HHS Public Access

Author manuscript

Int Rev Cell Mol Biol. Author manuscript; available in PMC 2018 February 27.

Published in final edited form as:

Int Rev Cell Mol Biol. 2012 ; 295: 175–198. doi:10.1016/B978-0-12-394306-4.00011-3.

New Insights into the Role of Angiogenin in Actin Polymerization

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Abstract

Angiogenin is a potent stimulator of angiogenesis. It interacts with endothelial cells and induces a wide range of cellular responses initiating a process of blood vessel formation. One important target of angiogenin is endothelial cell-surface actin, and their interaction might be one of crucial steps in angiogenin-induced neovascularization. Recently, it was shown that angiogenin inhibits polymerization of G-actin and changes the physical properties of F-actin. These observations suggest that angiogenin may cause changes in the cell cytoskeleton. This chapter reviews the current state of the literature regarding angiogenin structure and function and discusses the relationship between the angiogenin and actin and possible functional roles of their interaction.

Keywords

Angiogenin; Angiogenesis; Actin; Polymerization; Cytoskeleton; Tropomyosin

1. Introduction

Angiogenin, a 14-kDa basic protein, was originally isolated from the conditioned medium of human colon adenocarcinoma cells during a search for angiogenic factors secreted by cancer cells (Fett et al., 1985). Angiogenin is a potent inducer of angiogenesis, a process of blood vessel formation. The mechanism of angiogenin action is not yet fully understood, but it appears to involve several different pathways, including receptor binding on endothelial cells (Hu et al., 1997), nuclear transport (Hu et al., 2000; Moroianu and Riordan, 1994a), and activation of proteolytic enzymes and cascades (Hu et al., 1994). Angiogenin is homologous (33% sequence identity) to the well-studied bovine pancreatic RNase A (Strydom et al., 1985). Angiogenin belongs to the RNase A superfamily and is sometimes referred to as RNase 5 (Cho and Zhang, 2006). The ribonucleolytic activity of angiogenin, although several orders of magnitude weaker than that of RNase A (Harper and Vallee, 1989; Shapiro et al., 1986), is essential for its angiogenic effect (Shapiro and Vallee, 1989; Shapiro et al., 1989). Angiogenin interacts with endothelial and smooth muscle cells to induce a wide range of cellular responses including cell migration, invasion, proliferation, and formation of tubular structures. It is thought that binding of angiogenin to cell-surface actin results in activation of a cell-associated protease system that promotes cell invasion; therefore, formation of an angiogenin/actin complex promotes degradation of the basement membrane and extracellular matrix (ECM) (Hu et al., 1991, 1993, 1994).

Despite available evidence supporting the importance of the angiogenin/actin complex to angiogenesis, little is known about the molecular details of their binding interactions. It was reported that angiogenin promotes actin polymerization (Hu et al., 1993). However, the experiments were only done at low ionic strength conditions, and the structural nature of the polymers formed was not investigated. Recently, it was shown that, at low KCl concentrations, angiogenin induces formation of unstructured aggregates, and at subphysiological KCl concentrations, angiogenin does not promote but instead inhibits polymerization by sequestering G-actin (Pyatibratov et al., 2010). Additionally, it was shown that binding of angiogenin to preformed F-actin causes filament stiffening. These observations suggest that angiogenin may cause changes in the cell cytoskeleton by inhibiting polymerization of G-actin and changing the physical properties of F-actin.

Angiogenin has been the subject of many studies and recent reviews (Gao and Xu, 2008; Riordan, 1997; Shestenko et al., 2001; Strydom, 1998; Tello-Montoliu et al., 2006; Vallee and Riordan, 1997). In this review, we summarize the current status of angiogenin research, focusing on the relationship between the angiogenin and actin and the possible functional roles of these interactions.

2. Structural Properties of Angiogenin

2.1. Primary structure

The only gene that codes angiogenin in humans is located on chromosome 14q11 proximal to the alpha/delta T-cell receptor gene (Weremowicz et al., 1990). The first amino acid sequence of angiogenin was determined by sequencing human tumor-derived angiogenin (Strydom et al., 1985). Angiogenin is a single-chain protein consisting of 123 amino acids (Fig. 5.1A). Its sequence was found to be homologous to that of the pancreatic RNase A with 35% identity. Major active-site residues (His13, Lys40, His114) as well as other active-site residues (Gln12, Thr45, Asp116) are identical. Subsequently, complementary DNA for angiogenin was found in normal human liver (Kurachi et al., 1985). The primary translation product contains a signal peptide of 24 residues, which is consistent with angiogenin being a secreted protein. It is a minimally conserved protein; ortholog angiogenins (from birds to mammals) have ~60% identity.

2.2. Secondary and tertiary structure

Several crystal structures of angiogenins from different sources were determined, with the highest resolution of 1.5Å for the crystal structure of bovine angiogenin (Fig. 5.1B) (Acharya et al., 1994, 1995; Holloway et al., 2002, 2005; Leonidas et al., 1999, 2001; Papageorgiou et al., 1997). The overall structure features a kidney-shaped tertiary fold reminiscent of RNase A. The central core of the molecule consists of a β-structure with a pair of antiparallel twisted β-strands (residues 69–84 and 93–108) forming the main topology. Two additional strands on either side of these central strands (residues 41–47 and 111–116) complete the major sheet structure. There are three helices in the structure: H1 (residues 3–14), H2 (residues 22–33), and H3 (residues 49–58).

Angiogenin contains counterparts for the key catalytic residues of pancreatic RNase A and has similar catalytic properties. Like RNase A, angiogenin cleaves mainly on the 3′ side of pyrimidines using a transphosphorylation/hydrolysis mechanism. Comparison of the crystal structures of RNase and angiogenin shows that in angiogenin, Gln117 occupies the pyrimidine binding cleft and potentially hinders substrate binding (Acharya, 1994, 1995).

RNase A was demonstrated to dimerize by three-dimensional domain swapping of either the N-terminal helix (Liu et al., 1998) or its C-terminal β-strand and to form higher-order, possibly amyloid-like aggregates (Liu et al., 2001b). Angiogenin also forms dimers and possibly higher-order oligomers in solution, as shown by the dependence of the [^{15}N–^{1}H]-HSQC spectrum on the concentration of angiogenin (Pyatibratov et al., 2010). It was observed consistently that many residues affected by an increase in angiogenin concentration are localized in the first α-helix and at the C-terminus. The underlying RNase A–RNase A interaction is weak, and an apparent dissociation constant in solution was estimated as ~2mM (Park and Raines, 2000), similar to that observed for angiogenin (Pyatibratov et al., 2010).

3. Angiogenin Localization and Function

3.1. Localization

Following the original isolation of angiogenin from the conditioned medium of HT-29 human colon adenocarcinoma cells (Fett et al., 1985), angiogenin was also isolated from normal mammalian plasma (Bond and Vallee, 1988; Bond et al., 1993; Olson and Vallee, 1987; Shapiro et al., 1987), serum (Bond et al., 1993; Strydom et al., 1997), and milk (Maes et al., 1988). Remarkably, both plasma and serum contain angiogenin at a concentration of 100–400μgl^{-1} (Chang et al., 1997; Shapiro et al., 1987; Strydom, 1998), substantially greater than the 0.5μgl^{-1} found in HT-29 cell-conditioned medium (Fett et al., 1985). In cow's milk, its concentration is even higher, ranging from 2000 to 8000μgl^{-1} (Chang et al., 1997; Komolova and Fedorova, 2002). Although angiogenin mRNA was detected predominantly in the liver, it was also detectable at low levels in other tissues (Weiner et al., 1987). Cultures of vascular endothelial cells, aortic smooth muscle cells, fibroblasts, and tumor cells secrete angiogenin into the medium (Moenner et al., 1994).

In addition, angiogenin has been shown to undergo nuclear translocation in endothelial cells via receptor-mediated endocytosis and nuclear localization sequence-assisted nuclear import (Moroianu and Riordan, 1994a,b).

3.2. Mechanisms of angiogenin action

The angiogenic activity of angiogenin was confirmed using the chick embryo chorioallantoic membrane (CAM) assay and by the implantation of angiogenin into the cornea of a rabbit eye (Fett et al., 1985). The CAM of the chicken egg is commonly used to study blood vessel formation (Knighton et al., 1977). A window is cut into the eggshell, and the potential angiogenic substance is deposited on the surface of the exposed membrane. The appearance of blood vessels in response to the test substance is measured after several days. The implantation of an angiogenic material into the cornea of a rabbit eye, where the second eye

serves as the control, is an alternative but more sophisticated method (Langer and Folkman, 1976). When 35fmol of angiogenin was added per egg, the CAM assay demonstrated the angiogenic activity of angiogenin. 3.5pmol of angiogenin was required to induce extensive blood vessel growth in the rabbit cornea.

Angiogenin has been demonstrated to induce the main stages of angiogenesis by binding to endothelial cells (Badet et al., 1989; Hu et al., 1991, 1997; Moroianu et al., 1993), activating signaling transduction (Bicknell and Vallee, 1988; Hu et al., 1997; Kim et al., 2007; Liu et al., 2001a; Xu et al., 2001), promoting the adhesion of endothelial cells and fibroblasts (Soncin, 1992; Soncin et al., 1994), activating cell-associated proteases (Hu and Riordan, 1993), and stimulating cell invasion (Hu et al., 1994).

Angiogenin has been shown to enhance proliferation of bovine brain capillary endothelial cells (Chamoux et al., 1991), human endothelial cells in sparse cultures, and human umbilical artery smooth muscle cells (Hu et al., 1993, 2000). This activity of angiogenin depends on cell density. Jimi et al. (1995) have reported that bovine angiogenin stimulates migration of bovine aortic endothelial cells and their formation of tube-like structures in collagen gel.

Unlike RNase A, angiogenin has a specific nuclear localization signal consisting of ^{29}IMRRRGL^{35} (Moroianu and Riordan, 1994a,b) and undergoes nuclear translocation to the nucleus, via receptor-mediated endocytosis, in endothelial cells. The nuclear location appears to be necessary for its angiogenic activity (Moroianu and Riordan, 1994a; Nobile et al., 1998). The nuclear function of angiogenin relates to its capacity to induce rRNA synthesis, which is essential for the synthesis of new ribosomes, protein translation, and cell proliferation. The release of nuclear angiogenin from the nucleus by DNase I indicates that it can directly bind to nuclear DNA. However, it is unclear whether angiogenin acts directly on DNA to regulate gene transcription (Hu et al., 2000). It was reported that exogenous angiogenin binds to the promoter region of rDNA and enhances the production of ribosomal RNA in cells, whereas the reduction of endogenous angiogenin inhibits its transcription (Xu et al., 2002, 2003). HeLa cells engineered to express angiogenin antisense RNA show a decrease in rRNA transcription, ribosome biogenesis, proliferation, and tumorogenesis both *in vitro* and *in vivo* (Tsuji et al., 2005). Kishimoto et al. (2005) demonstrated that nuclear angiogenin is necessary for angiogenesis induced by other angiogenic proteins including fibroblast growth factors, epidermal growth factor, and vascular endothelial growth factor. Inhibitors of the nuclear translocation of angiogenin abolish the angiogenic activities of these factors.

3.3. Correlation between ribonucleolytic and angiogenic activities

Although angiogenin cleaves standard RNA substrates such as poly (C) and poly (U), it is 10^5–10^6 times less efficient than RNase A (Shapiro et al., 1986). Though weak, the endonucleolytic activity of angiogenin, demonstrated on rRNAs (Rybak and Vallee, 1988; Shapiro et al., 1986), as well as tRNAs (Lee and Vallee, 1989), appears to be essential for its biological functions. Angiogenin specifically cleaves 18S and 28S rRNA with major products 100–500 nucleotides in length contrasting with much smaller oligonucleotides generated by RNase A (Shapiro and Vallee, 1987; St. Clair et al., 1988). The activity of

angiogenin can be blocked by placental RNase inhibitor (PRI) (Lee et al., 1989). Highly basic proteins (poly-arginine, poly-lysine, poly-ornithine, core histones, spermatid-specific S1 protein, and the protamines HP3 and Z3) strongly inhibit angiogenin binding to the RNase inhibitor and may be potential regulators of angiogenin-induced angiogenesis (Moenner et al., 1999).

Yamasaki et al. (2009) have shown that angiogenin can be activated by stress, and angiogenin plays a role in mammalian stress response by cleaving tRNAs and inhibiting protein translation. Angiogenin is required for the production of tiRNAs (3′-tRNA fragments of tRNAs that are produced under stress conditions). Knockdown of angiogenin inhibits tiRNA production and translational arrest induced by arsenite stress, whereas knockdown of the placental RNase/angiogenin inhibitor causes the opposite effect. Recombinant angiogenin induces tiRNA production and inhibits protein synthesis in the absence of exogenous stress. Emara et al. (2010) have reported that angiogenin inhibits protein synthesis and promotes the assembly of stress granules, cytoplasmic structures that are formed synchronously with stress-induced translational arrest and contain mRNAs redirected from polyribosomes.

Extensive studies on site-directed mutagenesis have shown that mutations of His13, Lys40, or His114 (H13A, H13Q, K40Q, H114A, H114N) not only reduce the ribonucleolytic activity of angiogenin but also greatly decrease its angiogenic activity (Shapiro and Vallee, 1989; Shapiro et al., 1989). Mutations that enhance ribonuclease activity either increased (D116H) or did not alter (D116N, D116A, D116S, D116E) the angiogenic activity (Curran et al., 1993; Harper and Vallee, 1988).

Comparison of the sequences of RNase A and angiogenin reveals that one of the functionally important segments of RNase A, residues 63–74, differs from the corresponding segment in angiogenin. The RNase intersegment disulfide bridge between Cys 65 and Cys 72 forms an exposed, six-residue loop constituting one face of the purine-binding site (Wlodawer and Sjölin, 1983). Angiogenin lacks the Cys65–Cys72 disulfide bond and this segment is two residues shorter (Fig. 5.1A). However, this segment is highly conserved in angiogenin orthologs indicating its importance for specific angiogenin functions. In another experiment, a chimeric protein, in which angiogenin residues 58–70 (ENKNGNPHRENLR) were replaced with RNase A residues 59–73 (SQKNVACKNGQTNCY), showed dramatic increases in RNase-like enzymatic activity and reduced angiogenicity (Harper and Vallee, 1989).

3.4. Binding partners

Initially, three angiogenin-binding proteins were reported: human PRI (Shapiro and Vallee, 1987), actin (Hu et al., 1991, 1993), and a 170-kDa cell-surface protein with an unknown amino acid sequence expressed by endothelial cells (Hu et al., 1997).

Human PRI, a 50-kDa leucine-rich repeat protein, binds angiogenin with extraordinary affinity (K_i<1fM) and inhibits its ribonuclease and angiogenic activities (Lee et al., 1989). The ribonucleolytic activity of angiogenin is essential for its angiogenic function and is complemented by a putative receptor binding site. The site is located within a noncatalytic

region of the protein and comprises residues 58–70 and 108–111 (Hallahan et al., 1991, 1992; Harper and Vallee, 1989; Shapiro and Vallee, 1992). If the receptor-binding site is damaged or altered, the resulting variants of angiogenin lack angiogenic functions, while the enzymatic activity remains intact. A 170-kDa cell-surface protein that was identified as a potential angiogenin receptor is located on the endothelial cell surface and is expressed only on angiogenin-responsive but sparsely cultured endothelial cells ($<2\times10^4$cellscm^{-2}) (Hu et al., 1997). Surprisingly, so far the amino acid sequence and any structural features of the 170-kDa angiogenin receptor are unknown. Hu et al. (1991, 1993) found no high-molecular weight molecules bound to angiogenin in subconfluent cells ($5–10\times10^4$cellscm^{-2}). In other subconfluent cells, actin is expressed and binds to angiogenin specifically. It appears that the 170-kDa putative receptor and actin are not expressed concurrently on the endothelial cell surface. They seem to be expressed under different cellular conditions and play roles at different stages of angiogenin-induced angiogenesis.

Recently, a few other angiogenin-binding proteins were found using a two-hybrid yeast system: α-actinin-2 (Hu et al., 2005), follistatin (Gao et al., 2007), and fibulin 1 (Zhang et al., 2008). Angiogenin interaction with each of these three proteins was confirmed by immunoprecipitation experiments and the fluorescence resonance energy transfer analysis. Although biological significance of angiogenin interactions with α-actinin-2, follistatin, and fibulin is unclear, these findings offer new possibilities for further studies on the mechanisms of angiogenin-induced angiogenesis and cancer cell growth.

4. Interaction of Angiogenin with Actin *in vivo* and *in vitro*

Actin is an ubiquitous protein that plays a key role in cell structure, cell motility, and contraction in both muscle and nonmuscle cells. There are three classes (α, β, and γ) of actin isoforms. The α isoforms are found only in striated and smooth muscles, whereas the β and γ isoforms are prominent in nonmuscle cells. Actin may be in a monomeric (G-actin) or polymeric (F-actin) state. The ability of actin to form different polymeric structures defines its importance in cells. A large number of proteins bind to actin and participate in essential cellular functions, including cell motility, cytokinesis, maintenance of cell structure, and organelle movement (Dos Remedios et al., 2003).

4.1. Identification of an angiogenin-binding protein as an actin

The ability of angiogenin to bind actin was discovered by Vallee and his coauthors in the early 1990s (Hu et al., 1991, 1993). By this time, there was evidence indicating the presence of a binding site in angiogenin that was a hypothetical receptor for endothelial cells. Specific binding of angiogenin to calf pulmonary artery endothelial cells was first demonstrated by Badet et al. (1989). It was shown that angiogenin binding is concentration dependent, reversible, and saturable. Two apparent types of interaction were found: high-affine with a K_d=0.5nM and low-affine with a K_d=0.2μM. It was demonstrated that high-affinity angiogenin binding depends on cell density (Badet et al., 1989).

When angiogenin was mutated, by replacing residues 58–70 with the corresponding segment of RNase A (residues 59–73; Harper and Vallee, 1989), it lost angiogenic activity. At even a 20-fold molar excess, the mutated angiogenin could not compete with the native protein in

the CAM assay. Mutation of Arg66 leads to a similar effect (Shapiro and Vallee, 1992). Deamination experiments pointed to a second important residue, Asn61, and a possible second region containing Asn109 (Hallahan et al., 1992). Additional experiments were conducted in which angiogenin was cleaved by proteases at the peptide bonds between residues 60 and 68. Endoproteinase Lys-C cleaves angiogenin at the peptide bond between Lys60 and Asn61, and a baby hamster kidney cell protease cuts it between Glu67 and Asn68. In both cases, the two resulting proteolytic fragments (Ang-K and Ang-E, respectively) are held by disulfide bonds. These two angiogenin derivatives and the angiogenin in which both bonds were cleaved retain the ribonucleolytic activities but are not angiogenic on the CAM assay and do not inhibit angiogenin-induced angiogenesis.

In contrast, mutant angiogenins, in which the His13 or His114, that are involved in ribonucleolytic activity, were replaced by Ala, although inactive in the CAM assay, were competitive with the native angiogenin and at 20-fold molar excess inhibited angiogenesis in this assay (Shapiro and Vallee, 1989). Angiogenin residues 60–68 were identified as a part of a putative cell-surface receptor-binding site (Hallahan et al., 1991). A dual-site model was postulated in which both the catalytic site and the cell-binding site are required for the organogenic activity of angiogenin. Interaction of angiogenin with this putative cell-surface receptor was seen as a necessary step in angiogenesis.

A 42-kDa dissociable cell-surface component of calf pulmonary artery endothelial cells and a transformed bovine endothelial cell line, GM7373, was identified as an angiogenin-binding protein (AngBP) (Hu et al., 1991). Binding of ^{125}I-labeled bovine angiogenin to AngBP was specific, saturable, and inhibited by excess of unlabeled angiogenin. Binding of Ang-K and Ang-E to AngBP decreased drastically compared to binding to intact angiogenin. AngBP was isolated after treatment of cells with heparan sulfate using affinity chromatography on angiogenin-Sepharose. The authors noted the tendency of AngBP to dissociate from the endothelial cell surface particularly with heparan sulfate (Hu et al., 1991), which is sometimes used to release proteins that are noncovalently bound to a cell-surface glycosoaminoglycan (Höök et al., 1984).

In further experiments, AngBP was identified as a member of the actin family by peptide mapping and partial (~40% of the entire protein) amino acid sequencing. The amino acid composition of AngBP is closer to that for bovine brain capillary actin, which differs substantially in tyrosine and leucine content from bovine smooth muscle actin and is localized in the plasma membrane of the microvessel endothelial cells (Pardridge et al., 1989). It should be remembered that the complete amino acid sequence of the AngBP and its inclusion as a known actin isoform family has not yet been determined.

Immunofluorescent staining of cultured cattle pulmonary artery endothelial (CPAE) cells with monoclonal antibodies to a smooth muscle α-actin detected the presence of this actin type on the cell surface (Moroianu et al., 1993). Exposure of the cells to angiogenin decreased the cell-surface staining with these antibodies. These data indicate that AngBP (actin) may dissociate from the cell surface of cultured endothelial cells with the addition of angiogenin to the medium (Hu et al., 1993; Moroianu et al., 1993). As an alternative

explanation, angiogenin might block the anti-actin antibody recognition site on the cell surface.

Water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) cross-linked ^{125}I-angiogenin to AngBP present on the surface of CPAE and GM7373 cells (even in a crude cell lysate) causing a single major product of 58kDa detected by SDS/PAGE (Hu et al., 1991). It was demonstrated that complex formation of angiogenin with bovine smooth muscle actin is very similar to that with AngBP (Hu et al., 1993). In both cases, autoradiography revealed a 58-kDa band, indicating formation of a 1:1 complex between angiogenin (16kDa) and actin or isolated AngBP (42kDa).

An excess of unlabeled bovine or human angiogenin competed with ^{125}I-angiogenin for binding to AngBP, and the intensity of the 58-kDa bands was several fold reduced. RNase A competed with ^{125}I-angiogenin in cross-linking experiments with AngBP, though not as effectively as angiogenin. At the same time, only a relatively weak band was seen when ^{125}I-labeled RNase A was exposed with AngBP. Under the same conditions, lysozyme, which like angiogenin is a strongly basic protein, did not compete at all (Hu et al., 1991, 1993). Scatchard analysis yields K_d ~0.5nM for the binding of actin and angiogenin, identical to that obtained for the binding of AngBP and angiogenin (Hu et al., 1991; 1993). This value is in agreement with K_d=0.5nM for the high-affinity binding sites of angiogenin with calf pulmonary artery endothelial cells (Badet et al., 1989).

When angiogenin binds to actin, a 50% quenching of actin tryptophan fluorescence was observed in the angiogenin/actin complex (Chang et al., 1996). The angiogenin/actin interaction was estimated using fluorescence titration experiments performed in 50mM Tris–HCl at pH 7.5 and 1mM $CaCl_2$ at 25°C. The dissociation constant K_d=20±1nM for the angiogenin/actin complex was determined by adding increasing concentrations of bovine angiogenin (0–1.0μM) to a constant concentration of actin (0.1μM). This K_d value is different from that measured by Hu et al. (1993) possibly due to different experimental conditions and state of the actin used in the preparations.

Based on the data obtained, Chang et al. (1996) proposed that Trp80 in the small domain of bovine actin is responsible for the angiogenin binding. Attempts have been made to identify the actin-binding site on angiogenin (Hu et al., 1991, 1993). As mentioned, angiogenin derivatives, Ang-K and Ang-E, which were cleaved at residues 60–61 or 67–68, had considerably reduced affinity for actin, suggesting that actin interacts with angiogenin via the putative receptor-binding site. This region is not responsible for ribonuclease activity and is relatively well conserved among angiogenins from various species but differs markedly from pancreatic RNases (Fig. 5.1A). It is notable that none of these RNases is angiogenic and only dimeric RNase from bovine seminal plasma (RNase BS) binds to G-actin (Simm et al., 1987).

The interaction of angiogenin with actin occurred both in solution and on a polyvinylidene fluoride membrane (Hu et al., 1993). Preincubation of ^{125}I-angiogenin with human placental ribonuclease inhibitor (PRI) prevented subsequent formation of a cross-linked complex with AngBP. The cross-linking of angiogenin to actin is inhibited by protamine, a 4.3-kDa

arginine-rich basic protein known as an *in vivo* inhibitor of angiogenesis induced by embryologic, neoplastic, inflammatory, and immunogenic angiogenic stimuli (Taylor and Folkman, 1982). Another angiogenesis inhibitor, PF-4 (Maione et al., 1990), also inhibits the cross-linking of angiogenin to actin (Hu et al., 1993).

4.2. Effect of angiogenin on actin polymerization

Many actin-binding proteins promote or inhibit actin polymerization and may cause F-actin depolymerization (Lee and Dominguez, 2010; Uribe and Jay, 2009). When angiogenin was mixed with globular actin in 2mM Tris, pH 8.0, 0.2mM $CaCl_2$, 0.2mM ATP, 0.2mM dithiothreitol, visible turbidity develops within seconds (Hu et al., 1993). After centrifugation, the pellet appeared on SDS/PAGE as two bands corresponding to actin and angiogenin. It was concluded by the authors that angiogenin induces the polymerization of actin. The angiogenin mutants H13A and H114A, although lacking RNase activity (Shapiro and Vallee, 1989), have the same effect on actin as native angiogenin (Hu et al., 1993). However, the enzymatically active derivatives of angiogenin, Ang-K and Ang-E (Hallahan et al., 1991), were qualitatively less capable of inducing polymerization. These observations were consistent with previous evidence that Ang-K and Ang-E fail to compete efficiently for the binding of angiogenin to AngBP (Hu et al., 1991, 1993).

The conclusion that angiogenin induces actin polymerization was based only on the increased turbidity and the formation of actin sediments; the structural nature of the polymers formed was not investigated. Moreover, the experiments on angiogenin-induced actin polymerization described by Hu et al. (1993) were done at low ionic strength conditions in the absence of KCl and Mg^{2+}, which are essential for actin polymerization. When experiments with the same conditions were done using the pyrene-actin fluorescence assay to follow polymerization, an angiogenin-induced increase in fluorescence was observed; however, electron microscopy analysis of the samples revealed that under these conditions actin formed not regular filaments but unstructured aggregates (Pyatibratov et al., 2010). The aggregate formation explained the increased turbidity and the formation of actin sediments in experiments of Hu et al. (1993) as well as the increase of fluorescence in experiments of Pyatibratov et al. (2010). The ability of angiogenin to aggregate actin is most likely related to the ability of angiogenin to dimerize.

More interestingly, the presence of angiogenin in the G-actin solution inhibits the formation of filaments normally formed by G-actin in the presence of KCl and $MgCl_2$ (Pyatibratov et al., 2010). The maximal effect was reached at 1:1 ratio. Electron microscopy revealed the formation of both aggregates (though smaller than those formed in the absence of KCl) and very short actin filaments. In the presence of angiogenin, the amount of actin sedimented was much lower than that in control experiments (without angiogenin). Purportedly, most of actin remained in monomeric or olygomeric form.

This effect was similar to deoxyribonuclease I (DNase I) interaction with G-actin. DNase I also formed a 1:1 complex with G-actin and prevented actin polymerization (Hitchcock et al., 1976). Another effect of DNase I on actin was F-actin depolymerization. When F-actin was mixed with DNase I in 1:1 ratio, no filaments were left after 2h and only small aggregates similar to those formed in the angiogenin/G-actin mixture were seen. F-actin

sediment seen 10min after mixing with DNase I and containing almost no DNase I indicates that binding of DNase I to F-actin was weak.

Unlike DNase I, when angiogenin was added to F-actin, it could be coprecipitated with F-actin and no depolymerization was observed (Pyatibratov et al., 2010). However, the appearance of the actin filaments changed in the presence of angiogenin and showed visible filament straightening, implying an increase in mechanical stiffness. The biological significance of this effect of angiogenin is not clear, but the mechanical properties of both the actin cytoskeleton and the ECM are known to have profound effects on cell structure and function (Janmey, 1998; Janmey et al., 2009), and tissue morphogenesis (Patwari and Lee, 2008). Additionally, the angiogenic process can be modulated by mechanical forces arising in the physical interaction between cells and ECM, which alter cell shape and the structure of the cytoskeleton (Ingber and Folkman, 1989; Mammoto et al., 2009). It was demonstrated that some angiogenesis controlling transcription factors (TFII-I and GATA2) are regulated by mechanical signals conveyed by variations of endothelial cell matrix stiffness (Mammoto et al., 2009).

4.3. Influence of tropomyosin on angiogenin/actin interaction

Tropomyosins regulate many actin properties including stabilization of actin filaments by prevention of severing, branching and depolymerization. For example, in epithelial cells, short nonmuscle tropomyosin, Tm5a, is associated with actin filaments that regulate the insertion and/or retention of a membrane transporter into the plasma membrane and therefore can regulate the activity of the transporter (Gunning et al., 2008). The effect of two tropomyosin isoforms, long muscle α-tropomyosin and short nonmuscle α-tropomyosin, on angiogenin binding was studied using a cosedimentation assay (Pyatibratov et al., 2010). Results were the same for both isoforms. At saturating concentrations of angiogenin and tropomyosin, both tropomyosin and angiogenin had no effect on binding each other to F-actin. At lower angiogenin concentrations, tropomyosin, already bound to F-actin, impeded binding of angiogenin and was able to protect part of the actin filaments. However, when adding angiogenin first, tropomyosin added to angiogenin/F-actin was unable to remove angiogenin from the filaments. The inability of tropomyosin to completely prevent angiogenin binding may be crucial for the function of actin filaments associated with membranes.

4.4. Comparison with other nucleases

There are few actin-binding proteins that also possess RNase (like angiogenin) or DNase activity. RNase BS (D'Alessio et al., 1972) interacts with skeletal muscle actin with an apparent binding constant of $9.2\times l0^4 M^{-1}$ in 0.1M KC1 (Simm et al., 1987). According to the viscosimetric data, it induces the polymerization of actin below the critical concentration in a depolymerization buffer and accelerates the salt-induced polymerization of actin even at a molar ratio of RNase to actin below l/l00. RNase BS bundles F-actin filaments and the molar ratio of RNase to actin in the bundles is about 0.66. There is no evidence that RNase BS depolymerizes actin filaments like DNase I.

RNase BS consists of two identical subunits cross-linked by disulfide bridges, and it was concluded that the dimeric structure is responsible for the bundling activity and the accelerating effect on the polymerization of actin (Simm et al., 1987). A monomeric form of RNase BS does not cross-link actin filaments and has a much smaller effect on actin polymerization (Simm et al., 1987).

The RNase activity of both RNase BS and RNase A is inhibited by actin (Simm et al., 1987). The authors suggested that RNase A can bind to actin; however, neither these authors nor anyone else has checked it in direct actin-binding experiments. It was only demonstrated that RNase A does not cause the F-actin bundling (Simm et al., 1987) and very weakly binds to the AngBP (Hu et al., 1991).

The inhibition of the enzymatic activity of RNase BS by actin binding is a much weaker and slower process compared to the inhibition of DNase I activity by actin binding (Lazarides and Lindberg, 1974). DNase I is inhibited completely within a split second (Blikstad et al., 1978), whereas the maximum inhibition of RNase BS is reached only after 5min (Simm et al., 1987).

Note that the dissociation constants for angiogenin/actin (Hu et al., 1991, 1993) and DNase I/actin (Mannherz et al., 1980) complexes (0.05 and 0.5nM, respectively) are close to each other and differ by several orders from K_d of RNase BS/actin complexes (10μM) (Simm et al., 1987).

Chang et al. (1996), citing their preliminary data, reported that angiogenin competes with DNase I for binding to actin. Unfortunately, the authors have not published the detailed results of these experiments. At the same time, tropomyosin binding does not prevent F-actin depolymerization by DNase I (Hitchcock et al., 1976). The high-resolution structure of the actin–DNase I complex (Kabsch et al., 1990) shows that the main contact between actin and DNase I involves the loop formed by residues Arg39 to Gly46 of subdomain 2 in the small actin domain. As binding of tropomyosin and angiogenin to F-actin is not strictly competitive, it is possible that angiogenin and DNase I may bind to the same site of actin molecule.

4.5. Angiogenin/actin interplay *in vivo*

Both actin and an anti-actin antibody inhibit the angiogenic activity of angiogenin in the CAM assay (Hu et al., 1993). The results suggest that the reaction between angiogenin and cell-surface actin is an essential step in the angiogenesis process induced by angiogenin.

Although actin is more commonly known as an intracellular protein, it was found also on the external surface of not only endothelial cells (Hu et al., 1991, 1993; Moroianu et al., 1993) but also lymphocytes (Owen et al., 1978; Sanders and Craig, 1983), monocytes (Por et al., 1991), fibroblasts (Chen et al., 1978), and smooth muscle cells (Accinni et al., 1983; Jones et al., 1979). Despite strong evidence of actin presence on the cell surface, many aspects of this phenomenon, such as the precise structure of cell-surface actin and its translocation pathways, remain unclear (Smalheiser, 1996).

It has been suggested that the cell-surface actin of cultured endothelial cells may correspond to ECM–actin *in vivo*. It is thought that binding of angiogenin to ECM–actin molecules results in activation of a cell-associated protease system that could lead to cell detachment from the ECM followed by migration, proliferation, and differentiation into microvessels (Hu et al., 1993).

Actin accelerates plasmin generation by binding to a kringle (lysine-binding) region of both plasminogen and tissue plasminogen activator (tPA) thereby increasing a local concentration of protease (Lind and Smith, 1991). At the same time, actin inhibits the proteolytic activity of plasmin. G-actin and F-actin have the same inhibitory activity against plasmin. Both tranexamic acid and ε-aminocaproic acid prevent actin inhibition of plasmin, suggesting that accessible lysine residues of actin interact with the kringle region of plasmin. Actin-binding proteins such as gelsolin, vitamin D-binding protein, and DNase I do not prevent actin from inhibiting plasmin (Lind and Smith, 1991).

Formation of the angiogenin/actin complex significantly accelerates tPA-catalyzed generation of plasmin from plasminogen, and in contrast to actin, the angiogenin–actin complex does not inhibit plasmin activity (Hu and Riordan, 1993; Hu et al., 1994). In the presence of the angiogenin–actin complex, the overall proteolytic activity of a mixture of tPA and plasminogen is 11-fold higher than in its absence and sixfold higher than in its presence of actin alone. It was shown that in the presence of $1 pgml^{-1}$ bovine angiogenin, the proteolytic activity of cultured endothelial cells was 14-fold higher than in its absence (Hu et al., 1994).

Angiogenin promotes the degradation of basement membrane and ECM through the formation of the complex with actin and thus allows endothelial cells to penetrate and migrate into the perivascular tissue (Hu et al., 1994; Soncin, 1992). After the leading cells migrate away, the local density of the cells decreases. This triggers the expression of the 170-kDa putative angiogenin receptor on the remaining adjacent cells. Probably as a result of binding to a 170-kDa putative receptor on the endothelial cell surface (Hu et al., 1997), angiogenin induces second messenger responses, including diacylglycerol, prostacyclin (product of arachidonate metabolism; Bicknell and Vallee, 1988, 1989), and nitric oxide (Trouillon et al., 2010) and induces transient phosphorylation of extracellular signal related kinase Erk1/2 (Liu et al., 2001a) and protein kinase B/Akt (Kim et al., 2007). These cells become responsive to stimulation of angiogenin and will therefore divide to fill the space created by the migrating cells. The expression of the receptor may then be turned off when the cell density increases. It is speculated that such density-dependent receptor expression may regulate the angiogenin-induced growth of the new capillary network (Hu et al., 1997).

Cell-surface-associated actin binds not only angiogenin but also the angiogenesis inhibitor, angiostatin, a naturally occurring protein produced by autoproteolytic or elastase cleavage of plasminogen (Dudani et al., 2005). Angiostatin mimics plasminogen-binding properties but is not able to replace plasmin, since it does not contain a plasminogen protease domain (O'Reilly et al., 1994). Interestingly, elastase can cleave angiogenin at Ile29/Met30 to produce two disulfide-linked fragments. Elastase-cleaved angiogenin retains RNase activity but loses the ability for nuclear translocation that is essential for angiogenin activity (Hu,

1997). Thus, interactions and balance among plasminogen, tPA, elastase, actin, angiogenin, and angiostatin may be crucial in the regulation of angiogenesis.

Recently, the angiogenin upregulation of Kaposi's sarcoma (KS)-associated herpes virus (KSHV) was studied (Sadagopan et al., 2009). KSHV infection of human microvascular dermal endothelial cells results in increased secretion of angiogenin and other angiogenic factors, several growth factors, cytokines, and chemokines. KS tissue sections were positive for angiogenin, pointing to the importance of angiogenin in KS pathogenesis. It was demonstrated that KSHV-induced angiogenin was secreted from cells and bound to endothelial cell-surface actin. Upon KSHV infection, the actin–angiogenin complex forms under even confluent cell conditions. Potentially, the actin–angiogenin complex may be responsible for the activation of urokinase plasminogen activator resulting in the generation of plasmin from plasminogen, which is necessary for ECM degradation and cell migration.

As already mentioned, angiogenin is able to interact with intracellular actin indirectly through cytosolic α-actinin-2 (Hu et al., 2005). α-Actinin-2 is an actin-binding protein with specific structural and regulatory roles in different cell types. It forms an antiparallel rod-shaped dimer with one actin-binding domain at each end of the rod and bundles actin filaments. In skeletal, cardiac, and smooth muscles, α-actinin-2 is localized in the Z-disc, where it cross-links actin filaments from adjacent sarcomeres. In nonmuscle cells, α-actinin-2 was found along microfilament bundles and adherens-type junctions, where it is involved in binding actin to the membrane. In addition to actin filaments, α-actinin-2 interacts with a number of cytoskeletal and signaling molecules and receptors and integrates them to the actin cytoskeleton (Sjöblom et al., 2008). In the central nervous system, α-actinin-2 localizes to synaptic junctions in a complex with the NR1 and NR2B subunits of the *N*-methyl-D-aspartate (NMDA) glutamate receptors (Dunah et al., 2000; Wyszynski et al., 1997).

The recent discovery of missense variants in the angiogenin gene found in patients with amyotrophic lateral sclerosis (ALS) (Crabtree et al., 2007; Fernández-Santiago et al., 2009; Gellera et al., 2008; Greenway et al., 2004, 2006; Kishikawa et al., 2008) indicates an important role for angiogenin in motor neuron physiology and pathology. There is increasing evidence that mutations in both the signal peptide region (M24I, F13S, F13L, P4S) and in mature angiogenin (Q12L, K17E, K17I, S28N, R31K, C39W, K40I, I46V, P112L, V113I) play a role in ALS pathophysiology.

Mutations Q12L, C39W, and K40I, which are located in the catalytic center of the protein, undermine neurite extension, pathfinding, and survival of motor-neurons (Kieran et al., 2008; Subramanian et al., 2008). Mutations K17I, S28N, and P112L result in complete angiogenin loss-of-function, by reducing angiogenesis due to defects in ribonuclease activity, nuclear translocation, or a combination of both (Wu et al., 2007). For example, angiogenin containing the K17I mutation in the loop between the H1 and H2 helices lost angiogenic activity. It was shown that it had only 5–13% of the ribonucleolytic activity of the wild type, probably as a result of damaging a peripheral substrate binding site, and retained only a reduced capacity to translocate into the nucleus (Crabtree et al., 2007; Wu et al., 2007). This angiogenin missense variant led to neuronal intranuclear protein inclusions

that were immunoreactive for smooth muscle α-actin, but not for angiogenin. Meanwhile, expression of this actin was increased in the liver where severe steatosis was observed (Seilhean et al., 2009). Accumulation of actin due to the K17I angiogenin mutation is probably related to a disturbance of direct angiogenin–actin interaction, but the precise mechanisms of this phenomenon are not yet known.

5. Concluding Remarks

Since the discovery of angiogenin in 1985, views on its functional role have repeatedly been reconsidered. The initial view of angiogenin as a tumor-derived angiogenic factor has been refined and extended. Every year, new evidence of this protein's unique role in angiogenesis and other cellular processes is found.

Interaction of angiogenin with the cell-surface actin of endothelial cells was first detected about 20 years ago. The ingrained view of angiogenin as the inducer of actin polymerization has not received experimental confirmation. Instead, angiogenin has been shown to inhibit actin polymerization. It has been suggested that binding of angiogenin to G-actin as well as to F-actin may cause changes in cell cytoskeleton by inhibiting polymerization of G-actin and changing physical properties of F-actin.

Angiogenin/actin interaction stimulates the proliferation of cells in neovascularization but is not determinative in the process of angiogenesis, as initially thought. Nevertheless, one should not underestimate the importance of the interaction of angiogenin with actin for biological processes. Recent data have demonstrated that disruption of normal angiogenin/actin interactions may lead to different pathologies. Moreover, the inhibitory effect of actin on angiogenesis that is induced by angiogenin suggests a class of angiogenesis inhibitors that may have therapeutic benefit in pathological conditions characterized by excessive blood vessel growth.

The ubiquitous distribution of actin may provide a convenient and effective way to regulate angiogenesis. Two widely distributed multifunctional growth factors, basic fibroblast growth factor and tumor necrosis factor α, angiogenic molecules with distinct cellular receptors for different cellular responses, can also bind to actin. Therefore, this interaction may reflect a more general mechanism that is induced by different angiogenic molecules.

Acknowledgments

This work was partly supported by NIH grant GM081688 to A. S. K.

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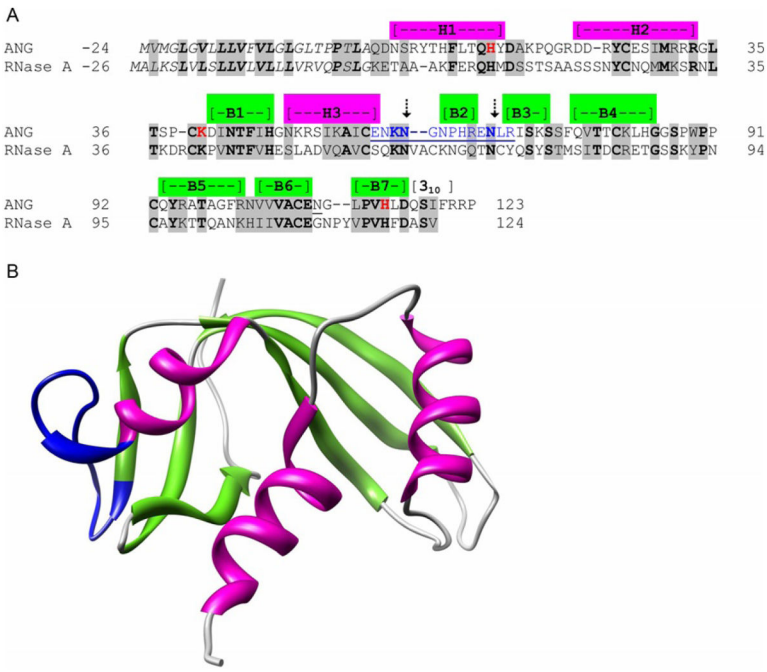

Figure 5.1.

(A) Alignment of human angiogenin (NP_001091046.1) and bovine pancreatic RNase A (NP_001014408.2) amino acid sequences. Conserved residues are shaded in gray and residues that are identical in both proteins are bold. Italic—leader peptides. Residues in red are the most critical for ribonucleolytic activity. The secondary structure (H—α-helix, B—β-strand) of human angiogenin is indicated in accordance with the crystallographic data (PDB 1B1I). (B) Ribbon model of bovine angiogenin (PDB 1AGI). Blue (underlined)—angiogenin segment that contains residues responsible for angiogenin binding to the cell receptor.