged arginines present in VEGF-A_{165} with neutral lysine-aspartic acid in VEGF-A_{165}b22. These differences have profound implications for structure (FIG. 4), receptor interaction (FIG. 5) and function (as discussed below). VEGF-A_{165} binding to VEGFR2 and neuropilin 1 induces a conformational change

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in VEGFR2 (REF. 23), which is thought to be similar to that of the ERBB2 receptor24, resulting in internal rotation of the intracellular domain. VEGF-A_{165} binding, after resulting in dimerization of the receptor, leads to re-positioning of the kinase domain by rotation to the inside of the dimer, and hence induces tyrosine autophosphorylation. By contrast, VEGF-A_{165}b is predicted not to have this full rotational effect and so autophosphorylation is not efficient (FIG. 5). Recent experiments from L. Claessen-Welsh's group in Uppsala, Sweden support this concept, showing that VEGFR2 is actively phosphorylated by VEGF-A_{165}b binding but on different tyrosine residues, suggesting it may not simply be an inactive competitive inhibitor25. Phospho-peptide mapping and site-specific phospho-antibody experiments show that VEGF-A_{165}b only partially activates VEGFR2 suggesting a partial intracellular rotation, such that the kinase domain is activated but tyrosine 1054, which is in the kinase regulatory site, is not phosphorylated, presumably due to insufficient torsional rotation. This results in rapid closure of the ATP binding site of the kinase and rapid inactivation25, leading to a poorly activated kinase and weak, transient phosphorylation of extracellular-signal-regulated kinase 1 (ERK1) and ERK2 (REF. 26) (FIG. 5). VEGF-A_{165} also stimulates robust phosphorylation of tyrosine 1175, resulting in activation of phospholipase Cγ, phosphoinositide 3-kinase and diacylglycerol production, and activation of the Raf-MEK-Erk pathway in a protein kinase C-dependent27, Ras-independent manner28 (FIG. 3). These events are crucial for the activation of pro-angiogenic gene expression in endothelial cells, particularly as they lead to the production of the matrix metalloproteinases that are required for invasion through the basement membrane and the initiation of endothelial cell migration and angiogenesis29. Notably, VEGF-A_{165}b does not bind neuropilin 1, as the basic carboxy-terminal amino acids essential for neuropilin 1 binding are absent22. The functional difference between VEGF-A_{165} and VEGF-A_{165}b might be determined by absence of neuropilin 1 co-signalling, or it might be due to unique downstream signalling resulting from the different tertiary structures of the neuropilin 1-VEGFR2-VEGF-A_{165} triple dimer complex and the VEGFR2-VEGF-A_{165}b double dimer. This question remains to be clarified.

VEGF-A functions as a dimer. The theoretical formation of heterodimers — either of paired isoforms (for example, VEGF-A_{165}-VEGF-A_{165}b) or non-paired isoforms (for example, VEGF-A_{121}-VEGF-A_{189}b) — adds yet more layers of potential complexity to the subject. The existence of heterodimers *in vivo* is unproven and their subsequent signalling and function is speculative.

Effect of VEGF-A_{xxx}b on non-endothelial cells

VEGF-A has long been regarded as a family of pro-angiogenic, pro-permeability vasodilator peptides. Two key discoveries have emerged in recent years that have resulted in a radical re-evaluation of VEGF-A biology. One was the identification of the anti-angiogenic VEGF-A_{xxx}b family. Although, 6 years after its description, the number of papers on this anti-angiogenic family equate to 40% of those published in the 6 years after the first discovery of VEGF, this group of isoforms has yet to attract the attention of the majority of VEGF investigators. The reasons for this are unclear but may simply be because it has been overlooked in the vast VEGF-A literature (approximately 200 publications per month) or because the identification of this group has unpalatable implications for all of us. Academically it suggests additional layers of complexity and, in terms of resource allocation, the existence of VEGF-A_{xxx}b suggests that many of the thousands of published manuscripts on VEGF-A may, at best, need re-interpretation or, at worst, require repeating with reagents that differentiate between isoform families. As tools for investigating VEGF-A_{xxx}b isoforms (for example, antibodies, probes and ELISA kits) have now become available, our understanding of the role of VEGF-A_{xxx}b should become clearer.

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The second revolution in VEGF-A biology has been that, despite its nomenclature, VEGF-A is not specific to endothelial cells and can also be vital in the function and maintenance of non-endothelial cells. Mutations in the hypoxia response element of the *Vegfa* promoter can, for example, result in a form of motor neuron disease in mice30, which is not associated with angiogenesis. VEGF-A_{165} was subsequently shown to be neuroprotective30. Inhibition of VEGF-A also results in retinal neurotoxicity both *in vitro* and *in vivo*[31] and proteinuria in humans32 and in rodents33. This latter effect could be due to podocyte cytotoxicity, for which both VEGF-A_{165} (REF. 34) and VEGF-A_{165}b18 are *in vitro* survival factors, perhaps through VEGF-A-dependent phosphorylation of nephrin35. VEGF-A_{165}b also acts as a paracrine or autocrine survival factor. Treatment of podocytes, retinal pigmented epithelial cells or colonic adenoma cells *in vitro* with a neutralizing antibody to VEGF-A_{165}b that does not bind VEGF-A_{165}, even when present in 50-fold excess18,20,36, results in increased cytotoxicity13,18,20. Conversely, treatment of these cell types with VEGF-A_{165}b reduces cytotoxicity when induced by multiple agents13,18,20. The receptor-mediated mechanism of action of VEGF-A_{165}b-dependent cytoprotection in epithelial cells has not been well defined and indeed may be cell-type-dependent and VEGFR-phenotype-dependent, but these results support the concept that VEGF-A_{165}b may elicit distinct signalling pathways.

Effect of VEGF-A_{165}b on angiogenesis and tumour growth

The properties of VEGF-A_{165}b have been published by nine laboratories worldwide using VEGF-A_{165}b-transfected cells20,36,37, VEGF-A_{165}b-encoding adenoviral constructs36 and recombinant human VEGF-A_{165}b38,39.

VEGF-A_{165} stimulates endothelial cell migration and proliferation *in vitro*, vasodilatation40, increased endothelial monolayer permeability *in vitro*18, chronically increased vascular permeability *in vivo*41, *in vivo* angiogenesis42 and pathological retinal neovascularization *in vivo*43. By stark contrast, VEGF-A_{165}b does not stimulate these responses, and inhibits several VEGF-A_{165}-mediated processes: endothelial cell migration *in vitro*11, proliferation *in vitro*11 and vasodilatation *ex vivo*11. VEGF-A_{165}b does not increase chronic microvascular permeability *in vivo*44 and reduces conditionally immortalized human glomerular endothelial cell monolayer permeability *in vitro*18. VEGF-A_{165}b also inhibits *in vivo* angiogenesis in the rat mesentery when VEGF-A_{165} overexpression is driven by an adenoviral vector36. In addition, VEGF-A_{165}b inhibits pathological angiogenesis in murine tumour models20,37,39, physiological angiogenesis in mammary tissue in transgenic mice45, VEGF-A_{165}-mediated angiogenesis in the chick chorioallantoic membrane assay26 and VEGF-A_{165}-mediated angiogenesis in the rabbit corneal eye pocket model36. Finally, recombinant human VEGF-A_{165}b inhibits hypoxia-mediated retinal angiogenesis *in vivo* in murine models of retinopathy of prematurity38 and human tumour growth in mice39.

Both VEGF-A_{165} and VEGF-A_{165}b bind VEGFR2 with equal affinity20,36 but VEGF-A_{165}b fails to stimulate angiogenesis *in vivo*26,36,45. These initial observations lent credence to the view that VEGF-A_{165}b was likely to demonstrate classical competitive ‘key-in-the-lock’ inhibition, even if the molecule was completely inert. However, further work by Ballmer-Hofer’s group26 has shown that a truncated isoform of VEGF-A_{165}, VEGF-A_{159}, which lacks the amino acids encoded by exons 8a and 8b, does not inhibit VEGF-A_{165}-mediated angiogenesis, despite binding to VEGFR2 and lacking angiogenic activity itself, which suggests that the presence of exon 8b in VEGF-A_{165}b may have a specific inhibitory contribution. Therefore, it is as yet unclear whether the profound difference in cellular behaviour induced by VEGF-A_{165}b relative to VEGF-A_{165} is due to a qualitative alteration in signalling (that is, differing signalling molecules are used) or a quantitative alteration in signalling (that is, the downstream signalling is insufficient), or whether both mechanisms are functional. There is data supporting the quantitative hypothesis25 but the qualitative hypothesis has only circumstantial evidence to date.

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In tumours, overexpression of transfected VEGF-A_{165}b delays the growth of melanoma36, kidney37, colon20, prostate37 and Ewing sarcoma37 tumours. Furthermore, recombinant human VEGF-A_{165}b inhibits developing and established solid tumour growth in nude mice when given subcutaneously or by intra-peritoneal injection39. Tumours treated with VEGF-A_{165}b are paler, less haemorrhagic and visibly less vascularized, with reduced microvascular density and increased necrosis39. Dose-response studies show complete inhibition of established tumour growth by 100 μg biweekly injection of recombinant VEGF-A_{165}b39. Furthermore, parenteral treatment with recombinant human VEGF-A_{165}b can reduce the growth of disseminated metastatic melanoma tumours46. All these data are consistent with a cancer-associated switch from anti- to pro-angiogenic VEGF-A isoform expression by alteration of splicing.

Heterogeneity of VEGF-A mRNA proximal splice site (PSS) selection in disease

Many cancers are associated with a switch from a VEGF-A_{xxx}b-dominated milieu in normal tissue to a proliferative phenotype in which VEGF-A_{xxx} isoforms dominate. In their study, Varey *et al.* showed by quantitative PCR and ELISA that the switch from VEGF-A_{xxx}b, which makes up 90% of the VEGF-A expressed by normal colonic tissue17, to VEGF-A_{xxx} (in other words, a switch from DSS to PSS selection) is variable in patients with colorectal cancer20. Approximately 30% of patients still have a modest excess of VEGF-A_{xxx}b over VEGF-A_{xxx}, about half an excess of up to threefold VEGF-A_{xxx}, and the remainder a much greater VEGF-A_{xxx} excess, up to 60-fold20. This switch to pro-angiogenic VEGF-A isoforms has also been shown at the mRNA level in prostate37, renal11 and bladder cancer21, and at the protein level in bladder cancer21 and metastatic but not non-metastatic melanoma47. The study in melanoma demonstrated that primary melanomas from patients that later developed distant metastases expressed less VEGF-A_{165}b than those from patients that were disease-free 8 years later47. Furthermore, a low level of VEGF-A_{165}b expression is a potential biomarker for poor prognosis in colonic carcinoma48.

Therapeutic implications

Interaction with established anti-VEGF-A agents

VEGF-A_{165}b contains binding domains for the vast majority of anti-VEGF-A antibodies, including therapeutic antibodies such as bevacizumab and most of the commercially available antibodies for laboratory use. Western blotting and Biacore experiments show that VEGF-A_{165}b binds bevacizumab with the same affinity as VEGF-A_{165} (REF. 20). However, a preliminary report suggests that pegaptinib, the VEGF-A aptamer (an oligonucleotide ligand that displays high-affinity binding to a molecular target), does not bind VEGF-A_{165}b49, although similar data are not yet available for most other anti-VEGF-A agents, including VEGF-TRAP (aflibercept — a unique fusion protein that has a high affinity for all isoforms of VEGF-A as well as for placental growth factor), and VEGFR tyrosine kinase inhibitors (TKIs), such as sunitinib and sorafenib. However, it is possible that the combined effect of recombinant VEGF-A_{165}b and TKIs that target VEGFR2 may have increased efficacy over treatment with a VEGFR TKI alone, as VEGF-A_{165}b is not simply a non-specific inhibitor of VEGFR2 but can actively antagonize VEGFR2 angiogenic signalling25 and possibly also VEGFR1 (REF. 44).

VEGF-A_{165}b expression has a profound effect on the efficacy of bevacizumab. In mice injected with VEGF-A_{165}b-expressing colonic cancer cells, the tumours grow more slowly than in those bearing VEGF-A_{165}-expressing cancer cells. However, the dose of bevacizumab required to prevent tumour growth in VEGF-A_{165}-expressing tumours had absolutely no effect on VEGF-A_{165}b-expressing tumours20. This startling finding suggests that treatment of patients with tumours expressing significant levels of VEGF-A_{xxx}b with

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bevaciumab may not be effective, because VEGF-A_{165}b will inhibit the effect of this anti-VEGF-A antibody. Conversely, this model would predict that bevacizumab treatment would be most effective in patients whose tumours produce an excess of VEGF-A_{xxx} isoforms.

Recombinant human VEGF-A_{165}b

It has yet to be established whether the inhibition of bevacizumab by VEGF-A_{165}b expression can be predicted by assessing the VEGF-A_{165}:VEGF-A_{165}b ratio in patients. If this is indeed the case, current assays for VEGF-A_{xxx}b will need to be developed for clinical use or standardized immunohistochemical procedures will be required. However, an alternative approach is that VEGF-A_{165}b (the most widely studied VEGF-A_{xxx}b isoform) or other VEGF-A_{xxx}b isoforms may be therapeutic themselves. In principle, VEGF-A_{165}b would have potential advantages over a number of existing anti-angiogenic therapies. These include its endogenous nature and the lack of side effects such as hypertension and proteinuria that are associated with the inhibition of VEGF-A_{xxx}39. Anti-VEGF-A therapy has been shown to cause normal capillary loss50, and endothelial cell-specific knockout of all VEGF-A isoforms (including VEGF-A_{165}b) results in adult mortality in mice due to endothelial cell apoptosis and subsequent haemorrhage51. There are therefore sound reasons to suspect that VEGF-A_{165}b-based therapy will be less problematic than agents that target all VEGF-A isoforms. Thus, the identification of VEGF-A_{xxx}-specific antagonists (for example, anti-exon 8a C'-terminal antibodies) may benefit from precise targeting. The next generation of anti-VEGF-A therapies might derive from such a design.

Tumour splicing hypothesis

The control of divergent physiological properties from one gene resides with mRNA splicing, stability and translation. The transcripts of the majority (70%) of human genes splice52, in that they code for multiple isoforms, many of which have strikingly different properties. Splicing is co-transcriptional, and the consensus sequences at the 5′ and 3′ sites are recognized by the splicing apparatus early in the splicing process — that is, the splicing choice occurs early in the birth of an RNA molecule. Splicing mechanisms in mammals are being elucidated using models such as fibronectin53 and β-globin[54] and there is now considerable evidence that regulation of splicing is a key event in cancer progression8,55. The process is mediated by splicing proteins, which form the spliceosome56, and is regulated by splicing regulatory factors. Progress made in defining the mechanisms of *VEGF-A* exon 8 splice site choice13 are summarized in FIG. 2. Alternate 5′ transcriptional start sites have been demonstrated for *VEGF-A*57, and these may result in alternative splicing through the recruitment of different splice factors. Other mechanisms, such as polypyrimidine tract binding protein-mediated repression of alternative exon splicing58, are also possible regulators of *VEGF-A* splicing59.

Thus, the cellular machinery underlying control of splice site choice, and hence which isoforms are expressed, are potential therapeutic targets, particularly as agents that inhibit the actions of specific splicing regulatory factors are now emerging60. Regulation of alternative splicing is unlikely to be restricted to VEGF-A during angiogenesis. Many proteins in the angiogenic cascade have alternative splice variants with antagonistic properties. In active angiogenesis they all splice such that the pro-angiogenic isoforms predominate. VEGFR1 (REF. 61), VEGFR2 (REF. 62), VEGFR3, platelet-derived growth factor receptor-β (PDGFRβ), fibroblast growth factor receptor 1 (FGFR1), FGFR2, FGFR4 (REF. 63) and neuropilin 1 (REF. 64) all have soluble splice variants that can, or are predicted to, act as natural inhibitors. Moreover, anti-angiogenic forms of collagen XVII (endostatin)65 and collagen IV (tumstatin)66 have also been characterized. Common splicing mechanisms allowing anti-angiogenesis to be switched to angiogenesis67 in disease

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or remodelling have been proposed67, and these may extend to many non-angiogenesis-related proteins that also exist as multiple isoforms and drive tumour progression.

Indeed there are many non-angiogenic cancer-related genes that have splice isoforms with antagonistic properties and it is becoming increasingly apparent that similar factors can orchestrate the splicing of angiogenic and non-angiogenic malignancy-associated genes8. For example, exclusion of exon 3 of the FGFR1 in gliomas produces the tumour-promoting isoform FGFR1β. This exon exclusion results from the loss of the splicing factor SRP55 (REF. 68), which also reduces *VEGF-A* C'-terminal DSS selection, shifting the VEGF-A_{xxx} versus VEGF-A_{xxx}b balance towards angiogenesis13.

Therefore, the dual phenomena of a molecular switch to stimulate unregulated malignant cell proliferation and the angiogenic switch as described by the late J. Folkman69 may have different cellular mechanisms, but alternate splicing may well provide a mechanism explaining the connection between malignancy and angiogenesis, as Folkman hypothesized.

In summary, VEGF-A_{xxx}b isoforms are key regulators of angiogenesis in health and disease. Exogenously (intravenously) administered recombinant VEGF-A_{165}b appears to accumulate in tumours (presumably because it targets VEGFR2-bearing tumour microvessels) and therefore has increased tumour bio-availability39. Thus, administration of recombinant VEGF-A_{xxx}b isoforms could be a novel therapeutic approach in the short term. However, the most effective but also the most challenging approach in the long term may be to allow cancer VEGF-A transcription to proceed unhindered but to control splicing such that the spliceosome opts for exon 8 DSS selection in place of exon 8 PSS selection. This would effectively cause the cancer to switch off its own nutrient supply. Indeed, given that the *VEGF-A* promoter contains a hypoxia response element70, the more hypoxic the tumour became, the more effective this switch might be.

DATABASES

National Cancer Institute: http://www.cancer.gov/

bladder cancer | colorectal carcinoma | Ewing sarcoma | kidney cancer | melanoma | prostate cancer

National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/

aflibercept | bevacizumab | sorafenib | sunitinib

UniProtKB: http://www.uniprot.org

β-globin | collagen IV | collagen XVII | FGFR1 | FGFR2 | FGFR4 | fibronectin | nephrin | neuropilin 1 | PDGFRβ | placental growth factor | SRP55 | VEGFA | VEGFR1 | VEGFR2 | VEGFR3

FURTHER INFORMATION

S. J. Harper's homepage: http://www.mvrl.org

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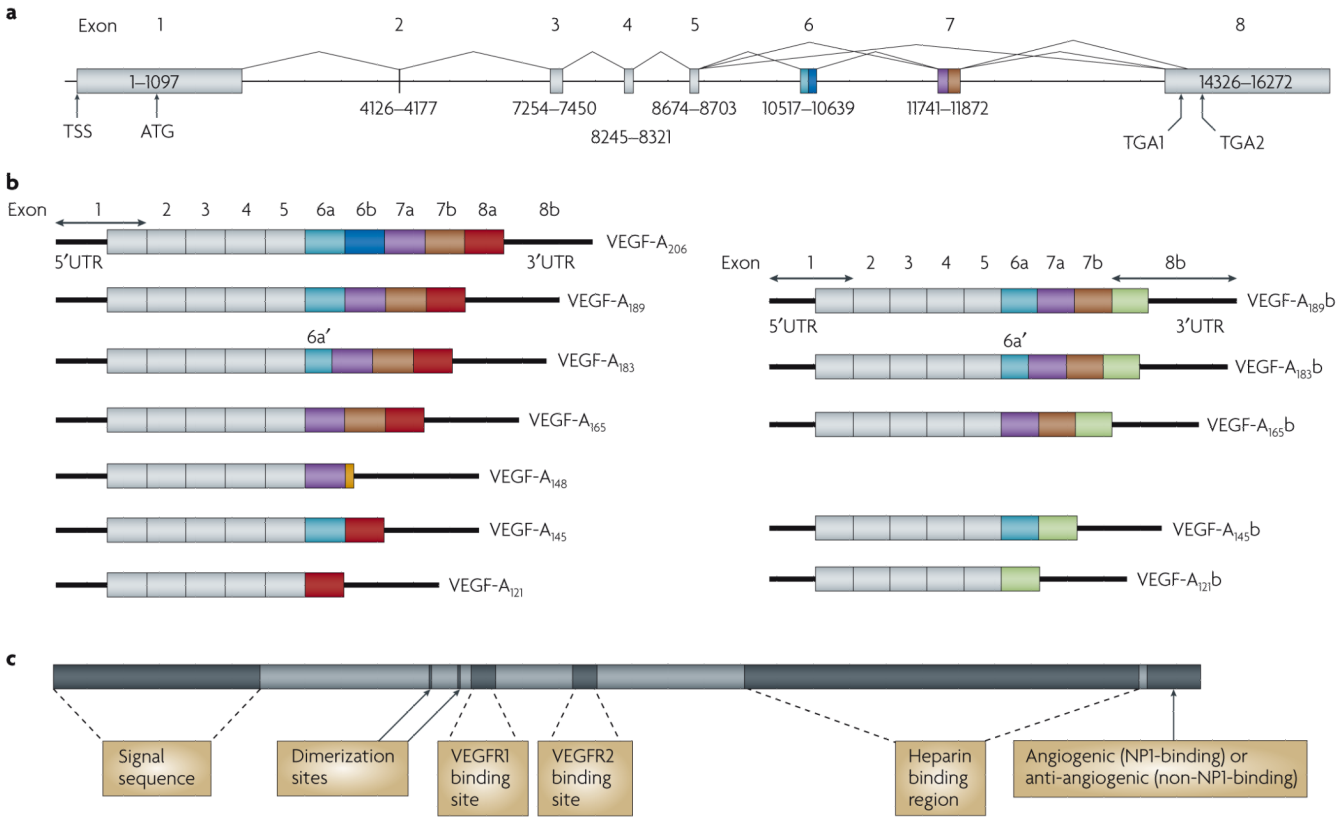

Figure 1. Protein and mRNA products of human vascular endothelial growth factor A (VEGF-A)

a | Gene structure of human *VEGF-A*. *VEGF-A* spans 16,272 bp of chromosome 6p12 and consists of eight exons. Alternate 5′ and 3′ splice site selection in exons 6, 7 and 8 generate multiple isoforms. Exons 6 and 7 encode heparin binding domains. The transcriptional start site (TSS) and translational start site (ATG) in exon 1 are indicated. Alternative stop codons within exon 8 are also indicated (TGA1 and TGA2). **b** | Alternative splicing can occur either at the 5′ donor splice site (for example, *VEGF-A*$_{189}$ versus *VEGF-A*$_{206}$) or the 3′ acceptor splice site (for example, *VEGF-A*$_{189}$ versus *VEGF-A*$_{165}$). Two mRNA isoform families are generated. The pro-angiogenic isoforms (*VEGF-A*$_{xxx}$, left) are generated by proximal splice site (PSS) selection in exon 8 and the anti-angiogenic family (*VEGF-A*$_{xxx}$*b*, right) from exon 8 distal splice site (DSS) choice. Thus, *VEGF-A*$_{165}$, formed by PSS selection in exon 8, has *VEGF-A*$_{165}$*b* as its DSS sister isoform11, the DSS-selected mRNA encoding a protein of exactly the same length. Exon 6a' occurs in *VEGF-A*$_{183}$ as a result of a conserved alternative splicing donor site in exon 6a and is 18 bp shorter than full-length exon 6a. *VEGF-A*$_{148}$ is a truncated isoform splicing from exon 7a into exon 8a out of frame and resulting in a premature stop codon71. *VEGF-A*$_{206}$*b* has not yet been identified. **c** | Protein structure of VEGF-A containing the dimerization sites and binding sites for heparin, VEGF-A receptor 1 (VEGFR1; encoded by exon 3) and VEGFR2 (encoded by exon 4), which are present in all isoforms. The six amino acids at the extreme carboxyl terminus of the protein can be either pro-angiogenic (CDKPRR, encoded by exon 8a) or anti-angiogenic (SLTRKD, encoded by exon 8b). The epitopes recognized by most commercial antibodies are in the region of the VEGF-A receptor-binding domains, present in VEGF-A isoforms of both families. UTR, untranslated region.

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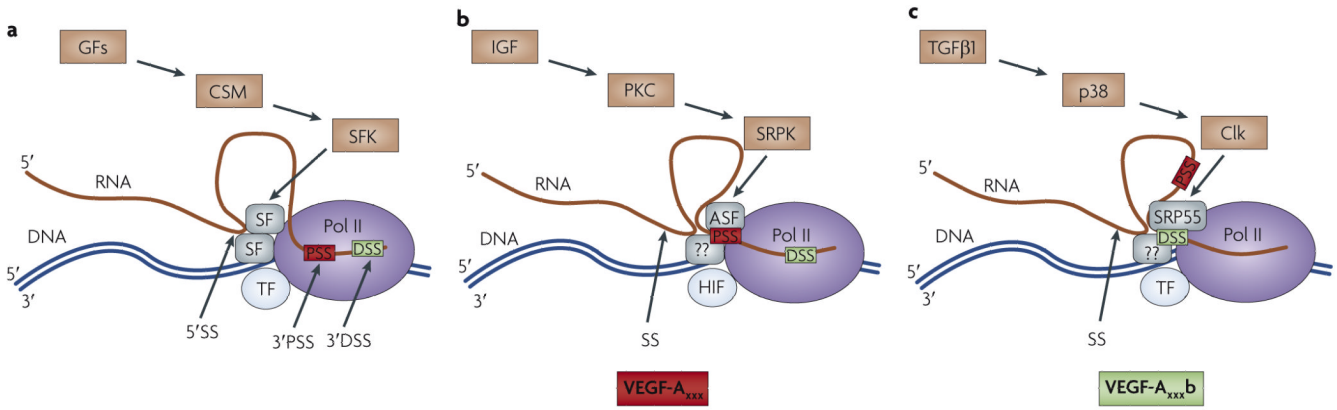

Figure 2. Vascular endothelial growth factor A (VEGF-A) C' terminal splicing regulation

a | The C' terminal domain of RNA polymerase II (Pol II) interacts with both transcription factors (TFs) and splicing factors (SFs). SFs are recruited to the transcriptional machinery owing to their interaction with Pol II72,73. These SFs recognize *cis*-acting RNA splicing sequences in the pre-mRNA and both splicing sites (SS) — 5′ donor (5′SS) or 3′ acceptor sites — can be recognized. Both 3′ proximal SS (3′PSS) and distal SS (3′DSS) are indicated. The particular splicing factors recruited are dependent on the sequence. These SFs can be regulated by SF kinases (SFKs), which are regulated by cell signalling molecules (CSMs) downstream of growth factors (GFs). **b** | Regulation of VEGF-A C' terminal PSS selection by insulin-like growth factor (IGF). IGF activates protein kinase C (PKC), which results in phosphorylation of SR protein kinases (SRPKs). These can activate the ASF-SF2 splicing factor, which favours PSS selection. This process may be dependent on the presence of hypoxia-inducible factor (HIF), a transcription factor involved in *VEGF-A$_{xxx}$* upregulation13. Other SFs and kinases may also be involved in PSS and DSS selection, denoted by '??'. **c** | Factors affecting VEGF-A C' terminal DSS selection. Transforming growth factor β1 (TGFβ1) results in p38 mitogen-activated protein kinase activation and subsequent activation of the kinases CLK1 and CLK4. CLK1 and CLK4 phosphorylate the splicing factor SRP55, resulting in DSS selection and production of *VEGF-A$_{xxx}$b*13. It is also possible that ASF-SF2 is inactivated by CLK1 and CLK4, or that phosphorylation of the SFs could change their location, degradation or binding affinity. This scheme summarizes the limited data available.

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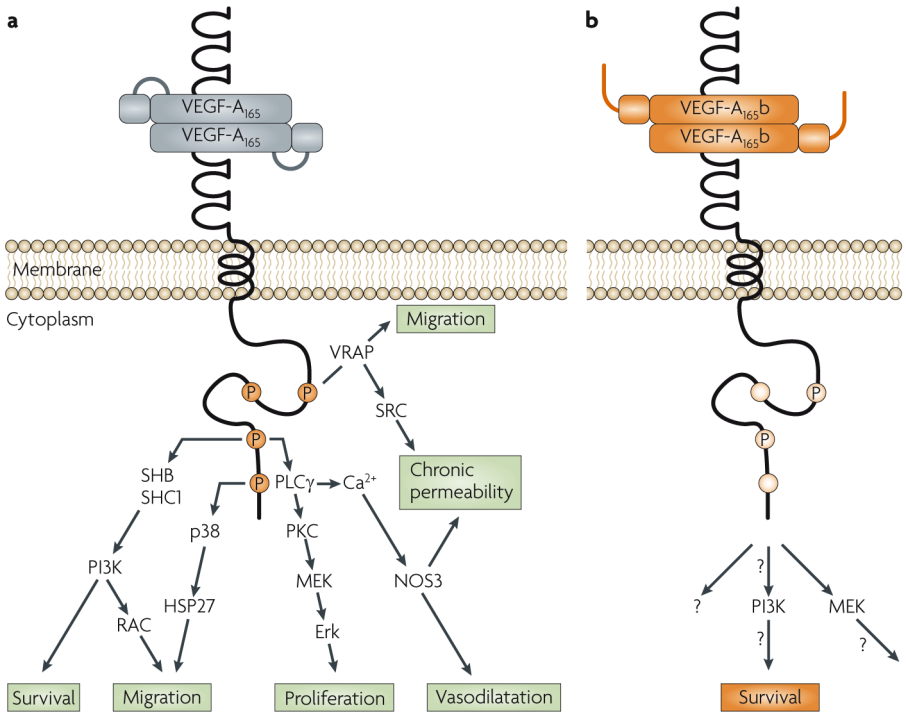

Figure 3. Signalling pathways downstream of vascular endothelial growth factor (VEGF-A)$_{xxx}$ and VEGF-A$_{xxx}$b

a | The VEGF-A$_{xxx}$-mediated angiogenic response acts primarily through VEGF receptor 2 (VEGFR2) to initiate multiple downstream pathways15,16. **b** | VEGF-A$_{165}$b results in transient, weak phosphorylation and the downstream signalling (denoted '?') from such qualitatively different phosphorylation is largely unknown (see REF. 25 for details). Erk, extracellular signal-regulated kinase; HSP27, heat shock protein 27; NOS3, endothelial nitric oxide synthase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; RAC, Ras-related C3 botulinum toxin substrate; PLC**γ**, phospholipase C**γ**; SHB, SH2 domain-containing adaptor protein B; SHC1, SH2 domain-containing transforming protein 1; VRAP, VEGF receptor-associated protein.

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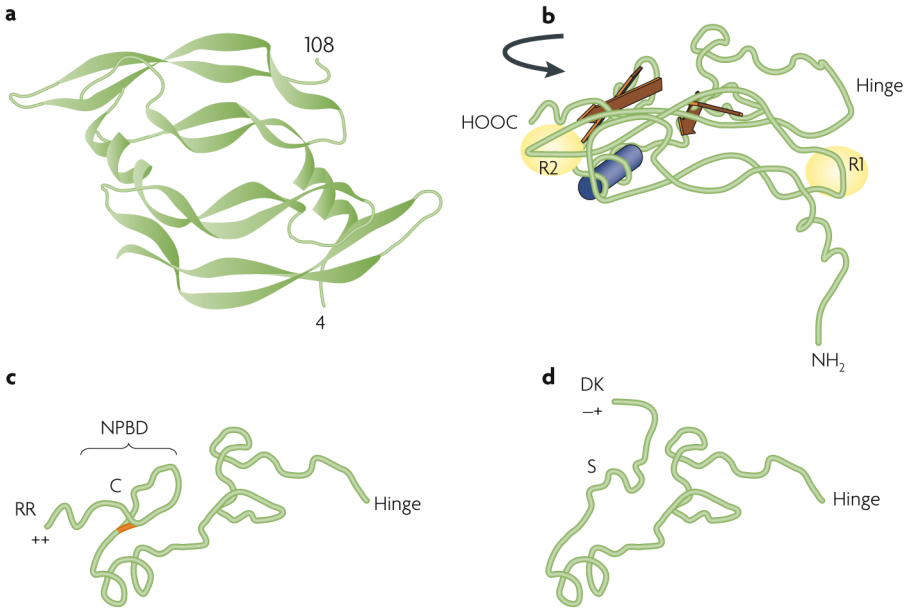

Figure 4. The structure of vascular endothelial growth factor A (VEGF-A)

a | Crystal structure of amino acids 4-108 of VEGF-A, which are present in all isoforms. The crystal structure of the full-length VEGF-A protein is not known as a hinge region after amino acid 108 prevents crystallization. Modified, with permission, from REF. 74 © 1999 Elsevier B.V. **b** | Amino acids 4-108 of VEGF-A are shown along with the crystal structure of the final 55 residues. Crystallization of the final 55 residues of VEGF-A_{165} indicates two cysteine (C)-bonded double anti-parallel β sheet structures (brown arrows) separated by an α helix (blue cylinder). This structure is highly mobile and rotates around the hinge, and could pass through the VEGF receptor 2 (VEGFR2) binding region but not the VEGFR1 region (yellow circles)[75,76]. **c** | Proposed structure of amino acids 108-165 of VEGF-A_{165}. The C' terminal six residues include a cysteine with two positively charged arginines (RR) that are proposed to interact with the VEGFR binding domain22 to activate intracellular torsional rotation of VEGFR2. The RR motif therefore acts as a molecular switch by inducing a conformational change in VEGFR2. A disulphide bond77 (shown in orange) between cysteines 146 and 160 is required for VEGF-A_{165} activity78 and ensures that the C terminus is maintained at close proximity to the neuropilin 1 binding domain (NPBD). **d** | Proposed structure of amino acids 108-165 of VEGF-A_{165}b. The C' terminal cysteine and the positively charged RR motif present in VEGF-A_{165} are replaced by a serine (S) and a neutral DK motif on VEGF-A_{165}b respectively. Although the VEGFR binding domain is present, the cysteine disulphide bond is absent. Thus, the molecular interaction with VEGFR is likely to be significantly different.

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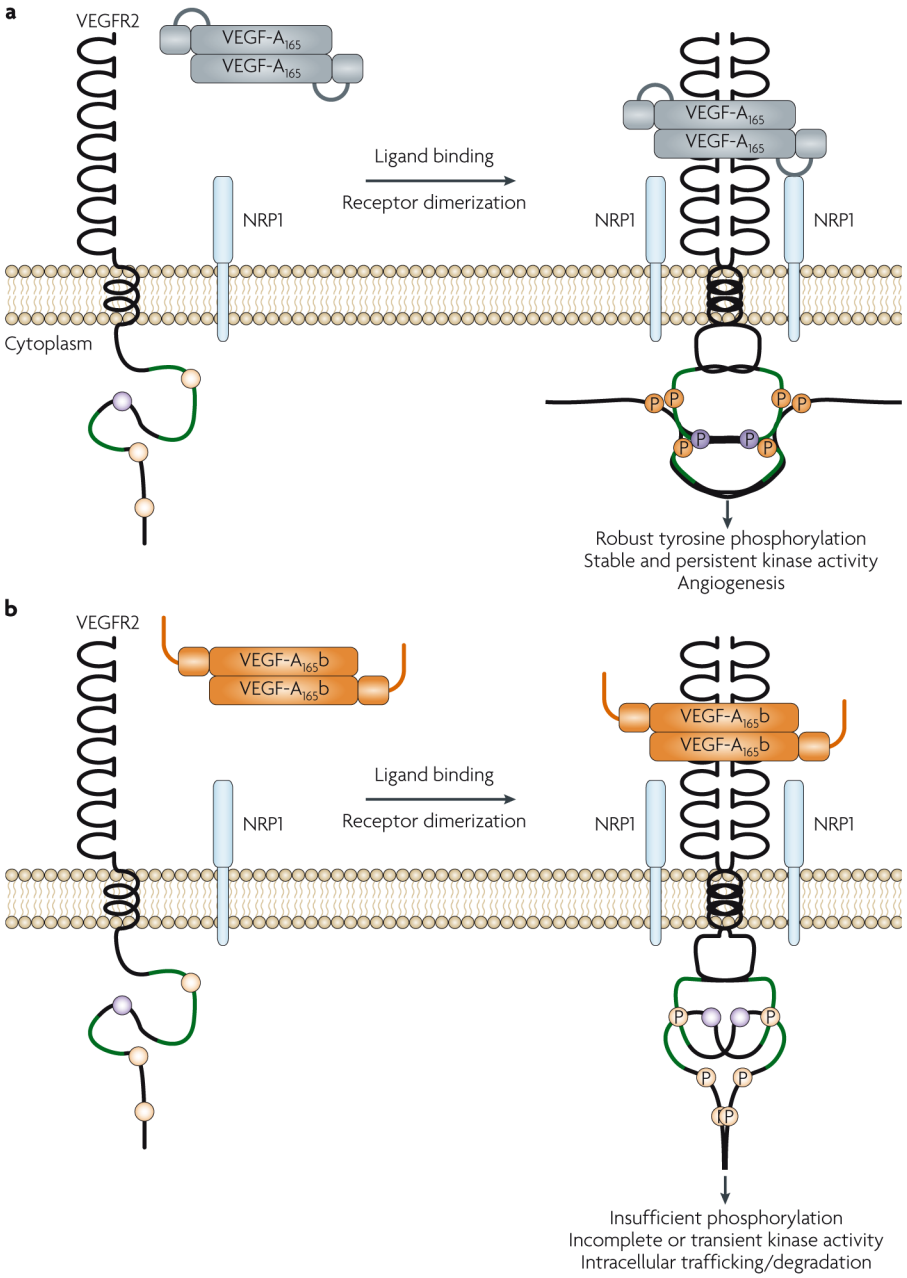

Figure 5. Vascular endothelial growth factor A (VEGF-A)$_{165}$b and VEGF-A$_{165}$ interaction with VEGF receptor 2 (VEGFR2)

a | The VEGFR2 binding site of VEGF-A$_{165}$ interacts with the VEGFR2 extracellular domain. VEGF-A$_{165}$ functions as a dimer and promotes the formation of VEGFR2 dimers (only one receptor is shown here for clarity) resulting in activation of the split kinase domains (green lines) and the phosphorylation of tyrosine residues 951, 1152 and 1214 (orange) and 1054 (purple). The charged residues at the carboxy-terminal end of the VEGF-A$_{165}$ molecule (omitted for clarity) are required for VEGFR activation and, in receptor tyrosine kinases, this is thought to occur through torsional rotation of the intracellular domain bringing together the split kinase domains. Tyrosine 1054 is located at the mouth of the ATP binding pocket of the tyrosine kinase and, once phosphorylated, prevents the binding pocket from closing, thus resulting in a stable open structure. This results in

formation of a persistently functional kinase from the split kinase domains, resulting in sustained *cis*- and *trans*- phosphorylation of the tyrosine residues on the intracellular tail, even in the presence of phosphatases. Robust tyrosine phosphorylation also results in the activation of angiogenic signalling pathways (FIG. 3). **b** | VEGF-A_{165}b binds the VEGFR2 binding site with equal affinity to VEGF-A_{165} but does not bind neuropilin 1 (NRP1). The C' terminus of VEGF-A_{165}b is neutral and there is insufficient torsional rotation for tyrosine 1054 to be phosphorylated, although weak phosphorylation of the other tyrosines can occur. Thus, the ATP binding pocket closes and the phosphorylated tyrosines can be rapidly dephosphorylated by phosphatases and trafficked much more quickly. As a result, angiogenic signalling pathways are not activated25.

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