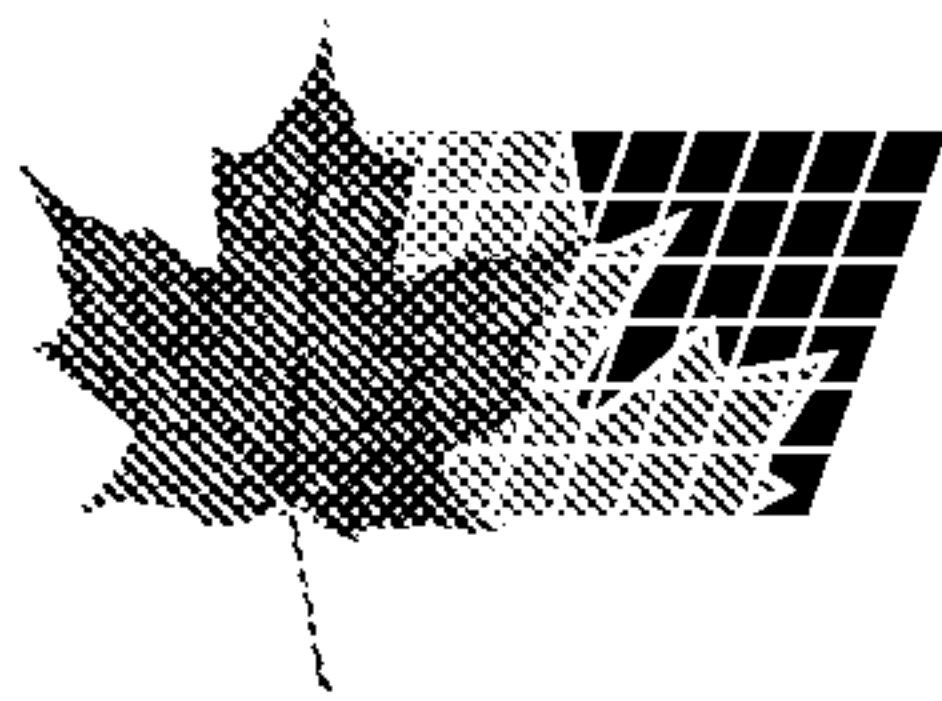




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(54) **VECTEURS D'EXPRESSION ET LIGNEES CELLULAIRES
EXPRIMANT LE FACTEUR DE CROISSANCE
ENDOTHELIAL VASCULAIRE D, ET PROCEDE DE
TRAITEMENT DES MELANOMES**

(54) **EXPRESSION VECTORS AND CELL LINES EXPRESSING
VASCULAR ENDOTHELIAL GROWTH FACTOR D, AND
METHOD OF TREATING MELANOMAS**

(57) Cette invention concerne des vecteurs d'expression qui comprennent le facteur de croissance endothélial vasculaire D ainsi que ses dérivés ayant une action biologique. Cette invention concerne également des lignées cellulaires qui expriment de manière stable le facteur de croissance endothélial vasculaire D ainsi que ses dérivés ayant une action biologique. Cette invention concerne en outre un procédé de production d'un polypeptide à l'aide de ces vecteurs d'expression et de cellules hôtes. Cette invention concerne enfin un procédé permettant de traiter et de réduire les mélanomes ou les tumeurs exprimant le facteur de croissance endothélial vasculaire D, ainsi que diverses autres maladies.

(57) This invention relates to expression vectors comprising VEGF-D and its biologically active derivatives, cell lines stably expressing VEGF-D and its biologically active derivatives, and to a method of making a polypeptide using these expression vectors and host cells. The invention also relates to a method for treating and alleviating melanomas or tumors expressing VEGF-D and various diseases.

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(54) Title: EXPRESSION VECTORS AND CELL LINES EXPRESSING VASCULAR ENDOTHELIAL GROWTH FACTOR D, AND METHOD OF TREATING MELANOMAS (57) Abstract This invention relates to expression vectors comprising VEGF-D and its biologically active derivatives, cell lines stably expressing VEGF-D and its biologically active derivatives, and to a method of making a polypeptide using these expression vectors and host cells. The invention also relates to a method for treating and alleviating melanomas or tumors expressing VEGF-D and various diseases.		

**EXPRESSION VECTORS AND CELL LINES EXPRESSING VASCULAR
ENDOTHELIAL GROWTH FACTOR D, AND METHOD OF TREATING MELANOMAS**

BACKGROUND AND SUMMARY OF THE INVENTION

5 This invention relates to expression vectors comprising
VEGF-D and its biologically active derivatives, cell lines stably
expressing VEGF-D and its biologically active derivatives, and
to a method of making a polypeptide using these expression
vectors and host cells. The invention also relates to a method
for treating and alleviating melanomas and various diseases.

10 **BACKGROUND OF THE INVENTION**

Angiogenesis is a fundamental process required for normal
growth and development of tissues, and involves the proliferation
of new capillaries from pre-existing blood vessels. Angiogenesis
is not only involved in embryonic development and normal tissue
15 growth, repair, and regeneration, but is also involved in the
female reproductive cycle, establishment and maintenance of
pregnancy, and in repair of wounds and fractures. In addition
to angiogenesis which takes place in the normal individual,
angiogenic events are involved in a number of pathological
20 processes, notably tumor growth and metastasis, and other
conditions in which blood vessel proliferation, especially of the
microvascular system, is increased, such as diabetic retinopathy,
psoriasis and arthropathies. Inhibition of angiogenesis is
useful in preventing or alleviating these pathological processes.

On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral
5 circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A
10 number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF α), and hepatocyte growth factor (HGF). See, for example, Folkman et al., J. Biol. Chem.,
15 1992 267 10931-10934 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain functions of the
20 differentiated cells. These factors are members of the PDGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs). Hitherto several vascular endothelial growth factor family members have been identified. Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein

that has been isolated from several sources. VEGF shows highly specific mitogenic activity against endothelial cells, and can stimulate the whole sequence of events leading to angiogenesis. In addition, it has strong chemoattractant activity towards
5 monocytes, can induce the plasminogen activator and the plasminogen activator inhibitor in endothelial cells, and can also influence microvascular permeability. Because of the latter activity, it is also sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF
10 have been reviewed; see Ferrara et al., J. Cellular Biochem., 1991 47 211-218 and Connolly, J. Cellular Biochem., 1991 47 219-223.

More recently, six further members of the VEGF family have been identified. These are designated VEGF-B, described in
15 International Patent Application PCT/US96/02957 (WO 96/26736) and in U.S. Patents 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C, described in Joukov et al., The EMBO Journal, 1996 15 290-298; VEGF-D, described in International Patent Application No. PCT/US97/14696
20 (WO 98/07832); the placenta growth factor (PlGF), described in Maglione et al., Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271; VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc; and VEGF3, described in International Patent Application No.
25 PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each

show between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six cysteine residues which form the cysteine knot motif. Functional characteristics of the VEGF family include
5 varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently
10 from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

15 VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique by screening for screening for cellular proteins which might interact with cellular retinoic acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02597 and in Olofsson et al.,
20 Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581.

VEGF-C was isolated from conditioned media of PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for ability of the medium to produce tyrosine phosphorylation of the endothelial

cell-specific receptor tyrosine kinase VEGFR-3 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was purified using affinity chromatography with recombinant VEGFR-3, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov et al., The EMBO Journal, 1996 15 290-298.

VEGF-D was isolated from a human breast cDNA library, commercially available from Clontech, by screening with an expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696.

In PCT/US97/14696, the isolation of a biologically active fragment of VEGF-D, designated VEGF-D Δ N Δ C, is also described. This fragment consists of VEGF-D amino acid residues 93 to 201 linked to the affinity tag peptide FLAG[®]. The entire disclosure of the International Patent Application PCT/US97/14696 (WO 98/07832) is incorporated herein by reference.

VEGF-D has structural similarities to other members of the VEGF family. However, despite these structural similarities, it is structurally and functionally distinguished from other members of VEGF family. Human VEGF-D is only 48% identical to VEGF-C,

which is the member of the family to which VEGF-D is most closely related.

5 The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

10 PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione et al., Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271. Presently its biological function is not well understood.

15 VEGF2 was isolated from a highly tumorigenic, oestrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 is unclear, and no characterization of the biological activity is disclosed.

20 VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding

VEGF3 is unclear and no characterization of the biological activity is disclosed.

Vascular endothelial growth factors appear to act primarily by binding to receptor tyrosine kinases. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos.

The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B. VEGF-C has been shown to be the ligand for VEGFR-3, and also activates VEGFR-2 (Joukov et al., The EMBO Journal, 1996 15 290-298). VEGF-D shares receptor specificity with VEGF-C (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553). A ligand for Tek/Tie-2 has been described (International Patent Application PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc.); however, the ligand for Tie has not yet been identified.

The primary translation products of VEGF-D and VEGF-C have long - and C-terminal polypeptide extensions in addition to a central VEGF homology domain (VHD). In the case of VEGF-C, these polypeptide extensions are propeptides which are proteolytically
5 cleaved to generate a secreted form which consists only of the VHD and is capable of binding to VEGFR-2 and VEGFR-3 (Joukov et al., The EMBO Journal, 1996 15 290-298; Joukov et al., EMBO J., 1997 16 3898-3911). Likewise, a recombinant form of VEGF-D, consisting only of the VHD, was shown to bind and activate these
10 receptors and to be mitogenic for endothelial cells, although VEGF-D processing was uncharacterized (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553).

Recently, a novel 130-135 kDa VEGF-A isoform specific receptor has been purified and cloned (Soker et al., Cell, 1998
15 92 735-745). The VEGF receptor was found to bind specifically the VEGF-A₁₆₅ isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker et al., Cell, 1998 92 735-745). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage
20 neuromorphogenesis. PlGF-2 also appears to interact with NP-1 (Migdal et al., J. Biol. Chem., 1998 273 22272-22278).

Gene targeting studies have demonstrated the absolute requirement of VEGFR-1, VEGFR-2 and VEGFR-3 for embryonic development. These studies show that VEGFR-1 plays a role in

vascular endothelial tube formation, VEGFR-2 is important for endothelial/hematopoietic cell differentiation and mitogenesis, and VEGFR-3 is involved in regulation of vascular remodeling, the formation of large vessels and in lymphangiogenesis. The
5 functions of these receptors are reviewed in Mustonen and Alitalo, J. Cell Biol., 1995 129 895-898.

The VEGFR-3 is expressed in venous and lymphatic endothelia in the fetus, and predominantly in lymphatic endothelia in the adult (Kaipainen et al., Cancer Res, 1994 54 6571-6577; Proc.
10 Natl. Acad. Sci. USA, 1995 92 3566-3570). VEGFR-3 has an essential role in the development of the embryonic cardiovascular system before the emergence of the lymphatic vessels (Dumont et al., Science, 1998 282 946-949). It has been suggested that VEGF-C may have a primary function in lymphatic endothelium, and
15 a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov et al., The EMBO Journal, 1996 15 290-298).

SUMMARY OF THE INVENTION

20 The invention generally provides expression vectors comprising VEGF-D and its biologically active derivatives, cell lines stably expressing VEGF-D and its biologically active derivatives, and a method of making a polypeptide using these expression vectors and host cells. The invention also generally

provides for a method for treating and alleviating melanomas or tumors expressing VEGF-D and various diseases.

According to a first aspect, the present invention provides a mammalian cell line stably expressing VEGF-D or a fragment or analog thereof having the biological activity of VEGF-D. 5 Optionally VEGF-D produced by the cell line of the invention is linked to an epitope tag such as FLAG[®], hexahistidine or I-SPY[™] to assist in affinity purification and in localization of VEGF-D. Preferably the mammalian cell line is the 293-EBNA human 10 embryonal kidney cell line. Preferably the VEGF-D expressed is VEGF-D_{Full}NFlag, VEGF-D_{Full}CFlag, VEGF-D Δ N Δ C, or VEGF-D Δ C, as described herein.

The expression "biological activity of VEGF-D" is to be understood to mean the ability to stimulate one or more of 15 endothelial cell proliferation, differentiation, migration, survival or vascular permeability.

A preferred fragment of VEGF-D is the portion of VEGF-D from amino acid residue 93 to amino acid residue 201 (i.e. the VEGF homology domain (VHD)) (SEQ ID NO:1) of SEQ ID NO:5 of 20 PCT/US97/14696, optionally linked to the FLAG[®] peptide. Where the fragment is linked to FLAG[®], the fragment is referred to herein as VEGF-D Δ N Δ C.

As used herein, the term "VEGF-D" collectively refers to any of the polypeptides of SEQ ID NO. 3, SEQ ID NO. 5, SEQ ID NO. 8 and SEQ ID NO. 9 as defined in International Patent Application PCT/US97/14696 and fragments or analogs thereof which have the biological activity of VEGF-D as herein defined.

According to a second aspect, the invention provides an expression vector comprising a sequence of human cDNA encoding VEGF-D, inserted into the mammalian expression vector Apex-3. Preferably the expression vector is pVDApexFullNFlag, VEGF-DFullCFlag, pVDApex Δ NAC or pVDApex Δ C, as described herein.

Preferably the expression vector also comprises a sequence encoding an affinity tag such as FLAG[®], hexahistidine or I-SPY[™].

The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing an expression vector of the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium. In one preferred embodiment of this aspect of the invention, the expression vector further comprises a sequence encoding an affinity tag, such as FLAG[®], hexahistidine or I-SPY[™], in order to facilitate purification of the polypeptide by affinity chromatography.

The polypeptides comprising conservative substitutions, insertions or deletions but which still retain the biological activity of VEGF-D are clearly to be understood to be within the scope of the invention. Persons skilled in the art will be well
5 aware of the methods which can be readily used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-
10 naturally occurring amino acid or an amino acid analog may retain the required aspects of the biological activity of VEGF-D. Such compounds can be readily made and tested by methods known in the art, and are also within the scope of the invention.

In addition, variant forms of the VEGF-D polypeptide which
15 result from alternative splicing, as are known to occur with VEGF and VEGF-B, and naturally-occurring allelic variants of the nucleic acid sequence encoding VEGF-D are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms of the encoded polypeptide.

20 Such variant forms of VEGF-D can be prepared by targeting non-essential regions of the VEGF-D polypeptide for modification. These non-essential regions are expected to fall outside the strongly-conserved regions. In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF,

VEGF-B, VEGF-C, VEGF-D, PDGF-A and PDGF-B show complete conservation of eight cysteine residues in the PDGF-like domains (Olofsson et al., Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581; Joukov et al., The EMBO Journal, 1996 15 290-298). These
5 cysteines are thought to be involved in intra- and inter-molecular disulfide bonding. Loops 1, 2 and 3 of each subunit, which are formed by intra-molecular disulfide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson et al., Growth Factors, 1995 12 159-
10 164). As noted above, the cysteines conserved in previously known members of the VEGF family are also conserved in VEGF-D.

Persons skilled in the art thus are well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2 and 3 also
15 should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can be readily tested for their ability to show the biological activity of VEGF-D by routine activity assay
20 procedures such as cell proliferation tests.

It is contemplated that some modified VEGF-D polypeptides will have the ability to bind to endothelial cells, i.e. to VEGF-D receptors, but will be unable to stimulate endothelial cell proliferation, differentiation, migration or survival, or induce

vascular permeability. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of VEGF-D, and to be useful in situations where prevention or reduction of VEGF-D action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing or non-survival promoting variants of VEGF-D are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive or interfering variants".

10 Likewise, it is contemplated that some modified VEGF-D polypeptides will have the ability to bind VEGF-D and will prevent binding of the dimer to VEGF-D receptors (e.g. VEGFR-2 and VEGFR-3) on endothelial cells. Thus these dimers will be unable to stimulate endothelial cell proliferation, 15 differentiation, migration or survival, or induce vascular permeability. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of VEGF-D, and to be useful in situations where prevention or reduction of VEGF-D action is desirable. Thus such VEGF-D-binding but non- 20 mitogenic, non-differentiation inducing, non-migration inducing or non-survival promoting variants of VEGF-D are also within the scope of the invention, and are referred to herein as "VEGF-D-binding but otherwise inactive or interfering variants".

According to a third aspect, the invention provides a method of treatment or alleviation of malignant melanoma or tumors expressing VEGF-D, comprising the step of inhibiting the expression or activity of VEGF-D in the vicinity of the melanoma or tumor. Local inhibition of VEGF-D expression may be achieved for example by the use of anti-sense nucleic acid or triple-stranded DNA encoding VEGF-D. Alternatively a VEGF-D variant polypeptide, as described above, which has the ability to bind to VEGF-D and prevent binding to the VEGF-D receptors or which bind to the VEGF-D receptors, but which is unable to stimulate endothelial cell proliferation, differentiation, migration or survival may be used as a competitive or non-competitive inhibitor of VEGF-D. Small molecule inhibitors to VEGF-D, VEGFR-2 or VEGFR-3 and antibodies directed against VEGF-D, VEGFR-2 or VEGFR-3 may also be used.

Use of the above method is also contemplated in non-malignant conditions, where there is increased or continuous expression of VEGF-D, such as in psoriasis. Based on the distribution of VEGF-D in the skin of the developing mouse embryo it is possible that VEGF-D plays a role in the initiation or continuation of high epidermal cell turnover dermatoses such as psoriasis, where vascular proliferation in the upper dermis is a consistent and prominent histopathological feature.

In an additional aspect of the invention, VEGF-D is conjugated to toxins or drugs which have endothelial cell inhibiting activity that would be targeted to proliferating vascular and lymphatic endothelial cells which express VEGF-D
5 receptors, e.g. VEGFR-2 and VEGFR-3. Thus, growth of vessels, which is important for numerous pathological conditions, such as tumor growth, could be blocked.

According to a fifth aspect, the invention provides a method of enhancing the acceptance and/or healing of a skin graft,
10 comprising the step of stimulating angiogenesis and lymphangiogenesis with an effective dose of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

According to a sixth aspect, the invention provides a method of stimulating the healing of a surgical or traumatic wound to
15 the skin, comprising the step of stimulating angiogenesis and lymphangiogenesis with an effective dose of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

It is contemplated that the latter two aspects of the invention will be particularly useful in the treatment of burns
20 and in plastic surgery.

In another aspect of the invention a method is provided for stimulating lymphangiogenesis for treatment or alleviation of

lymphedema, comprising the step of stimulating lymphangiogenesis with an effective dose of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D. Few diseases are as disfiguring as lymphedema. Lymphedema occurs when there is
5 obstruction of the lymphatic vessels which are involved in the draining of fluid bathing the tissues. As a result of this obstruction, lymph or fatty fluid accumulates within the tissues and results in limb and tissue engorgement. The end result is often grotesque and severely incapacitating due to local
10 infections, discomfort and deformity. There are several causes of lymphedema. Most notable is breast cancer associated with either lymph node obstruction or removal during surgery. Recurrent infections and other forms of surgery are also associated with lymphedema. A significant proportion of patients
15 with lymphedema have no identifiable precipitant. Increasing the amount of VEGF-D should induce lymphangiogenesis and alleviate lymph and fatty fluid accumulation.

Inappropriate down-regulation of VEGF-D synthesis during embryogenesis may also be important in adnexal structure
20 maldevelopment including anhydrotic ectodermal dysplasia. Normally the sweat glands in the dermis are surrounded by vascularized fatty connective tissue. If the vascular supply is compromised or unable to replenish due to a possible lack of VEGF-D, it will lead to sweat gland hypoxia and malfunction.
25 These lesions may be due to lack of ability of differentiated

cells at some stage of development to produce VEGF-D or production of blocking agents to the VEGF-D receptors on blood vessels adjacent to differentiating adnexal cells producing such blocking agents. Thus the invention provides a method for
5 treating or alleviating anhydrotic ectodermal dysplasia by stimulating vascularization of fatty connective tissue, comprising the step of administering an effective dose of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

10 A further disease which may be related to a lack of VEGF-D or lack of response to VEGF-D is sclerodema. Scleroderma is an uncommon disorder of connective tissue characterized by thickening and increased collagenization of the skin that is thought to be due to changes in vascularization and/or fibroblast
15 function. Damage to the endothelial cells due to a lack of VEGF-D or to a failure to response to VEGF-D may be a contributing factor to the inability of the vessels to repair leading to the continued platelet aggregation observed and subsequent release of growth factors having a mitogenic action on fibroblasts. This
20 results in increased collagen production. The same considerations apply to systemic organ involvement in scleroderma. Thus the invention provides a method for treating or alleviating scleroderma by stimulating proliferation of vascular endothelial cells, comprising the step of administering an effective dose of

VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

According to a seventh aspect, the invention provides a method for stimulating at least one bioactivity of VEGF-D
5 selected from endothelial cell proliferation, migration, survival and differentiation, and lymphangiogenesis without inducing vascular permeability, comprising the step of administering a bioactivity stimulating amount of fully processed VEGF-D.

A further aspect of the invention provides a method for
10 regulating receptor-binding specificity of VEGF-D, comprising the steps of expressing an expression vector comprising a nucleotide sequence encoding an unprocessed VEGF-D and supplying a proteolytic amount of at least one enzyme for processing the encoded VEGF-D to generate a proteolytically processed form of
15 VEGF-D.

It will be clearly understood that for the purposes of this specification the phrase "fully processed VEGF-D" means a VEGF-D polypeptide without the N- and C-terminal propeptides, the phrase "proteolytically processed form of VEGF-D" means a VEGF-D
20 polypeptide without the N- and/or C-terminal propeptide, and the phrase "unprocessed VEGF-D" means a VEGF-D polypeptide with both the N- and C-terminal propeptides.

The invention also provides a method of detecting tumors expressing VEGF-D in a biological sample, comprising the steps of contacting said sample with a specific binding reagent for VEGF-D, allowing time for a binding of said specific binding reagent to VEGF-D, and detecting said binding. In a preferred embodiment the specific binding reagent for VEGF-D is an antibody and the binding and/or extent of binding is detected by means of an antibody with a detectable label. Quantitation of VEGF-D in cancer biopsy specimens will be useful as an indicator of future metastatic risk.

Antibodies according to the invention may be labeled with a detectable label, and utilized for diagnostic purposes. The antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays, radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as ^{125}I or ^{32}P . Examples of non-radioactive labels include enzyme labels, such as horseradish peroxidase, or fluorimetric labels, such as fluorescein-5-isothiocyanate (FITC). Labeling may be direct or indirect, covalent or non-covalent.

The polypeptides or antibodies which induce the biological activity of VEGF-D may be employed in combination with a suitable pharmaceutical carrier. The polypeptides, VEGF-D antagonists or antibodies which inhibit the biological activity of VEGF-D also

may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the antibody, and a pharmaceutically acceptable carrier or adjuvant. Examples of such a carrier include, but are not limited to, saline, buffered saline, mineral oil, talc, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, ointments or other conventional forms. The formulation is selected to suit the mode of administration. W h e r e polypeptides, VEGF-D antagonists or antibodies are to be used for therapeutic purposes, the dose and route of application will depend upon the nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include subcutaneous, intramuscular, intraperitoneal or intravenous injection, topical application, implants etc. Topical application of VEGF-G may be used in a manner analogous to VEGF.

It will be clearly understood that for the purposes of this specification the word "comprising" means "including but not limited to". The corresponding meaning applies to the word "comprises".

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic maps of the Apex-3 plasmid constructs for expression of human VEGF-D derivatives in 293-EBNA cells;

5 Figure 2 shows the expression of VEGF-D derivatives by 293-EBNA cells;

Figure 3 shows the precipitation of VEGF-D by soluble VEGF receptor-immunoglobulin fusion proteins;

10 Figure 4 shows the nucleotide sequence of a cDNA encoding mouse VEGF-D1 (SEQ ID NO:2), isolated by hybridization screening from a commercially-available mouse lung cDNA library;

Figure 5 shows autoradiographs taken after two days of exposure to mouse 15.5 days post-coital tissue sections hybridized with VEGF-D antisense and sense RNAs;

15 Figure 6 shows the results of analysis of the distribution of VEGF-D mRNA in the post-coital day 15.5 mouse embryo by *in situ* hybridization;

Figure 7 shows the analysis of human malignant melanoma by immunohistochemistry with VEGF-D monoclonal antibodies;

20 Figure 8 provides a schematic representation of the structural domains of VEGF-D and some VEGF-D derivatives

Figure 9 shows the analyses of VEGF-D derivatives secreted by 293-EBNA cells expressing VEGF-D_{FullN}Flag (A, B and C) and VEGF-D_{ΔC} (D and E);

25 Figure 10 shows the analysis of VEGF-D_{ΔNΔC} by size exclusion chromatography and SDS-PAGE; and

Figure 11 provides a schematic representation of the mode of VEGF-D processing.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1 Cell Lines Stably Expressing VEGF-D Derivatives

5 In order to generate cell lines constitutively expressing derivatives of VEGF-D, regions of the human VEGF-D cDNA were inserted into the mammalian expression vector Apex-3 (Evans et al, Mol. Immunol., 1995 32 1183-1195). This vector is maintained episomally when transfected into 293-EBNA human embryonal kidney
10 cells. For expression of VEGF-D Δ N Δ C, the region of pEFBOSVEGF-D Δ N Δ C containing the sequences encoding the IL-3 signal sequence, the FLAG[®] octapeptide and VEGF-D Δ N Δ C was inserted into the XbaI site of Apex-3 (see Example 9 in International Patent Application PCT/US97/14696). The resulting plasmid was designated
15 pVDApex Δ N Δ C, and is illustrated schematically in Figure 1. Similar types of constructs were made for expression of VEGF-DFullNFlag, a derivative of full-length human VEGF-D which had been tagged with FLAG[®] at the N-terminus, and for expression of a truncated derivative of human VEGF-D, consisting of amino
20 acid residues 2 to 202, designated VEGF-D Δ C. The expression constructs for these VEGF-D derivatives were designated pVDApexFullNFlag and pVDApex Δ C respectively, and are also shown schematically in Figure 1. IL-3 SS denotes the interleukin-3 signal sequence, and the arrows indicate the direction of
25 transcription proceeding from the cytomegalovirus promoter (CMV)

through the expression cassettes. These vectors were transfected into cells of the human embryo kidney cell line 293-EBNA by the calcium phosphate method, and stable transfectants were selected in the presence of hygromycin. Cell lines expressing high levels
5 of VEGF-DFullNFlag, VEGF-DAC and VEGF-DANAC were subsequently identified by metabolic labeling, immunoprecipitation and Western blot analysis, as shown in Figure 2.

In Figure 2, the 293-EBNA cell lines expressing VEGF-DFullNFlag, VEGF-DANAC and VEGF-DAC were metabolically
10 labeled, and proteins in conditioned medium samples were immunoprecipitated with anti-FLAG antibody (M2) or with antiserum specific for the VEGF homology domain of VEGF-D (A2). The precipitated proteins were analyzed by SDS-PAGE and visualized by autoradiography in the case of VEGF-DFullNFlag and VEGF-DANAC
15 or detected in Western blot analysis with M2 antibody in the case of VEGF-DAC. Arrows denote the positions of VEGF-D derivatives. These derivatives were not detected from control supernatants derived from parental 293-EBNA cells (data not shown). The positions of molecular weight markers (in kDa), are shown to the
20 right of each panel. The band at approximately 50 kDa detected by Western blot analysis of VEGF-DAC corresponds to the immunoglobulin heavy chain.

Numerous VEGF-D derivatives were detected in the supernatants of cells expressing VEGF-DFullNFlag and VEGF-DAC.

These derivatives are formed as a result of proteolytic processing which occurs as part of the biosynthesis of VEGF-D. The cell lines expressing VEGF-DN^{Full}Flag, VEGF-DAC and VEGF-DANAC have been maintained under hygromycin selection while
5 being passaged at least twenty times, and continue to express the VEGF-D derivatives.

Example 2 Binding of VEGF-DANAC to soluble VEGF receptors To further assess the interactions between VEGF-D and the VEGF receptors, VEGF-DANAC was tested for its capacity to bind to
10 soluble immunoglobulin fusion proteins comprising the extracellular domains of human VEGFR-1, human VEGFR-2 and human VEGFR-3. The corresponding fragment of VEGF-C, VEGF-CANAC, was used for comparison. For binding experiments, 293T human embryonal kidney cells were transfected with plasmids encoding
15 the soluble receptor-immunoglobulin fusion proteins VEGFR-1-Ig, VEGFR-2-Ig or VEGFR-3-Ig using the calcium-phosphate (Ca-phosphate) method. In these fusion proteins, the extracellular domain of the relevant VEGF receptor is fused to the Fc portion of human IgG₁. The cells were incubated for 24
20 hours after transfection, washed with Dulbecco's Modified Eagle's Medium (DMEM) containing 0.2% bovine serum albumin (BSA) and starved for 24 hours. Media were then collected and clarified by centrifugation, and fusion proteins were precipitated using protein A Sepharose beads. The Sepharose beads were then
25 incubated at room temperature for 3 hours with 900 µl of

metabolically ^{35}S -labeled medium from 293-EBNA cells which had been transfected with expression plasmids encoding human VEGF-DANAC, human VEGF-CANAC or human VEGF₁₆₅ using the Ca-phosphate method. Metabolic labeling of 293-EBNA cells was
5 carried out essentially as described (Joukov et al., 1997). The Sepharose beads were then washed twice with binding buffer (0.5% BSA, 0.02% Tween 20, 1 $\mu\text{g}/\text{ml}$ heparin in phosphate buffered saline (PBS)) at 4°C and once with PBS, boiled in Laemmli sample buffer, and proteins were then analyzed by SDS-PAGE. The results are
10 shown in Figure 3.

In Figure 3, precipitation of labeled VEGF₁₆₅, VEGF-CANAC and VEGF-DANAC by VEGFR-1-Ig, VEGFR-2-Ig and VEGFR-3-Ig was carried out as described above. The fusion proteins used for the
15 precipitations are shown to the right. "Vector" denotes results of precipitations from medium derived from cells transfected with expression vector lacking sequence encoding the VEGFs. The molecular weight markers are indicated in kDa

A polypeptide of the size expected for VEGF-DANAC
20 (approximately 22 kDa) was precipitated by VEGFR-2-Ig and VEGFR-3-Ig from the medium of cells expressing VEGF-DANAC. In contrast, no protein of this size was precipitated from the same medium by VEGFR-1-Ig. Essentially the same results were observed for precipitation of VEGF-CANAC. As expected, a predominant
25 polypeptide of approximately 24 kDa was precipitated by

VEGFR-1-Ig and VEGFR-2-Ig from the medium of cells expressing VEGF₁₆₅, but was not precipitated by VEGFR-3-Ig. No labeled polypeptides were precipitated by the three fusion proteins from the medium of cells transfected with the expression vector
5 lacking sequences encoding the VEGFs. These data indicate that VEGF-DANAC can bind to VEGFR-2 and VEGFR-3 but not to VEGFR-1. Thus VEGF-DANAC resembles VEGF-CANAC in the receptor-binding specificity to VEGFR-2 and VEGFR-3.

Example 3 In Situ Hybridization Studies of VEGF-D Gene

10 Expression in Mouse Embryos

The pattern of VEGF-D gene expression was studied by *in situ* hybridization using a radiolabeled antisense RNA probe corresponding to nucleotides 1 to 340 of the mouse VEGF-D1 cDNA, whose sequence is shown in Figure 4. The antisense RNA was
15 synthesized by *in vitro* transcription with T3 RNA polymerase and [³⁵S]UTPs. Mouse VEGF-D is fully described in International Patent application PCT/US97/14696. This antisense RNA probe was hybridized to paraffin-embedded tissue sections of mouse embryos at post-coital day 15.5. The labeled sections were subjected
20 to autoradiography for 2 days. The resulting autoradiographs for sections hybridized to the antisense RNA and to complementary sense RNA (as negative control) are shown in Figure 5. In Figure 5, "L" denotes lung and "Sk" denotes skin, and the two tissue sections shown are serial sections. Strong signals for VEGF-D

mRNA were detected in the developing lung and associated with the skin. No signals were detected using the control sense RNA.

In Figure 6, sagittal tissue sections were hybridized with VEGF-D antisense RNA probe and subsequently incubated with photographic emulsion, developed and stained. Microscopic analysis revealed that VEGF-D mRNA was abundant in the mesenchymal cells of the developing lung (Figure 6A-C). In contrast, the epithelial cells of the bronchi and bronchioles were negative, as were the developing smooth muscle cells surrounding the bronchi. The endothelial cells of bronchial arteries were also negative. In Figure 6A, the dark field micrograph shows a strong signal for VEGF-D mRNA in lung (Lu). Liver (Li) and ribs (R) are also shown. Figure 6B shows a higher magnification of the lung. The light field micrograph shows a bronchus (Br) and bronchial artery (BA). The black outline of a rectangle denotes the region of the section shown in Figure 6C but at a higher magnification. Figure 6C shows the epithelial cells of the bronchus (Ep), the developing smooth muscle cells (SM) surrounding the epithelial cell layer and the mesenchymal cells (Mes). The abundance of silver grains associated with mesenchymal cells is apparent. In Figure 6D, a dark field micrograph shows a limb bud. A strong signal was located immediately under the skin in a region of tissue rich in fibroblasts and developing melanocytes. The magnification for

Figures 6A and D is x40, for Figure 6B, it is x200 and for Figure 6C, it is x500.

5 The results presented here suggest that VEGF-D may attract the growth of blood and lymphatic vessels into the developing lung and into the region immediately underneath the skin. Due to the expression of the VEGF-D gene adjacent to the skin, it is considered that VEGF-D could play a role in inducing the angiogenesis that is associated with malignant melanoma. Malignant melanoma is a very highly vascularized tumor. This suggests that local inhibition of VEGF-D expression, for example using VEGF-D or VEGF receptor-2 or VEGF receptor-3 antibodies, is useful in the treatment of malignant melanoma. Other suitable inhibitors of VEGF-D activity, such as anti-sense nucleic acids or triple-stranded DNA, may also be used.

15 Example 4 Production of monoclonal antibodies that bind to
human VEGF-D

Monoclonal antibodies to VEGF-DANAC were raised in mice. VEGF-DANAC includes the amino acid sequence of the VHD of VEGF-D and is similar in sequence to all other members of the VEGF family. Therefore, it is thought that the bioactive portion of VEGF-D likely resides in the VHD. A DNA fragment encoding a truncated portion of human VEGF-D from residue 93 to 201, i.e. with the N- and C-terminal regions removed, was amplified by polymerase chain reaction (PCR) with *Pfu* DNA polymerase, using

as template a plasmid comprising full-length human VEGF-D cDNA. The amplified DNA fragment, the sequence of which was confirmed by nucleotide sequencing, was then inserted into the expression vector pEFBOSSFLAG (a gift from Dr. Clare McFarlane at the Walter and Eliza Hall Institute for Medical Research (WEHI), Melbourne, Australia) to give rise to a plasmid designated pEFBOSVEGF-D Δ N Δ C. The pEFBOSSFLAG vector contains DNA encoding the signal sequence for protein secretion from the interleukin-3 (IL-3) gene and the FLAG[®] octapeptide (Sigma-Aldrich). The FLAG[®] octapeptide can be recognized by commercially available antibodies such as the M2 monoclonal antibody (Sigma-Aldrich). The VEGF-D PCR fragment was inserted into the vector such that the IL-3 signal sequence was immediately upstream from the FLAG[®] octapeptide, which was in turn immediately upstream from the truncated VEGF-D sequence. All three sequences were in the same reading frame, so that translation of mRNA resulting from transfection of pEFBOSVEGF-D Δ N Δ C into mammalian cells would give rise to a protein which would have the IL-3 signal sequence at its N-terminus, followed by the FLAG[®] octapeptide and the truncated VEGF-D sequence. Cleavage of the signal sequence and subsequent secretion of the protein from the cell would give rise to a VEGF-D polypeptide which is tagged with the FLAG[®] octapeptide adjacent to the N-terminus. This protein was designated VEGF-D Δ N Δ C. VEGF-D Δ N Δ C was purified by anti-FLAG[®] affinity chromatography from the medium of COS cells which had been transiently transfected with

the plasmid pEFBOSVEGF-DANAC. (see Example 9 in International Patent Application No. PCT/US97/14696).

Purified VEGF-DANAC was used to immunize female Balb/C mice on day 85 (intraperitoneal), 71 (intraperitoneal) and 4 (intravenous) prior to the harvesting of the spleen cells from the immunized mice and subsequent fusion of these spleen cells to mouse myeloma P3X63Ag8.653 (NS-1) cells. For the first two immunizations, approximately 10 µg of VEGF-DANAC in a 1:1 mixture of PBS and TiterMax adjuvant (#R-1 Research adjuvant; CytRx Corp., Norcross, GA) were injected, whereas for the third immunization 35 µg of VEGF-DANAC in PBS was used.

Monoclonal antibodies to VEGF-DANAC were selected by screening the hybridomas on purified VEGF-DANAC using an enzyme immunoassay. Briefly, 96-well microtiter plates were coated with VEGF-DANAC, and hybridoma supernatants were added and incubated for 2 hours at 4°C, followed by six washes in PBS with 0.02% Tween 20. Incubation with a horse radish peroxidase conjugated anti-mouse Ig (Bio-Rad, Hercules, CA) followed for 1 hour at 4°C. After washing, the assay was developed with an 2,2'-azino-di-(3-ethylbenz-thiazoline sulfonic acid) (ABTS) substrate system (Zymed, San Francisco, CA), and the assay was quantified by reading absorbance at 405 nm in a multiwell plate reader (Flow Laboratories MCC/340, McLean, VA). Six antibodies were selected for further analysis and were subcloned twice by limiting

dilution. These antibodies were designated 2F8, 3C10, 4A5, 4E10, 4H4 and 5F12. The isotypes of the antibodies were determined using an Isostrip™ isotyping kit (Boehringer Mannheim, Indianapolis, IN). Antibodies 2F8, 4A5, 4E10 and 5F12 were of the IgG₁ class whereas 4H4 and 3C10 were of the IgM class. All six antibodies contained the kappa light chain.

Hybridoma cell lines were grown in DMEM containing 5% v/v IgG-depleted serum (Gibco BRL, Gaithersburg, MD), 5mM L-glutamine, 50 µg/ml gentamicin and 10 µg/ml recombinant IL-6. Antibodies 2F8, 4A5, 4E10 and 5F12 were purified by affinity chromatography using protein G-Sepharose according to the technique of Darby et al., J. Immunol. Methods, 1993 159 125-129, and the yield assessed by measuring absorption at 280nm.

Example 5 Use of monoclonal antibodies to human VEGF-D for immunohistochemical analysis of human tumors

In order to assess the role of VEGF-D in tumorigenesis, the above described MAbs were used for immunohistochemical analysis of a human malignant melanoma. Four VEGF-D MAbs, 2F8, 5F12, 4A5 and 4E10, were used for the analysis. A MAb raised to the receptor for granulocyte colony-stimulating factor, designated LMM774 (Layton et al., Growth Factors, 1997 14 117-130), was used as a negative control. Like the VEGF-D MAbs, LMM774 was of the mouse IgG₁ isotype and therefore served as an isotype-matched control antibody. The MAbs were tested against two randomly

chosen invasive malignant melanomas by immunohistochemistry. Five micrometer thick sections from formalin fixed and paraffin embedded tissue of the cutaneous malignant melanomas were used as the test tissue. The sections were dewaxed and rehydrated and then washed with PBS. Normal rabbit serum diluted 1:50 was applied to each section for 20 minutes. The excess serum was blotted off and the primary antibodies, i.e. the VEGF-D MAbs and LMM774 at crudely optimized dilutions of 1:100 and 1:200, were applied to the sections and incubated in a moist chamber at room temperature overnight. The sections were again washed in PBS for 5 minutes followed by the application of biotinylated rabbit anti-mouse antibodies (DAKO Corp., Carpinteria, CA) at a 1:400 dilution in PBS for 35 minutes at room temperature. The sections were then washed in tris buffered saline (TBS) for 5 minutes and then streptavidin-alkaline phosphatase (Silenus, Australia) was applied at a 1:500 dilution in TBS. The sections were washed in TBS for 5 minutes and the fast red substrate (Sigma, St. Louis, MO) was applied at room temperature for 20 minutes. The sections were washed in water and then mounted. The red reaction product was used to avoid confusion in interpretation of those tumors producing melanin. A step omission control, in which the VEGF-D MAbs were omitted, was included as were isotype-matched controls with the LMM774 antibody.

Figures 7A-C show results with the same melanoma sample whereas Figures 7D and 7E show results for a different tumor

stained in the same batch. Islands of immunoreactive melanoma cells are indicated by a one (1) inside the arrows in Figures 7A and 7B, and immunoreactive blood vessels are indicated a two (2) inside the arrows in 7C. Melanoma cells with varying levels of VEGF-D are apparent in 7E. The magnification in Figures 7A, 7D and 7E is approximately x60, and in Figure 7B and 7C, it is approximately x300.

Positive reactions were seen with all four VEGF-D MAb with essentially the same staining patterns. The results shown in Figures 7A-C and 7E were with MAb 2F8. Assessment of the staining patterns by light microscopic examination showed variable staining through the bulk of the melanomas. In the larger tumor, staining was more pronounced in small islands of tumor cells at the periphery of the invasive portions (Figures 7A and 7B) and in the intraepidermal nests of tumor cells, being less intense or undetectable in the central invasive portion of the tumor. Small capillary sized vessels in the papillary and reticular dermis adjacent to positive reacting tumor cells showed variable granular reaction to the antibodies in the cytoplasm of endothelial cells (Figure 7C). The reaction for the smaller tumor was more even in distribution throughout the tumor mass (Figure 7E). Blood vessels at a variable distance lateral to the tumor, and in the mid and deep reticular dermis and subcutaneous tissue away from the immunoreactive tumor cells did not show any reaction with the VEGF-D MAb. In contrast to the results with

the VEGF-D MAbs, the LMM774 control in the same tumor was negative (Figure 7D) as were the step omission controls.

It has been shown for some tumors that VEGF synthesis and secretion can be switched on in hypoxic tumor cells and that the tumor can also induce expression of VEGFR-2 in the endothelial cells of nearby blood vessels (Plate et al., Cancer Res., 1993 53 5822-5827). In this way a paracrine system is established for inducing tumor angiogenesis whereby VEGF, secreted in the tumor, diffuses through interstitium and binds to VEGFR-2 on target endothelial cells and thereby induces endothelial cell proliferation. The results for VEGF-D localization in melanomas indicate that VEGF-D may be fulfilling a similar function in malignant melanomas. The VEGF-D MAbs detected VEGF-D in melanoma cells in both clinical samples tested. These tumor cells are most likely producing VEGF-D. In addition, VEGF-D was detected on the endothelial cells of blood vessels in the vicinity of the producer tumor cells but not on more distant vessels. The VEGF-D is probably localized on these endothelial cells due to interaction with VEGFR-2, a receptor for VEGF-D which is often expressed on tumor blood vessels (Plate et al., Cancer Res., 1993 53 5822-5827). Further immunohistochemical analyses will be required to assess if VEGF-D is also localized on lymphatic vessels in the vicinity of the tumor. Such a scenario is feasible because lymphatic endothelial cells express VEGFR-3, a

high affinity receptor for VEGF-D (Joukov et al., The EMBO Journal, 1996 15 290-298).

The results indicate that melanoma cells can express the VEGF-D gene. Analysis of mouse embryos at post-coital day 15.5
5 by *in situ* hybridization showed expression of the VEGF-D gene immediately under the developing skin, in a region rich in developing melanocytes and fibroblasts (Example 3 and Figures 5 and 6). Therefore it may be that transformed melanocytes have re-acquired the capacity to express the gene for VEGF-D, as was
10 the case during embryogenesis. If events other than oncogenic transformation can induce VEGF-D gene expression in melanocytes, this protein could be involved in other types of skin disorders characterized by inflammation or proliferation of blood vessels and/or lymphatic vessels. In a therapeutic setting, the
15 application of VEGF-D in response to tissue damage may be useful for stimulating the growth of blood and lymphatic vessels adjacent to regenerating skin. Similarly, application of VEGF-D to stimulate angiogenesis and lymphangiogenesis is useful to enhance the success of skin grafting procedures. These are used
20 in the treatment of a variety of conditions such as burns and other traumatic injuries, in avoiding or reducing surgical scarring, in cosmetic surgery, and the like.

Example 6 Testing Antibodies for the Capacity to Bind to
VEGF-C

The enzyme immunoassay as described above was used to test the six VEGF-D MAb's for the capacity to bind to VEGF-CANAC. VEGF-CANAC consists of the VEGF homology domain of VEGF-C (residues 103 to 215) and is the region of VEGF-C which is most similar to VEGF-DANAC. VEGF-CANAC, to which a 6X histidine tag had been added at the C-terminus, was expressed in strain GS115 of the yeast *P. pastoris* using the expression vector pIC9 (Invitrogen, San Diego, CA) according to manufacturer's instructions and purified using Ni-NTA Superflow resin (QIAGEN, Valencia, CA). Of the six antibodies tested by this immunoassay, only 4E10 bound to VEGF-CANAC.

Example 7 VEGF-D is proteolytically processed in a similar fashion to VEGF-C

In order to investigate the proteolytic processing of VEGF-D, 293-EBNA cells were stably transfected with pVDApex Δ C, pVDApexFullNFlag, pVDApex Δ NAC (Example 1 and Figure 1) and pVDApexFullCFlag. These expression constructs encode VEGF-D Δ C, VEGF-DFullNFlag, VEGF-DANAC (Example 1 and Figure 1) and VEGF-DFullCFlag respectively (Figure 8). The VEGF-D structural domains are shown at the top of Figure 8. "SS" denotes the signal sequence for protein secretion, N-terminal pro and C-terminal pro denote the propeptides and VHD denotes the VEGF homology domain. Beneath are shown the characterized and putative proteolytic cleavage sites in VEGF-D marked by arrows. The potential N-linked glycosylation sites are marked with asterisks. The region of VEGF-D used as the immunogen to generate the A2 antiserum (described below) is shown by a black

bar. The bottom half of the figure shows the primary translation products for the VEGF-D derivatives expressed in 293-EBNA cells. For simplicity, the signal sequences for protein secretion have been omitted. The FLAG octapeptide epitope is denoted by an encircled "F". The plasmid pVDApexFullCFlag was constructed in a similar fashion as pVDApexFullNFlag (Example 1) except that the DNA for the endogenous VEGF-D signal sequence for protein secretion had been retained and the "Kozak" consensus sequence for translation initiation had been optimized which necessitated insertion of the three amino acids "A-R-L" immediately after the initiation codon of VEGF-D. This construct also encoded the amino acids "A-R-Q" followed by the FLAG octapeptide sequence at the C-terminus of the protein. Since the 293-EBNA cell line is capable of proteolytically processing VEGF-C (Joukov et al., EMBO J., 1997 16 3898-3911), this allows analysis of the VEGF-D derivatives derived from these transfected cells to be followed during cellular biosynthesis and processing.

The VEGF-D derivatives were purified from the conditioned medium of stably transfected 293-EBNA cells by affinity chromatography on M2 (anti-FLAG) gel (Sigma-Aldrich) and eluted using the FLAG[®] peptide according to the manufacturer. The FLAG[®] peptide was removed using a centrifugal concentrator (Amicon, Beverly, MA). Aliquots of the fractions eluted from the M2 affinity columns were analyzed by SDS-PAGE and silver staining or immunoblotted with the M2 antibody (Sigma-Aldrich) to confirm the identity of the purified species.

Analysis of the 293-EBNA cells expressing the various VEGF-D derivatives by SDS-PAGE show that the VEGF-D polypeptide is proteolytically processed. Purification using medium from 293-EBNA cells expressing VEGF-D_{FullN}Flag allowed specific analysis
5 of only those VEGF-D polypeptides with the FLAG octapeptide at the N-terminus or of derivatives bound covalently or non-covalently to the FLAG[®]-tagged polypeptides (Figure 8 and Figure 11).

The polyclonal antiserum designated A2 was raised in rabbits
10 against a synthetic peptide corresponding to the region of human VEGF-D from residues 190 to 205, KCLPTAPRHPYSIIRR (SEQ ID NO:3), which are in the VHD (SEQ ID NO:5 of PCT/US97/14696).

For the SDS-PAGE and Western Blot analysis, samples containing the purified VEGF-D derivatives were combined 1:1 with
15 2X SDS-PAGE sample buffer, boiled and resolved by SDS-PAGE (Laemmli, Nature, 1970 227 680-685). The proteins were then transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and non-specific binding sites were blocked by incubation in 3% BSA, 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.02% Tween 20.
20 Blots were then incubated with a 1:2000 dilution of A2 antiserum for 2 hours at room temperature or alternatively with the M2 (anti-FLAG) antibody as described by the manufacturer. After washing in buffer (3% BSA, 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.02% Tween 20) the blots were probed with anti-rabbit Ig
25 horseradish peroxidase (HRP) conjugate or anti-mouse Ig HRP

conjugate (Biorad, Hercules, CA) and developed using chemiluminescence (ECL, Amersham, UK).

Analysis of proteins secreted by cells expressing VEGF-
DFullNFlag by SDS-PAGE under reducing conditions and silver
5 staining revealed a species of approximately 53 kDa, the expected
size of unprocessed VEGF-D, as well as two polypeptides of
approximately 31 and 29 kDa (Figure 9A). The sizes of molecular
weight markers (in kDa) are shown to the left of each panel and
the positions of the VEGF-D derivatives (with molecular weights
10 in kDa) are marked by arrows to the right. This result is
consistent with proteolytic cleavage events occurring near the
C-terminus of the VHD. According to this model, the
approximately 53 kDa polypeptide would represent unprocessed
VEGF-D and the approximately 31 kDa polypeptide would consist of
15 the N-terminal propeptide and the VHD (i.e. lacking the C-
terminal propeptide). The expected size of a polypeptide
consisting of the N-terminal propeptide and the VHD is indeed
approximately 31 kDa because the VHD, which is glycosylated, was
shown previously to be approximately 21 kDa (Achen et al., Proc.
20 Natl. Acad. Sci. USA, 1998 95 548-553; Figure 12b of
PCT/US97/14696) and the expected size of the FLAG-tagged N-
terminal extension is approximately 10 kDa. If processing of
VEGF-D involves cleavage near the N-terminus of the VHD, in
addition to the C-terminus of the VHD, cells expressing VEGF-
25 DFullNFlag should also secrete a 10 kDa FLAG-tagged polypeptide
consisting only of the N-terminal extension. Although a 10 kDa
polypeptide was not detected among the VEGF-D derivatives

secreted by these cells as assessed by silver staining (Figure 9A), it was clearly detected by Western blot analysis of the same material using the M2 antibody (Figure 9B). The approximately 29 kDa polypeptide detected by silver staining was not detected
5 in the same sample by Western blot with the A2 polyclonal antiserum (Figure 9C) and therefore would represent the C-terminal propeptide. This was confirmed by N-terminal amino acid of this polypeptide which identified the N-terminal sequence as "SIQIPEED" (SEQ ID NO:4), which is immediately adjacent to the
10 predicted C-terminal cleavage site of the VHD based on comparison with VEGF-C. Therefore the C-terminal cleavage site in VEGF-D is located immediately after arginine 205 ("R↓SIQIPEED") (SEQ ID NO:5). It is most likely that this approximately 29 kDa polypeptide was present in the affinity-purified material because
15 of the interchain disulfide bonds between the - and C-terminal propeptides (see Figure 11 for a scheme for VEGF-D processing).

To further examine the possibility of proteolytic cleavage of VEGF-D near the N-terminus of the VHD, proteins secreted by 293-EBNA cells expressing VEGF-DAC were purified and analyzed as
20 above. The construct for VEGF-DAC drives expression of a VEGF-D derivative in which the C-terminal extension has been deleted and replaced with FLAG (Figure 8). Conditioned medium from these cells contained two FLAG-tagged polypeptides of approximately 31 and 21 kDa as assessed by silver staining (Figure 9D). This
25 result is consistent with an N-terminal cleavage event which occurs near the N-terminus of the VHD, approximately 10 kDa from the N-terminus of unprocessed VEGF-D. Thus the approximately

31 kDa polypeptide would consist of the N-terminal extension and the VHD, whereas the approximately 21 kDa polypeptide would consist of the VHD alone. Consistent with this model were the findings that the both the approximately 31 and approximately 21 kDa bands were detected by Western blot analysis with M2 antibody (Figure 9E). Also as expected, both bands were detected by Western blot analysis with the A2 antiserum (data not shown).

To determine the exact position of the N-terminal proteolytic cleavage site in VEGF-D, the approximately 21 kDa polypeptide purified from the supernatants of cells expressing VEGF-DAC was subjected to N-terminal amino acid sequencing. N-terminal amino acid sequencing of affinity-purified protein was carried out using a Hewlett-Packard Protein Sequencer, model G1000A (Hewlett-Packard, Palo Alto, CA). The N-terminal sequence of this polypeptide was heterogeneous. The predominant sequence, representing approximately 80% of the material began as "FAATFY" (SEQ ID NO:6) and a minor sequence, representing 10-15% of the material began with "KVIDEE" (SEQ ID NO:7). Thus, as expected, the N-terminus of the approximately 21 kDa polypeptide is located at about the same position as the N-terminus of the VHD. The major N-terminal cleavage site in VEGF-D is located immediately after arginine 88 ("R↓FAATFY") (SEQ ID NO:8) and the minor cleavage site is immediately after leucine 99 (L↓KVIDEE) (SEQ ID NO:9) (Figure 8).

Example 8 VEGF-DANAC exists predominantly in the form of a non-covalent dimer

In general, VEGF family members exist as disulfide-bonded homodimers. However, VEGF-CANAC exists predominantly in the form of a non-covalent dimer (Joukov et al., EMBO J., 1997 16 3898-3911). The mature form of VEGF-D, VEGF-DANpro Δ Cpro, is also not a disulfide-linked dimer because this polypeptide migrates almost identically under reducing and non-reducing conditions in SDS-PAGE. In order to test the nature of the mature form of VEGF-D, affinity-purified VEGF-DANAC was subjected to size exclusion chromatography. Size exclusion chromatography was carried out by loading the affinity-purified protein onto a TSKG2000SW (7.5 x 60mm Id) column (LKB Bromo, Sweden). The column was equilibrated with PBS. Proteins were eluted with a flow rate of 0.25 ml/min and 1 minute fractions collected. The protein elution was monitored at 215 nm. Three major peaks were eluted from the column with apparent molecular weights (shown above each peak in brackets) of 73 kDa (peak 1), 49 kDa (peak 2) and 25 kDa (peak 3) and the ratio of total protein in these peaks was estimated spectrophotometrically to be approximately 1: 2.1:0.9 (Figure 10A). The apparent molecular weights were determined using a calibration curve constructed from known proteins: bovine serum albumin dimer, bovine serum albumin, ovalbumin and trypsin inhibitor (Sigma Aldrich Pty Ltd, Australia). The fractions corresponding to these peaks were pooled, concentrated to 100 μ l using centrifugal concentrators and analyzed by SDS-PAGE under reducing conditions and silver stained (Figure 10B). Tracks 1, 2 and 3 correspond to protein from peaks 1, 2 and 3 respectively.

The position of the VEGF-DANAC subunit is shown in Figure 10B to the left and the positions of molecular weight markers (in kDa) are shown to the right.

5 The VEGF-DANAC subunit (approximately 21 kDa) was most abundant in peak 2, was easily detectable in peak 3 and was undetectable from peak 1 (Figure 10B). The predominant species in peak 1 was a 73 kDa protein which is a contaminant that is often detected in samples of protein purified by M2 affinity chromatography and which cannot be detected by Western blot
10 analysis with either M2 antibody or A2 antiserum (data not shown). The 73 kDa protein was also observed in control M2 affinity purifications using the supernatants from 293-EBNA cells which had been transfected with Apex-3 plasmid lacking sequence encoding VEGF-D (data not shown). The apparent molecular weights
15 determined from the size exclusion chromatography indicated that the proteins in peaks 2 and 3 were a VEGF-DANAC dimer and the VEGF-DANAC monomer respectively. Therefore, a non-covalent dimer, the subunits of which separate in SDS-PAGE under reducing or non-reducing conditions, was the predominant molecular species
20 in the affinity-purified preparations of VEGF-DANAC.

The capacities of the dimeric and monomeric forms of VEGF-DANAC to bind VEGFR-2 were assessed with fractions eluted from the column and assaying for the capacity to bind VEGFR-2 using the Ba/F3 cell bioassay described in International Patent
25 application PCT/US95/16755. The VEGFR-2-binding activity in peak 3 was approximately 2% of that in peak 2, indicating that the

VEGF-DANAC non-covalent homodimer is much more bioactive than the monomer. The VEGFR-2 binding activity in peak 1 was approximately 1% of that in peak 2, presumably reflecting a small amount of the VEGF-DANAC non-covalent homodimer in this peak. Clearly the dimeric form of VEGF-DANAC binds far better to VEGFR-2 than does the monomeric form.

The data presented in Example 6 demonstrates that VEGF-D is proteolytically processed and that the sites of proteolytic cleavage are similar in location, but not identical, to those in VEGF-C. The proteolytic processing is likely to be of considerable biological importance because different VEGF-D derivatives have different capacities for activating VEGF receptors. Whereas fully processed VEGF-D binds and activates both VEGFR-2 and VEGFR-3 (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553) the unprocessed form of VEGF-D activates VEGFR-3 but not VEGFR-2 (Figures 14 and 15 of PCT/US97/14696). Therefore step-wise proteolytic processing may be a way to regulate the receptor-binding specificity of VEGF-D *in vivo*.

Size exclusion chromatography also demonstrated that affinity-purified VEGF-DANAC is predominantly a non-covalent dimer but that a small proportion is monomeric. Only the dimeric form could strongly activate a chimeric receptor containing the extracellular domain of VEGFR-2. This finding was expected, given that activation of cell surface receptor tyrosine kinases involves receptor dimerization. Presumably the dimeric ligand provides two receptor binding sites per molecule whereas the

monomeric form provides only one. Thus the dimeric ligand can induce receptor dimerization but the monomeric ligand cannot.

A scheme for the processing of VEGF-D as carried out by 293-EBNA cells which would give rise to monomers and dimers is shown in Figure 11. Two distinct forms of unprocessed VEGF-D are secreted from the cell: a monomer (left side) and an anti-parallel disulfide-linked dimer with disulfide bridges between the - and C-terminal propeptides (right side). Arrows lead from the intracellular forms to the products of stepwise proteolytic processing at the - and C-termini of the VHD which ultimately give rise to mature forms of VEGF-D that consist of a non-covalent dimer and a monomer of the VHD. Analyses of VEGF-D derivatives from the cell lines described here suggest that cleavage of the C-terminal propeptide from the VHD is more efficient than cleavage of the N-terminal propeptide. For simplicity, not all possible derivatives arising from proteolytic processing are shown. In Figure 11, N-pro denotes N-terminal propeptide; C-pro, the C-terminal propeptide; VHD, the VEGF homology domain; grey boxes, non-covalent interactions between domains; -S-, intersubunit disulfide bridges; N-, the N-termini of polypeptides; and the arrowheads represent the approximate locations of proteolytic cleavage sites.

Example 9 VEGF-D and vascular permeability

Affinity-purified human VEGF-DANAC was tested for the capacity to induce vascular permeability using the Miles assay. The Miles assay (Miles, A.A. and Miles, E.M., J. Physiol., 1952

118 228-257) was performed using anesthetized guinea pigs. For quantitation of extravasation induced by permeability factors, the area of sample injection was excised and the Evans Blue dye extracted by a three day incubation in formamide at 42°C. The amount of dye extracted was quantitated spectrophotometrically by reading the absorbance of the samples at 620 nm. VEGF-DANAC was used because the VHD of human VEGF-C (VEGF-CANAC) is known to induce vascular permeability (Joukov et al., EMBO J., 1997 16 3898-3911). Purified mouse VEGF₁₆₄ was included as a positive control. As expected, mouse VEGF₁₆₄ strongly induced vascular permeability. The lowest concentration of mouse VEGF₁₆₄ which induced detectable vascular permeability was 60 ng/ml. Likewise, human VEGF-CANAC also induced vascular permeability, however the lowest concentration with detectable activity was 250 ng/ml. In contrast, VEGF-DANAC showed no activity, even at protein concentrations as high as 1 µg/ml. These results indicate that human VEGF-DANAC is not an inducer of vascular permeability in guinea pigs.

VEGF-D and VEGF-C are considered members of a sub-family of the VEGF family (Achen et al., Proc. Natl. Acad. Sci. USA, 1998 95 548-553) because of similarities in primary structure and receptor-binding specificity. The mechanisms of processing of these two molecules are similar, but not identical. However, these two growth factors exhibit differences in bioactivities as illustrated by the finding that VEGF-DANAC does not induce vascular permeability. In contrast, VEGF-CANAC does induce

vascular permeability although not as potently as VEGF (Joukov et al., EMBO J., 1997 16 3898-3911).

5 The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.

SEQUENCE LISTING

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<120> EXPRESSION VECTORS AND CELL LINES EXPRESSING VASCULAR
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MELANOMAS

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WO 99/33485

PCT/US98/27373

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WO 99/33485

PCT/US98/27373

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WO 99/33485

PCT/US98/27373

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1 5

WHAT IS CLAIMED IS:

1. A mammalian cell line stably expressing VEGF-D or a fragment or analog thereof having the biological activity of VEGF-D.
- 5 2. A mammalian cell line according to Claim 1, in which the VEGF-D produced by said cell line is linked to an epitope tag.
3. A mammalian cell line according to Claim 2, in which the epitope tag is FLAG[®], hexahistidine or I-SPY[™].
4. A mammalian cell line according to Claim 1, Claim 2 or
10 Claim 3 which is the 293-EBNA human embryonal kidney cell line.
5. A mammalian cell line according to Claim 1, Claim 2, Claim 3 or Claim 4 in which the VEGF-D expressed is VEGF-DFullNFlag, VEGF-DFullCFlag, VEGF-DANAC, or VEGF-DAC.
6. A mammalian cell line according to Claim 1 in which the
15 fragment of VEGF-D is a portion of VEGF-D from amino acid residue 93 to amino acid residue 201 (SEQ ID NO:1) of (SEQ ID NO:5 of PCT/US97/14696).
7. A mammalian cell line according to Claim 1 in which the
20 fragment of VEGF-D is a portion of VEGF-D from amino acid residue 93 to amino acid residue 201 (SEQ ID NO:1) of (SEQ ID NO:5 of PCT/US97/14696) linked to a FLAG[®] peptide.

PCT/US 98/27373
IPEA/US 22 JUL 1999

8. An expression vector comprising a sequence of human DNA encoding a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or a fragment or analog thereof having the biological activity of VEGF-D, inserted into a mammalian expression vector.

9. An expression vector according to Claim 8, wherein the expression vector is Apex-3.

10. An expression vector according to Claim 8 or Claim 9, wherein the expression vector is pVDApex Δ NAC.

11. An expression vector according to Claim 8 or Claim 9, further comprising a sequence encoding an affinity tag.

12. An expression vector according to Claim 11, wherein the affinity tag is FLAG[®], hexahistidine, or I-SPY[™].

13. A host cell transformed or transfected with vector according to Claim 8 or Claim 9 or Claim 10 or Claim 11 or Claim 12.

14. A method of making a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or a fragment or analog thereof having the biological activity of VEGF-D, comprising the steps of expressing an expression vector according to Claim 8 or Claim 9 in a host cell, and isolating the

PCT/US 98/27373

IPEA/US 22 JUL 1999

polypeptide from the host cell or from the host cell's growth medium.

15. A method according to Claim 14 in which the expression vector further comprises a sequence encoding an affinity tag.

16. A method according to Claim 15, in which the affinity tag is FLAG[®], hexahistidine or I-SPY[™].

17. A method of treatment or alleviation of malignant melanoma, comprising the step of administering an effective inhibitory amount of a VEGF-D antagonist to inhibit the expression or activity of VEGF-D in the vicinity of the melanoma.

18. A method according to Claim 17 in which the VEGF-D antagonist is an anti-sense nucleic acid or a triple-stranded DNA encoding VEGF-D.

19. A method according to Claim 17 in which the VEGF-D antagonist is a VEGF-D variant polypeptide.

20. A method according to claim 17 in which the VEGF-D antagonist is a composition of matter comprising an antibody or F(ab')₂, F(ab') or F(ab) fragment thereof specifically reactive with VEGF-D.

21. A method according to claim 20 in which the antibody is a monoclonal antibody, $F(ab')_2$, $F(ab')$, $F(ab)$ fragment or chimeric monoclonal antibody thereof.

22. A method of treatment or alleviation of a tumor which
5 expresses VEGF-D, comprising the step of administering an effective inhibitory amount of a VEGF-D antagonist in the vicinity of the tumor.

23. A method of treatment or alleviation of a tumor which
expresses VEGF-D, comprising the step of administering an
10 effective inhibitory amount of VEGF-D, or a fragment or analog thereof capable of binding to a VEGF-D receptor binding activity conjugated to toxins or drugs having tumor inhibiting activity.

24. A method of enhancing the acceptance and/or healing of a skin graft, comprising the step of administering an effective
15 angiogenesis and lymphangiogenesis stimulating amount of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

25. A method of stimulating the healing of a surgical or traumatic wound to the skin, comprising the step of administering
20 an effective angiogenesis and lymphangiogenesis stimulating amount of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

26. A method for regulating receptor-binding specificity of VEGF-D, comprising the steps of expressing an expression vector comprising a nucleotide sequence encoding an unprocessed VEGF-D and supplying a proteolytic amount of at least one enzyme
5 for processing the expressed unprocessed VEGF-D to generate a proteolytically processed form of VEGF-D.

27. A method for creating proteolytic fragments of VEGF-D, comprising the steps of expressing an expression vector comprising a nucleotide sequence encoding an unprocessed VEGF-D
10 and supplying a proteolytic amount of at least one enzyme for processing the expressed unprocessed VEGF-D to generate a proteolytically processed fragments of VEGF-D.

28. A method of detecting tumors expressing VEGF-D in a biological sample, comprising the steps of contacting said
15 sample with a specific binding reagent for VEGF-D, allowing time for a binding of said specific binding reagent to VEGF-D, and detecting said binding.

29. A method of detecting tumors expressing VEGF-D in a biological sample according to Claim 28, wherein said specific
20 binding reagent is a first antibody.

30. A method of detecting tumors expressing VEGF-D in a biological sample according to Claim 29, wherein the binding is detected by means of a second antibody with a detectable label.

31. A method of treatment or alleviation of lymphedema, comprising the step of administering an effective lymphangiogenesis stimulating amount of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

5 32. A method of treatment or alleviation of psoriasis, comprising the step of administering an effective inhibitory amount of an VEGF-D antagonist.

33. A method of treatment or alleviation of scleroderma, comprising the step of administering an effective endothelial
10 proliferation stimulating amount of VEGF-D, or a fragment or analog thereof having the biological activity of VEGF-D.

34. A method of treatment or alleviation of anhydrotic ectodermal dysplasia, comprising the step of administering an effective vascularization stimulating amount of VEGF-D, or a
15 fragment or analog thereof having the biological activity of VEGF-D.

35. A method for stimulating at least one bioactivity of VEGF-D selected from endothelial cell proliferation, migration, survival and differentiation, and lymphangiogenesis without
20 inducing vascular permeability, comprising the step of administering a bioactivity stimulating amount of fully processed VEGF-D.

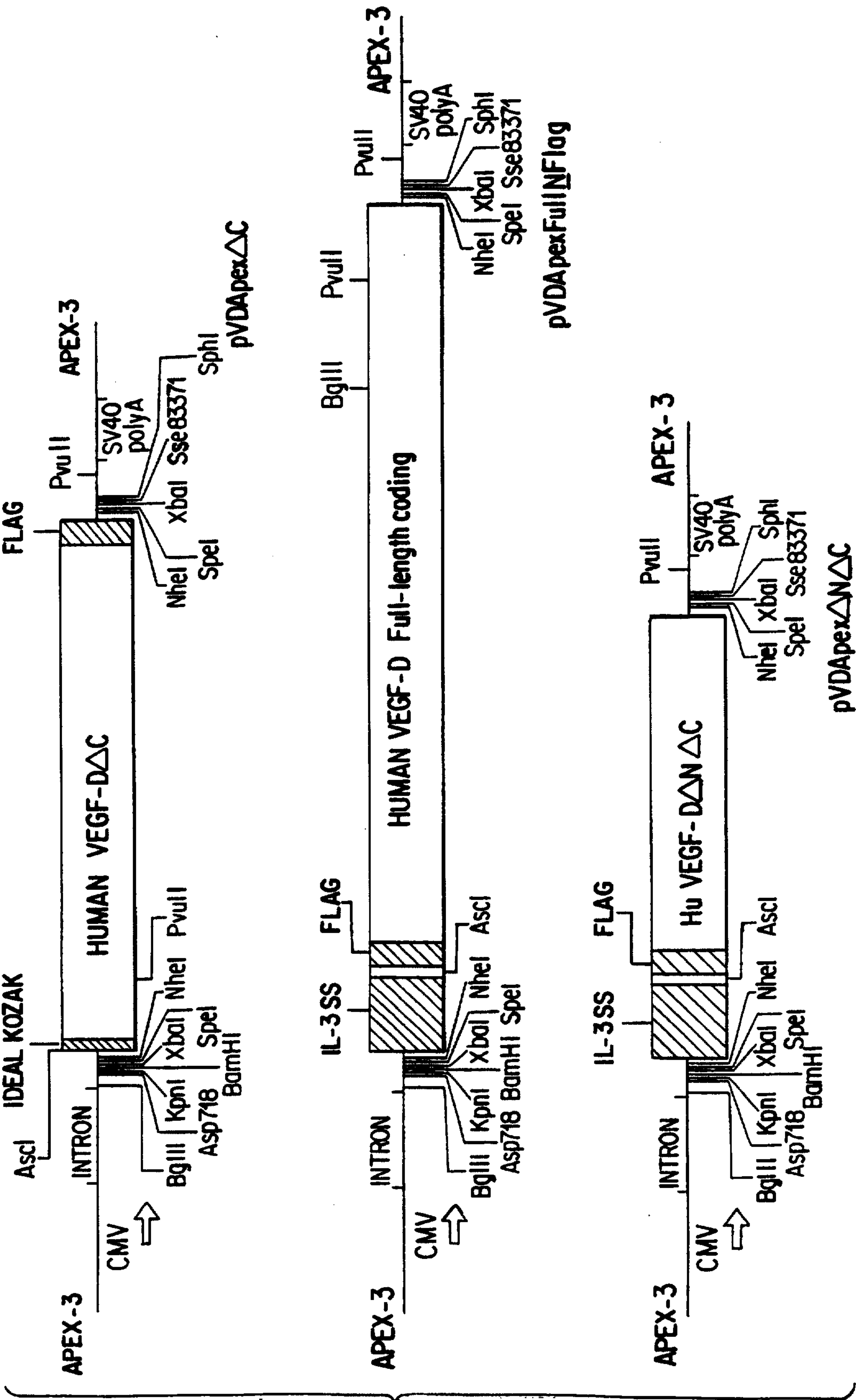
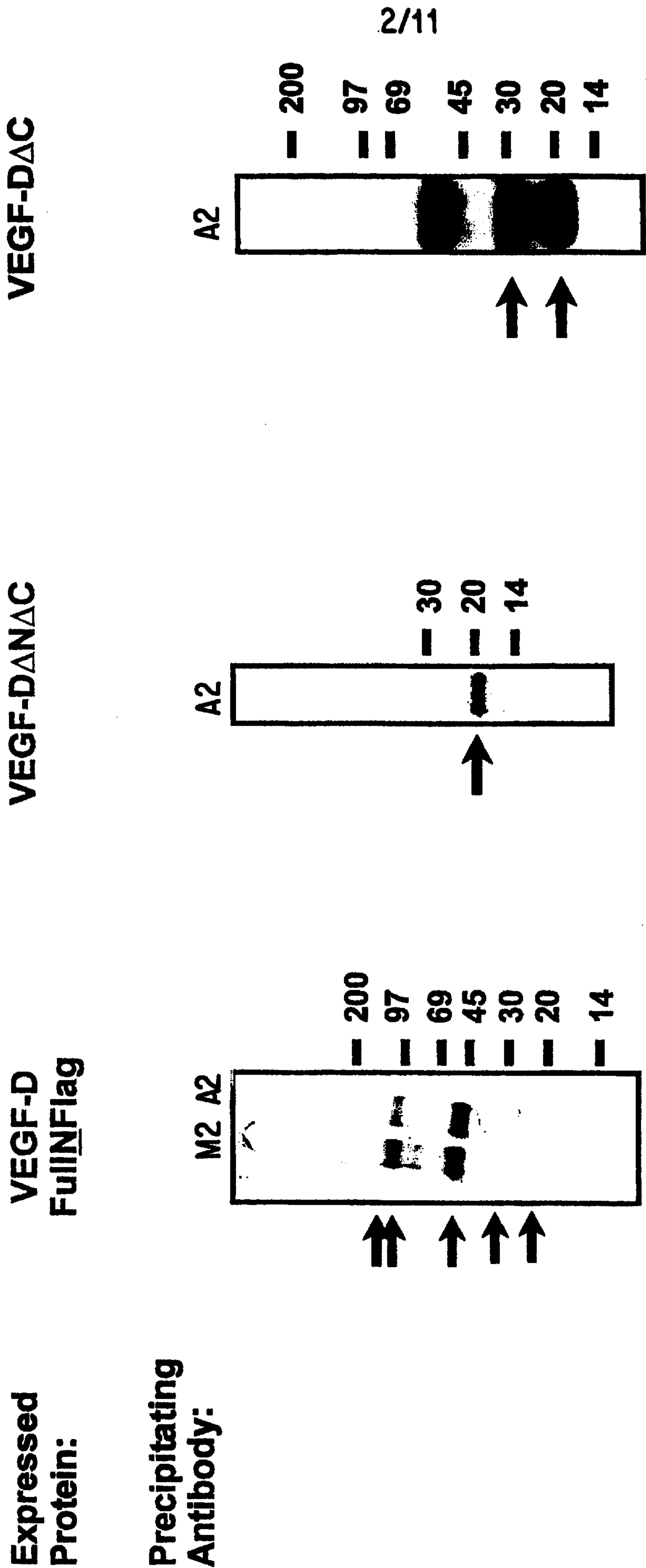


FIG. 1



3/11

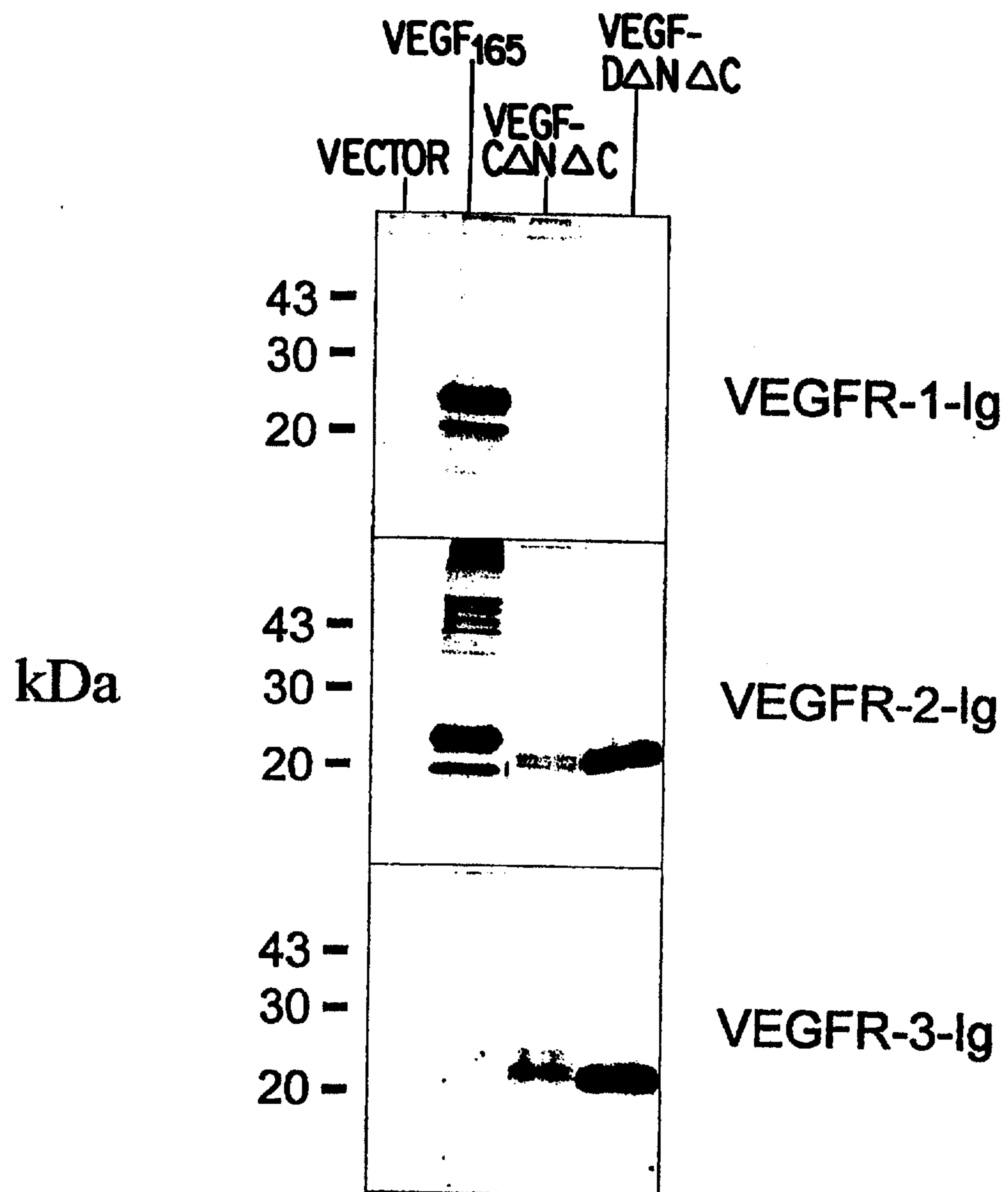


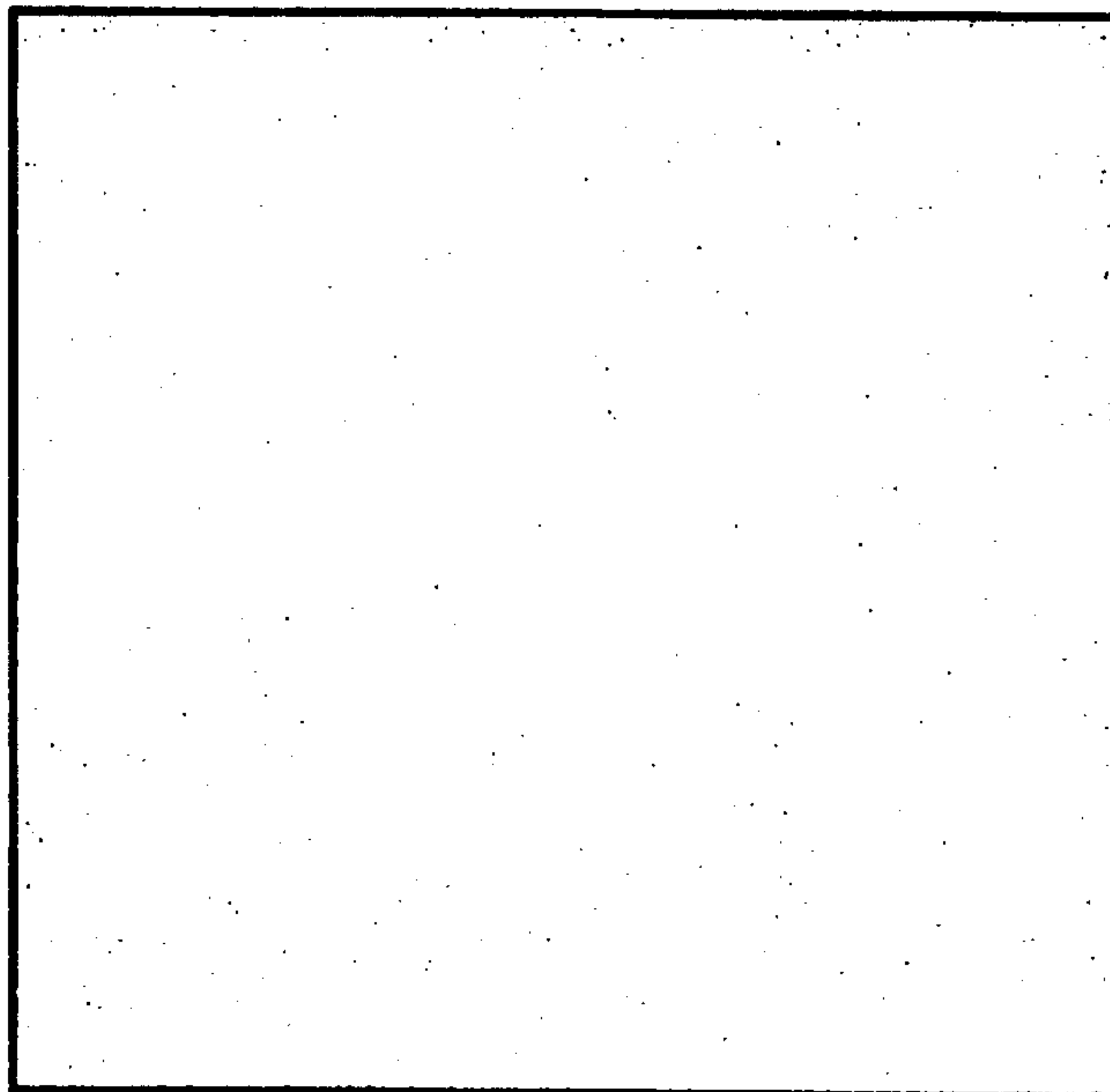
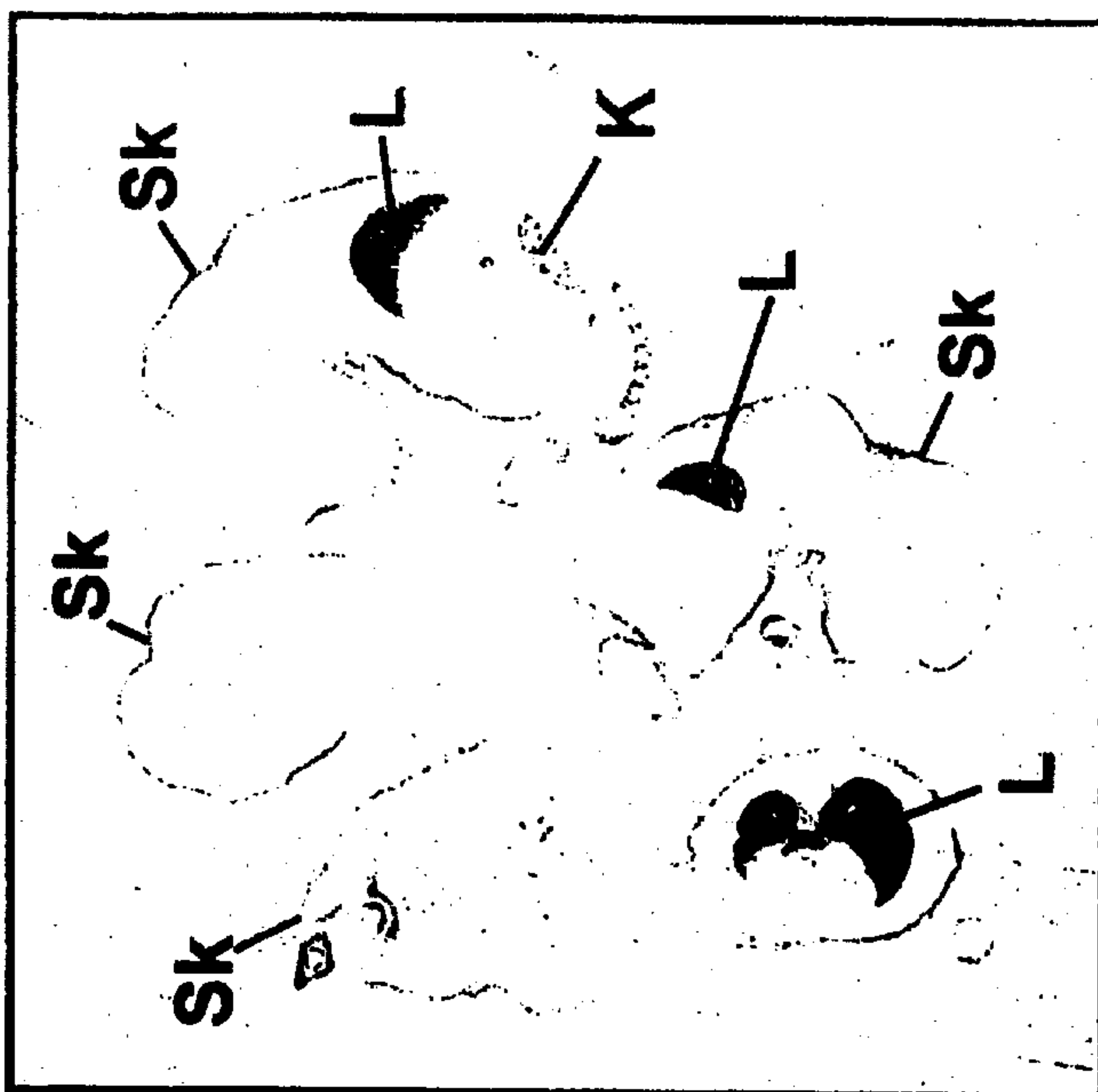
FIG. 3

4/11

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CATGTGCAAA CACAGACACA CACACACACA CACACACAGA GCAACTAGGA
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FIG. 4

5/11

VEGF-D Sense**VEGF-D Antisense****FIG. 5**

6/11

FIG. 6B

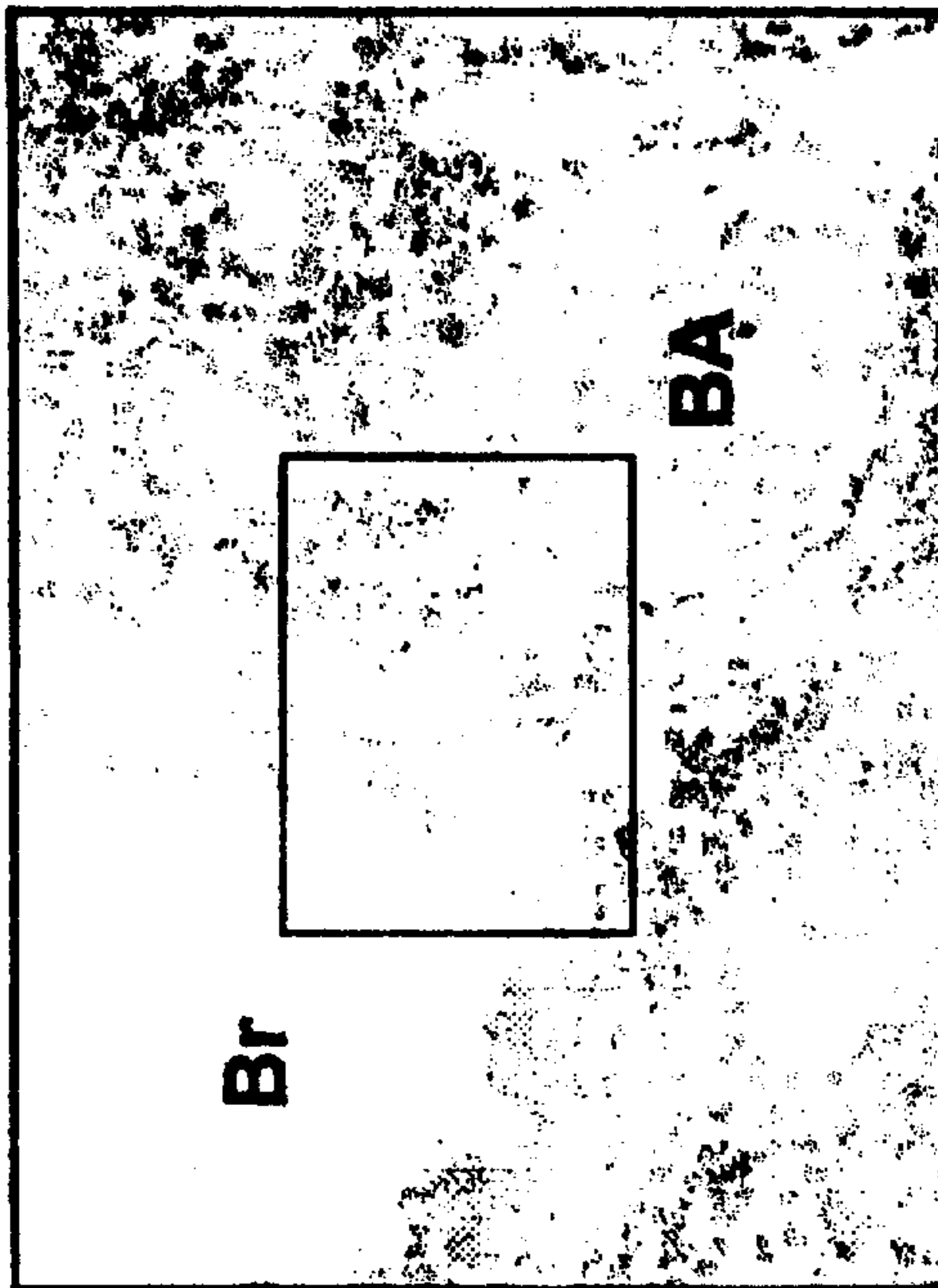


FIG. 6D



FIG. 6A



FIG. 6C



7/11

FIG. 7C

FIG. 7B

FIG. 7A



FIG. 7E

FIG. 7D

8/11

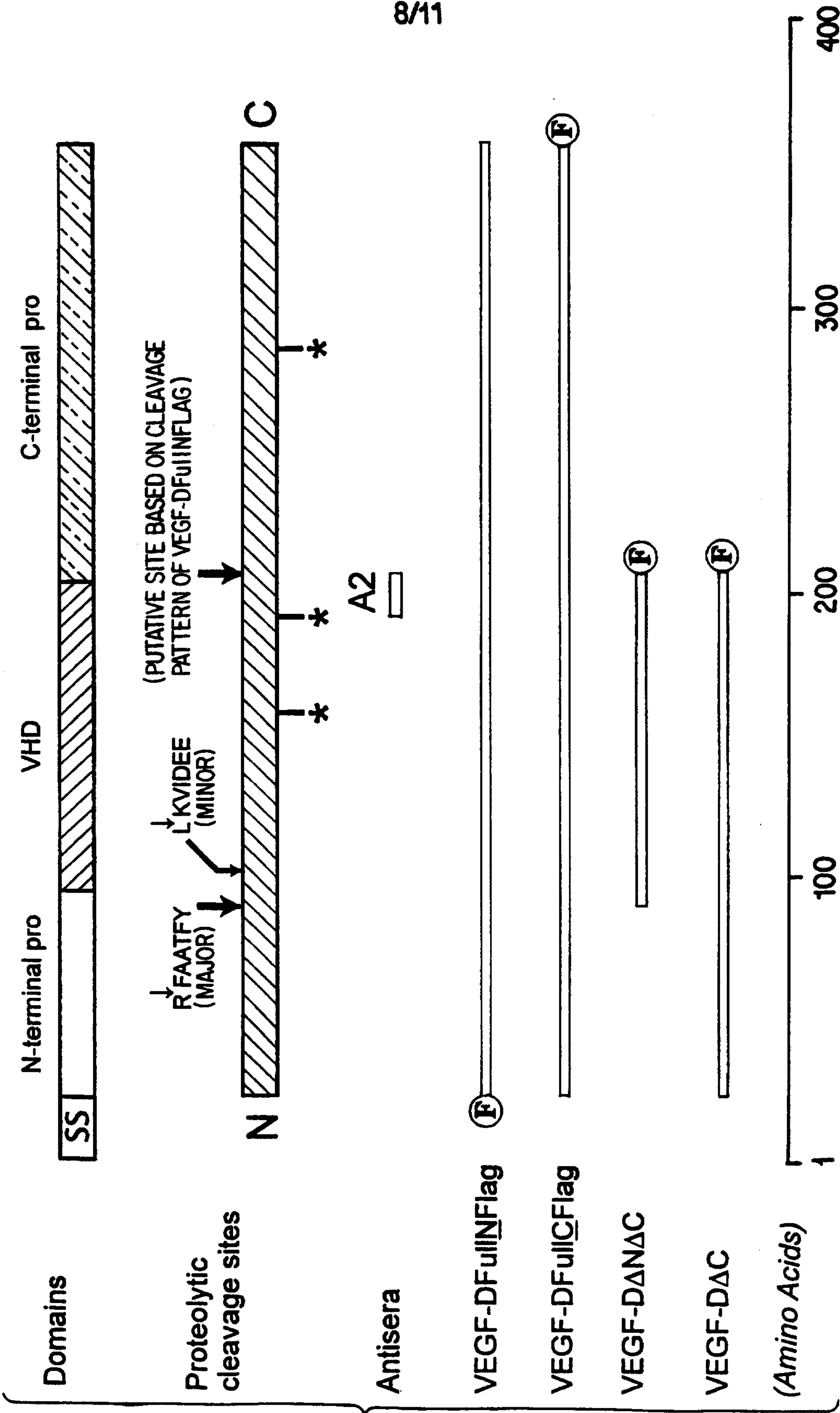


FIG. 8

VEGF-D Δ C

VEGF-DFuIIINFlag



10/11

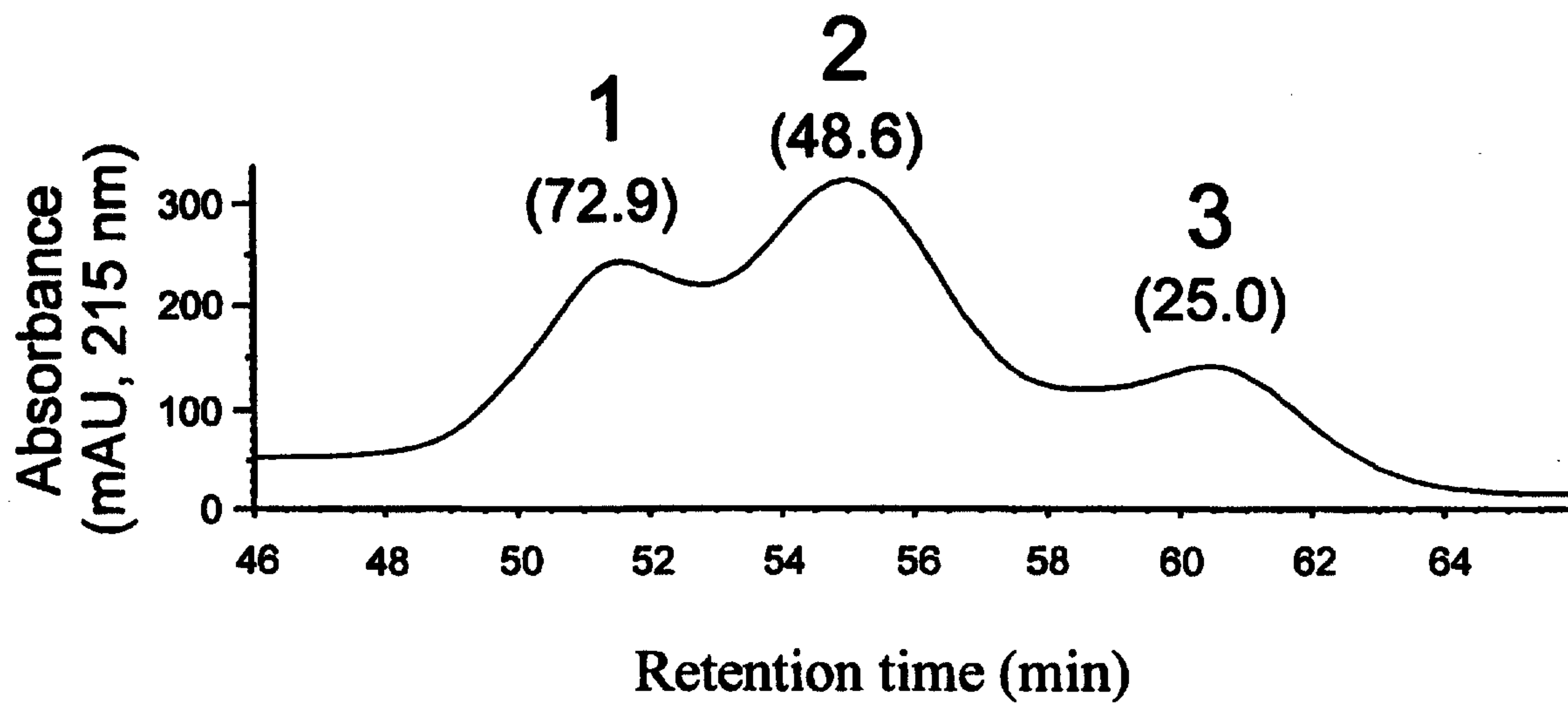


FIG. 10A

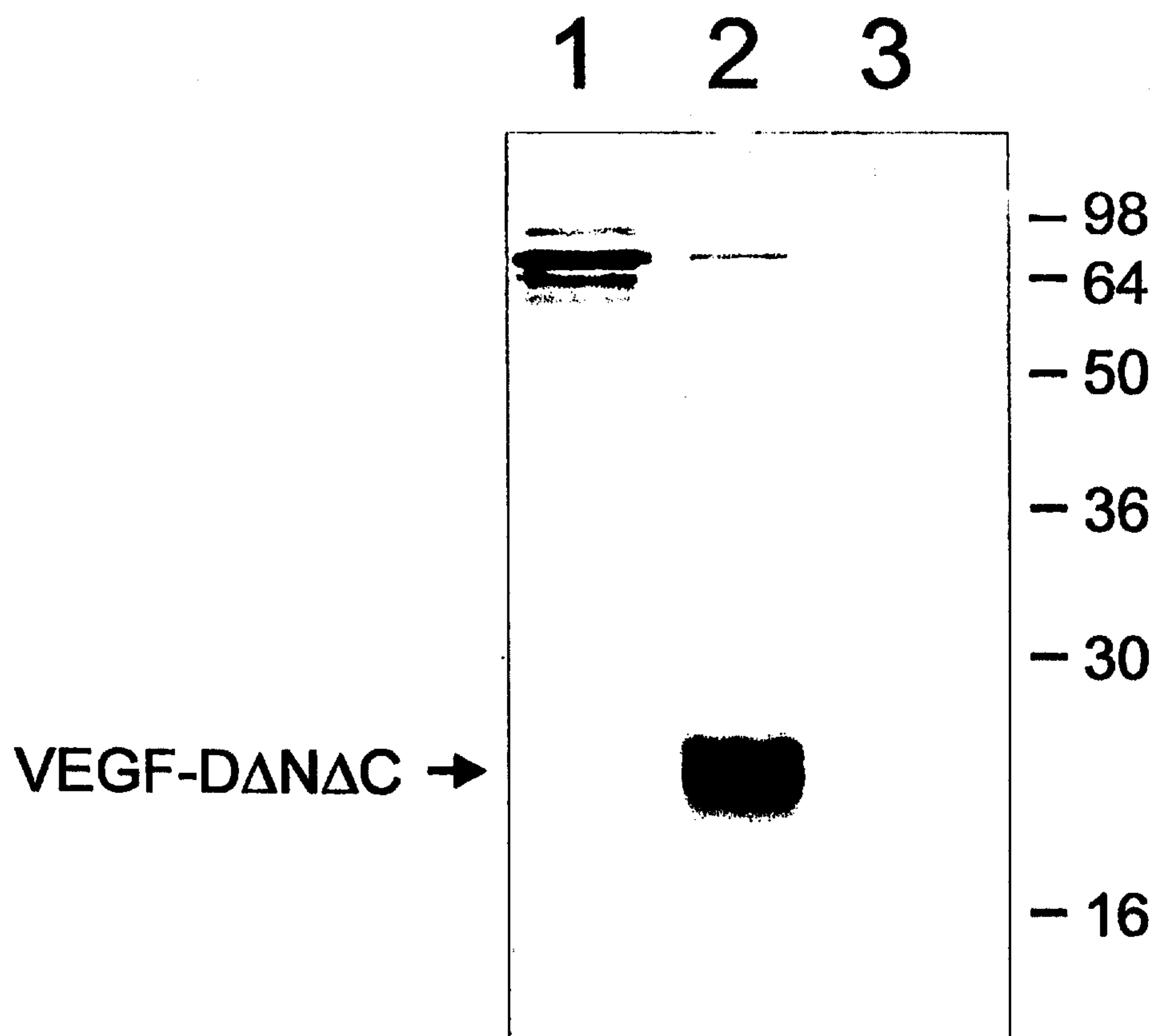
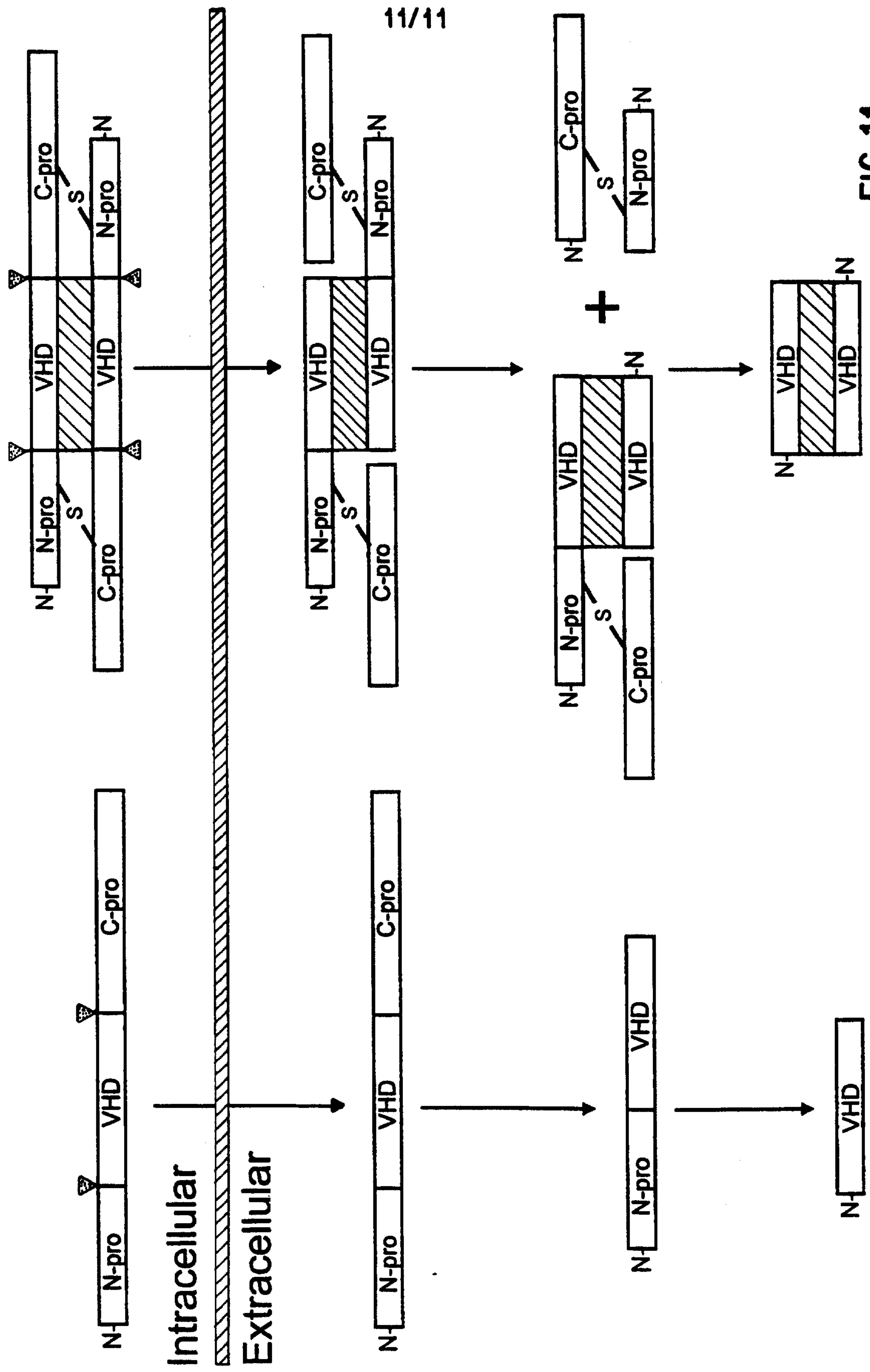


FIG. 10B



11/11

FIG. 11