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Angiogenin

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Synonym
DIP, degranulation inhibiting protein; RNase 5, pancreatic-type ribonuclease 5.

Definition
Ribonuclease with angiogenic activity

Introduction
Angiogenin is a plasma protein [1] with angiogenic [2] and ribonucleolytic [3] activities. It is the first angiogenic molecule to be isolated and characterised from a human tumour. The strategy of purification used by Professor Vallee and his co-workers was based on the observation by Professor Folkman that tumour growth depends on neovascularization, and their project was inspired by the hypothesis that anti-angiogenesis might be used as a therapeutic approach [4]. Angiogenin was originally purified from 2000 litres of serum-free medium conditioned by HT-29 human adenocarcinoma cells (yield: 500 ng/L), on the basis of its ability to induce neovascularization [2].
Angiogenin elicits new blood vessel formation in the chick chorioallantoic membrane, where only femtomole amounts were needed to induce angiogenesis [2], and in the rabbit cornea [2] and meniscus [5].
Angiogenin is also present in normal human tissues and fluids such as plasma [1, 6] and amniotic fluid [7]. Angiogenin is secreted in culture by vascular endothelial cells, aortic smooth muscle cells (SMC), fibroblasts and tumour cells [2, 8].
Angiogenin is a ribonuclease-related molecule. This cationic single-chain protein has 35% amino acid sequence identity with human pancreatic ribonuclease (RNase), many of the remaining residues being conservatively replaced [9]. Angiogenin also displays ribonuclease activity, albeit markedly different in both its specificity and magnitude [3]. Angiogenin has ribonucleolytic specificity for ribosomal and transfer RNA [3]. The limited cleavage of 18S rRNA seems to be responsible for the inhibition of cell-free protein synthesis by specific inactivation of the 40S ribosomal subunit [10]. Angiogenin blocks protein synthesis when injected into *Xenopus* oocytes [11]. Its physiological substrate remains to be identified. In addition, angiogenin binds to a protein RNase inhibitor (RI) first isolated from placenta, which abolishes both its ribonucleolytic and angiogenic activities ([12, 13], for reviews).
The integrity of the catalytic site and a cell-binding domain are required for its capacity to induce neovascularization [14, 15]. Indeed, angiogenin binds to high-affinity receptors on subconfluent endothelial cells [16-18], activates cell-associated proteases

[19, 20] and triggers several intracellular events [21, 22]. It has been reported to stimulate proliferation of endothelial cells [17, 18]. In aortic smooth muscle cells, angiogenin activates phospholipase C and induces cholesterol esterification [23]. Angiogenin binds copper [24]. The presence of copper, a modulator of angiogenesis *in vivo*, enhances angiogenin binding to endothelial cells *in vitro* [16, 24].
Angiogenin suppresses, *in vitro*, the proliferation of stimulated human lymphocytes [25] and, under the name DIP, at concentrations in the nanomolar range, inhibits degranulation of polymorphonuclear leukocytes (PMNL) [26].
Angiogenin supports the adhesion of endothelial cells, fibroblasts and tumour cells [27, 28].
Angiogenin is a heparin-binding protein [29].
Besides its angiogenic potency, the possible involvement of angiogenin in the development of tumours is suggested by the demonstration that angiogenin antagonists prevent the growth of human tumour xenografts in athymic mice [30]. In addition, angiogenin expression increases in pancreatic cancer [31] and serum angiogenin concentrations increase in cancer patients [31-35]. As angiogenin is not a tumour-specific product, these data point to potential modulatory mechanisms of angiogenin functions.

Characteristics

Molecular Weight

Calculated relative molecular mass are 14 124, 14 595, 14 059 and 14 362 for the human [1], bovine [36], pig and rabbit [37] angiogenins, respectively.

Domains

The similarity of angiogenin with ribonuclease has been used to define structure/function relationships through existing information on RNase. However, only angiogenin is able to induce angiogenesis, which suggests that its biological activities result from structural characteristics. A tremendous effort has been made, mainly by Professor Vallee's group, to identify the regions of the molecule that are critical for its activities. Domains or residues corresponding to those known to be important for the enzymatic activity of RNase have been modified chemically or by mutagenesis. Only part of this work is reported here. The reader is also referred to recent reviews [38, 39].

Receptor binding domains: The putative receptor binding domain (Figures 1, 2, 4; see conformation ß) includes two segments on adjacent loops (segment 58-70: loops 4 and 5 and ß strands B_2 and B_3; segment 107-110: loop 9) containing residues 60-68 [15] and Asn-109 [40] respectively. This location was deduced from studies on proteolysis [15], deamidation [40] and mutagenesis [41]. The segment 58-70 in angiogenin contains two fewer residues than RNase, and angiogenin lacks the two cysteines (replaced by Pro-64 and Leu-69 in human angiogenin) that form the disulphide loop in RNase A (positions 65 and 72) involved in purine binding. Replacement of the segment 58-70 by the corresponding sequence of RNase A by means of regional mutagenesis causes a reduction in angiogenic potency [41]. Substituting the surface loop of RNase A (residues 59-73) with residues 58-70 of angiogenin endows ribonuclease with angiogenic activity [42]. Furthermore, peptide ANG(58-70) inhibits endogenous angiogenesis in mice [42]. The segment of bovine angiogenin includes an Arg-Gly-Asp sequence (67-69) which is replaced by Arg-Glu-Asn in the human molecule. Arg-66

has been identified as an essential component of this site, as its mutation reduces the angiogenic potency of angiogenin [43].
Angiogenin inhibits the degranulation of PMNL [26]. A similar but weaker inhibitory effect has been obtained with the synthetic peptide Leu^{83}-His-Gly-Gly-Ser-Pro-Trp-Phe-Phe-Cys-Gln-Tyr-Arg^{95}, which might be the domain of angiogenin involved in this biological activity. Indeed, the segment 83-95 includes loop 7 (connecting ß sheets B_4 and B_5), a region that diverges from that of RNase [44]. Moreover, this domain is exposed to solvent, and nuclear magnetic resonance (NMR) studies of bovine angiogenin have shown increased disorder in this domain, which is likely to reflect greater flexibility [45].
Epitope: Loop 7 (residues 85-92) forms part of the epitope for a monoclonal antibody that has been shown to prevent the establishment of human tumours in athymic mice [30].

Cell adhesion domains: The endothelial cell adhesion domain might include the Arg^{67}-Gly-Asp^{69} segment of bovine angiogenin, which is replaced by Arg^{66}-Glu-Asn^{68} in human angiogenin [27]. Although these segments do not have a conformation typical of an integrin recognition site when analysed by X-ray crystallography [46], in solution the Arg-Gly-Asp sequence of bovine angiogenin forms a short loop at the apex of the B_2-B_3 sheet exposed to solvent, with signs of flexibility in proton NMR. These characteristics are shared with many integrin binding proteins [45]. Human angiogenin also supports human adenocarcinoma HT-29 cell adhesion. The domains involved might include Arg^{31}-Arg-Arg^{33}, Arg-^{66} and Arg^{70}, as mutation of these basic amino acids decreases the capacity of angiogenin to mediate cell adhesion [28].
The heparin-binding domain includes the segment Arg^{31}-Arg-Arg^{33} and Arg^{70} [29].
Nuclear localisation signal: Segment Arg^{31}-Arg-Arg-Gly-Leu^{35} of human angiogenin has been identified as the nuclear localisation signal responsible for nucleolar targeting of angiogenin in calf pulmonary and human umbilical artery endothelial cells. Arg-33 is the essential amino acid [47].

Actin-binding domain: The actin-binding domain of angiogenin might involve the segment 60-68, as the two derivatives, cleaved at residues 60-61 and 67-68 respectively, fail to bind to angiogenin-binding protein [48], a dissociable cell-surface component of endothelial cells and a member of the actin family [49].

Enzymatic active site: As in RNase A, the ribonucleolytic active site of angiogenin consists of several subsites [44]. The catalytic centre (P_1), at which phosphodiester bond cleavage occurs, involves the three catalytic residues: His-13, Lys-40 and His 114. The B_1 site, for binding the pyrimidine, whose ribose donates its 3' oxygen to the scissile bond, corresponds to Gln-12, Thr-44, Ser-118; and the B_2 site, that preferentially binds a purine, corresponds to Glu-108. The side-chain of Gln-117 forms two hydrogen bonds with Thr-44 and obstructs the pyrimidine binding site B_1. Ile-129 and Phe-120, in the middle of the C-terminal helix, make intramolecular hydrophobic interactions that stabilise the conformation. The terminal segment of angiogenin, Arg^{121}-Arg-Pro^{123}, does not seem to form contacts with the rest of the molecule and might be a subsite for binding polymeric substrates [50]. Thus, the C-terminal region might play a key role in angiogenin activity. The C-terminal peptide ANG(108-121) inhibits both the enzymatic and angiogenic activities of angiogenin. It is devoid of activity by itself.

The N-terminal region of angiogenin ANG(1-21) is highly conserved in angiogenins from five mammalian species and differs from the corresponding region of RNase A. It might be involved in the biological activities of angiogenin (Figure 4).

RI binding site: Human angiogenin binds human placental RNase inhibitor with the extremely low Ki value of 0.7 fM ([12, 13], for reviews). The tight interaction results from a large contact surface involving 24 residues distributed over seven segments of angiogenin [51]. The domains of the molecule involved in this complex include the catalytic site (mostly residue Lys-40) and the surface loop 84-89 [12, 13, 51].

Binding Sites and Affinity

Cell-surface receptors: Specific binding sites of angiogenin are present on endothelial cells from calf pulmonary artery, and bovine aorta, cornea and adrenal cortex capillary [16], bovine brain capillary [17], human umbilical artery and vein, and human foreskin dermal tissue [18]. They were not detected on Chinese hamster lung fibroblasts [16]. Cell binding of iodinated angiogenin at 4°C is time- and concentration-dependent, reversible, saturable and specific, whereas iodinated RNase does not bind to endothelial cells [16, 17]. Scatchard analyses of binding data reveal two apparent types of specific interaction with CPAE cells. High-affinity binding sites with an apparent dissociation constant (Kd_1) of 1-5 x 10^{-9} M bind about 2 x 10^5 molecules per cell [16, 24]. Low-affinity/high-capacity binding sites with a Kd_2 of 0.2 x 10^{-6} M are associated with pericellular components and bind several million molecules [16, 24]. Angiogenin that is bound with low affinity to extracellular matrix and cells is released by a wash at 0.6 M NaCl. On bovine brain capillary endothelial cells, high-affinity binding sites with an apparent dissociation constant of 0.5 x 10^{-9} M bind about 11 x 10^3 molecules of bovine angiogenin per cell [17]. The high-affinity binding sites are likely cell-surface receptors, as angiogenin triggers a series of intracellular events when added at 1 ng/ml to cultured CPAE cells [21], and a concentration of 100-1000 ng/ml enhances [^{3}H]thymidine incorporation and proliferation in human umbilical venous and microvascular endothelial cells by up to 50% [18]. Furthermore, bovine angiogenin at 100 ng/ml induces [^{3}H]thymidine incorporation and proliferation in bovine brain capillary endothelial cells [17].

Density-dependent regulation of angiogenin receptors has been observed on endothelial cells. High-affinity binding sites decrease as cell density increases [16, 17] and are not detected at confluence [16, 18].

Characterisation of angiogenin binding sites on bovine brain capillary endothelial cells by ligand-blotting of iodinated-angiogenin to solubilized membrane proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose identifies a labelled component with an apparent molecular mass of 49 kDa [17]. In contrast, affinity labelling of human umbilical venous, artery and microvascular endothelial cells with iodinated angiogenin using the homobifunctional cross-linker bi(sulfosuccinimidyl)suberate yields a cross-linked band of 180 kDa on SDS-PAGE autoradiography [18]. When biotinylated surface molecules are purified by affinity on an angiogenin-Sepharose column, and the final product is analysed by SDS-PAGE and western-blotting with alkaline phosphatase-labelled streptavidine, a band is detected at 170 kDa [18].

Among the factors that modulate angiogenesis, protamine, an inhibitor of angiogenesis, competes for angiogenin binding to CPAE cell receptors, whereas heparin interacts to a lesser extent [16]. Placental RI, a tight-binding inhibitor of both the ribonucleolytic and

angiogenic activities of angiogenin ([12, 13], for reviews), abolishes its binding to CPAE cells at a molar ANG/placental RI ratio of 1[16]. Cu^{2+}, a modulator of angiogenesis *in vivo*, increases by 4-fold the number of angiogenin molecules bound to high-affinity receptors on CPAE cells at saturation, at a concentration of 0.1 mM [16, 24]. Specific binding is also increased in the presence of Zn^{2+}, whereas Ni^{2+}, Co^{2+} and Li^{+} have no effect. Specific angiogenin binding to the low-affinity/high-capacity sites is increased about 2-fold in the presence of Cu^{2+} and Zn^{2+} respectively [24]. Furthermore, in the presence of Cu^{2+} or Zn^{2+}, no inhibitory effect of RI on cell binding was observed. Metal ions do not irreversibly inactivate placental RI, as their action can be reversed by adding diethyldithiocarbamate, a metal chelator [16, 52].

Copper binding: Metal ion affinity chromatography and atomic absorption spectrometry have been used to show the direct interaction of angiogenin with copper and zinc ions. Angiogenin binds 2.4 mol of copper per mol protein, *in vitro* [24]. Cu^{2+} inhibits angiogenin-catalysed cleavage of tRNA, with an IC_{50} of 0.03 mM [53].

Interaction with PMNL: Angiogenin inhibits granule discharge from PMNL at concentrations between 7 and 70 nM [26].

Cell adhesion: Angiogenin supports the adhesion of endothelial cells, fibroblasts [27] and tumour cells [28] when coated at concentrations $\geq$ 100 ng/cm^2. It has no effect on cell adhesion when in solution. Both human and bovine angiogenins promote adhesion of CPAE cells and Chinese hamster lung fibroblasts, whereas RNase A does not. Endothelial cell adhesion on bovine and human angiogenin is inhibited by the peptide Arg-Gly-Asp-Ser. Adhesion of endothelial cells is Ca^{2+} and Mg^{2+}-dependent but the addition of collagens I and IV, fibrinogen, fibronectin or vitronectin in solution at concentrations up to 0.01 mg/ml has no effect [27]. In contrast, adhesion of HT-29 human adenocarcinoma cells on human angiogenin is not inhibited by the Arg-Gly-Asp-Ser peptide, does not require Ca^{2+} or Mg^{2+} but is affected by heparin, which has no effect on endothelial cell adhesion at concentrations up to 0.01 mg/ml [28]. Platelet factor-4 also reduces tumour cell adhesion by 60% at 0.01 mg/ml, but protamine has no effect at concentrations up to 0.5 mg/ml. Finally, placental RI prevents tumour cell adhesion. Adhesion of tumour cells to angiogenin is sensitive to heparinase and heparitinase treatment, and a cell-surface proteoglycan of apparent molecular mass > 200 kDa has been isolated by angiogenin-affinity chromatography [28].

Heparin: Angiogenin binds to heparin-Sepharose and is eluted by 0.78 M NaCl [28]. The stoichiometry of the angiogenin-heparin interaction, estimated by light-scattering measurements, is 1 heparin chain (molecular mass 16.5 kDa):$\approx$ 9 angiogenin molecules [29]. Heparin partially protects angiogenin from tryptic cleavage at Arg-31, Lys-60 and Arg-101 [29]. It inhibits angiogenin-catalysed cleavage of tRNA at pH 5.5 with an IC_{50} value of 0.7 mg/ml [53]. Adhesion of HT-29 human adenocarcinoma cells to human angiogenin is reduced by 60% in the presence of 0.05 mg/ml heparin [28]. The minimum heparin fragment required for inhibition is the dodecamer [29].

Actin: An angiogenin-binding protein of 42 kDa is released from CPAE and GM7373 fetal bovine aortic endothelial cells by incubating starved subconfluent monolayers with 1 mg/ml heparan sulphate. It has been cross-linked to ^{125}I-angiogenin in a crude cell lysate and in heparan sulphate-released material by using 10 mM EDC (1-ethyl-3 (3-dimethylaminopropyl) carbodiimide). The formation of a 58-kDa complex is inhibited

by a 50-fold molar excess of either bovine or human unlabelled angiogenin, and is reduced by a factor of 3.4 in the presence of RNase A. Pre-incubation of ^{125}I-angiogenin with placental RI prevents the formation of the complex, whereas co-incubation does not [48]. Among the angiogenin-binding molecules obtained by a purification procedure comprising angiogenin-affinity chromatography, a 42-kDa protein has been purified and further identified as a member of the actin family [49]. A 42-kDa band has also been revealed by immunoblotting with a monoclonal antibody to smooth-muscle alpha-actin of CPAE cell-surface proteins selected by biotinylation of the cell surface and further isolation by avidin affinity chromatography. In addition, immunoreactivity has been detected at the surface of CPAE cells by immunofluorescence with a monoclonal antibody specific for smooth muscle alpha-actin. Angiogenin binds to bovine muscle actin and induces its polymerisation *in vitro*. The cross-linking of ^{125}I-angiogenin to actin is inhibited by platelet factor-4 (400 ng/ml) and protamine (0.01 mg/ml) [49]. Finally, both actin at a 100-fold molar excess and anti-actin antibody at a 10-fold molar excess inhibit the angiogenesis induced by 10 ng of bovine angiogenin in the chick chorioallantoic membrane assay [49].

Ribonuclease inhibitor: Placental RI, a tight-binding inhibitor of both the ribonucleolytic and angiogenic activities of angiogenin ([12, 13], for reviews), abolishes angiogenin binding to CPAE cells [16] and angiogenin-induced increase of diacylglycerol in cultured CPAE cells [21]. It also prevents tumour cell adhesion to angiogenin [28]. The stoichiometry of binding between angiogenin and placental RI is 1:1. The apparent association rate constant of 1.8×10^{8} $M^{-1}s^{-1}$ and the dissociation rate constant of the complex of $1.3\ 10^{-7}$ s^{-1} result in an extremely low calculated Ki of 7.1×10^{-16} M. The half-life of the complex is about 60 days. Inhibition is competitive and reversible, and 1 mM p-(hydroxymercuri) benzoate dissociates the complex to yield active angiogenin. The human RI gene (RNH) is located on the terminal part of the short arm of chromosome 11, subband 11p15.5, within 90 kb of the Harvey-ras protooncogene (HRAS). Human placental RI is an acidic protein (pI 4.7) composed of 460 amino acids with a calculated molecular mass of 49 847 Da; it is detected in western blot analysis as a single polypeptide chain of 51 kDa ([12, 13], for reviews). Human RI has a high leucine (92 residues, 20%) and cysteine (32 residues, 7%) content organised in fifteen alternate homologous leucine-rich repeats. Each repeat corresponds to a single right-handed ß-α structural unit [54]. The amino acid sequences of RIs are highly conserved, as the human, pig and rat species are 75-77% identical, with no insertions or deletions, except for a short insertion at the N-terminus of human RI. Crystal structure studies of porcine RI at a resolution of 2.5 Å reveals a horseshoe structure with overall dimensions 70 Å x 62 Å x 32 Å (Figure 3, [54]). The 16 helices align on the outer circumference and 17 ß-strands form a curved parallel ß-sheet on its inner circumference exposed to the solvent. Extensive mutagenesis studies of the human RI-angiogenin complex [12] and X-ray crystallographic analysis at 2.0 Å reveal that the tight interaction results from a large binding interface involving 26 human RI residues from 13 of the 15 repeat units of RI and a total of 124 contacts [51]. The interaction is predominantly electrostatic with a high chemical complementarity, mainly in the C-terminal segment 434-460 of human RI. Angiogenin binds to RI with its catalytic site covering the C-terminal part of the inhibitor; the ε-amino group of Lys-40 forming two salt bridges with the carboxylate of Asp-435. About one-third of angiogenin is located inside the central cavity of RI and the other part of the molecule lies over it. Finally, the complex crystallises as a dimer [51]. RI mRNA is ubiquitously expressed at ≈ 2 kb in the 16 normal human tissues so far tested [55].

Immunoreactivity, using a monoclonal antibody against placental RI, has been detected in normal human serum and would correspond to 2-3 mg/L, but there is no evidence that an active form is present. The absence of disulphide bonds in RI is consistent with its cytoplasmic location; it is irreversibly inactivated by sulfhydryl reagents.

RNA: Angiogenin has the same general catalytic properties as RNase A. It cleaves RNA preferentially on the 3' side of pyrimidine by a transphosphorylation/hydrolysis mechanism. However, its activity differs markedly in both its magnitude and specificity [3, 41]. Angiogenin is 10^5 to 10^6-fold less active than RNase A on mono-, di- and polynucleotide substrates [41, 53]. It is inactive on various dinucleotides and polynucleotides used as substrates for RNase [3]. The order of reactivity for angiogenin is CpA>CpG>UpA>UpG [41]. The base-cleavage specificity towards RNA has been determined with *Saccharomyces cerevisiae* and *Escherichia coli* 5S RNAs [56]. Like RNase A, phosphodiester bond cleavage occurs at the 3' side of cytidylic or uridylic acid residues when the pyrimidine is followed by adenine, but not at all the potential cleavage sites [56]. Angiogenin cleaves 28S and 18S rRNAs to give a mixture of products 100 to 500 nucleotides long. The overall pattern is different from that of RNase, and it requires 10^4-10^5 as much angiogenin to degrade the same amount of rRNA as with RNase A [3, 14]. Like RNase A, angiogenin prefers single-stranded RNA as substrate. It does not cleave single-stranded DNA. However, angiogenin has been reported to bind to single-stranded DNA, double-stranded plasmid DNA and high-molecular-weight DNA [56]. The optimal parameters for yeast tRNA cleavage by angiogenin are pH ≈ 6.8, 15-30 mM NaCl, and ≈ 55°C [53].

Additional Features

Both the ribonucleolytic and angiogenic activities of angiogenin are abolished by reagents that modify histidine, lysine, or arginine residues, but not those that modify tyrosine, aspartate, glutamate or methionine residues. The activities are also abolished by reduction of the three disulphide bonds, and are restored by reoxidation [14].

Fluorescence changes on binding of angiogenin to placental RI: Placental RI has six tryptophans and a fluorescence spectrum maximum at 338 nm. Human and rabbit angiogenins with one tryptophan (Trp-89) exhibit a weaker fluorescence spectrum with a maximum at 343 nm when excited at 285 nm. An enhancement of fluorescence is observed upon binding of angiogenin to placental RI. This property has been used as a probe to study placental RI binding to angiogenin ([12, 13], for reviews).

Adsorption of angiogenin to surfaces: Loss of angiogenin due to adsorption to container surfaces is significant. Conditions used to minimise this interaction are as follows: polypropylene containers and an angiogenin concentration above 300 nM (≈ 4 mg/L). Lysozyme or serum albumin should be added as carriers at 0.1 g/L to more dilute angiogenin solutions.
The sequence data are available in the Swiss-Prot database under the accession number placental RI, P13489. The nucleotide sequence accession number in the EMBL/Genbank Data Library is M22414.

Structure

Sequence and Size

Human angiogenin is a single-chain protein of 123 amino acids and has a calculated relative molecular mass of 14 124. The amino-acid composition of the mature protein is 15 Asp/Asn, 7 Thr, 9 Ser, 10 Glu/Gln, 8 Pro, 8 Gly, 5 Ala, 6 Cys, 5 Val, 1 Met, 7 Ile, 6 Leu, 4 Tyr, 5 Phe, 7 Lys, 6 His, 13 Arg, 1 Trp. It has the following sequence: <Glu^{1}-Asp-Asn-Ser-Arg-Tyr-Thr-His-Phe-Leu^{10}-Thr-Gln-His-Tyr-Asp-Ala-Lys-Pro-Gln-Gly^{20}-Arg-Asp-Asp-Arg-Tyr-Cys-Glu-Ser-Ile-Met^{30}-Arg-Arg-Arg-Gly-Leu-Thr-Ser-Pro-Cys-Lys^{40}-Asp-Ile-Asn-Thr-Phe-Ile-His-Gly-Asn-Lys^{50}-Arg-Ser-Ile-Ile-Lys-Ala-Ile-Cys-Glu-Asn-Lys^{60}-Asn-Gly-Asn-Pro-His-Arg-Glu-Asn-Leu-Arg^{70}-Ile-Ser-Lys-Ser-Ser-Phe-Gln-Val-Thr-Thr^{80}-Cys-Lys-Leu-His-Gly-Gly-Ser-Pro-Trp-Pro^{90}-Pro-Cys-Gln-Tyr-Arg-Ala-Thr-Ala-Gly-Phe^{100}-Arg-Asn-Val-Val-Val-Ala-Cys-Glu-Asn-Gly^{110}-Leu-Pro-Val-His-Leu-Asp-Gln-Ser-Ile-Phe^{120}-Arg-Arg-Pro^{123}-OH. Three disulphide bonds link Cys^{26}-Cys^{81}, Cys^{39}-Cys^{92} and Cys^{57}-Cys^{107}. The protein is free of the glycosylation signal sequence Asn-X-Ser/Thr [9].
Angiogenin isolated from normal human plasma and from medium conditioned by HT-29 human adenocarcinoma cells has identical chromatographic behaviour, molecular weight, amino-acid composition, ribonucleolytic and angiogenic activities, and immunoreactivity [1].

Homologies
Angiogenins: Angiogenin, that can be referred to as angiogenin-1, has been isolated from HT-29 human adenocarcinoma cells [2] and from human plasma [1]. It has also been purified from mouse, rabbit and pig sera [37] and from bovine serum and milk [57, 58]. The mouse protein sequence has been deduced from the genomic DNA sequence. Mouse, rabbit, pig and bovine angiogenins have 75, 73, 66 and 64% sequence identity to human angiogenin, respectively (Figure 4). Most of the differences are the result of conservative substitutions. Apart from cyclization of the N-terminal glutamyl residue in the human, mouse and rabbit proteins, there is no evidence of post-translational modification. There is no Asn-X-Thr/Ser site for potential N-linked glycosylation and no evidence of O-linked glycosylation. All five angiogenins contain the essential catalytic residues His-13, Lys-40 and His-114 (human angiogenin-1 numbering), and three disulphide bonds. They induce neovascularization *in vivo* and display very low ribonucleolytic activities [1, 37, 59]. Human, rabbit, pig and bovine angiogenins all bind human placental RI with 1:1 stoichiometry [1, 12, 37, 59].
Angiogenin-2 isolated from bovine serum and milk is 57% identical to bovine angiogenin-1, with an overall similarity of 71%. It is glycosylated at Asn-33 and contains 2-3 glucosamine, 5-6 mannose, 1-2 galactosamine and 0-1 xylose. Its apparent molecular mass is 20 kDa [60]. Its ribonucleolytic activity is lower than that of bovine angiogenin-1. Angiogenin-2 is a less potent inducer of angiogenesis than angiogenin-1. The conserved Asn-109 of angiogenin-1 is replaced by Asp-108 in bovine angiogenin-2. The same replacement by site-directed mutagenesis in human angiogenin-1 (replacement of Asn-109 by Asp-109) abolishes angiogenic activity, and the aspartyl derivative inhibits angiogenin-induced angiogenesis [40].

Angiogenin-related family: By screening a mouse genomic library with an angiogenin-1 gene probe, an angiogenin-related protein gene with 88% nucleotide sequence identity to the BALB/c mouse angiogenin gene has been identified [59]. The mouse angiogenin-related protein (Angrp), produced in *E. coli*, is 78% identical to mouse angiogenin-1. It is free of consensus sequences for N- and O- linked carbohydrate chains. It has higher ribonucleolytic activity than angiogenin-1 and is inhibited by human placental RI. Angiogenin-related protein lacks angiogenic activity in the chick embryo

chorioallantoic membrane assay, which could result from poor conservation of the receptor-binding domain. Angiogenin-related protein does not inhibit angiogenin-1-induced angiogenesis. It has not been detected in mouse serum [59].
EF-5 induced by E2a-Pbx1 in mouse NIH 3T3 fibroblasts encodes another member of the angiogenin gene family (EF5/angiogenin-3) that is 74% identical to mouse angiogenin-1 and 81% identical to mouse angiogenin-related protein [61]. Its transcript is expressed in adult liver and on day 7 of development. It is not expressed in NIH 3T3 fibroblasts, exhibits dose-dependent expression in response to E2a-Pbx1, and slight upregulation in Abl-transformed cells. It is not induced by oncogenic Lck, Ras, Neu, Src or Sis, or in myeloblasts immortalised by E2a-Pbx1 [61]. It has the residues required for ribonucleolytic activity but diverges at residues in the receptor-binding domain.

RNase family: The human RNase family is composed of related structurally proteins that cleave ribonucleic acids on the 3' side of pyrimidine and have a variety of distinct biological activities ([62, 63], for reviews). In human angiogenin (RNase-5), 43 of the 123 amino acids are identical to those of human pancreatic RNase (RNase-1) at the corresponding positions, and many of the remaining residues are conservatively replaced [9]. Thus, angiogenin has 35% sequence identity and a similarity of 68% with human pancreatic RNase-1. RNase-1 has also been purified from urine, seminal plasma, brain and kidney ([62, 63], for reviews). Angiogenin has 28% sequence identity with RNase-2 (also named EDN, for eosinophil-derived neurotoxin). RNase-2 occurs predominantly in eosinophils, liver, spleen and placenta. It is known for its neurotoxicity, due to its ribonuclease activity. RNase-2 is 35% identical to RNase-1. Angiogenin is 27% identical to RNase-3 (also named eosinophil cationic protein or ECP). RNase-3, isolated from granulocytes, is highly similar to RNase-2 (70% identity) but less neurotoxic than RNase-2. It is a helminthotoxin with antibacterial activity as well as cytotoxic activity for mammalian cells *in vitro*. Angiogenin is 40% identical to RNase-4. First isolated from tumour-cell-conditioned medium, RNase-4 has been purified from plasma and is highly similar to bovine and porcine liver RNase (≈ 90% identity). RNase k6 has been amplified from human genomic DNA and its mRNA transcript has been detected in all the human tissues so far tested (predominantly in lung). The deduced amino acid sequence of RNase k6 is 30% identical to that of angiogenin ([62, 63], for reviews). None of the ribonucleases tested (human RNase-1, -2 and -4 and bovine RNase A) are angiogenic, emphasising the peculiarity of angiogenin.
" Fibroblast growth factor receptor ligand 2 " cloned *Xenopus* gene (FRL2) encodes a protein that is 21% identical to angiogenin/RNase, with no homology with typical FGF family members. FRL2 increases protein-tyrosine phosphorylation in yeast cells expressing FGF receptors and binds to the extracellular domain of the FGF receptor [64].
A gene differentially expressed in two types of v-myb-transformed avian myelomonocytic cells (clone 462) encodes an RNase/angiogenin-related protein that shows 34% sequence identity with angiogenin [65].
The RNase-superfamily-related gene (RSFR), highly expressed in chicken bone marrow cells, codes for a protein that shows 32% identity with angiogenin and differs fully in the angiogenin receptor-binding domain [66].
Several RNases were first isolated on the basis of their biological properties and subsequently identified as RNases [67]. Angiogenin, EDN (RNase-2) and ECP (RNase-3) belong to this family, together with bovine seminal ribonuclease (BS-RNase), frog

onconase and bullfrog lectin. The three last are 32%, 27% and 30% identical to human angiogenin, respectively. BS-RNase exhibits antitumour, immunosuppressive and antispermatogenic actions. Onconase from *Rana pipiens* and the sialic-acid-binding lectin purified from *Rana catesbeiana* oocytes possess antitumour activity [67].

Other homologies: Bovine prochymosin has 6 residues identical to residues 103-108 of human angiogenin and 9 of the 14 in positions 103 to 116 are also identical. Residues 1136-1149 of DNA-dependent RNA polymerase align with 7 of the 14 C-terminal residues of human angiogenin [9].

Conformation

Human Met-(-1) angiogenin crystallises in the orthorhombic system, space group $\underline{C}222_1$, with unit cell dimensions a = 83.4 Å, b = 120.6 Å and c = 37.7 Å (one molecule per asymmetric unit, specific volume 3.3 $Å^3$/Da, 63% solvent content). When determined at 2.4-Å resolution, the three-dimensional structure of human angiogenin is kidney-shaped with dimensions 38 Å x 43 Å x 34 Å, and consists of three helical regions and seven ß-strands (Figure 1, [44]). The secondary-structure elements in human angiogenin are helix 1 (H1, residues 3-14), helix 2 (H2, residues 21-33), ß-sheet 1 (B1, residues 41-47), helix 3 (H3, residues 49-58), ß-sheet 2 (B2, residues 62-65), ß-sheet 3 (B3, residues 69-73), ß-sheet 4 (B4, residues 76-84), ß-sheet 5 (B5, residues 93-101), ß-sheet 6 (B6, residues 103-108), ß-sheet 7 (B7, residues 111-116) and 3_{10}-helix (residues 117-121). The central core of angiogenin is organised around the two antiparallel twisted ß-strands B3-B4 and B5-B6. It is completed by two additional strands on either side (B1 and B7) and the short strand B2. The N-terminal helix H1 is close to the short C-terminal 3_{10}-helix. Helix 2 and H3 are oriented at ≈ 70° relative to the plane of the ß-sheet. Three disulphide bridges are present in angiogenin, linking H2 to B4 (Cys^{26}-Cys^{81}), B1 to B5 (Cys^{39}-Cys^{82}) and H3 to B6 (Cys^{57}-Cys^{107}) [44]. The overall structure of human angiogenin is similar to that of RNase A but differs in the putative receptor binding site and the ribonucleolytic active centre. The 1.5-Å-resolution crystal structure of bovine angiogenin confirms that the site spatially analogous to that for pyrimidine binding in RNase A is obstructed by Gln-117 and Glu-118 in human and bovine angiogenin, respectively [44, 46]. The properties of crystalline angiogenin are conserved in solution, as shown by ^{1}H NMR spectroscopy of bovine angiogenin [45]. Five loops and one helix show a larger dispersion, *viz.* the N- and C- terminal segments, the Arg^{67}-Gly-Asp^{69} loop covering part of the putative receptor binding domain [15] and the endothelial cell adhesion domain [27], and the 35-42 and 86-94 segments covering part of the domains involved in the inhibition of PMNL degranulation by angiogenin [26].

Additional Features

Isoelectric point: Angiogenin elutes after lysozyme (pI 10.5) in cation-exchange high-performance liquid chromatography [2] suggesting a pI > 10.5.
Ultraviolet absorption: Angiogenin has a molar absorptivity of 12,500 M^{-1} cm^{-1} at 280 nm in 20 mM Tris, pH 7.5 [68].

Metal content: Atomic absorption spectrometry of plasma-derived angiogenin at a concentration of 0.07 mM showed that less than 0.01 mol/mol of copper, iron, manganese, and zinc is present [1].

Deamidation of asparagine residues is a non enzymatic post-translational protein modification that occurs in angiogenin, BS-RNase, RNase A, human RNase-4 and other proteins such as human growth hormone, mouse epidermal growth factor, interleukin-1ß, calmodulin, adrenocorticotropin and lysozyme. In human angiogenin, treatment at alkaline pH or long-term storage results in deamidation of Asn-61 and Asn-109 [40]. The desamido derivatives mainly contain isoaspartic acid, exhibit nearly full enzymatic activity, have low angiogenic activity on the chick embryo chorioallantoic membrane, and do not inhibit angiogenin-induced neovascularization. The aspartic acid derivatives, obtained by site-directed mutagenesis, differ from the isoaspartic derivatives by their inhibition of angiogenin-induced angiogenesis. This underlines the importance of Asn-61 and Asn-109 for the angiogenic activity of human angiogenin [40].

The sequence data are available in the Swiss-Prot database under the following accession numbers: human RNase-1, P07998; bovine RNase-1, RNase A, P00656; human RNase-2, EDN, P10153; human RNase-3, ECP, P12724; RNase-4, P34096; RNase-5, human angiogenin, P03950; rabbit angiogenin, P31347; pig angiogenin, P31346; bovine angiogenin-1, P10152; bovine angiogenin-2, P80929; mouse angiogenin-1, P21570; mouse angiogenin-related protein, Q64438; EF5/mouse angiogenin-3, P97802; chicken clone 462 RNase/angiogenin, P27043; chicken G. gallus RSFR, P30374; RNase k6, Q93091; BS-RNase, P00669; onconase, p30 (*Rana pipiens*), P22069; sialic acid-binding lectin, SBL-C (*Rana catesbeiana*), P14626.
The atomic coordinates are available in the Protein Data bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973, under the following entry codes: human angiogenin, 1ANG; bovine angiogenin, 1AG1; RNase A, 7RSA.
The proton NMR coordinates of bovine angiogenin are available from the Brookhaven Protein Data bank under the file name 1GIO.

Gene

Gene Structure

The gene for angiogenin is devoid of introns in the coding and 3'-noncoding regions of the gene ([69], Figure 5). A consensus sequence for a 3' boundary suggests the presence of an intron that exceeds 1700 base pairs in the 5' untranslated region [69, 70]. The presence of an intron in the 5' untranslated region and the absence of introns in the coding sequence are common features in many members of the RNase A family. The angiogenin gene contains a leader sequence coding for a signal peptide of 24 (or 22) amino acids, indicating that angiogenin is a secreted protein, 369 nucleotides coding for the mature protein of 123 amino acids, a stop codon, a 3'-noncoding sequence of 175 nucleotides and a poly(A) tail of 36 nucleotides. A potential TATA box is present. Two Alu sequences flank the gene, 400 base pairs upstream and 300 base pairs downstream of the coding region, respectively. These two Alu repeats are in the same inverted orientation. At 1100 base pairs downstream of the gene, a third Alu sequence is present in the typical orientation. The coding region of the mouse angiogenin gene is 82% identical to the human region.
Southern blotting of human leukocyte DNA has suggested that only one copy of the angiogenin gene is present in human DNA [69]. However, 2-3 copies of the gene have been detected in various experimental conditions [70].

Chromosomal Location

The human angiogenin gene is located on chromosome band 14q11 [71]. It resides proximal to a translocation breakpoint within the T cell receptor α/δ locus, upstream of this locus. Chromosome translocations in peripheral T lymphocytes are frequently observed in patients with the autosomal recessive disease ataxia telangiectasia. The excessive vascularization observed in this disease points to the involvement of an angiogenic factor [71].
In mice, the angiogenin gene is assigned to chromosome 14.

Gene Expression
Angiogenin mRNA is widely distributed, in both tissues and cultured cells. Angiogenin mRNA is expressed predominantly in human and rat liver [55, 72]. The ≈ 1-kb angiogenin messenger has also been detected by northern blotting in human pancreas, lung, prostate, testis, ovary, small intestine, colon, heart, kidney, skeletal muscle and peripheral blood leukocytes [55]. In the rat, it is also present in small intestine, colon, heart, kidney, adrenal, spleen, ovary, brain, lung and skeletal muscle [72]. Larger transcripts have been detected in human liver [55] and in HT-29 human colon adenocarcinoma cells, at 1.6, 2.7, 3.5, 5.2 and 6 kb [73].
A 0.8- to 1.1-kb angiogenin mRNA is present in normal and tumour cells [8, 72, 73]. Angiogenin transcripts have been detected in human tumour cell lines such as lung carcinoma A549 cells [8, 73], the colon lines HT-29, SW620, SW480 and WiDr, the medulloblastoma line TE-671, the fibrosarcoma line HT-1080, SK-HEP hepatoma cells, embryonic tumour cells from rhabdomyosarcoma, and bladder carcinoma HT-1376 cells [72, 73], as well as in T-cell leukaemia CEM cells [73] and MT4 cells [8]. They are also present in normal cells such as epithelial cells from colon [72, 73] and liver [72, 73], mesothelial cells [73], and fibroblasts from embryonic lung, foreskin [73], adult saphenous vein [8], human umbilical endothelial cells and endothelium-derived EA.hy926 cells [8]. The abundance of angiogenin RNA transcripts is increased in stimulated peripheral blood lymphocytes [73]. Angiogenin transcripts have not been detected in promyelomonocytic HL-60 and U-937 cells, which is consistent with the absence of immunoreactivity in cell-conditioned media [8].
Angiogenin immunoreactivity is widely and differentially found in anchorage-dependent growing cells such as vascular endothelial cells from saphenous and umbilical veins, SMC, fibroblasts from embryos (WS1 and WI-38 cells), new-borns (AG1523) and adults (cells from saphenous vein), and tumour cells (A-431, A549, HT-29 and HeLa). The secretion of angiogenin can reach ng/10^6 cells/day. As compared to anchored cells, normal peripheral blood cells and tumour cells such as myelomonocytes (HL-60, U-937) and megakaryocytes (Dami) do not secrete angiogenin or secrete low levels (<30 pg/10^6 cells/day), but myeloma RPMI 8226 cells produce as much angiogenin cross-reactivity as anchored cells. Among the tumour T-cells tested, Jurkat and MT4 cells express cross-reactivity, while H9 and HuT 78 cells do not [8].
In vivo, angiogenin is present in normal human plasma at 110-380 ng/ml (n = 65) [6] and in human amniotic fluid [7]. The bovine angiogenin concentration has been estimated at 100-180 ng/ml of serum and 4-8 mg/L of milk [74]. Immunohistochemical studies have identified angiogenin in epithelial and secretory cells in sections of cow mammary gland, visceral peritoneum and gallbladder [74].

Gene Regulation
The angiogenin concentration in human serum increases in the perinatal period. A statistically significant increase in angiogenin concentrations has been reported in day 4 neonatal serum relative to umbilical cord serum, with values reaching maternal levels

[75]. Similarly, expression of the angiogenin gene in rat liver is developmentally regulated: the mRNA level is low in the foetus, increases in the neonate and is predominantly expressed in the adult [72].
The distribution of 5-methyldeoxycytidine in 5'-CG-3'-rich sequences can be related to the degree of transcriptional activity, or to replication or recombination in the genome. The human angiogenin gene has three Alu sequences (see Gene Structure §). The Alu element located upstream of the transcription start site in the angiogenin gene is unmethylated in the DNA of haploid spermatozoa, while it is highly methylated in the DNA of differentiated diploid human cells. This is surprising, because DNA from sperm is usually highly methylated. Cell-free transcription experiments suggest that 5'-CG-3' methylation can lead to transcriptional inactivation [76].

Additional Features
Pseudogenes: Screening of a 129-strain mouse genomic DNA library with a BALB/c angiogenin gene probe yielded angiogenin pseudogene 1 (Ang-ps1, [77]). Ang-ps1 contains a single insertion resulting in a frameshift mutation in the early part of the coding region. Polymerase chain reaction cloning has also yielded a second angiogenin-like pseudogene, Ang-ps2 [77].

Nucleotide sequence accession numbers (EMBL/Genbank Data Library): human RNase-1, X62946; human RNase-2, EDN, M24157; human RNase-3, ECP, X15161; human RNase-4, U36775; RNase-5: human angiogenin, M11567; mouse angiogenin-1, U22516; mouse angiogenin-related protein, U22519; EF5/mouse angiogenin-3, U72672; chicken clone 462 RNase/angiogenin, X 61192; chicken G gallus RSFR, X64743; mouse Ang-ps1, U22517; mouse Ang-ps2, U22518; human RNase k6, U64998.
NBRF accession numbers of sequences: angiogenin, NRHUAG; RNase-2, EDN, A35328; RNase-3, ECP, B35328; RNase A, A32471; onconase, p30 (*Rana pipiens*), A39035; sialic acid-binding lectin, SBL-C (*Rana catesbeiana*), A27121.

Processing and Fate
Nuclear translocation of angiogenin has been studied by means of indirect immunofluorescence microscopy in CPAE cells grown for one day in minimal essential medium containing 20% heat-inactivated fetal bovine serum [78]. Bright staining was observed in the nucleoli of cells exposed to 100 or 1000 ng/ml (≈ 7 or 70 nM) of human Met-(-1)angiogenin and native <Glu-angiogenin for 30 min at 37°C in serum-free medium. Immunostaining was not observed in the presence of a 10-fold excess of bovine angiogenin with mAb 26-2F, a monoclonal antibody against human angiogenin which does not react with the bovine protein. Neither lysozyme nor RNase A affects nuclear translocation of human angiogenin in endothelial cells. Exogenous smooth muscle alpha-actin (0.001 or 0.01 mg/ml), a monoclonal antibody to smooth muscle cell alpha-actin (0.1 mg/ml), heparin (0.0001, 0.001 or 0.01 mg/ml), and an 8-fold molar excess of FGF-2 all inhibit this process, but chondroitin sulphate does not. CPAE cells do not internalise enzymatically active angiogenin derivatives with an altered cell-binding site, such as angiogenin K (Met-(-1)angiogenin cleaved at residues 60-61), ARH-1 (Met-(-1)angiogenin/RNase hybrid protein in which the angiogenin segment 58-73 is replaced by residues 59-73 of RNase A), R66A and N109D. In contrast, two enzymatically inactive mutants, K40Q and H13A, whose cell-binding site is intact, are translocated to the nucleus. Like the angiogenin derivatives angiogenin K, ARH-1, R66A and N109D, the R33A mutant is not translocated to the nucleus and lacks

angiogenic activity [78]. In digitonin-permeabilised endothelial cells, angiogenin mutants with an altered cell-binding domain (angiogenin K, ARH-1 and R66A) are imported into the nucleus and targeted to the nucleolus, whereas the R33A derivative is not. Segment Arg^{31}-Arg-Arg-Gly-Leu^{35} of human angiogenin has been identified as the nuclear localisation signal responsible for nucleolar targeting of angiogenin. The corresponding peptide Arg-Arg-Arg-Gly-Leu targets non nuclear proteins and the R33A derivative to the nucleolus of digitonin-permabilised endothelial cells, while the peptide Arg-Arg-Ala-Gly-Leu does not [47].
Nuclear translocation has also been observed in GM7373 and human umbilical artery endothelial cells [47, 78]. No staining was observed in confluent CPAE cells [78].

Biological Activity

Angiogenin is a potent blood vessel-inducing factor: 0.5 ng (35 fmol) induces angiogenesis in the chick embryo chorioallantoic membrane assay [2], 50 ng (3.5 pmol) in the rabbit corneal assay [2], 100 ng (7 pmol) in the rabbit meniscus [5], and 1 nmol in the disc angiogenesis assay in mice [42]. Placental RI blocks angiogenin-induced angiogenesis in the chick embryo chorioallantoic membrane assay [12]. In the disc angiogenesis assay in mice, placental RI also inhibits the angiogenesis induced by FGF-2 and sodium orthovanadate. In the mouse cornea assay, placental RI inhibits FGF-2-induced angiogenesis [79].
During angiogenesis, quiescent endothelial cells lining microvessels are induced to invade the extracellular matrix. In an experimental model, microvascular endothelial cells grown as a monolayer on a three-dimensional matrix can be stimulated to invade the underlying matrix and to form capillary-like tubular structures. Unlike FGF-2 and VEGF, human angiogenin does not induce cloned bovine microvascular endothelial cells from the renal cortex to form capillary-like tubules in a collagen invasion assay (R. Montesano, M. Moenner and J. Badet, unpublished observation). However, bovine angiogenin stimulates the cell-associated proteolytic activity of CPAE cells and GM 7373 cells, a foetal bovine aortic endothelial cell type transformed by benzo[α]pyrene, as determined by using a tissue-type plasminogen activator (t-PA)-specific peptide as substrate. Stimulation is maximal at an angiogenin concentration of 500 ng/ml [19].
Angiogenin induces fibrinolytic activity in GM 7373 cells grown on fibrin gels. Maximal stimulation was observed in the presence of angiogenin at $\approx$ 1000 ng/ml and resulted in small focal defects of the fibrin gel [19]. Angiogenin also induces endothelial cells to invade Matrigel. Invasion is inhibited by a rabbit polyclonal anti-angiogenin antibody and α_2-antiplasmin [19]. Although actin has been shown to inhibit angiogenin-induced angiogenesis *in vivo* [49], it does not inhibit angiogenin-induced invasion of endothelial cells into Matrigel [19]. On the contrary, the presence of both angiogenin and actin induces more invasion than does angiogenin alone [19]. In fact, angiogenin interacts with actin. It enhances actin acceleration of plasmin generation from plasminogen by t-PA and abolishes actin inhibition of plasmin [48, 49].
When confluent bovine aortic endothelial cells cultured on collagen-coated dishes are wounded, bovine angiogenin at 10 or 100 ng/ml induces cell migration [20].
Angiogenin upregulates the mRNA of both urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor 1 (PAI-1). Both cell lysates and conditioned media express u-PA activity. It has been reported that angiogenin at 10 and 100 ng/ml induces the formation of tube-like structures by endothelial cells on type-I collagen gel, an effect blocked by aprotinin, an inhibitor of serine proteases [20]. Finally, bovine angiogenin at 100 ng/ml induces an increase in c-fos and c-jun mRNA levels 30 min after stimulation [20].

Angiogenin has been reported to stimulate the proliferation of endothelial cells [17, 18]. Human angiogenin at concentrations ranging from 100 to 1000 ng/ml enhances [^{3}H]thymidine incorporation and cell proliferation in human umbilical venous and microvascular endothelial cells by up to 50% [17, 18]. Bovine angiogenin at 100 ng/ml induces [^{3}H]thymidine incorporation and cell proliferation in bovine brain capillary endothelial cells [17].

Angiogenin interacts with cultured endothelial cells to activate second messengers. Angiogenin at concentrations in the ng/ml range, is a weak inducer of diacylglycerol formation in CPAE and BACE cells [21]. Higher concentrations of angiogenin (> 100 ng/ml) are required to induce diacylglycerol in HUVE cells [21]. Angiogenin seems to activate phospholipase C; however, the concomitant 20% increase in inositol triphosphate is small compared to the 500% increase induced by the endothelial cell agonist bradykinin [21]. Chemical modification of one or both His residues in the catalytic site (His-13 and His-114) abolishes the ability of angiogenin to increase CPAE cellular diacylglycerol. Placental RI also completely abolishes the response induced by angiogenin. Angiogenin at 1 ng/ml activates BACE and HUVE cells but not CPAE cells to secrete prostacyclin, a potent vasodilator and inhibitor of platelet aggregation [22]. Angiogenin does not stimulate the secretion of the angiogenic prostaglandins PGE_1 and PGE_2. The secretion of prostacyclin by BACE cells is blocked by pre-treating the cells with indomethacin and tranylcypromine (inhibitors of prostacyclin synthesis), quinacrine (a specific inhibitor of phospholipase A_2), RHC 80267 (a diglyceryl and monoglyceryl lipase inhibitor), H7 (a protein kinase inhibitor) and also phorbol 12-myristate 13-acetate (that down-regulates protein kinase C). Angiogenin-induced prostacyclin secretion by BACE cells is also blocked by pre-treatment with pertussis toxin, which has no effect on the induction of diacylglycerol by angiogenin. This suggests that a putative angiogenin receptor is coupled by a pertussis-sensitive G protein to phospholipase A_2. No calcium mobilisation has been detected in BACE cells after exposure to angiogenin, either by fura-2 labelling and fluorescent measurements or by determining $^{45}Ca^{2+}$ efflux [22].

In cultured rat SMC, angiogenin activates phospholipase C [23]. Angiogenin at 1 ng/ml induces a transient increase in inositol triphosphate to a maximum of 400% of control, and also generates a transient increase in 1,2-diacylglycerol. However, the authors observed no detectable internal Ca^{2+} release by fura-2-labelled cells [23]. Angiogenin transiently depressed rat aortic smooth muscle cell cAMP levels by a pertussis toxin-sensitive mechanism. It has no effect on cellular cGMP. In addition, angiogenin stimulates rapid incorporation of fatty acids into cholesterol esters, which might have important physiological implications [23].

Angiogenin supports cell adhesion (see Binding sites and affinity §) [27, 28].

A degranulation inhibiting protein (DIP) purified from ultrafiltrates of plasma from patients with uraemia, has been shown to be identical to angiogenin by amino acid sequence determination, immunoblotting and assays of inhibitory effects on leukocyte degranulation [26]. DIP at concentrations in the nanomolar range inhibits spontaneous degranulation by 40% in PMNL, and by 70% in cells previously stimulated with the chemotactic peptide formyl-norLeu-Leu-Phe-norLeu-Tyr-Leu [26].

Human angiogenin suppresses [^{3}H]thymidine incorporation in human lymphocytes stimulated by phytohemagglutinin or concanavalin A, and in allogenic human lymphocytes in mixed lymphocyte culture. This immunosuppressive activity has been observed with an angiogenin concentration of 0.02 mg/ml [25].

Angiogenin has ribonucleolytic activity on both 18S and 28S ribosomal RNAs, as well as transfer RNA [3]. At a concentration of 40-60 nM, angiogenin abolishes cell-free

protein synthesis by ribonucleolytic inactivation of the 40S ribosomal subunit. It generates limited cleavage products from reticulocyte RNAs. Kinetic studies of protein synthesis indicate that angiogenin inhibits either the chain elongation or termination step of protein synthesis, not its initiation [10]. Angiogenin blocks protein synthesis when injected into *Xenopus* oocytes, by acting as a tRNA-specific ribonuclease [11]. Protein synthesis, *in vitro* and *in vivo*, is restored in the presence of RI [10, 11].

Role in Vascular Biology

Physiological Function

Angiogenin is a potent inducer of blood vessel formation in experimental models, *in vivo* [2, 5, 42]. However, the widespread expression of angiogenin suggests a physiological function not restricted to the neovascularization process [8, 72]. The presence of angiogenin in normal plasma [1] suggests that it may be involved in vascular homeostasis.

The angiogenin concentration in plasma is in the range 8-30 nM [6, 8]. At such concentrations, angiogenin inhibits the degranulation of PMNL *in vitro*, as described by Tschesche and colleagues, who suggested that angiogenin might participate in an endogenous inhibitory mechanism to counterbalance plasma-derived molecules released during inflammatory responses [26].

Angiogenin expression is developmentally regulated in rat liver [72]. In human serum, the angiogenin concentration increases in the perinatal period [75]. These two observations might provide clues to the physiological function(s) of angiogenin.

Pathology

By *in situ* hybridisation, increased angiogenin mRNA expression has been detected in human colonic adenocarcinoma, gastric adenocarcinoma and pancreatic cancer tissues compared to corresponding normal tissues [31, 80]. Over-expression of angiogenin in pancreatic cancer tissue is associated with cancer aggressiveness [31]. There is a significant increase in serum levels of angiogenin in patients with pancreatic [31], breast [35], ovarian [32], endometrial [33] and cervical [34] cancer. The possible involvement of angiogenin in tumour development is suggested by the demonstration that angiogenin antagonists prevent the growth of human tumour xenografts in athymic mice [30].

An increase in serum levels of angiogenin has also been reported among children and adolescent with insulin-dependent diabetes mellitus [81] and women with severe ovarian hyperstimulation syndrome [82].

Angiogenin is regulated as an acute-phase protein in experimental models *in vivo* [83].

Clinical Relevance and Therapeutic Implications

Correlation between angiogenin concentrations in biological fluids and disease processes might provide prognostic information. For example, increased angiogenin concentrations in midtrimester amniotic fluid has been shown to be a marker of preterm delivery [7]. Similarly, progression of endometrial and pancreatic cancer is associated with increased serum levels of angiogenin [31, 33].

Inhibition of angiogenesis might be a therapeutic target in cancer. Angiogenin-induced angiogenesis is inhibited by angiogenin derivatives such as angiogenin H13A and H114A, N61D and N109D, and the C-terminal peptide ANG(108-121). The peptide ANG(58-70), corresponding to the putative receptor binding domain, and two peptides complementary to the receptor-binding domain of angiogenin are also inhibitors [42,

84]. The amino acid sequences of these latter two peptides have been deduced from the antisense RNA sequence corresponding to the putative receptor binding site of angiogenin (residues 58-70) in either the 5'→3' (chANG) or the 3'→5' (chGNA) direction [84]. They have the following sequences: Val-Phe-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe (chANG) and Leu-Leu-Phe-Leu-Pro-Leu-Gly-Val-Ser-Leu-Leu-Asp-Ser (chGNA).

The monoclonal antibody mAb 26-2F, that recognises both Trp-89 and the 38-41 segment of human angiogenin, neutralises the angiogenic activity of angiogenin. It has been shown to prevent or delay the appearance of HT-29 tumours in athymic mice. Similarly, mAb 36u, that interacts with segment 58-73, prevents the appearance of tumours [30].

The activity of angiogenin (10 ng) in the chick embryo chorioallantoic membrane assay is also inhibited by actin (3000 ng) and anti-actin antibody (1000 ng) [49]. On the basis of the angiogenin-actin interaction [48, 49], angiogenin antagonists have been screened from a phage-displayed peptide library. ANI-E peptide (sequence Ala-Gln-Leu-Ala-Gly-Glu-Cys-Arg-Glu-Asn-Val-Cys-Met-Gly-Ile-Glu-Gly-Arg), that contains a disulphide bond, blocks the neovascularisation induced by angiogenin in the chick chorioallantoic membrane assay [85]. The disulphide bond and the glutamic acid inside the disulphide ring are both required for the activity of the peptide. The peptide alone does not induce or inhibit embryonic angiogenesis. ANI-E peptide does not inhibit the adhesion of angiogenin-secreting PC3 human prostate adenocarcinoma cells to angiogenin, but does block the angiogenesis induced by these cells [85].

Because the integrity of the catalytic site is required for angiogenin to express its angiogenic properties, the homology of angiogenin with RNases generates novel approaches to antitumoral therapy. Homology modelling can serve as a guide for the design of tight-binding inhibitors of angiogenin. For example, kinetic and modelling studies of the catalytic centre subsites of angiogenin suggest that 5'-diphosphoadenosine 2'-phosphate might be a potent inhibitor [86].

Specific ligands for angiogenin have been selected by using the procedure of systematic evolution of ligands by exponential enrichment. An oligodeoxynucleotide aptamer inhibits both the enzymatic and biological activities of angiogenin [87].

The cytotoxic potential of RNases has been explored with a view to designing targeted drugs, named immunotoxins, by coupling non cytotoxic RNases to cell-binding ligands. The RNase chimeras display receptor-mediated cytotoxicity. On the basis of the ability of angiogenin to block protein synthesis, angiogenin fused to a single-chain antibody against the human transferrin receptor has been expressed as a targeted immunofusion protein. Angiogenin single-chain immunofusion proteins that retain both enzymatic and biological activity might form a new class of therapeutic agents [88].

Abbreviations:

ANG, angiogenin; BACE cells, bovine adrenal capillary endothelial cells; BS-RNase, bovine seminal ribonuclease; CPAE cells, calf pulmonary artery endothelial cells; DIP, degranulation inhibiting protein; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; FGF, fibroblast growth factor; FRL2, FGF receptor ligand 2; HUVE cells, human umbilical vein endothelial cells; NMR, nuclear magnetic resonance; PA, plasminogen activator; t-PA, tissue-type PA; u-PA, urokinase-type PA; PMNL, polymorphonuclear leukocytes; PG, prostaglandin; RI, ribonuclease inhibitor; RNase, ribonuclease; RNase A, bovine RNase-1; SDS-PAGE, sodium dodecyl sulphate - polyacrylamide gel electrophoresis; RSFR, RNase super-family-related gene; SMC, aortic smooth muscle cells; VEGF, vascular endothelial growth factor.

Mutant proteins are designated by the single-letter code for the original amino acid followed by its position in the sequence and the single letter code for the new amino acid. "Protein(n-n')" refers to a peptide whose N- and C- terminal residues, denoted by n and n' respectively, are the positions in the primary structure of the protein.

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