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C-TERMINAL ANGIOGENIN PEPTIDES INHIBIT THE BIOLOGICAL AND ENZYMATIC ACTIVITIES OF ANGIOGENIN

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Synthetic peptides corresponding to the C-terminal region of angiogenin (Ang) inhibit the enzymatic and biological activities of the molecule while peptides from the N-terminal region do not affect either activity. The peptide Ang(108-121) transiently abolishes the inhibition of cell-free protein synthesis caused by angiogenin coincidentally with its cleavage of reticulocyte RNA. Several C-terminal peptides also inhibit nuclease activity of angiogenin when tRNA is the substrate. Furthermore, peptide Ang(108-123) significantly decreases neovascularization elicited by angiogenin in the chick chorioallantoic membrane assay.

Angiogenin derived from tumor cell-conditioned medium (1), plasma (2), or recombinant sources (3) is a potent inducer of neovascularization. Structurally it is highly homologous to pancreatic RNase A, but functionally they differ distinctly (4,5). Angiogenin also potently inhibits cell-free protein synthesis in rabbit reticulocyte lysates (6) through specific inactivation of the 40S ribosomal subunit (7). C-terminal peptides of angiogenin, themselves devoid of any biological or

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enzymatic effects, inhibit both the capacity of angiogenin to induce neovascularization and to cleave various RNA substrates. Such peptides may modulate the biological actions of angiogenin and clarify their structural basis.

MATERIALS AND METHODS

Materials. Angiogenin was isolated from media conditioned by genetically altered baby hamster kidney cells as described (3). Reticulocyte lysate translation components and placental RNase inhibitor (PRI) were obtained from Promega Biotec. The 5' DNA terminus labeling system was from Bethesda Research Laboratories. tRNA (Type X from yeast), human serum albumin and phenylmethylsulfonyl floride (PMSF) were obtained from Sigma. [^{35}S]-Methionine (1200 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear.

Peptide Synthesis and Purification. Peptides were synthesized by solid phase methodology using Boc-amino acids (Penninsula Laboratories) (8). The chloromethylstyrene-divinylbenzene resin was purchased from Lab Systems, Inc. Trifluoroacetic acid (Halocarbon) and dichloromethane (E.M. Science) were distilled prior to use. Triethylamine and dimethylformamide were sequanal grade (Pierce Chemical Co.). Coupling and deblocking of the N-terminus were monitored by a ninhydrin test (9). Details of the synthetic procedure have been reported (10).

The HF treated resin was extracted with ethyl acetate and a 1% solution of acetic acid. The crude peptides were purified on a Waters HPLC unit connected to a Foxy fraction collector. A linear gradient of acetonitrile or methanol in H_2O/0.1% trifluoroacetic acid (TFA) on a Waters μBondpak C18, 19 X 150 mm column separates the peaks. All amino acids were within 5% of the theoretical value as verified by amino acid analysis.

In Vitro Translation Assay. Methods used to study the effects of angiogenin on cell-free protein synthesis have been detailed (6). In experiments where reticulocyte ribosomes were the target for angiogenin, the lysate was preincubated with angiogenin in the presence or absence of peptide and then angiogenin was inhibited with PRI before translation. Translation was initiated by the addition of reticulocyte lysate and/or mRNA in the presence of [^{35}S]-methionine and other components as specified by the manufacturer. 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.

tRNA Assay. The details of the assay which measures formation of acid-soluble fragments from tRNA have been described (11). Conditions were optimized for this study as follows. The 100 μL reaction mixture contained 30 mM Hepes, pH 7, 30 mM NaCl, 0.01 percent human serum albumin, 40 μM tRNA and 1x10^{-5}M PMSF. The reaction was stopped by the addition of 120 μL of ice cold 6% perchloric acid. After 10 min on ice the samples were centrifuged for 10 min at 4°C in an Eppendorf centrifuge. A 170 μL aliquot of supernatant was withdrawn, mixed with 130 μL of water and the absorbance of the solution was measured at 260 nm. Any absorbance contribution from acid solubilized angiogenin or peptide was corrected for by subtracting the absorbance of the appropriate control reaction mixture.

Isolation of Reticulocyte RNA. Reticulocyte RNA was dissolved in 0.5% $NaDodSO_4$ in 10 mM Tris-HCl, pH 7.5, 10mM NaCl,

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 water and stored at -70°C.

Angiogenesis Assay. The chick chorioallantoic membrane (CAM) assay of Knighton (12) was employed as described (1,2). In the case of mixtures of angiogenin with C-terminal peptide, the two were mixed just prior to application to the disks. Statistical analyses were performed on data recorded at 68 ± 2 h after sample implantation. In control assays for these experiments 13 out of 63 eggs were positive, a frequency of 0.21. Employing this as the probability of an egg being positive in a Bernoulli experiment, the probability for each assay was calculated from the appropriate cumulative binomial distribution. These probabilities were converted to normal deviates employing formula 26.2.23 of Zelen and Severo (13). One-way analysis of variance of the latter values was carried out according to Kendall and Stuart (14). Newman-Keuls ranking for multiple comparisons of the group means obtained from the analysis of variance was carried out as described by Keuls (15). These group means were converted back to probabilities employing formula 26.2.17 of Zelen and Severo (13). A significance level of $\leq 5\%$ has to be attained for a sample to be considered active.

RESULTS

A synthetic peptide corresponding to residues 108-121 of angiogenin, Ang(108-121), abolishes the inhibition of protein synthesis resulting from treatment of the rabbit reticulocyte lysate system with angiogenin. This effect is concentration dependent: peptide, 30 μM, almost completely prevents the inhibition of protein synthesis caused by angiogenin (Table 1). The ribonucleolytic action of angiogenin is responsible for the inhibition of protein synthesis in the rabbit reticulocyte lysate (6). A 5 min exposure to angiogenin inactivates globin mRNA which is then unable to support protein synthesis (Fig. 1A, lane

TABLE 1. Effect of Ang(108-121) on the Inhibition of Protein Synthesis Caused by Angiogenin in the Rabbit Reticulocyte Lysate Assay[a]

	Incorporation of [^{35}S]-methionine	
Ang(108-121), μM	Protein Synthesis Inhibition	% Decrease of Angiogenin's Inhibitory Activity
0	100	0
7.5	45	55
15.0	38	62
30.0	7	93

[a]Average values from two experiments. Angiogenin was preincubated in the presence or absence of Ang(108-121) for 30 min. Final concentration of angiogenin was 0.03 μM. Protein synthesis was initiated by the addition of the reticulocyte lysate containing mRNA and quantitated as described in Methods. 100% incorporation = 1.9×10^4 cpm.

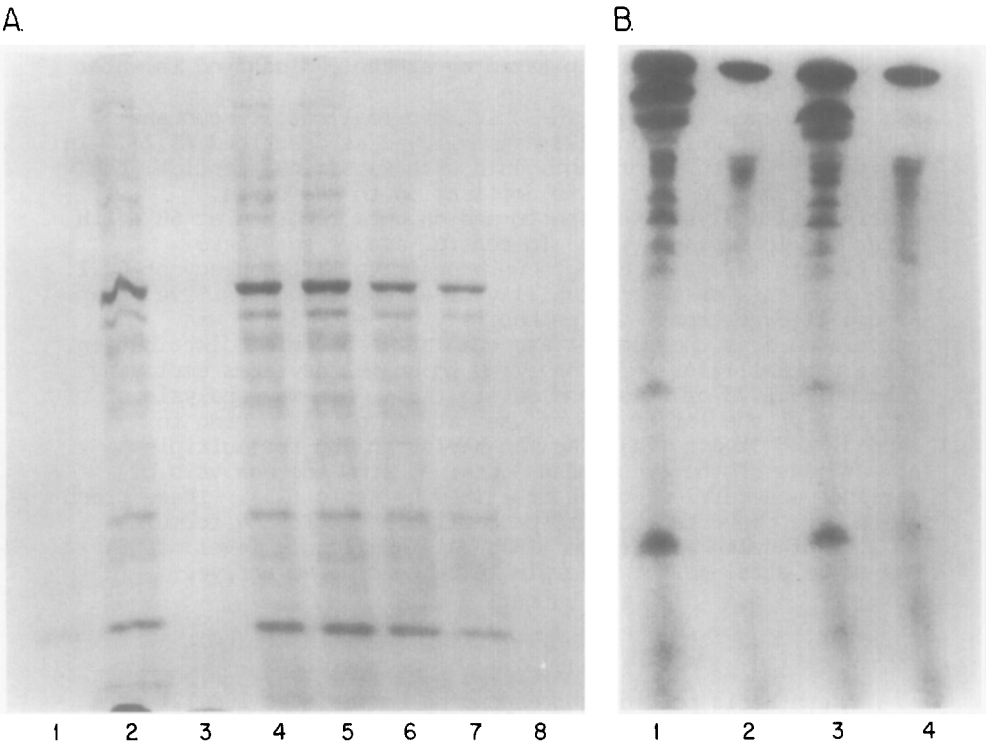

Figure 1

(A) Autoradiograph of a 10% polyacrylamide gel of in vitro translation products demonstrating the ability of Ang(108-121) to prevent degradation of globin mRNA caused by angiogenin. Angiogenin was pre-incubated with peptide at 37°C for 30 min. Globin mRNA (0.2 μg) was added for an additional 5, 10, 15 or 30 min after which time the reaction was stopped by the addition of PRI (40 units). Protein synthesis was determined as described in Methods. Lane 1, mRNA-dependent rabbit reticulocyte lysate translation without exogenous mRNA; lane 2, same as 1 but with addition of 0.2 μg of globin mRNA; lane 3, same as 2 but with angiogenin (0.03 μM) added for 5 min; lane 4, same as 2 but with Ang(108-121) (30 μM) for 5 min; lanes 5-8, same as lane 3 but with peptide for 5, 10, 15 and 30 min.

(B) Autoradiograph of a 10% 7 M urea/acrylamide gel showing cleavage of [γ-^{32}P]ATP labeled reticulocyte RNA by angiogenin and the transient inhibition of this by Ang(108-121). Angiogenin (2 μM) was preincubated with peptide (3.4 mM) for 30 min at 37°C. Reticulocyte RNA was treated with or without angiogenin (2 μM) or with angiogenin plus peptide. Lane 1, RNA treated with buffer for 30 min; lane 2, same as lane 1, but treated with angiogenin for 5 min; lanes 3 and 4, same as lane 2 but treated with peptide for 5 min (lane 3) or 30 min (lane 4).

3). Although Ang(108-121) itself has no effect (Fig. 1A, lane 4), it prevents the angiogenin-induced inhibition of protein synthesis in a time dependent manner. Incubation of mRNA with angiogenin and the peptide for 10 min diminishes protection, and 30 min abolishes it (Fig. 1A, lanes 5-8). Ang(108-121), 30 μM, also prevents the effects of angiogenin on isolated reticulocyte ribosomes (not shown).

To visualize directly the inhibition of the ribonucleolytic activity of angiogenin by Ang(108-121), RNA was isolated from the rabbit reticulocyte lysate, labeled by T4 kinase at the 5′ end with [γ-^{32}P]ATP and treated with angiogenin in the presence or absence of peptide. An analysis of this RNA by gel electrophoresis demonstrates significant degradation of reticulocyte RNA after 5 min with angiogenin (Fig. 1B, lane 2). Inclusion of the C-terminal peptide in the 5 min incubation mixture prevents this degradation (Fig. 1B, lane 3). However, as is the case with the effect of the peptide on protein synthesis (Fig. 1A) the capacity of Ang(108-121) to inhibit the nuclease activity of angiogenin is transient (Fig. 1B, lane 4).

The substrate independent inhibition of the ribonucleolytic activity of angiogenin by Ang(108-121) prompted a more analytic examination of this phenomenon by means of an assay which measures formation of acid-soluble fragments from tRNA. In the tRNA assay extending Ang(108-121) by one, Ang(108-122), or two, Ang(108-123), amino acids does not greatly change the inhibitory activity among the C-terminal peptides (Table 2). The peptides themselves do not degrade tRNA. Although the inhibitory effect of all C-terminal peptides disappears completely within 60 min in this assay as well (not shown), PMSF extends the period of inhibition, presumably by inhibiting serine proteases contaminating the tRNA. This results in substantial inhibition of angiogenin's activity by Ang(108-123) up to 270 min (Table 3). Peptide Ang(108-123) inhibits the initial rate of degradation of tRNA catalyzed by angiogenin in a concentration dependent manner yielding an apparent K_i value of 278 μM (Fig. 2). The N-terminal peptides Ang(1-21) and Ang(6-21) do not inhibit hydrolysis of tRNA (Table 2).

The results depicting the inhibition of the activity of angiogenin on the CAM assay by the C-terminal peptide

TABLE 2. Effect of Synthetic Angiogenin Peptides on the Ribonucleolytic Activity of Angiogenin using tRNA as Substrate[a]

		%Inhibition
Ang(108-121)	^{108}E N G L P V H L D Q S I F R^{121}	35
Ang(108-122)	^{108}E N G L P V H L D Q S I F R R^{122}	39
Ang(108-123)	^{108}E N G L P V H L D Q S I F R R P^{123}	39
Ang(1-21)	^{1}Q D N S R$_{6}$Y T H F L T Q H Y D A K P Q G R^{21}	0
Ang(6-21)	Y T H F L T Q H Y D A K P Q G R^{21}	0

[a]Average values from four to six experiments in which angiogenin, 1 μM, was incubated in the presence of 100 μM peptide with 40 μM tRNA at pH 7, 37°C for 10 min as described in Methods. The decrease in absorbance change in the presence of peptide x 100 is expressed as % inhibition.

TABLE 3. Time Course of the Inhibition by Ang(108-123) of Angiogenin's Activity in the tRNA Assay[a]

Time (min)	Activity (% inhibition)
10	42
30	30
60	27
150	20
270	9

[a]Angiogenin (1 μM) was incubated with 40 μM tRNA in the absence or presence of peptide (100 μM) for varying times at 37°C. Under these conditions the absorbance change with angiogenin alone ranged from 0.3 (10 min) to 0.8 (240 min). The decrease in the absorbance change in the presence of peptide x 100 is expressed as % inhibition. Data represent average values of duplicate incubations.

Ang(108-123) are shown in Table 4. The group with significant positive results ($\bar{p}<0.05$) is that for angiogenin alone (Group IV). Moreover, one-way analysis of variance of the CAM results yielded an F value of 17.297 corresponding to a probability $<<0.001$. Since this demonstrates a significant difference among the groups, the Neuman-Keuls method was applied to the group means. This shows that Group IV differs from Groups I, II, and III with an associated probability $<<0.001$ whereas Groups I, II, and III cannot be regarded as differing significantly from each other ($p > 0.10$). Thus, while angiogenin alone is highly active (Group IV) addition of Ang(108-123) to angiogenin effectively inhibits the latter's activity (Group III). Indeed, the assay results cannot be distinguished from those of the control (Group I) or of Ang(108-123) itself (Group II).

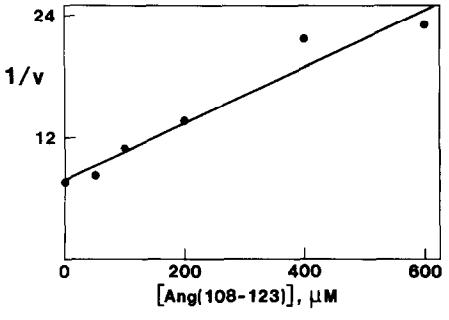

Figure 2
Inhibition of the ribonucleolytic activity of angiogenin by Ang(108-123). Angiogenin (1 μM) was incubated with tRNA (40 μM) in the presence or absence of peptide for 30 min at 37°C. Under these conditions the absorbance change at 260 nm in the absence of peptide was 0.4. The rate of the reaction, v, is expressed as the change in absorbance per min. The data represent average values from duplicate incubations.

TABLE 4. Effect of Ang(108-123) on the Activity of Angiogenin in the CAM Assay

Group	Sample	Sets of Assays[a]	Average Probabilities[b]
I	Control[c]	5	0.60
II	Ang(108-123) (500 pmol)	7	0.55
III	Ang(108-123) (500 pmol) plus Angiogenin (3.5 pmol)	7	0.25
IV	Angiogenin (3.5 pmol)	7	0.02

[a]Each individual set of assays contained between 9 and 15 eggs.
[b]Average probability that a given group result would occur with this frequency if the underlying control frequency is 0.21.
[c]Water.

DISCUSSION

Peptide inhibition of receptor-ligand interactions is well documented (16-18) and synthetic peptides derived from amino acid sequences implicated in the association of enzymatic subunits also have been shown to inhibit enzymatic activity (19,20). The data presented herein demonstrate the effect of a synthetic peptide on the association of a protein with RNA. In both the tRNA and protein synthesis assays peptides derived from the C-terminus of angiogenin appear to be much more potent than those derived from the N-terminal region of the molecule (Table 2).

Structure/function studies have demonstrated that the consequences of chemical modification of angiogenin are much like those of RNase A with respect to the critical histidyl and lysyl residues involved in catalysis (11). These results as well as similarities in conformation deduced from energy minimization (21) suggest that previous work with RNase A may be useful in understanding structural aspects of angiogenin. In this regard the importance of the C-terminal region to RNase activity is underscored by studies which showed that synthetic C-terminal peptides of RNase can bind non-covalently to a shortened, inactive RNase component to regenerate nearly full activity (22). In a similar fashion C-terminal angiogenin peptides restore RNase-like activity to inactive fragments of RNase (10). Thus, the peptide inhibition of the enzymatic activity of angiogenin could be due to disruption of the critical structural features of regions of angiogenin which form the active site of the enzyme. However, since the substrates for angiogenin are macromolecular it is also conceivable that the peptide binds to nucleotides themselves. The fact that the concentration, 30μM, of Ang(108-121) needed to prevent angiogenin's ability to inhibit protein synthesis is approximately 10-fold lower than the K_i, 278

μM, for the inhibition of tRNA degradation may reflect the difference in the binding affinity of the peptide for the different forms of RNA substrates. The possibility that this is merely an electrostatic interaction with RNA seems unlikely since adding an additional C-terminal Arg to Ang(108-121) did not greatly increase the inhibitory effect. Furthermore, basic peptides from the N-terminal region had no effect on the capacity of angiogenin to degrade tRNA (Table 2).

Irrespective of mechanism, this work further links the ribonucleolytic activity of angiogenin to its capacity to induce neovascularization in the CAM. In addition, it suggests that inhibition of neovascularization by peptides is feasible and that rapid and easy-to-perform nuclease assays may be very useful in screening for potential inhibitors. This work also emphasizes the usefulness of synthetic peptides in probing sites critical to the action of angiogenin.

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