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Emerging biological functions of ribonuclease 1 and angiogenin

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Abstract

Pancreatic-type ribonucleases (ptRNases) are a large family of vertebrate-specific secretory endoribonucleases. These enzymes catalyze the degradation of many RNA substrates and thereby mediate a variety of biological functions. Though the homology of ptRNases has informed biochemical characterization and evolutionary analyses, the understanding of their biological roles is incomplete. Here, we review the functions of two ptRNases: RNase 1 and angiogenin. RNase 1, which is an abundant ptRNase with high catalytic activity, has newly discovered roles in inflammation and blood coagulation. Angiogenin, which promotes neovascularization, is now known to play roles in the progression of cancer and amyotrophic lateral sclerosis, as well as in the cellular response to stress. Ongoing work is illuminating the biology of these and other ptRNases.

Keywords

Blood coagulation; endoribonuclease; extracellular RNA; inflammation; pancreatic–type ribonuclease; stress

Introduction

Ribonucleases (RNases) are omnipresent enzymes that operate at the crossroads of transcription and translation (Figure 1) (D'Alessio and Riordan, 1997; Nicholson, 2011). By catalyzing the cleavage of RNA, RNases oversee a myriad of biological niches. For example, RNA primers that promote the replication of DNA are broken down by RNase H, tRNAs for the ribosomal translation of proteins are matured by RNase P, and miRNAs that modulate the expression of other RNA transcripts are processed by Dicer and Drosha. All of these functions are highly conserved and essential for eukaryotic cells (Bernstein et al., 2003; Wang et al., 2007; Altman, 2011; Reijns et al., 2012).

Vertebrates express a unique RNase superfamily, termed the vertebrate secretory or pancreatic-type ribonucleases (ptRNases) (Beintema et al., 1988; Beintema and Kleineidam, 1998). In comparison to intracellular RNases, our understanding of the biological function

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of ptRNases is immature. The imperative for clarification has intensified because RNAs in the extracellular space are now known to act as messengers, inflammatory mediators, and enzyme activators (Lomax et al., 2017; Lu et al., 2018).

The pancreatic-type ribonuclease superfamily

Pancreatic-type ribonucleases are perhaps the most well-studied enzyme family. The familiarity with ptRNases is due to extensive research on the prototypical member of this enzyme family, RNase A. After its detection in porcine pancreatic tissue over a century ago (Jones, 1920), RNase A became the favorite model protein of the foundational years of biochemistry (Richards and Wyckoff, 1971; Blackburn and Moore, 1982; Raines, 1998; Marshall et al., 2008). For example, RNase A was the source of the first fragment complementation system (Richards and Vithayathil, 1959), the first enzyme for which an amino acid sequence was determined (Hirs et al., 1960), the first enzyme for which a mechanism of catalysis was identified (Findlay et al., 1961), the third enzyme for which a crystal structure was determined (Kartha et al., 1967), and the object of Nobel-prize-winning studies on amino acid sequencing (Moore and Stein, 1973), protein folding (Anfinsen, 1973), and solid-phase synthesis (Merrifield, 1986). In addition to their seminal contributions to biochemistry as a field, these studies on RNase A laid the groundwork for understanding other ptRNases.

Characteristic biochemical features of ptRNases

ptRNases are small enzymes. RNase A is composed of 124 amino acid residues, with a molecular mass of 13.7 kDa, which is similar to that of other family members (Cho et al., 2005). Each ptRNase contains a signal peptide that directs the secretion of the protein (Zhang et al., 2002). Despite the low sequence identity between ptRNase genes (Cho et al., 2005), the three-dimensional structure of their encoded enzymes is highly conserved (Figures 2A and 2B). An invariant catalytic triad of two histidine residues and a lysine residue endows ptRNases with the ability to catalyze the cleavage of the P–$O^{5'}$ bond on the 3′ side of pyrimidine residues (Figure 3). ptRNases are cationic in character, which encourages binding to their anionic RNA substrates (Park and Raines, 2001), and they have high thermostability, owing largely to the presence of intramolecular disulfide bonds (Klink et al., 2000; Ruoppolo et al., 2000; Schulenburg et al., 2010).

ptRNases are inhibited by ribonuclease inhibitor (RI), which is a 50-kDa protein that is likewise conserved in vertebrates (Lomax et al., 2014) and is expressed ubiquitously and abundantly in the cytosol of cells (Lee and Vallee, 1993; Dickson et al., 2005; Sarangdhar and Allam, 2021). RI comprises leucine-rich repeats (Hofsteenge et al., 1988; Kobe and Deisenhofer, 1993) and is anionic, facilitating Coulombic interactions with cationic ptRNases (Johnson et al., 2007) (Figure 2C). The two proteins form a 1:1 complex with a K_d value near 10^{-15} M (Lee et al., 1989; Vicentini et al., 1990; Lomax et al., 2014). This extraordinary protein–protein interaction blocks access to the active site of ptRNases and squelches catalytic activity (Domachowske et al., 1998b; Spencer et al., 2014). Consequently, RI is capable of protecting cells from the action of endocytosed

ptRNases, which would otherwise degrade cellular RNAs and cause cell death (Thomas et al., 2016; Thomas et al., 2018).

ptRNases have been identified in all vertebrate species evaluated to date. Humans express genes encoding 13 such enzymes, clustered near one another on chromosome 14 (Zhang et al., 2002). Eight of these ptRNases are classified as “canonical” (Sorrentino, 2010). The remaining five exhibit more unusual features, such as the absence of one or more catalytic residues or the lack of cationic character (Cho et al., 2005). Other species also express multiple ptRNases, with gene duplication resulting in larger numbers of enzymes in some species. For example, mice express about 20 ptRNase genes, owing to duplication of the ribonucleases 2 and 3 and ribonuclease 5 (angiogenin) subfamilies (Cho et al., 2005).

Biological functions of the ptRNase superfamily

The first report on the function of RNase A claimed that the enzyme participated in ruminant digestion but was vestigial in non-ruminants (Barnard, 1969). The appreciation of other biological roles has been growing for decades (Benner and Allemann, 1989; D’Alessio, 1993; Wang et al., 2018a; Lee et al., 2019). Antimicrobial activity has been described for a cluster of closely related ptRNases, including ribonucleases 2 and 3, as well as 6, 7, and 8 (Sorrentino, 2010), which are expressed in eosinophil granules, placenta, and skin and exhibit relatively broad-spectrum cytotoxicity (Ackerman et al., 1985; Molina et al., 1988; Lehrer et al., 1989; Venge et al., 1999; Harder and Schroder, 2002; Boix and Nogués, 2007; Rosenberg, 2008; Attery and Batra, 2017) and antiviral activity (Domachowske et al., 1998a; Domachowske et al., 1998b; Ilinskaya and Mahmud, 2014; Li and Boix, 2021). Angiogenins have well-documented angiogenic activity, but some subfamily members have also been reported to exhibit immune activity. Ribonucleases 9 and 10, which are non-canonical members of the ptRNase superfamily, play roles in the maturation of sperm (Krutskikh et al., 2012; Westmuckett et al., 2014). For other members of the ptRNase superfamily, however, biological functions are unclear. Recent research on two particular family members, RNase 1 and angiogenin (RNase 5), has revealed numerous new functions for these enzymes in various biological niches, ranging from vascular homeostasis and inflammation to cellular growth and quiescence. Here, we focus on these newly identified biological roles.

Ribonuclease 1

RNase 1 is the most active catalyst of the ptRNase superfamily in humans. Its activity against single-stranded RNA is nonspecific, degrading poly(C) > poly(U) >> poly(A) as well as double-stranded RNA and RNA:DNA hybrids (Sorrentino and Libonati, 1994). RNase 1 is also the most broadly expressed ptRNase, with *Rnase1* mRNA being detectable in all tissue types (Futami et al., 1997). In healthy humans, RNase 1 circulates in serum at a concentration of 250 ng/mL (Weickmann et al., 1984); this serum RNase 1 is produced predominantly by vascular endothelial cells (Landre et al., 2002; Barrabés et al., 2007). Though secreted constitutively, RNase 1 also accumulates in Weibel–Palade bodies in endothelial cells (Fischer et al., 2011). Additionally, RNase 1 can be *N*-glycosylated at Asn34, Asn76, and Asn88 (Figure 2A) (Beintema, 1986), a phenomenon that appears to vary with the tissue of origin (Mizuta et al., 1990; Yasuda et al., 1993; Ribó et al.,

1994). A glycan at any of the three residues attenuates catalytic activity but enhances both thermostability and resistance to proteolysis (Ressler and Raines, 2019). The sequon that includes Asn34 is highly conserved among vertebrate species, and a glycan there donates hydrogen bonds that "cap" the carbonyl groups of an α-helix (Kilgore et al., 2020). RNase 1 also binds to some cell-surface glycans with micromolar affinity (Eller et al., 2014), and RNase A exhibits an especially specific interaction with Globo H (Eller et al., 2015).

Early biological studies

Human RNase 1 first elicited biomedical interest when its upregulation in the plasma of patients with pancreatic cancer spurred excitement about its potential utility as a biomarker (Reddi and Holland, 1976). Subsequent studies showed that RNase 1 levels were elevated not only by pancreatic cancer, but also by other malignancies (Maor and Mardiney, 1978; Isaacs, 1981; Weickmann et al., 1984). Ultimately, the idea of using RNase 1 as a cancer biomarker was abandoned because of its upregulation in other disease states, including bacterial and viral infection (Kutas et al., 1969), smoking (Maor et al., 1978), burn injury (Coombes et al., 1978), surgery (Barlow et al., 1979), myocardial infarction (Sznajd et al., 1981), ageing (Coombes et al., 1977), Alzheimer's disease (Sajdel-Sulkowska and Marotta, 1984), malnutrition (Sigulem et al., 1973), and arthritis (Oribe, 1984). Nonetheless, a recent study demonstrated that RNase 1 promotes breast tumor initiation by activating signaling via tyrosine kinase receptor EphA4 and that high RNase 1 levels correlate with poor clinical outcomes for patients with breast cancer (Lee et al., 2021). These findings could revive interest in the utility of RNase 1 as a biomarker for cancer.

ptRNases returned to the forefront of cancer research with the identification of ranpirnase (Onconase®), a ptRNase in the northern leopard frog (*Rana pipiens*) that exhibited toxicity against cancer cell lines (Darzynkiewicz et al., 1988). This cytotoxicity was attributed to its ability to enter the cytosol and degrade cellular RNA, while being insensitive to inhibition by human RI (Wu et al., 1993; Lee and Raines, 2008). These key attributes of ranpirnase were presaged in a ruminant ptRNase: bovine seminal ribonuclease (BS-RNase) (Dostál and Matoušek, 1973; Matoušek, 1973; D'Alessio et al., 1991). BS-RNase is, in its essence, a natural dimer of RNase 1. The dimer interface is unusual. Two cysteine residues evolved in this ruminant enzyme: Cys31 and Cys32. Their sulfhydryl groups form disulfide bonds with those of another monomer. The enforced proximity facilitates the swapping of N-terminal helices within the covalent dimer (Figure 4). Because dimeric BS-RNase evades RI but monomeric BS-RNase does not (Murthy and Sirdeshmukh, 1992), this domain swap is critical to biological function (Cafaro et al., 1995; Kim et al., 1995a; Kim et al., 1995b; Gotte et al., 2012). That is because the Cys31–Cys32′ and Cys32–Cys31′ disulfide bonds are solvent-exposed and vulnerable to reduction by endogenous β-glutathione in the cytosol. Even after the reduction of these two disulfide bonds, the dimer can remain intact and thus RI-evasive because of the noncovalent interactions that ensue from the domain swap. Monomers of BS-RNase that are engineered to evade RI are highly cytotoxic, as are dimers of those monomers (Lee and Raines, 2005).

The attributes of BS-RNase and ranpirnase inspired the creation of RNase 1 variants that evade association with RI (Rutkoski and Raines, 2008; Lomax et al., 2012). These variants

have shown promise as anti-cancer agents and led to the introduction of a clinical drug candidate (Strong et al., 2012a, 2012b). Variants of RNase 1 that enter the nucleolus, which is devoid of RI, provide an alternative strategy (Castro et al., 2013; Castro et al., 2021). Circular zymogens of RNase 1 that are activated by a pathogenic protease could extend the utility of cytotoxic ribonucleolytic activity beyond cancer (Windsor et al., 2019; Windsor et al., 2021).

Extracellular RNA

The utility of RNase 1 variants as cytotoxins is established and continues to grow. Nonetheless, insight on the biological functions of RNase 1 has remained sparse. The discovery that RNA exists in the extracellular space—where RNase 1 is free to act without inhibition by RI—has led to the emergence of new ideas (Figure 5).

RNA was once thought to be unstable outside of the cellular environment (Benner, 1988). After all, ptRNases reside in the extracellular space and could degrade extracellular RNA (eRNA) species (Kamm and Smith, 1972). Long ago, however, RNA was demonstrated to be released actively by frog and human cells (Stroun et al., 1978). More recent studies have shown that RNA can persist in serum long enough to permit analysis and elicit biological functions. For example, EBER1 RNAs from Epstein–Barr virus, as well as tyrosinase mRNA from melanoma cells, were isolated from serum and plasma (Lo et al., 1999; Hasselmann et al., 2001). Moreover, cancer cells release RNA at a significantly higher rate than do noncancerous cells (Wieczorek et al., 1985; Fischer et al., 2013).

The characterization of eRNAs has revealed that almost every known class of RNA exists outside the cell. The bulk are miRNAs, mRNA, and tRNA; though, other types of RNA, such as rRNA, piwiRNA, lncRNA, and sncRNA, have also been detected in varying proportions (Huang et al., 2013; Yuan et al., 2016; Savelyeva et al., 2017). These eRNAs are transported in a variety of contexts, including within membranous compartments such as microvesicles, exosomes, and apoptotic bodies (Yáñez-Mó et al., 2015), as ribonucleoprotein complexes (Turchinovich et al., 2011), and bound to high-density lipoproteins (Vickers et al., 2011).

eRNA can act as a messenger between cells, driving changes in gene expression (Valadi et al., 2007; Mittelbrunn et al., 2011). eRNA does not need to encode genetic information to exert a biological function. Instead, eRNA can stimulate lymphocyte adhesion to smooth muscle by upregulating the production of adhesion molecules such as Vcam-1, Icam-1, P-selectin, and Ccl2 in smooth muscle cells (Fischer et al., 2012). eRNA can drive macrophage polarization to an M1 (proinflammatory) phenotype, increasing the production of inflammatory markers such as TNFα, IL-1β, IL-6, IL-12, and iNOS (Simsekyilmaz et al., 2014). eRNA also potentiates blood coagulation by acting as an anionic scaffold for the activation of the intrinsic coagulation proteases FXI, FXII, and plasma prekallikrein. These proteases are also known as “contact factors”, as their activation has long been known to be potentiated by artificial anionic substrates, such as glass and kaolin. The finding that eRNA acts as an *in vivo* activator has been appreciated, however, more recently (Nakazawa et al., 2005; Kannemeier et al., 2007). At least for its procoagulant activities, the size and secondary structure of eRNA appears to be as important as its sequence, with coagulation

protease activity only potentiated with RNAs greater than 100 nucleotides in size, and potentiated more strongly with RNAs that form a stable hairpin structure (Gansler et al., 2012). Poly(G) and poly(I) RNAs have been reported to be efficacious activators of intrinsic proteases *in vitro* (Gajsiewicz et al., 2017).

New biological roles

The discovery and characterization of eRNAs is spawning interest in ptRNases (Lomax et al., 2017; Lu et al., 2018). For example, wild-type RNase A has been shown to reduce tumor growth in mice when administered exogenously (Fischer et al., 2013). This effect could be mediated by eRNA, which drives TNFα production via activation of TNFα-converting enzyme (TACE) in macrophages to produce an inflammatory environment conducive to tumor growth (Fischer et al., 2012).

Other studies have shown similar effects of RNase 1 on inflammation in other contexts. In a mouse model of atherosclerosis, eRNA accumulated in atherosclerotic plaques and recruited macrophages to these plaques (Simsekyilmaz et al., 2014). Elevated plasma eRNA was also associated with the incidence of ischemia-reperfusion injury in patients undergoing heart surgery (Cabrera-Fuentes et al., 2015) and with edema and tissue death in a mouse model of myocardial infarction (Cabrera-Fuentes et al., 2014). These adverse effects of eRNA were blocked by the administration of exogenous RNase 1. Notably, however, RNase 1 therapy was reported to be most effective when given prophylactically, as changes engendered by eRNA exposure were irreversible by RNase 1 after the occurrence of an injury (Stieger et al., 2017). Additionally, a TLR7-overproduction mouse model of systemic lupus erythematosus suffered fewer inflammatory symptoms when RNase A was knocked-in (Sun et al., 2013), resulting in extended lifespan, reduced myeloid cell number, and reduced inflammatory deposits in the kidneys and liver of mice. The pre-treatment of an ischemic murine stroke model with RNase A also resulted in less edema, smaller brain lesions, and reduced vascular permeability in treated animals than controls (Fischer et al., 2007; Walberer et al., 2009). Likewise, the treatment of rats undergoing heart transplantation surgery with RNase 1 improved graft survival and reduced edema and thrombus formation (Kleinert et al., 2016).

RNase A is now known to exhibit anticoagulant activity. Treating a mouse model of arterial thrombosis resulted in the clearance of eRNA from the thrombus, delay of vascular occlusion, and reduction in thrombus size (Kannemeier et al., 2007). RNase 1-knockout mice, though viable and fertile, have high plasma eRNA levels (Garnett et al., 2019). Moreover, their plasma forms blood clots much more rapidly than does the plasma from wild-type animals. The mechanism of action of eRNA in blood clotting entails activation of intrinsic coagulation factors FXII and FXI, as well as the upstream protease plasma kallikrein (Kannemeier et al., 2007; Gajsiewicz et al., 2017). This process is linked closely to inflammation, as plasma kallikrein is a protein that, when cleaving plasma kininogen, releases bradykinin, a potent vasodilator and vascular permeabilizing agent. Interestingly, eRNA alone spurs vascular permeabilization in a manner mediated by VEGF and nitric oxide synthase, leading to disintegration of tight junctions in cells (Fischer et al., 2007).

Given the myriad pro-inflammatory functions ascribed to eRNA and the studies conducted to date on RNase 1, the biological function of this enzyme appears to be more complex

and multifaceted than appreciated previously. Early studies demonstrating the upregulation of RNase 1 in many disease states point toward a general role for RNase 1 as a regulator of inflammatory processes, particularly in the cardiovascular system. A role for RNase 1 in this niche reflects its high compatibility with the pH of the vascular environment (Eller et al., 2014; Lomax et al., 2017), and its colocalization in endothelial cells with other vascular regulators, such as von Willebrand factor and P-selectin (Fischer et al., 2011). RNase 1 could modulate the effects of RNA accumulation in the extracellular space secondary to tissue damage, malignancy, or death and thus prevent excess activation of macrophage activity, blood coagulation, and vascular permeabilization. Future studies of RNase 1 are likely to uncover not only new functions of the enzyme, but also the biological roles of now unknown substrate RNAs.

Angiogenin

Angiogenin is named for its ability, which is unique among ptRNases, to stimulate blood vessel growth (Fett et al., 1985). This activity coincides with other unusual features. For example, angiogenin has catalytic activity against simple substrates that is 10^4-fold lower than that of RNase 1 and other ptRNases (Shapiro et al., 1986; Leland et al., 2002). This deficiency is due, in part, to the occlusion of the enzymic active site by Gln117 (Acharya et al., 1994; Russo et al., 1994). Angiogenin has a well-known ability to cleave tRNAs (Saxena et al., 1992; Lyons et al., 2017).

Structural and signaling properties

Unlike RNase 1, angiogenin acts mainly within cells (Figure 5) (Sarangdhar and Allam, 2021). Residues 60–68 compose a receptor-binding site that is necessary for cellular internalization (Hallahan et al., 1991; Raines et al., 1995). The circulating concentration of the enzyme (~400 ng/mL) is much higher than that required to stimulate its angiogenic activity (Bicknell and Vallee, 1988), and this activity occurs in normal cells in a density-dependent manner (Hu et al., 2000). Several different proteins have been identified as receptors that mediate angiogenin activity, including syndecan-4 in astrocytes (Skorupa et al., 2012) and plexin B2 in multiple cell types (Yu et al., 2017). On the cell surface, angiogenin also binds to the EGFR and activates the ERK pathway (Liu et al., 2001; Wang et al., 2018b; Lee et al., 2019).

Angiogenin contains a nuclear localization signal (Arg31–Arg32–Arg33) (Moroianu and Riordan, 1994), which drives the accumulation of the enzyme in the nucleolus and is required for its angiogenic activity (Xu et al., 2002). Normally, the nuclear localization of angiogenin would be prevented by the association of the enzyme with cytosolic RI, to which angiogenin binds with femtomolar affinity (Lee et al., 1989). Angiogenin avoids association with RI by the disruption of favorable Coulombic interactions through phosphorylation, which is known to occur at Ser28, Ser37, and Ser87 (Figure 2B) and is mediated by PKC and CDK (Hoang and Raines, 2017).

In the nucleus, angiogenin has been shown to cleave pRNA, which normally associates with TIP5, a member of the NoRC chromatin remodeling complex. pRNA is responsible for the recruitment of TIP5 and NoRC to the rDNA promoter, which results in repression of

rDNA transcription (Mayer et al., 2006; Mayer et al., 2008). Cleavage of pRNA prevents its association with TIP5 and impairs recruitment of NoRC to the rDNA promoter, which results in derepression of rDNA and cell growth (Hoang and Raines, 2017). This signaling pathway is unique among transcription factors, as no other proteins are known to reach the nucleus from the extracellular space and effect transcriptional change.

Angiogenin-mediated cell growth

In 1985, angiogenin was identified as a protein capable of stimulating blood vessel growth from human adenocarcinoma cells (Fett et al., 1985). Subsequently, angiogenin was shown to be secreted by multiple types of tumors, including those from prostate cancer (Yoshioka et al., 2006), oral squamous cell carcinoma (Kishimoto et al., 2012), and melanoma (Hartmann et al., 1999). Its production from cancer cells appears to be regulated by HIF-1α (Kishimoto et al., 2012), suggesting a mechanism of action for tumor neovascularization in response to oxygen starvation. Angiogenin has also been studied as a biomarker in human colorectal cancer (Ramcharan et al., 2013), bladder cancer (Urquidi et al., 2012), and breast cancer (He et al., 2015), and its levels have been correlated with tumor aggressiveness. Consequently, angiogenin is a therapeutic target for the treatment of cancer. The knock-down of angiogenin in bladder cancer cells using siRNA did diminish tumor growth in a mouse xenograft model (Shu et al., 2015), and the neutralization of angiogenin with an antibody reduced the *in vitro* migration of triple-negative breast cancer cells (Dutta et al., 2014). An experimental small-molecule drug, 8-amino-5-(4′-hydroxybiphenyl-4ylazo)naphthalene-2-sulfonate, inhibits catalysis by angiogenin and deters the growth and vascularization of tumors in mouse xenograft models (Kao et al., 2002).

Angiogenin also acts in the placenta, endometrium, and ovarian follicle, tissues that normally undergo extensive angiogenesis in adults (Reynolds et al., 2002). Angiogenin is present in ovarian follicular fluid. There, its production is stimulated by human chorionic gonadotropin—a hormone that promotes luteinization and angiogenesis during the formation of the corpus luteum (Koga et al., 2000). In studies of ovarian follicle transfer, angiogenin was identified to be a crucial factor in mediating the angiogenic and follicle-preserving effect of co-transplanted mesenchymal stem cells (Zhang et al., 2017), underscoring the importance of the ribonuclease for follicle survival and function. Angiogenin levels in the endometrium reflect the menstrual cycle, approximately doubling in the mid-to-late secretory phases relative to the proliferative phase (Koga et al., 2001). In pregnancy, angiogenin levels increase over time in the human placenta, reaching a maximum at term (Rajashekhar et al., 2002; Pavlov et al., 2003). In the placenta, angiogenin is found alongside markers of early vasculogenesis, such as VE-cadherin, CD34, and von Willebrand factor, and is present in developing fetal blood vessels (Pavlov et al., 2014).

Angiogenin can promote cell growth elsewhere than in the vascular endothelium. This versatility was observed first in the context of cancer; HeLa cells both secrete angiogenin and take it up, with angiogenin localizing to the nucleus and stimulating cell proliferation independent of cell density (Tsuji et al., 2005). Angiogenin has also demonstrated this activity in prostate cancer cells (Yoshioka et al., 2006). In addition, angiogenin appears to promote the growth of hepatocellular carcinoma cells in paracrine. These cells secrete

angiogenin, which is taken up by tumor-associated hepatic stellate cells, spurring their activation and promoting the growth and metastasis of hepatocellular carcinoma cells (Han et al., 2014; Bárcena et al., 2015). Angiogenin is also expressed in the nervous system during embryonic development in mice and is localized to axonal growth cones, where it appears to promote axonal growth and neurite pathfinding (Subramanian and Feng, 2007).

Angiogenin-mediated protection from cell stress

Loss-of-function mutations in the human *ANG* gene are associated with amyotrophic lateral sclerosis (ALS). In addition to its stimulation of neuronal growth, angiogenin has also been observed to have a protective effect on motoneurons in the context of ALS. Similar to the action of angiogenin in hepatocellular carcinoma, this activity is mediated in paracrine, by astrocyte uptake of motoneuron-secreted angiogenin (Skorupa et al., 2012). Angiogenin-stimulated astrocytes are then able to protect motoneurons against excitotoxic stress through a mechanism that involves protein expression changes in astrocytes largely related to modulation of the extracellular matrix (Skorupa et al., 2013). This finding helps to explain many earlier studies that linked ALS to *ANG* mutations, many of which interfere with the catalytic activity of the enzyme (Greenway et al., 2006; Gellera et al., 2008). Nrf2 is a transcription factor that regulates the response of neurons and other cell types to oxidants and is likewise implicated in ALS. Angiogenin activates the Nrf2 pathway and thereby counteracts the deleterious consequences of reactive oxygen species. This link between ANG and Nrf2 provides a clear molecular basis for the neuroprotective activity of ANG and highlights the potential utility of angiogenin as a treatment for ALS (Hoang et al., 2019), though more data are necessary (Aluri et al., 2020).

A possible effector substrate for angiogenin in neurons is tRNA, which has been known to be an angiogenin substrate for many years (Saxena et al., 1992). This activity was first reported as cytotoxicity, as cleaved tRNAs arrest protein translation. Yet, it has been appreciated only recently that some, but not all (Su et al., 2019), tiRNAs (which are tRNAs cleaved at the anticodon loop) are a product of catalysis by angiogenin that is induced by cellular stress (Yamasaki et al., 2009; Fu et al., 2009; Su et al., 2020). These tiRNAs promote the formation of stress granules and inhibit protein synthesis, which is suggested to occur via displacement of the initiation factor eIF4G/A from mRNA (Ivanov et al., 2011). Nonetheless, tiRNAs also exert a pro-survival effect in cells by binding to apoptotic protease-activating factor 1 (APAF1) in a manner that competitively blocks the binding of cytochrome c and the formation of the apoptosome (Saikia et al., 2014). SLFN2 binds to and protects tRNAs from oxidative stress-induced cleavage by the angiogenin, allowing activated T cells to maintain protein synthesis in the presence of reactive oxygen species that would otherwise inhibit translation (Yue et al., 2021).

Angiogenin has also been implicated to play roles in stem cell self-renewal. In hematopoetic stem/progenitor cells (HPSCs), angiogenin has been found to generate tiRNAs and reduce protein synthesis, which is suggested to promote the stemness and quiescence of these cells while having a proliferative effect on myeloid progenitor cells (Goncalves et al., 2016). The mechanism by which angiogenin differentially regulates these two cell types is unknown.

Angiogenin has antimicrobial and anti-inflammatory activities. For example, angiogenin has been reported to be toxic to *Candida albicans*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* at micromolar concentrations (Hooper et al., 2003; Noschka et al., 2021), though its antimicrobial activity has been called into question (Avdeeva et al., 2006). Closely related proteins in the angiogenin subfamily in mice could have broader-spectrum antimicrobial activity (Hooper et al., 2003). Further, angiogenin is an acute-phase protein (Olson et al., 1998) that suppresses the effects of TNFα in fibroblasts, reducing levels of NFκB, TNFαR1, TNFαR2, IL6, and IL8 and exerting a net anti-inflammatory effect (Lee et al., 2014; Park et al., 2020).

Emerging research on angiogenin suggests broader roles in cell growth and survival that extend beyond well-characterized roles in vascular growth. Future research on angiogenin might offer a better understanding of the mechanisms that determine whether angiogenin drives cellular proliferation or quiescence. Angiogenin is an attractive drug target for both antiangiogenic therapy in cancer (Kao et al., 2002) and protection from cellular stress in neurodegeneration (Hoang et al., 2017), though more knowledge of the regulators of its activity could enable more selective targeting of the enzyme in specific biological niches.

Connections to vertebrate physiology

New roles identified for RNase 1 and angiogenin cluster broadly into the areas of regulating vascular homeostasis and cell growth/survival. Interestingly, some of these roles are tied to the innate physiology of vertebrates. ptRNase are exclusively present in vertebrates and are the only enzyme family limited to that subphylum (International Human Genome Sequencing Consortium, 2001). The distribution of ptRNase subfamilies within vertebrates does, however, vary. Some vertebrate species, such as fish, express only angiogenin-like enzymes (Pizzo et al., 2006; Kazakou et al., 2008), and the diversification of the RNase 2 and 3 subfamilies occurs only in rodents (Cho et al., 2005). Some highlights of this evolutionary path are depicted in Figure 6.

Angiogenin could be the primordial ptRNase. This hypothesis is supported by the presence of enzymes in fish that are more similar to human angiogenin than to RNase 1 or other ptRNases (Kazakou et al., 2008). Additionally, angiogenin has three intramolecular disulfide bonds in contrast to the four in other ptRNases (Sorrentino, 2010) (Figures 2A and 2B). That fourth disulfide bond is reduced more readily than the other three (Zhou and Strydom, 1993), suggesting that it is less important to enzymic structure and function and was likely a later addition (Strydom, 1998). Angiogenin is, then, likely to be nearly as old as vertebrates themselves (Figure 6), a reflection of its fundamental roles in cell growth and survival. The functions of angiogenin in the brain highlight this centrality, as the neural crest (which gives rise to protective glial cells) is not present in invertebrates (Green et al., 2015).

One of the other hallmarks of vertebrate physiology is the presence of a closed circulatory system with a continuous endothelial barrier (Muñoz-Chápuli et al., 2005), which requires a specialized blood clotting system (Doolittle and Surgenor, 1962). In humans, the coagulation of blood plasma is achieved through a complex network of coagulation factor proteases, which are produced as zymogens and require activation from an upstream factor or series of factors. As a consequence of mechanical injury or inflammation, tissue factor (also

known as Factor III) enters the bloodstream and forms a complex with the zymogen form of Factor VII, which potentiates its proteolytic activation. This TF·FVIIa complex acts as a "tenase," performing proteolytic activation of FX to FXa. In parallel, intrinsic factor proteases, including FXII and prekallikrein, are activated in a protease-independent manner through contact with a variety of factors (such as proteins, glycans, or eRNA). FXIIa catalyzes the activation of FXI to FXIa, which in turn activates FIX to FIXa. Both FXIa and FIXa perpetuate further activation of FVII and of themselves, and FIXa forms a tenase complex with Factor VIII and Factor V. Both cascades result in the generation of thrombin (*i.e.*, Factor II), which catalyzes the subsequent cleavage and cross-linking of fibrinogen, ultimately yielding a stable fibrin clot (Smith et al., 2015).

Some vertebrates share the common thrombin-catalyzed coagulation pathway of humans but lack intrinsic factor proteases such as FXII, FXI, and prekallikrein (Jiang and Doolittle, 2003). Intrinsic factors emerged with the evolution of amphibians, with FXI appearing only in mammals (Doolittle, 2009). Interestingly, the emergence of these RNA-sensitive coagulation factors mirrors the presence of RNase 1. RNase 1 is more abundant in reptiles and birds than in fish that do not express these intrinsic coagulation factors, and much more highly expressed in mammals (Zendzian and Barnard, 1967), suggesting that the evolution of RNA-responsive coagulation proteins and a cognate extracellular ribonuclease to regulate this activity were simultaneous.

Vertebrates also exhibit a much more specialized immune system than do invertebrates, with the emergence of major histocompatibility complex proteins, the T-cell receptor, and immunoglobulins. The rapid evolution of ptRNases has been used to suggest that these enzymes also contribute to the vertebrate-specific immune response (Beintema et al., 1988; Rosenberg et al., 1995; Rosenberg, 2008; Goo and Cho, 2013), as many family members (including angiogenin) are cytotoxic against a variety of microorganisms (*vide supra*). RNase 1 alone, however, is not cytotoxic (Eller and Raines, 2020). Instead, RNase 1 modulates the activity of a broad array of cytokines and affects the interplay between innate and adaptive immunity via eRNA-mediated macrophage polarization, which influences T and B cell activity (Kleinert et al., 2016). The polarization of macrophages also appears to be a vertebrate-specific phenomenon, having been observed in fish but not other animals (Chettri et al., 2014). These multiple avenues implicate eRNA (and, by extension, RNase 1) as a regulator of the inflammation associated with cancer, autoimmune disease, and infectious disease.

Prospectus

Numerous new biological functions of pancreatic-type ribonucleases have been revealed in the past decade. Still, our appreciation of the functions of these enzymes in their endogenous environment has lagged. The effects of RNase 1 as an exogenous agent have been characterized in multiple models of cancer, vascular homeostasis, and injury. Angiogenin has been studied more thoroughly, but little information about the phenotype of constitutive knockout mice is available. Further study on the biological functions of these enzymes—RNase 1 outside of cells and angiogenin inside of cells—would advance

our knowledge of the roles of the ptRNase superfamily and the functions of novel RNA populations and could guide the development of new therapeutic regimens.

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Abbreviations:

CDK	cyclin-dependent kinase
EGFR	epidermal growth factor receptor
EphA4	ephrin A4
ERK	extracellular signal-regulating kinase
eRNA	extracellular RNA
HIF-1α	hypoxia-inducible factor 1-alpha
HPSC	hematopoetic stem/progenitor cell
lncRNA	long non-coding RNA
miRNA	microRNA
iNOS	inducible nitric oxide synthase
NoRC	nucleolar remodeling complex
piwiRNA	piwi-interacting RNA
PKC	protein kinase C
poly(A)	polyadenylic acid
poly(C)	polycytidylic acid
poly(G)	polyguanylic acid
poly(I)	polyinosinic acid
poly(U)	polyuridylic acid
pRNA	promoter-associated RNA
ptRNase	pancreatic-type ribonuclease
rDNA	ribosomal DNA
RI	ribonuclease inhibitor protein
RNase	ribonuclease

SLFN2	Schlafen 2
sncRNA	small non-coding RNA
TIP5	termination factor I-interacting protein 5
TLR7	Toll-like receptor 7

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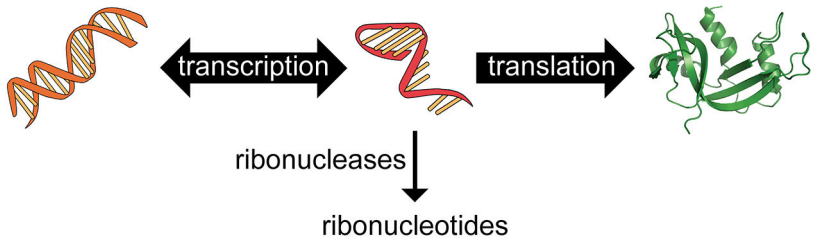

Figure 1.
Ribonucleases function at the crossroads between transcription and translation.

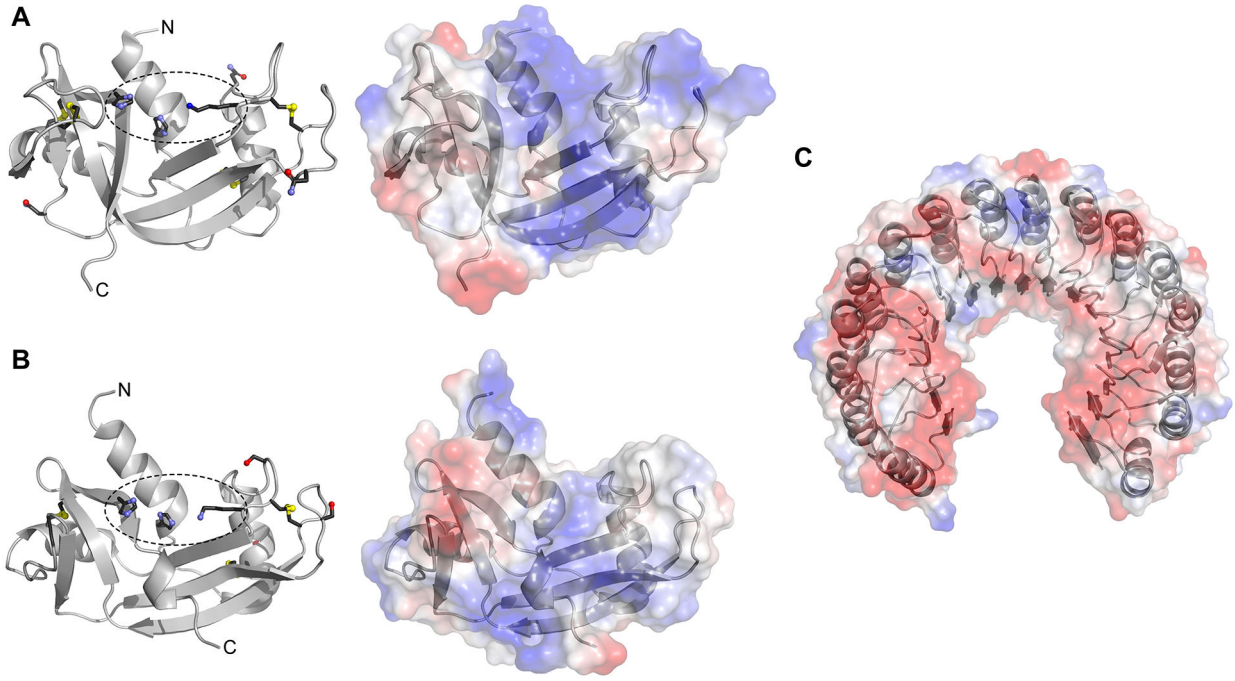

Figure 2.

Structures of human RNase 1 (UniProtKB P07998), angiogenin (UniProtKB P03950), and RI (UnitProtKB P13489). (A) Ribbon diagram of RNase 1. The active-site residues (His12, Lys41, and His119; circled), four disulfide bonds, and three sites of asparagine glycosylation (Asn34, Asn76, and Asn88) are shown explicitly with CPK colors. Right, surface charges (blue, positive; red, negative). The cationic binding cleft for RNA is apparent across the middle of the structure. (B) Ribbon diagram of angiogenin. The active-site residues (His13, Lys40, and His114; circled), three disulfide bonds, and three sites of serine phosphorylation (Ser28, Ser37, and Ser87) are shown explicitly. Right, surface charges. (C) Surface charges of RI, which is highly anionic. Images were made with the program PyMOL from Schrödinger (New York, NY). Atomic coordinates: RNase 1 and RI, PDB entry 1z7x (Johnson et al., 2007); angiogenin, PDB entry 1ang (Acharya et al., 1994).

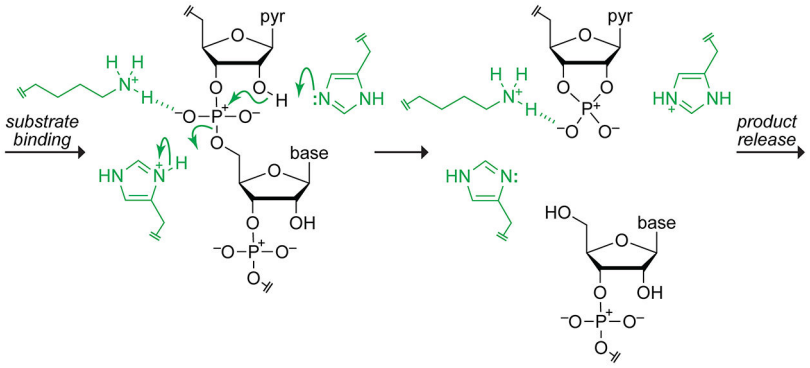

Figure 3.

Putative mechanism for catalysis of RNA cleavage by ptRNases, highlighting the roles of the three active-site residues (green) depicted in Figure 2 (Findlay et al., 1961; Cuchillo et al., 2011). The 2′,3′-cyclic phosphodiester product can be hydrolyzed by ptRNases in a separate step (Cuchillo et al., 1993; Thompson et al., 1994).

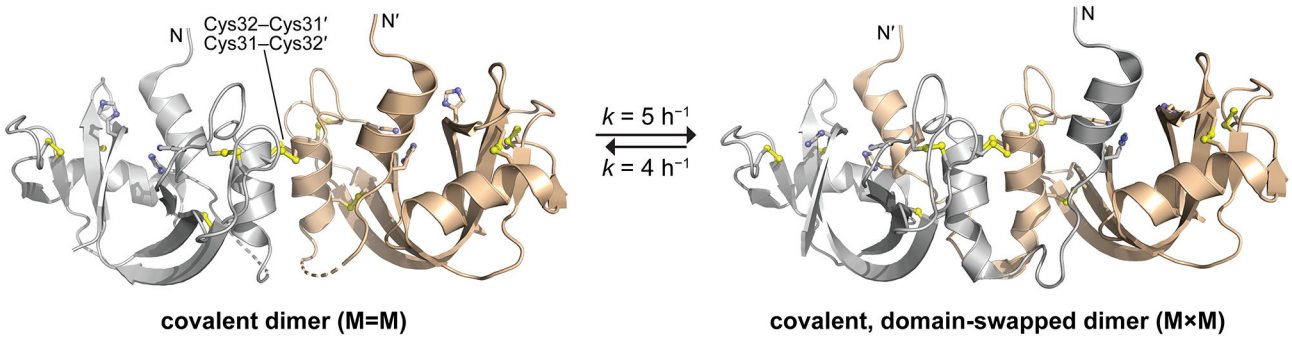

Figure 4.

Domain swapping in the BS-RNase dimer (Kim et al., 1995a). The active-site residues (His12, Lys41, and His119) and six disulfide bonds are shown explicitly. Images were made with the program PyMOL and PDB entries 3bcm and 3bco (Merlino et al., 2008).

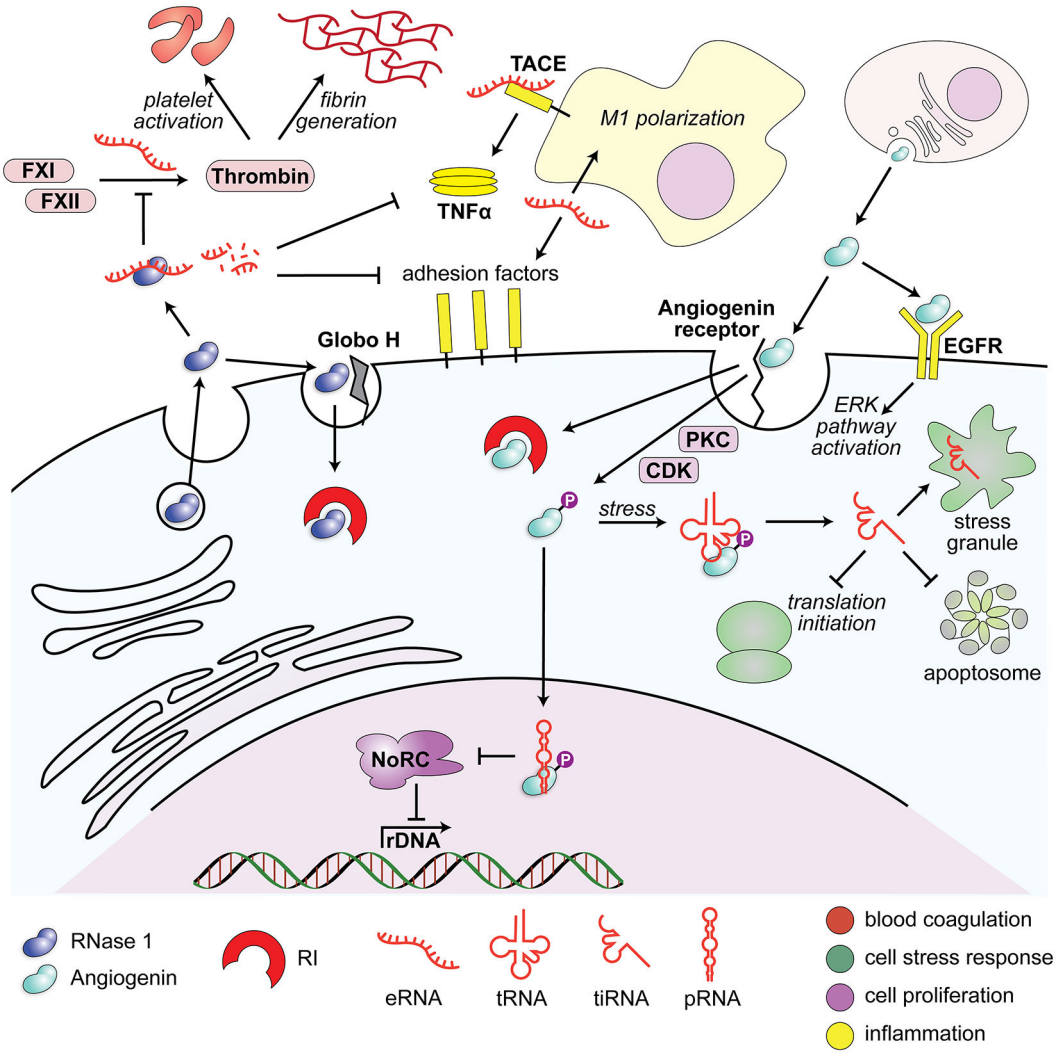

Figure 5.

Schematic of RNase 1 and angiogenin activities and substrates. RNase 1 acts primarily on targets outside of cells, where it degrades eRNAs. These eRNAs would otherwise act as activators of coagulation and inflammatory pathways. Angiogenin, in contrast, mediates its effects largely within cells, driving both cellular quiescence in response to stress via tRNA degradation and proliferation via pRNA degradation.

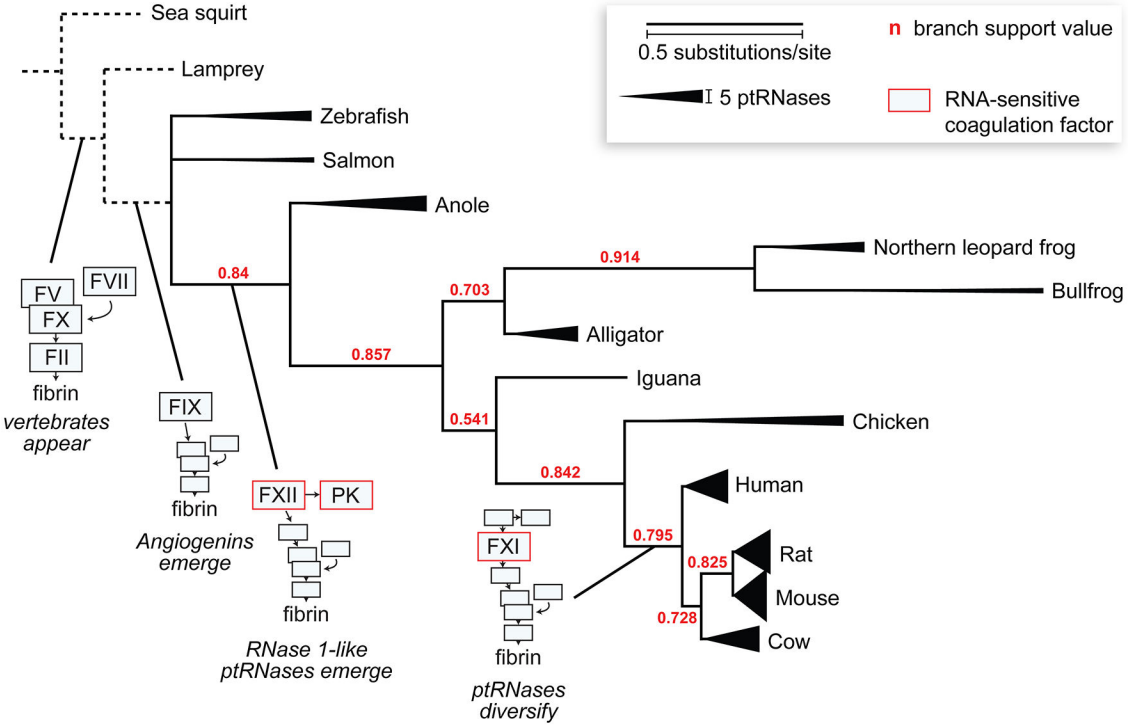

Figure 6.

Phylogenetic tree of homologous ptRNases expressed in vertebrate species, with annotations highlighting the co-emergence of coagulation factors in given groups of species. Protein sequences of RNase 1 homologs from UniProtKB P07998 (human), P00683 (mouse), P00684 (rat), P61823 (cow), P30374 (chicken), P80287 (iguana), A0A151LY34 (alligator), P11916 (bullfrog), Q8UVX5_LITPI (Northern leopard frog), H9GD73_ANOCA (anole), A0A1S3RRZ8 (salmon), and A5HAK0 (zebrafish) were used to generate a phylogenetic tree based on protein sequence similarity with the program phylogeny.fr (Castresana, 2000; Edgar, 2004; Chevenet et al., 2006; Anisimova and Gascuel, 2006; Guindon et al., 2010). The branch support value (red) is shown for each junction, and the number of substitutions per site is represented by line length. Line thickness at the termini of each leaf represents the number of ptRNase proteins reported to exist in each organism (Zhao et al., 1994; Irie et al., 1998; Beintema and Kleineidam, 1998; Cho et al., 2005; Pizzo et al., 2006; Kazakou et al., 2008; Pizzo et al., 2008). Species without identified ptRNases are represented by dashed lines that are not to scale; these species are included to highlight the emergence of vertebrate coagulation factors. The emergence of angiogenins in fish, RNase 1-like proteins in amphibians, and RNA-sensitive coagulation factors (FXII, FXI, and prekallikrein [PK]) in amphibians and mammals is highlighted at the appropriate junctions (Doolittle and Surgenor, 1962; Doolittle, 2009).