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Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes

(RNase/RNA cleavage)

DARET K. ST. CLAIR*, SUSANNA M. RYBAK*†, JAMES F. RIORDAN*‡, AND BERT L. VALLEE*§

*Center for Biochemical and Biophysical Sciences and Medicine, and Departments of †Pathology and ‡Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

Contributed by Bert L. Vallee, August 14, 1987

ABSTRACT **Angiogenin is a potent inhibitor of cell-free protein synthesis. When incubated with rabbit reticulocyte lysate at a concentration of 40–60 nM, it completely abolishes the capacity of the lysate to support protein synthesis. The inhibition appears to be due to its ribonucleolytic activity since it (*i*) generates limited cleavage products from reticulocyte RNA and (*ii*) is prevented from both cleaving RNA and inhibiting protein synthesis by placental RNase inhibitor. The ribonucleolytic activity of angiogenin toward the reticulocyte RNA system is highly specific. Thus, under conditions where angiogenin totally abolishes protein synthesis, an equivalent concentration of pancreatic RNase A inhibits it only partially. In contrast, RNase A is a much more effective enzyme than angiogenin using isolated RNA as substrate. Angiogenin inhibits protein synthesis by cleaving rRNA, thereby inactivating the protein synthesis machinery. Addition of isolated reticulocyte ribosomes to an angiogenin-treated lysate restores the capacity for protein synthesis, whereas addition of tRNA or mRNA does not. This potent effect on protein synthesis suggests a possible physiological function of angiogenin whose overall relevance and implications should become evident as the mechanisms of neovascularization are deciphered. The use of angiogenin may also further elucidate ribosome structure and its role in protein synthesis.**

Angiogenin is a 14-kDa protein, isolated initially from tumor cell-conditioned medium (1) and subsequently from human plasma (2), that induces neovascularization in the chicken chorioallantoic membrane and the rabbit cornea. Its primary structure is highly homologous to that of pancreatic RNase A (3, 4). While angiogenin does not exhibit enzymatic activity in standard pancreatic RNase A assays with substrates such as cyclic CMP or poly(C), it exhibits characteristic ribonucleolytic activity toward isolated 28S and 18S rRNA (5). To delineate the ribonucleolytic activity of angiogenin and to differentiate it further from that of pancreatic RNase A, we have examined its consequent effects on the translational capacity of the rabbit reticulocyte lysate. In this system, angiogenin proves to be a potent inhibitor of cell-free protein synthesis, and its principal target is the reticulocyte ribosome. These results may bear importantly on the physiological mode of action of angiogenin.

MATERIALS AND METHODS

Materials. Angiogenin, free of any contaminating RNase, was purified from growth medium conditioned by cells from a human colon adenocarcinoma cell line (HT-29), as described by Shapiro *et al.* (5). The concentration of angiogenin was determined by amino acid analysis. Bovine pancreatic RNase A was purchased from Cooper Biomedical (Freehold, NJ). Its concentration was determined by measuring absorbance at 280 nm using A_{280} = 9800 $M^{-1} \cdot cm^{-1}$ (6). Globin mRNA was obtained from Bethesda Research Laboratories and Brom mosaic virus RNA was from Promega Biotec (Madison, WI). HT-29 mRNA was isolated from HT-29 cells according to the procedure of Chirgwin *et al.* (7) and poly(A)$^+$ RNA was purified by chromatography on oligo-d(T)-cellulose (8). Human placental RNase inhibitor (PRI) and rabbit reticulocyte lysate, with or without previous micrococcal nuclease treatment, were purchased from Promega Biotec, and [^{35}S]methionine (1200 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham.

***In Vitro* Translation Assay.** Rabbit reticulocyte lysate was incubated with or without angiogenin or RNase A at the concentrations specified in the experimental data for 15 min at 30°C. The action of angiogenin and RNase was stopped by addition of the same molar concentration of PRI, which is sufficient for complete inhibition. Translation was initiated by the addition of exogenous mRNA in the presence of [^{35}S]methionine. The reaction contained 33 μl of lysate, 0.8 μg of mRNA, 1 μl of [^{35}S]methionine (4.4 μM), and 1 μl of a mixture of the other 19 amino acids (each 1 mM), all in a total volume of 50 μl. The amount of protein synthesis was determined by the incorporation of [^{35}S]methionine into products precipitable by 10% trichloroacetic acid and by autoradiography of the proteins separated by polyacrylamide gel electrophoresis.

Isolation and Reticulocyte Ribosomes. Reticulocyte ribosomes were obtained by centrifugation through a sucrose gradient, as described by Wreschner *et al.* (9). The lysate was layered onto 1 M sucrose/5 mM Tris·HCl, pH 7.6/1 mM dithiothreitol/0.1 mM EDTA and centrifuged for 3 hr at 150,000 × *g*. The ribosomes (10 mg/ml) were resuspended in 0.25 M sucrose/5 mM Tris·HCl, pH 7.6/1 mM dithiothreitol/0.1 mM EDTA.

RESULTS

The Effect of Angiogenin on the Translational Capacity of the Rabbit Reticulocyte Cell-Free System. Incubation of previously frozen rabbit reticulocyte lysate, containing mRNA and all other components necessary for protein synthesis at 30°C, results in the incorporation of added [^{35}S]methionine into newly synthesized protein. Treatment of the lysate with increasing concentrations of angiogenin, prior to the addition of [^{35}S]methionine, inhibits protein synthesis in a concentration-dependent manner (Table 1). An angiogenin concentration of 64 nM completely abolishes cell-free protein synthesis by disrupting the function of one or more components of the reticulocyte lysate system.

Counteraction of the Inhibitory Effect of Angiogenin by PRI. PRI forms an exceedingly tight complex with angiogenin

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Abbreviation: PRI, human placental RNase inhibitor.

§To whom reprint requests should be addressed.

Table 1. Effect of angiogenin on translational capacity of rabbit reticulocyte lysate

Angiogenin, nM	Incorporation of [^{35}S]methionine	
	% cpm	% inhibition
0	100	0
43	39	61
64	0	100
83	0	100

Average values from two experiments. The reticulocyte lysate was preincubated with angiogenin at the concentrations shown for 15 min. Protein synthesis was quantitated by addition of [^{35}S]methionine described. 100% = 7.6×10^4 cpm.

thereby abolishing its ribonucleolytic action toward RNA (10). Accordingly, PRI was tested to determine whether it would also eliminate the effect of angiogenin on protein synthesis using the mRNA-requiring *in vitro* translation system. Fig. 1 (lane 3) demonstrates that incubation of the lysate mixture with angiogenin completely inhibits protein synthesis. Prior treatment with PRI abolishes the ability of angiogenin to inhibit protein synthesis (lane 4). The amount and pattern of [^{35}S]methionine-labeled proteins synthesized after this treatment are identical to those of the control when examined by trichloroacetic acid precipitation and NaDodSO$_4$ gel electrophoresis (lanes 2 and 4). These results indicate that the inhibition of protein synthesis by angiogenin is a consequence of its ribonucleolytic activity. Since the reticulocyte lysate contains multiple RNA species, it was necessary to investigate each of them to determine which one might be the specific target of angiogenin.

The Effect of Angiogenin on mRNA. Isolated globin mRNA was incubated for 15 min with increasing concentrations of angiogenin followed by the addition of PRI to arrest further ribonucleolytic action. The mRNA that was treated with 300 nM angiogenin is no longer capable of directing the synthesis of new protein (Fig. 2). Similar effects were observed when viral RNA and mRNAs from a human colon carcinoma cell line (HT-29) were treated with angiogenin. Direct analysis of the angiogenin-treated globin mRNA on an ethidium bromide-stained urea/acrylamide gel (not shown) reveals that it is indeed degraded.

Despite the susceptibility of mRNA to nucleolytic cleavage by angiogenin, this does not appear to be the primary reason for the loss of protein synthesis. Thus, a micrococcal nuclease-treated reticulocyte lysate—composed of ribosomes, tRNA, and all other components necessary for the translation of protein but lacking exogenous mRNA—was incubated with angiogenin for 15 min followed by the addition of PRI. *In vitro* translation was then initiated by exogenous mRNA together with [^{35}S]methionine, and newly synthesized protein was subsequently measured by NaDodSO$_4$ gel electrophoresis. Fig. 3 (lanes 4–7) shows that no protein is synthesized; hence, angiogenin still abolishes the capacity for protein synthesis. Addition of as little as 40 nM angiogenin to the lysate completely destroys its capacity to support protein synthesis after mRNA supplementation. In contrast, under identical conditions, 40 nM pancreatic RNase A diminishes translational capacity by only 50%, as determined both by trichloroacetic acid-precipitable radioactivity and autoradiography (lanes 8–11).

The Effect of Readdition of tRNA and/or Ribosomes on the Translational Capacity of Angiogenin-Treated Lysate. In an attempt to restore protein synthesis, reticulocyte ribosomes and tRNA were added to an angiogenin-treated lysate, either as individual components or in combination. Addition of tRNA alone is totally ineffective (Table 2). Addition of reticulocyte ribosomes, however, does reactivate the translational capacity of the system (Table 2). Since ribosomes that have undergone an isolation procedure provide a less active system than the original unfractionated system (11), the incorporation of [^{35}S]methionine in the angiogenin-treated ribosome-supplemented lysate is less than that in the

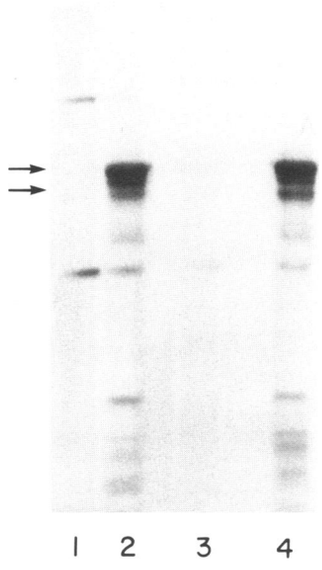

FIG. 1. Autoradiograph of a 10% polyacrylamide gel of *in vitro* translation products showing the effect of PRI on the protein synthesis inhibitory activity of angiogenin. Angiogenin (40 nM) was preincubated for 5 min with 40 units of PRI and the mixture was added to the rabbit reticulocyte lysate system. *In vitro* translation was performed for 60 min as described. Lane 1, mRNA-dependent rabbit reticulocyte lysate translation without added mRNA. Lane 2, the same as lane 1 but with 0.8 μg of globin mRNA added. Lane 3, the same as lane 2 except that the lysate was incubated with 40 nM angiogenin for 15 min at 30°C; it was then treated with 40 units of PRI before addition of globin mRNA. Lane 4, the same as lane 3 except that the angiogenin was preincubated with 40 units of PRI before being added to the lysate. The upper and lower arrows refer to the positions of β- and α-globin, respectively.

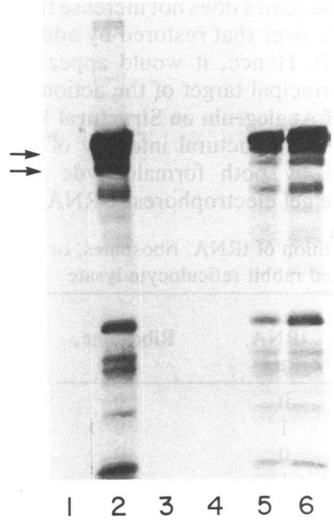

FIG. 2. Autoradiograph of a 10% polyacrylamide gel of *in vitro* translation products showing the effect of angiogenin on mRNA. Globin mRNA (0.8 μg) was preincubated with angiogenin. The enzyme reaction was stopped by addition of 40 units of PRI per 300 nM angiogenin. Protein synthesis was conducted as described. Lanes: 1, mRNA-dependent rabbit reticulocyte lysate translation without exogenous mRNA; 2, the same as lane 1 but with 0.8 μg of globin mRNA; 3–6, the same as lane 2 except that mRNA was preincubated with 1400, 300, 69, and 14 nM angiogenin, respectively. The arrows are the same as in Fig. 1.

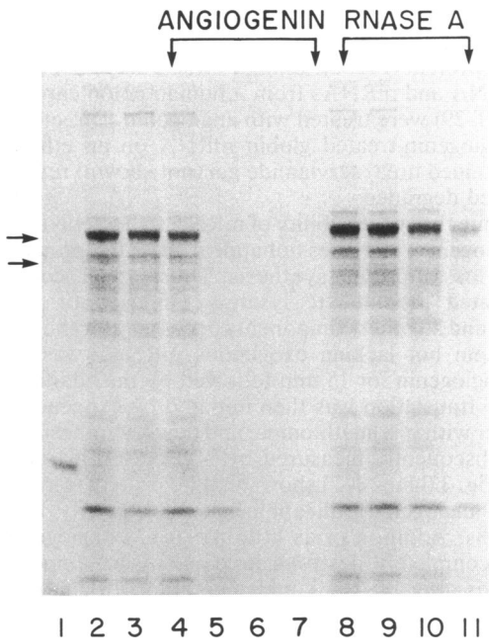

FIG. 3. Autoradiograph of a 10% polyacrylamide gel of *in vitro* translation products showing the effects of angiogenin and pancreatic RNase A on the translational capacity of an mRNA-dependent rabbit reticulocyte lysate system. Reticulocyte lysate was treated with the indicated amount of angiogenin or RNase A for 15 min at 30°C. Ribonucleolytic activity was inactivated by addition of 40 units of PRI to the incubation mixture or preincubation of the enzyme with the inhibitor. Lanes: 1, translation without exogenous mRNA; 2, translation with 0.8 μg of globin mRNA; 3, the same as lane 2 but preincubating the lysate at 30°C for 15 min; 4–7, the same as lane 3 but the lysate was preincubated with 1, 10, 20, or 40 nM angiogenin, respectively; 8–11, the same as lanes 4–7 but with the same molar concentrations of RNase A instead of angiogenin. The arrows are the same as in Fig. 1.

unfractionated lysate. Addition of tRNA in combination with reticulocyte ribosomes does not increase the incorporation of [^{35}S]methionine over that restored by addition of ribosomes alone (Table 2). Hence, it would appear that reticulocyte rRNA is the principal target of the action of angiogenin.

The Effect of Angiogenin on Structural RNA. The effect of angiogenin on the structural integrity of reticulocyte RNA was examined by both formaldehyde agarose and urea polyacrylamide gel electrophoresis. RNA degradation is not readily discernible on a formaldehyde agarose gel (Fig. 4*A*). The material isolated from an angiogenin-treated lysate and labeled at the 5′ end with [γ-^{32}P]ATP and T4 kinase appears to be unchanged compared to the control (lanes 1–3). On the other hand, RNA isolated from a RNase A-treated lysate has clearly undergone extensive degradation (lane 4). In contrast, the generation of degradation products in an angiogenin-treated lysate can be detected readily using a 10% urea/polyacrylamide gel to analyze fragments of <1 kilobase (Fig. 4*B*). The newly formed products migrate in the region corresponding to 40- to 90-base oligonucleotides, indicating that angiogenin cleavage is quite limited compared to the extensive degradation that occurs with RNase. It would appear that these products arise from the action of angiogenin on reticulocyte rRNA.

Table 2. Readdition of tRNA, ribosomes, or both to angiogenin-treated rabbit reticulocyte lysate

Treatment	tRNA, μg	Ribosome, μg	Incorporation of [^{35}S]methionine, % cpm
None	0	0	100
	1	0	100
Angiogenin (40 nM)	0	0	0
	1	0	0
	1.5	0	0
	2.0	0	0
	0	10	5
	0	15	30
	0	50	29
	1	15	29

An mRNA-dependent lysate was incubated with 40 nM angiogenin at 30°C for 15 min. The ribonucleolytic activity of angiogenin was stopped by addition of 40 units of PRI. Translation was initiated by addition of 0.8 μg of globin mRNA plus tRNA and/or ribosomes in the amounts listed. 100% = 6.9×10^4 cpm.

DISCUSSION

The amino acid sequence of angiogenin contains homologs of three major structural segments that are thought to constitute the catalytic and substrate-binding regions of RNase (3, 4, 12). This extensive homology prompted a detailed examination of the potential ribonucleolytic activity of angiogenin, which, as previously reported (5), was found to catalyze the cleavage of 28S and 18S rRNA, although it lacked detectable activity toward standard substrates for RNase A.

As a first approach to determine the possible relationship between the angiogenic and ribonucleolytic activities of angiogenin, we have examined its action on *in vitro* protein synthesis. The results presented here show clearly that angiogenin is a potent and specific inhibitor of protein synthesis in an mRNA-dependent rabbit reticulocyte lysate

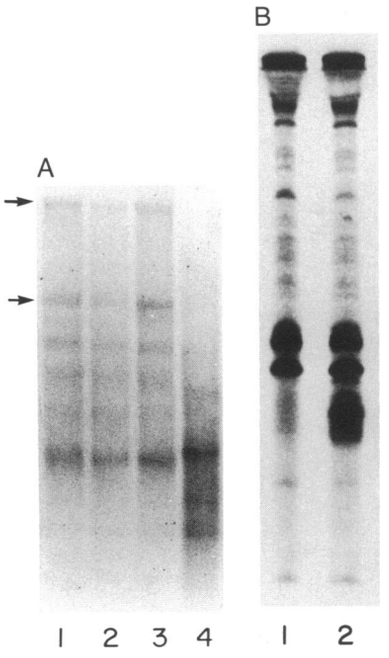

FIG. 4. (*A*) Autoradiograph of an agarose gel electrophoresis of RNA isolated from an angiogenin- or RNase A-treated reticulocyte lysate. Lanes: 1, RNA from untreated lysate; 2, the same as lane 1 but incubated at 30°C for 15 min; 3, the same as lane 2 but incubated with 40 nM angiogenin; 4, the same as lane 3 but incubated with 40 nM RNase A. The upper and lower arrows indicate the positions of 28S and 18S rRNA, respectively. (*B*) Autoradiograph of a 10% 7 M urea/polyacrylamide gel electrophoresis showing cleavage of reticulocyte RNA by angiogenin. RNAs isolated from rabbit reticulocyte lysate treated with buffer (lane 1) or 40 nM angiogenin (lane 2). The angiogenin-treated lysate was totally unable to translate protein from exogenous mRNA.

system, and that this inhibition may be due to limited cleavage of reticulocyte RNA, since it is prevented by PRI. Moreover, the readdition of various lysate components to the angiogenin-treated lysate and the subsequent reactivation of the system by non-angiogenin-treated ribosomes support the conclusion that cleavage of rRNA is responsible for protein synthesis inhibition. Thus, angiogenin appears to be a secreted human ribonucleolytic enzyme that specifically affects the eukaryotic ribosome *in vitro*.

Analysis of RNA isolated from angiogenin-treated lysates has revealed that the action of angiogenin is highly specific and clearly distinct from that of known degradative ribonucleases such as pancreatic RNase A. Under conditions in which angiogenin inhibits protein synthesis completely and without major destruction of rRNA, RNase A inhibits protein synthesis only partially and does so by causing extensive degradation of rRNA (Fig. 4*A*).¶ Earlier work has already established that random cleavage of rRNA by RNases at nonspecific sites does not significantly alter the normal activity of the ribosome (14).

The ribonucleolytic activity of angiogenin was first demonstrated using RNA isolated from HT-29 cells (5). Monitoring hydrolysis by agarose gel electrophoresis revealed that both the 18S and 28S rRNA bands were degraded into products 100–500 nucleotides long. Similar results have also been obtained with rRNA isolated from rabbit reticulocyte lysate (data not shown).‖ This is in marked contrast to the limited cleavage products seen on urea polyacrylamide gels after treating the unfractionated reticulocyte lysate with angiogenin (Fig. 4). These observations indicate that the specificity of angiogenin toward rRNA depends critically on the structural integrity of the ribosome. It is not known at this point whether it acts on only one particular species of rRNA and, if so, which one it is. It may induce lesions at specific sites, which cause an irreversible conformational change in the ribosome, thus rendering it inactive, or it may cleave sites directly responsible for either formation of the initiation complex, chain elongation, or chain termination. This suggests that angiogenin may be useful for probing the structure–function relationship of the eukaryotic protein synthesis machinery much like colicin E_3 (17) and α-sarcin (18).

Does the ribonucleolytic activity of angiogenin *in vitro* provide clues to its mechanism of action *in vivo* and what, if any, are the implications of protein synthesis inhibition to angiogenesis? To answer such questions, it would be necessary to establish, among other things, what the relationship between the angiogenic and ribonucleolytic activities of angiogenin might be; whether angiogenin inhibits protein synthesis in intact and/or damaged cells or *in vivo*; whether the inhibition is specific to certain physiological (or pathological) states of a cell; whether this relates to its angiogenic activity; or whether it signals an as yet undiscovered physiological activity of angiogenin.

While it is uncertain at present whether the ribosome is the normal target of angiogenin, there is compelling evidence that some form of RNA is the specific substrate for this enzyme and that the ribonucleolytic activity of angiogenin correlates to its angiogenic activity. Thus, PRI abolishes both the angiogenic and the ribonucleolytic activities of angiogenin (10) as well as its capacity to cleave rRNA and inhibit protein synthesis. PRI and related inhibitors are present in the cytoplasm of virtually every cell (19), and it is thought that the concentration and ratios of RNases to inhibitor are important in the regulation of protein synthesis (20). Therefore, it is plausible that particular alterations in cellular physiology—e.g., tissue trauma—could alter the levels of inhibitor and the extent of its potential complex formation with angiogenin to allow angiogenin to enter damaged cells where it could encounter ribosomes.

Our paradoxical finding that a potent inducer of angiogenesis causes significant inhibition of *in vitro* protein synthesis underscores the complexity of regulation of neovascularization and may therefore be only a seeming paradox. Blood vessel cells are extremely quiescent and do not proliferate under normal conditions. However, when neovascularization does take place, as in wound healing or tumor proliferation, it has been variously thought to involve the disappearance of pericytes and basement membrane, degradation of other existing vascular components, and stimulation of endothelial cell migration and proliferation, all of which lead to differentiation and morphogenesis. Clearly, such a complex process must involve the cooperative interaction of multiple agents and undoubtedly, as with other similarly regulated metabolic events, be initiated by more than one mechanism. Indeed, it is now known that a host of well-characterized substances, most of them proteins, are capable of inducing new blood vessel growth. With the exception of angiogenin, these proteins had long been studied for a wide variety of other biological actions in many different systems (see ref. 21 for review). Which, if any, of these other activities are relevant to neoangiogenesis, whether and how they relate to and interact with one another is unknown as yet: in no case is there direct evidence for how these proteins act by themselves or in concert *in vivo*, nor is there established a clear connection or relationship between various angiogenic stimulators and their mode of action. It may be pertinent to note, however, that type β transforming growth factor, an inducer of blood vessel growth in newborn mice, inhibits endothelial cell division in culture (22), and that tumor necrosis factor, which kills tumor cells, also seems to be angiogenic (23).

More than 35 years ago Glucksmann (24) pointed out that regression phenomena are common if not typical at some point during the development of virtually all biological (including vascular) systems. Therefore, since local regression of capillaries occurs during morphogenesis (25), angiogenin could, among its other actions, promote remodeling of the vasculature. Yet another possibility is that inhibition of protein synthesis may, in fact, constitute a step in maintaining the final morphology and function of blood vessels.

Finally, it is pertinent to raise questions concerning both the source and the target of angiogenin. Initially isolated from human tumor cell-conditioned medium it was cloned from normal human liver and has since been found in appreciable concentrations in normal human plasma (2); clearly, it is not a tumor-specific protein. Neither the function of plasma angiogenin nor the tissue of origin has yet been determined. It does not appear to be a primary secretory product of platelets since its concentrations are similar in serum and plasma. Both lymphocytes and macrophages are known to secrete angiogenic substances, but these are still structurally unidentified.

The present data show that in the reticulocyte lysate the principal site of action of angiogenin is the ribosome. However, there is no evidence thus far to indicate that angiogenin actually inhibits protein synthesis *in vivo* or that it enters undamaged cells. Therefore, it would be premature to conclude that such inhibition is either necessary or sufficient to induce new blood vessel formation. Other forms of RNA

¶A RNase other than angiogenin, isolated from HT-29-conditioned medium (13), as well as several other RNases from other sources were also tested and found to inhibit protein synthesis by causing extensive damage to reticulocyte RNA (unpublished results). Moreover, when lower concentrations of these RNases were used, RNA was still degraded but without affecting protein synthesis.

‖Reticulocyte membrane-bound RNase M (15) and an interferon-regulated nuclease (16) can also specifically degrade rRNA from isolated ribosomes and inhibit protein synthesis, but both of these enzymes differ distinctly from angiogenin (S.M.R., unpublished data).

including small nuclear RNAs, naturally occurring anti-sense RNAs (26), other oligoribonucleotides (27), or similar nucleotides have not been ruled out as possible substrates, products, or metabolites of angiogenin, nor have they been examined as likely participants in neovascularization. However, it is worth considering that any or all of these could play an important regulatory role. It is also possible, of course, that under certain circumstances the nucleolytic activity of angiogenin toward one such RNA might be highly specific and selective in a manner reminiscent of DNA restriction enzymes. Preliminary studies of the base-specific cleavage pattern of angiogenin indicate that it hydrolyzes many, although not all, of the CpN and UpN phosphodiester bonds in 5S RNA from *Saccharomyces cerevisiae* and *Escherichia coli* with a preference for those in which N is adenine (S.M.R., unpublished data).

Among the possible roles for angiogenin would be to aid in normal wound healing or to participate in maintaining the integrity of the microvasculature. The extent to which its interaction with potential modifiers, activators, and amplifiers or specific cell receptors, as well as inhibition by PRI, determine its functional expression are all questions yet to be explained and answered. As the mechanisms regulating angiogenesis are deciphered, facilitated by the availability of highly purified and characterized angiogenic mediators, the overall relevance and mechanistic implications of an isolated biochemical event such as the inhibition of protein synthesis in reticulocyte lysates—however exciting and provocative—should become evident.

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