Angiogenin Abolishes Cell-Free Protein Synthesis by Specific Ribonucleolytic Inactivation of 40S Ribosomes†

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ABSTRACT: The translational capacity of a rabbit reticulocyte lysate is rapidly abolished on treatment with angiogenin, an effect that is due to cleavage of rRNA [St. Clair, D. K., Rybak, S. M., Riordan, J. F., & Vallee, B. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8330–8334]. The same time course of inhibition is seen when isolated ribosomes are treated with angiogenin prior to being added to a ribosome-dependent lysate system. In both cases, the onset of inhibition occurs at a rate similar to that seen on addition of puromycin, a known inhibitor of elongation, suggesting that this is the step in the protein synthesis machinery that is inactivated by angiogenin. The action of angiogenin on ribosomes is quite specific: both 28S and 18S rRNAs are cleaved whereas 5.8S and 5S rRNAs are not. Moreover, 28S and 18S rRNAs are affected differently. Prolonged incubation with angiogenin degrades 28S rRNA extensively but only causes limited cleavage of 18S rRNA. Remarkably, it is the effect of angiogenin on 18S rRNA that seems to be responsible for the inhibition of protein synthesis rather than the nucleolytic degradation of 28S rRNA. This has been demonstrated by separating the isolated ribosomes into their 40S and 60S subunits and treating them individually with angiogenin. The pattern of rRNA cleavage is the same with the separated subunits as with intact ribosomes, but translation is abolished only on treatment of the 40S, not the 60S, subunit with angiogenin. These results confirm our previous observations on the effect of angiogenin on the rabbit reticulocyte cell-free translation system and extend the understanding of its mechanism of action on the ribosome.

Angiogenin is a potent stimulator of blood vessel formation initially isolated from tumor-conditioned media (Fett et al., 1985) and subsequently from human plasma (Shapiro et al., 1987). Its sequence is 35% identical with that of pancreatic ribonuclease A (RNase A)[1] (Strydom et al., 1985; Kurachi et al., 1985), and it has a unique ribonucleolytic activity (Shapiro et al., 1986; St. Clair et al., 1987). Angiogenin is essentially inactive toward classic RNase A substrates, but it does catalyze the hydrolysis of isolated 28S and 18S rRNA (Shapiro et al., 1986). This activity differs significantly from that of pancreatic RNase in two respects: (a) it requires up to 10^5 as much angiogenin to obtain the same degree of rRNA degradation as with RNase; and (b) the products are much larger, i.e., from 100 to 500 nucleotides. Also, angiogenin significantly hydrolyzes certain bonds in isolated 5S rRNA that are apparently not altered by RNase A and vice versa (Rybak & Vallee, 1988).

The cleavage specificities of angiogenin and RNase A may be responsible for their different biological activities. Thus, angiogenin totally abolishes protein synthesis in the rabbit reticulocyte lysate system at concentrations where an equivalent amount of RNase A inhibits it only partially (St. Clair et al., 1987). This inhibition of cell-free protein synthesis by angiogenin has been shown to be due to the specific ribonucleolytic inactivation of reticulocyte rRNA (St. Clair et al., 1987).

In the present study, we have further characterized the action of angiogenin with intact reticulocyte ribosomes as well as ribosomal subunits and demonstrate that in both cases angiogenin cleaves rRNA to yield a characteristic pattern of discrete fragments. This specificity of angiogenin toward reticulocyte rRNA is a property of both angiogenin and the ribosomal structure.

Materials and Methods

Angiogenin, free of trace amounts of nonspecific ribonuclease, was purified from human plasma as described by Shapiro et al. (1987). Bovine pancreatic RNase A was purchased from Cooper Biomedical (Freehold, NJ). The concentration of stock solutions of angiogenin was determined by amino acid analysis and of RNase A by the absorbance at 280 nm using A_{280} = 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957). Placental ribonuclease inhibitor (PRI) was isolated by the method of Blackburn (1979), and its concentration was determined by titration of a standard RNase A solution (F. S. Lee, personal communication). The mRNA-dependent rabbit reticulocyte cell-free translation system was purchased from Promega Biotec (Madison, WI), [^{35}S]methionine (1200 Ci/mmol) was from New England Nuclear (Boston, MA), and reticulocyte-rich whole blood was from Pel-Freeze (Rogers, AR).

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[1] Abbreviations: RNase, ribonuclease; PRI, human placental ribonuclease inhibitor; TCA, trichloroacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ATA, aurintricarboxylic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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In Vitro Translation Assay. The in vitro translation assay was performed as previously described (St. Clair et al., 1987). Briefly, rabbit reticulocyte lysate was incubated with or without angiogenin at the concentration specified for 15 min at 30 °C followed by addition of the same molar concentration of PRI. Translation was initiated by addition of Brom mosaic virus RNA in the presence of [^{35}S]Met. The amount of protein synthesis was determined by the incorporation of [^{35}S]Met into products precipitable by 10% trichloroacetic acid (TCA).

Preparation of Rabbit Reticulocyte Ribosomes. Rabbit reticulocyte-rich blood was filtered through cotton gauze and centrifuged at 2000*g* for 10 min. The cell pellet was washed 3 times with several volumes of 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$. The layer of lymphocytes was removed and the reticulocytes lysed with 1.5 volumes of H_2O and centrifuged at 25000*g* for 10 min. The supernatant was layered on top of 1 M sucrose containing 5 mM Tris-HCl, pH 7.4, 1 mM DTT, and 0.1 mM EDTA and centrifuged for 3 h at 150000*g* at 4 °C. Intact ribosomes were resuspended in 0.25 M sucrose, 5 mM Tris-HCl, pH 7.4, 1 mM DTT, and 0.1 mM EDTA and stored in liquid nitrogen.

Isolation of Ribosomal Subunits. Ribosomes (100–200 mg) were suspended in about 2 mL of 0.3 M KCl, 3 mM $MgCl_2$, 1 mM DTT, and 20 mM Tris-HCl, pH 7.4, allowed to dissolve (up to 18 h) at 0 °C, and adjusted to 20 mL with a solution containing KCl, guanosine triphosphate, and puromycin (final concentrations 0.5 M, 0.2 mM, and 0.2 mM, respectively). After incubation at 37 °C for 30 min, the solution was made 5% (w/v) in sucrose and cooled in an ice bath, and 10 A_{260} units were layered onto a 15–45% sucrose gradient in 0.3 M KCl, 3 mM $MgCl_2$, 1 mM DTT, and 20 mM Tris-HCl, pH 7.4, buffer. Centrifugation in an SW-40 rotor was for 16 h at 22 000 rpm. The A_{260} profile of the gradient was recorded continuously using a flow cell mounted in a type 4710A LKB UVICORD optical unit. Fractions corresponding to the 60S and 40S peaks were collected, and the subunits were obtained by centrifugation of the fractions for 2 h at 49 000 rpm (type 50 rotor). The subunit pellets were resuspended in 0.1 M NH_4Cl, 2 mM magnesium chloride, 1 mM DTT, 20 mM Tris-HCl, pH 7.5, and 15% (v/v) glycerol.

Preparation of the Ribosome-Dependent Translation System. The endogenous reticulocyte ribosomes were inactivated by incubating the lysate with 40 nM angiogenin for 15 min at 30 °C and subsequently inhibiting the ribonucleolytic action of angiogenin with an equimolar concentration of PRI. Previous results have demonstrated that the translational capacity of an angiogenin-treated lysate can be restored by the addition of isolated, untreated ribosomes (St. Clair et al., 1987).

Angiogenin Treatment of Ribosomes or Ribosomal Subunits. To determine the effect of angiogenin on the translational capacity of reticulocyte ribosomes or ribosomal subunits, isolated ribosomes (or ribosomal subunits) were incubated with various concentrations of angiogenin as indicated in the figure legends; the ribonucleolytic action of angiogenin was stopped by the addition of an equimolar concentration of PRI. The functional integrity of the reticulocyte ribosomes or ribosomal subunits was then determined by the amount of protein synthesis that occurred when they were added to the ribosome-dependent lysate system described above, as indicated either by acid-precipitable radioactivity or by autoradiography of the translation products separated by $NaDodSO_4$ gel electrophoresis.

Characterization of rRNA. rRNA from control and angiogenin-treated ribosomes or separated subunits was dissolved in 0.5% $NaDodSO_4$ in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1.5 mM $MgCl_2$ followed by sequential extraction with phenol saturated with this buffer and with chloroform/isoamyl alcohol (24:1). The RNA was precipitated by ethanol, dissolved in H_2O, and stored at −70 °C. Electrophoretic analysis was accomplished by formaldehyde/agarose gels or 7 M urea/acrylamide gels. After electrophoresis, RNA was stained with ethidium bromide and visualized under ultraviolet light. Scanning was accomplished with an LKB Ultrascan XL laser spectrophotometer.

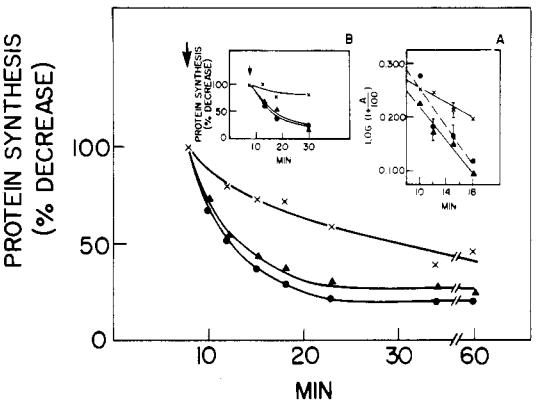

FIGURE 1: Translation of mRNA in the presence of protein synthesis inhibitors. Protein synthesis was monitored in each of four translation mixtures, all containing 0.5 μg of Brom mosaic viral RNA, 33 μL of rabbit reticulocyte lysate, 5 μCi of [^{35}S]Met, and 1 mM each of the other 19 amino acids. The acid-insoluble ^{35}S-labeled proteins were determined from 5 μL of each reaction as described under Materials and Methods. The results are expressed as percentages of a control reaction to which no inhibitor was added. ATA, 0.1 mM (×), angiogenin, 0.04 μM (▲), or puromycin, 1 mM (●), was added 8 min after initiation of translation (arrow), and aliquots were removed as a function of time thereafter. Insert A: Semi-log plot of percent decrease in protein synthesis activity (*A*) vs time; data from five separate experiments and their statistical analysis. A *t* test of the slope of the regression line for angiogenin vs ATA indicates a significant difference at $p = 0.007$, while a similar *t* test for angiogenin vs puromycin shows no significant difference ($p = 0.355$). Insert B: Effect of inhibitors on protein biosynthesis in the ribosome-dependent rabbit reticulocyte system. A mRNA-dependent rabbit reticulocyte lysate was made dependent on exogenous ribosomes by treatment with angiogenin as described under Materials and Methods. The system was reactivated by adding 20–50 μg of isolated ribosomes, and the resultant translational capacity was quantitated by measuring acid-insoluble radioactivity generated at the indicated times. ATA (0.1 mM), angiogenin (0.25 μM), or puromycin (1 mM) was added 8 min (arrow) after the addition of ribosomes. Data represent one of three experiments.

RESULTS

Stage of Translation Affected by Angiogenin. The rate at which an inhibitor abolishes protein synthesis can provide an indication of the particular stage of translation affected by that inhibitor. Initiation inhibitors, such as aurintricarboxylic acid (ATA), inhibit protein synthesis slowly since ribosomes already translating continue to do so, but no new initiation occurs (Eller et al., 1984). In contrast, elongation inhibitors, such as puromycin, halt protein synthesis rapidly (Eller et al., 1984). When angiogenin (final concentration 40 nM) is added to the in vitro translation assays 8 min after initiation of translation, protein synthesis ceases very quickly (Figure 1). The production of acid-insoluble radioactivity declines sharply within 20 min after addition of angiogenin. Virtually identical results are obtained with puromycin. On the other hand, the production of acid-insoluble radioactivity in the ATA-treated sample declines more slowly and remains consistently higher than that seen with either angiogenin or puromycin throughout the period of the experiment. Linear regression analysis of the initial slopes obtained after addition of inhibitor (Figure

Table I: Effect of Angiogenin on the Translational Capacity of Exogenous Rabbit Reticulocyte Ribosomes

[angiogenin] (nM)	incorpn of [^{35}S]Met[a]	
	% cpm	% inhibn
0	100	0
25	26	74
50	12	88
100	5	95
250	0	100

[a] Average value from two experiments. The ribosomes were preincubated with angiogenin at the concentration shown for 15 min. Protein synthesis was quantitated by addition of [^{35}S]Met as indicated under Materials and Methods. 100% = 1.5×10^5 cpm.

1, insert A) indicates a significant difference between the rates of inhibition by ATA and angiogenin or puromycin but not between those of the latter two. These results suggest that angiogenin acts to inhibit protein synthesis at the level of elongation or termination.

Effect of Angiogenin on the Translational Capacity of Reticulocyte Ribosomes. Previous work demonstrated that the loss of protein synthesis in the rabbit reticulocyte lysate caused by angiogenin could be restored by addition of isolated reticulocyte ribosomes (St. Clair et al., 1987). Therefore, it was of interest to examine the ribonucleolytic action of angiogenin on the isolated ribosomes directly. Ribosomes were prepared from reticulocyte-rich rabbit blood for such direct treatment with angiogenin. The reticulocyte translation system was made dependent upon exogenous ribosomes by preincubating it with 40 nM angiogenin for 15 min at 30 °C. The angiogenin was then inactivated by adding an equimolar concentration of PRI. The resultant system will translate exogenous mRNA only if isolated rabbit reticulocyte ribosomes are added to it. However, if the isolated ribosomes are first treated with angiogenin for 15 min at 30 °C, the reconstituted lysate loses its ability to carry out mRNA translation in proportion to the angiogenin concentration employed (Table I).

Angiogenin affects the same stage of translation in the ribosome-dependent system as it does in the intact lysate system described in Figure 1. Thus, when angiogenin is added to the reconstituted ribosome-dependent system, protein synthesis ceases rapidly in a manner identical with puromycin (Figure 1, insert B), confirming the effect of angiogenin on the translational capability of reticulocyte ribosomes and indicating that its mode of inhibition in the two systems is the same.

Ribosomal RNA Cleavage by Angiogenin. Concentrations of angiogenin that totally abolish in vitro protein synthesis generate low molecular weight products from reticulocyte lysate RNA as discerned by urea/acrylamide gel electrophoresis (St. Clair et al., 1987). RNA isolated from angiogenin-treated ribosomes and then 5′ labeled also appears as low molecular weight cleavage products on these gels (not shown).

Since angiogenin appears to inhibit peptide chain elongation (Figure 1), inactivation of only a few ribosomes could block protein synthesis completely (Eller et al., 1984). Under such circumstances, it might be difficult to identify the specific source of the cleavage products, and, in fact, it is not even possible to detect rRNA degradation by analysis on formaldehyde/agarose gels. Therefore, 10–100-fold higher concentrations of angiogenin than needed to inhibit protein synthesis completely were used to modify the ribosomes (Figure 2). The electrophoretic pattern of RNA isolated from intact ribosomes treated with these high concentrations of angiogenin

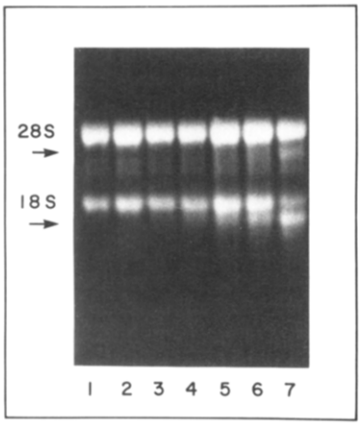

FIGURE 2: Angiogenin-generated cleavage products from isolated reticulocyte ribosomes. Electrophoretic pattern in a 1.1% formaldehyde/agarose gel of RNA isolated from ribosomes after incubation for 15 min at 30 °C with increasing concentrations of angiogenin. (Lanes 1–7) Angiogenin: 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 4 μM/50 μg of ribosome, respectively. Arrows indicate new RNA bands appearing after treatment with angiogenin. The positions of intact 28S and 18S rRNAs are also indicated. Note: the concentration of angiogenin required to inhibit protein synthesis completely under these conditions is 0.04 μM.

reveals the presence of RNA fractions moving faster than both 28S rRNA and 18S rRNA, and diminished amounts of intact 28S and 18S rRNA. RNA fragments generated by angiogenin treatment of intact ribosomes also were separated by electrophoresis on a 10% urea/polyacrylamide gel to analyze fragments of less than 1 kb (Figure 3A). Two interesting results are evident: the 5.8S and 5S rRNAs resist degradation by angiogenin when they are part of the ribosome, and angiogenin generates a new fragment that contains approximately 230 bases. In order to demonstrate this more clearly, the gel photograph was scanned with a densitometer, and the results were aligned with the apparent band on the gel (Figure 3B,C).

The nature of this activity was investigated further by prolonged incubation of the ribosome with angiogenin (Figure 4). Angiogenin was dissolved in 50 μL of 25 mM HEPES, pH 7.5, and incubated with ribosomes (0.7 μg/5 mg) at 30 °C. Sequential aliquots (5 μL) of the ribosome mixture were removed after 1, 15, 30, 60, 120, 240, and 480 min, and the integrity of the RNA was examined by electrophoresis in a 1.1% formaldehyde/agarose gel. Both 28S and 18S rRNAs decrease with time of incubation, and a new, high molecular weight band of RNA migrating just ahead of 18S rRNA increases concomitantly and persists after 240 min, at which point all intact 28S and 18S rRNA has disappeared. In fact, the new band resists further degradation, even during incubations of up to 480 min.

Ribonucleolytic Effect of Angiogenin on RNA in Isolated Ribosomal Subunits. Ribosomal subunits were prepared in order to further characterize the ribonucleolytic action of angiogenin toward rRNA in ribosomes and determine the source of the angiogenin-resistant, high molecular weight RNA fragment (see Figure 4). Intact 40S and 60S subunits were prepared by sucrose gradient centrifugation following treatment of ribosomes with KCl and puromycin. Incubation of the 40S subunit with 1 μM angiogenin/50 μg of subunit in 25 mM HEPES, pH 7.5, at 30 °C for 60 min completely transforms the constituent 18S RNA into a faster migrating RNA which is identical in size with that seen when the intact ribosome is treated with angiogenin (Figure 5). Since this fragment arises from and is only slightly smaller than 18S rRNA, it appears that the 18S rRNA must be cleaved by angiogenin in a limited manner at either the 5′ or the 3′ end,

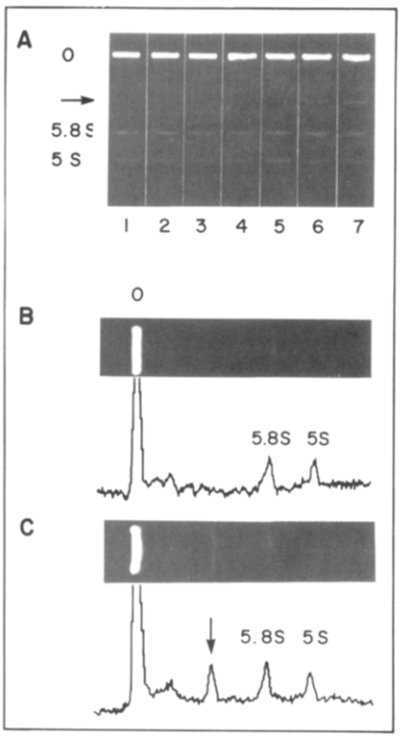

FIGURE 3: (A) Angiogenin-generated low molecular weight RNA. A 10% urea/polyacrylamide gel electrophoretic pattern of RNA isolated from ribosomes that had been incubated for 15 min at 30 °C in the presence of the indicated amounts of angiogenin. (Lanes 1–7) Angiogenin: 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 4 μM/50 μg of ribosome, respectively. The arrow indicates a band that appears with increasing concentrations of angiogenin. The gel was stained with ethidium bromide before being photographed. (B) Spectrophotometric scanning of RNA isolated from reticulocyte ribosomes and separated on a 10% urea/polyacrylamide gel. RNA from lane 1 described in the legend to (A). The densities of the RNA bands were determined by scanning with an LKB Ultrascan XL laser spectrophotometer. An enlargement of the photograph corresponding to the scan is presented above the scan data. The material which did not enter the gel is indicated by 0. (C) The same as (B), except that angiogenin (4 μM, lane 7) was included in the incubation mixture. The arrow indicates the specific band formed after incubation with angiogenin also represented by the arrow in (A).

or both, when it is contained within the ribosome. In contrast, 28S rRNA in either the intact ribosome (Figure 4) or the 60S subunit (not shown) is cleaved into small fragments not discernible by agarose gel electrophoresis although readily visualized by 10% urea/polyacrylamide gel electrophoresis. Only 28S rRNA from the 60S subunit is catalytically attacked in this way by angiogenin. The 5.8S and 5S rRNAs remain resistant to degradation up to 4 μM angiogenin (Figure 3A–C).

Effect of Angiogenin on Isolated Ribosomal Subunit Function. Ribosomal subunits were individually treated with angiogenin in order to determine if ribosome inactivation is due to a specific effect of angiogenin on one, the other, or both. Incubation of either 40S or 60S subunits was carried out with 1 μM angiogenin (10 and 20 μg of 40S and 60S ribosomal subunits, respectively) in 25 mM HEPES, pH 7.5, at 30 °C for 15 min. The translational capacity of the treated and untreated subunits was then determined by reconstituting the lysate with the treated and the corresponding untreated subunits. Neither subunit alone was able to support protein synthesis (Figure 6, lanes 2 and 3). However, when added together, they were able to reconstitute the protein synthesis capacity of the intact lysate providing they had not been exposed to angiogenin (Figure 6, lane 4). Treatment of just the 40S subunit with angiogenin results in almost complete loss of protein synthesis capability (Figure 6, lane 5), whereas

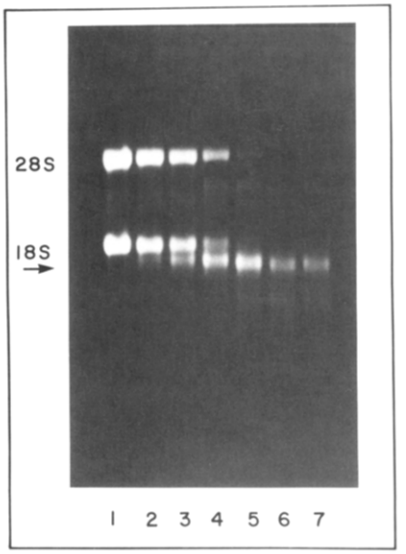

FIGURE 4: Effect of prolonged exposure of intact ribosomes to angiogenin. Electrophoretic pattern in a 1.1% formaldehyde/agarose gel of RNA isolated from intact ribosomes after incubation with angiogenin (1 μM) at 30 °C for 15, 30, 60, 120, 240, and 480 min, respectively (lanes 2–7). (Compare to control, no angiogenin, lane 1.) The arrow indicates the fragment that resists further angiogenin cleavage after prolonged incubation for 480 min. The positions of 28S and 18S rRNA are indicated.

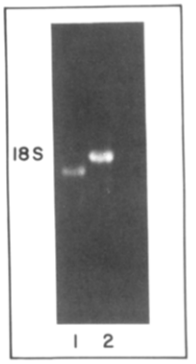

FIGURE 5: Limited cleavage of 18S rRNA by angiogenin in the 40S ribosomal subunit. Ribosomal subunits were prepared as described under Materials and Methods and incubated with angiogenin (1 μM) at 30 °C for 60 min (lane 1). Intact 18S rRNA from untreated 40S subunits is also shown (lane 2).

Table II: Effect of Angiogenin (Ang) on the Ability of 40S and 60S Ribosomal Subunits To Reconstitute the Translational Capacity of a Ribosome-Dependent Rabbit Reticulocyte Lysate System

	protein synthesis (cpm × 10^{-3})
lysate	170
lysate + Ang	1.5
(lysate + Ang) + (40 S/60 S)	16
(lysate + Ang) + (40 S[a]/60 S)	2.1
(lysate + Ang) + (40 S/60 S[a])	15
(lysate + Ang) + (40 S[a]/60 S[a])	2.2

[a] Subunits treated with angiogenin as described in the text.

treatment of the 60S subunit apparently has no effect on its ability, in conjunction with untreated 40S subunits, to support protein synthesis (Figure 6, lanes 5 and 6). These results were confirmed in a separate experiment in which the acid-insoluble radioactivity of the translation products was measured (Table II).

The pattern of cleavage fragments seen in these experiments is characteristic of angiogenin and seems to reflect its specificity rather than rRNA exposure: when the ribosomes are treated with RNase A, random degradation of all rRNA into

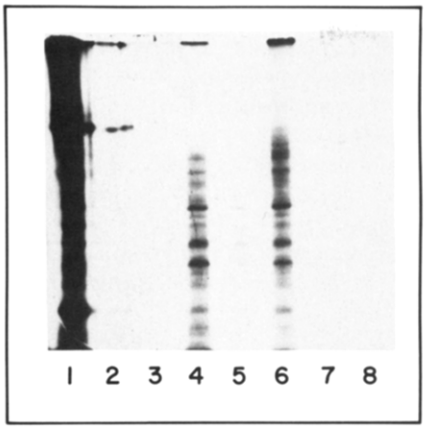

FIGURE 6: Autoradiograph of a 10% polyacrylamide gel of in vitro translation products showing the effect of angiogenin on the ability of reticulocyte ribosomal subunits to reactivate translation by the ribosome-dependent translation system. Lane 1, translation of Brom mosaic viral RNA. Lanes 2 and 3, translation of viral RNA in the ribosome-dependent translation system when only the 40S (lane 2) or the 60S (lane 3) subunit was added. Lane 4, the same as lane 2 except that both subunits were added. Lanes 5 and 6, the same as lane 4 except that one subunit (40 S for lane 5 and 60 S for lane 6) was pretreated with angiogenin. Lane 7, the same as lane 5, but both subunits were pretreated with angiogenin. Lane 8, the same as lane 7 except that no ribosomal subunits were added.

oligonucleotides is observed (not shown). Moreover, there is a marked difference in the susceptibility of rRNAs to the ribonucleolytic action of angiogenin when these are structurally arranged within the ribosome as compared to when they are in the isolated state. Separating the ribosome into its constituent subunits does not alter this susceptibility to attack. Presumably, it is the conformation of 18S rRNA within the 40S ribosomal subunit, together with the specificity of angiogenin, that determines the uniqueness of the cleavage pattern.

Discussion

Inhibition of in vitro protein synthesis by angiogenin in the rabbit reticulocyte lysate system appears to be due to cleavage of rRNA (St. Clair et al., 1987). PRI prevents the inhibition, and readdition of intact ribosomes to an angiogenin-treated lysate restores the capacity of the lysate to support protein synthesis (St. Clair et al., 1987). These conclusions are confirmed and extended by the results presented here, which directly demonstrate that angiogenin cleaves rRNA in the intact ribosome. Furthermore, angiogenin-treated ribosomes do not support protein synthesis when added to a ribosome-dependent translation system. In addition, the time course for inhibition of protein synthesis in both the lysate and the ribosome-dependent translation systems is the same. These results suggest that inactivation of ribosomes by cleavage of ribosomal RNA is responsible for the inhibition of in vitro protein synthesis caused by angiogenin.

The concentration of angiogenin needed to cause complete cleavage of rRNA is much greater than that needed to inhibit protein synthesis. This is not surprising since the kinetic results suggest that angiogenin may act at the elongation or termination step of peptide synthesis (Figure 1). Complete inhibition could therefore be caused by inactivation of only a few ribosomes which would effectively block the others from advancing along the polysome. The same phenomenon has previously been described for the effect of α-sarcin on yeast ribosomes (Schindler & Davies, 1977) and ribonuclease M on reticulocyte ribosomes (Wreschner et al., 1978).

The angiogenin cleavage site(s) has (have) not yet been identified precisely. However, it is apparent that the action of angiogenin on rRNA is distinctly different from that of known toxins such as α-sarcin (Schindler & Davies, 1977) or colicin E (Bowman et al., 1971), both of which generate single fragments from 28S or 18S rRNA. The effect of angiogenin on reticulocyte rRNA is more reminiscent of the rRNA cleavage induced by either interferon-activated intracellular RNase L (Wreschner et al., 1981) or RNase M, a component of reticulocyte membranes (Wreschner et al., 1978). Both of these enzymes inhibit protein synthesis in reticulocytes by digesting RNA. The enzyme regulated by interferon cleaves rRNA in intact ribosomes to yield a characteristic pattern of discrete products which differs from that generated by angiogenin. Interestingly, RNase M, like angiogenin, causes extensive cleavage of 28S rRNA while converting 18S rRNA into an only slightly smaller species. The cleavage specificity of RNase M differs from that of angiogenin, however, since it is independent of whether the substrate is free or ribosomally bound. The possibility exists, of course, that some of the rRNA cleavage products observed with angiogenin may arise from secondary RNase activity on the ribosome following initial cleavage by angiogenin.

Other possible mechanisms that might result in loss of translational capacity include a conformational change in the ribosome resulting from cleavage of rRNA by angiogenin which causes the release of some ribosome-associated, low molecular weight RNA. The regulation of protein synthesis by small RNAs is a concept that has acquired considerable support. Inhibitory RNAs have been isolated from several systems including rabbit reticulocytes (Dionne et al., 1982). Such RNAs could act by the well-characterized mechanism of double-stranded RNA which can inhibit translation by activation of a nuclease (Kerr & Brown, 1978; Clemens & Vaquero, 1978) or by promoting phosphorylation of the protein synthesis machinery (Farrell et al., 1977; Rosen et al., 1981).

Regardless of the mechanism of protein synthesis inhibition, the nature of the cleavage activity of angiogenin toward RNA in the intact ribosome reveals interesting properties of both the ribosome and the enzyme. Its effects on 28S and 18S rRNAs are strikingly different; i.e., prolonged incubation of ribosomes with angiogenin results in extensive degradation of 28S rRNA whereas 18S rRNA is converted to a slightly smaller species that has probably been trimmed at either one or both ends and then resists further degradation. Moreover, neither the 5.8S nor the 5S rRNAs are affected by angiogenin. This pattern of cleavage also occurs when the component ribosomal subunits are individually treated with angiogenin, indicating that the angiogenin-susceptible sites are not protected by subunit association. However, the effects of angiogenin on rRNAs in the ribosome are quite different from those on isolated 18S and 28S rRNAs (Shapiro et al., 1986) or isolated eukaryotic 5S rRNA (Rybak & Vallee, 1988), all of which are cleaved by angiogenin, to fragments of 100–500 nucleotides for the large rRNAs and of 5–12 nucleotides for *Saccharomyces cerevisiae* 5S rRNA. These results are consistent with observations that RNA structure contributes importantly to the cleavage specificity of angiogenin (Rybak & Vallee, 1988).

The present data demonstrate clearly that in the reticulocyte lysate system the specificity of nucleolytic cleavage by angiogenin is a function of both the ribosome and the enzyme. In this regard, angiogenin might prove useful for studying ribosomal structure and for preparing fragments of rRNA for sequencing, hybridization, and reconstitution studies of rRNA. Indeed, the observation that the effect of angiogenin on the 40S ribosomal subunit is responsible for the major inhibition of protein synthesis may lead to the identification of a pre-

viously unrecognized site critical to ribosomal function.

Inhibition of protein synthesis and an inherent ribonucleolytic activity are particularly interesting properties of angiogenin. In this regard, it resembles other physiologically important proteins such as eosinophil-derived neurotoxin and eosinophil cationic protein which also hydrolyze RNA and have sequence homology to RNase A (Gleich et al., 1986; Gullberg et al., 1986). Angiogenin and these proteins may comprise part of a family of RNases that have evolved important metabolic functions as contrasted with conventional degradative or processing functions. The possible connection of diverse physiological activities with ribonucleolytic activity promises to elucidate new pathways in cellular biology and, with regard to angiogenin, new mechanisms regulating the induction of neovascularization. The importance of this is underscored in view of the unique features of angiogenin among other molecules reported to have angiogenic properties (Folkman & Klagsbrun, 1987).

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