Angiogenin Is a Cytotoxic, tRNA-specific Ribonuclease in the RNase A Superfamily*

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Angiogenin is a 14.4-kDa human plasma protein with 65% homology to RNase A that retains the key active site residues and three of the four RNase A disulfide bonds. We demonstrate that recombinant angiogenin functions as a cytotoxic tRNA-specific RNase in cell-free lysates and when injected into Xenopus oocytes. Inhibition of protein synthesis by angiogenin correlates with degradation of endogenous oocyte tRNA. Exogenous, radiolabeled tRNA is also hydrolyzed by angiogenin, whereas oocyte tRNA and mRNA are not detectably degraded by angiogenin. Protein synthesis was restored to angiogenin-injected oocytes by injecting the RNase inhibitor RNasin™ plus total Xenopus or calf liver tRNAs, thereby demonstrating that the tRNA degradation induced by angiogenin was the sole cause of cytotoxicity. A similar tRNA-reversible inhibition of protein synthesis was seen in rabbit reticulocyte lysates. Angiogenin therefore appears to be a specific cellular tRNase, whereas five homologues in the RNase A superfamily lack angiogenin's specificity for tRNA. One of these homologues purified from human eosinophils, eosinophil-derived neurotoxin, nonspecifically degrades oocyte RNA similar to RNase A and is also cytotoxic at very low concentrations.

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**RESULTS AND DISCUSSION**

EDN-mediated Disappearance of Cellular RNA Correlates with Inhibition of Protein Synthesis—Injected EDN abolished oocyte protein synthesis as determined by trichloroacetic acid precipitation of labeled proteins (Fig. 2A) and EDN-mediated disappearance of cellular RNA correlates with inhibition of protein synthesis (Fig. 2B). Oocyte tRNA, 5S RNA, and 5.8S RNA were also hydrolyzed by EDN (not shown). EDN is nearly as effective as RNase A, ricin, or diphtheria toxin (2) in abolishing oocyte protein synthesis. Inhibition of protein synthesis at 10^{-5} mg/ml EDN corresponds to 29 pm or 9 x 10^6 molecules/oocyte. Injected angiogenin does not hydrolyze rRNA (2), whereas EDN exhibited a similar mechanism and cytotoxic properties with RNase A when injected into Xenopus oocytes. Both EDN and angiogenin possess similar sequence homology with RNase A, yet these two human proteins sharply differ in their intracellular nuclease activity.

Unlike EDN, Angiogenin-(M-,) Abolishes Protein Synthesis by Specifically Hydrolyzing Cellular tRNA—a Sarcin and recombinant angiogenin containing a single additional amino-terminal methionine (angiogenin-(M-)) both abolished oocyte protein synthesis when present at ~2 nm (2). Angiogenin-(M-) did not hydrolyze phosphodiester bonds in the α-sarcin domain of 28 S RNA, nor did it cause generalized degradation of cellular RNA like RNases A, B, C, T1, T2, or U2 (2) or EDN (Fig. 2B). However, injected angiogenin-(M-) hydrolyzed virtually all oocyte tRNA (Fig. 3, B and C), and this hydrolysis directly correlated with protein synthesis inhibition (Fig. 3A). This nearly complete hydrolysis of oocyte tRNA (60 ng or ~10^{12} molecules/oocyte; Ref. 11) is particularly significant because other injected cytotoxins such as α-sarcin (6) or ricin (4) cleave less than 5% of total 28 S RNA (~10^{12} molecules/oocyte). Therefore, angiogenin-(M-) functions as a very potent and specific tRNase when injected into Xenopus oocytes.

**Angiogenin-(M-) Hydrolysis of Endogenous tRNA Can Occur in the Absence of Functional Ribosomes**—One explanation for angiogenin’s specificity for cellular tRNA may be that translating ribosomes present only the tRNA as a substrate to angiogenin. We tested this possibility by injecting oocytes with protein synthesis inhibitors or toxins and then subsequently injected angiogenin. Cycloheximide, puromycin, diph-

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**Fig. 1.** Angiogenin and EDN are members of the RNase A superfamily. Amino acid sequence alignment of human (7) and bovine angiogenin, human EDN, and bovine RNase A (31-34) were done with the GCG programs GAP and Bestfit. The amino acids identical to human angiogenin in either bovine angiogenin, EDN, or RNase A are shown in boldface letters; amino acids closely related (35) to angiogenin are shown in boldface lowercase letters. Periods represent gaps in amino acid alignment. Active site amino acids in RNase A (36) are indicated with an asterisk. Note that identical or closely related amino acids among bovine angiogenin, EDN, or RNase A are not shown in bold type unless also closely related to human angiogenin.

**Fig. 2.** Injected EDN abolishes protein synthesis as effectively as diphtheria toxin or ricin. A, injected EDN (28) hydrolyzes oocyte RNA similar to RNase A. EDN injected at 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}, and 10^{-1} mg/ml in lanes C-I, respectively; A, uninjected; B, deionized H2O-injected; J, background trichloroacetic acid precipitation from labeled medium. Injections and analyses as previously described (2). B, recovered RNA corresponding to lanes A-J (respectively) in A analyzed on 1.4% agarose gel.
Angiogenin-(M-\textunderscore) hydrolyzed exogenous tRNA is inhibited by RNasin\textsuperscript{TM}. A, oocytes injected with kinase-labeled calf liver tRNA (Boehringer Mannheim) were incubated 4 h before injection of deionized H\textsubscript{2}O (lanes A and G) or angiogenin-(M-\textunderscore) at 10\textsuperscript{-3}, 10\textsuperscript{-4}, 10\textsuperscript{-5}, and 10\textsuperscript{-6} mg/ml (lanes B-F, respectively). B, lanes A and F, same as A and G in panel A; B, angiogenin-(M-\textunderscore) injected 4 h after tRNA; C, RNasin\textsuperscript{TM} injected 4 h after angiogenin-(M-\textunderscore), then tRNA injected 3 h after RNasin; D, RNasin\textsuperscript{TM} plus tRNA injected 4 h after angiogenin-(M-\textunderscore); E, angiogenin-(M-\textunderscore) combined with tRNA and then immediately injected. Angiogenin-(M-\textunderscore) injected at 0.1 mg/ml; tRNA or 5 S or 18/28 S injected at 0.5 mg/ml; RNasin\textsuperscript{TM} injected at 28 units/\textmu l. tRNA was labeled as described previously (4).
presumably because angiogenin-(M-)’s tRNase activity is so efficient that it degrades both the endogenous and injected tRNAs in the absence of RNasin™. Consistent with the model that angiogenin-(M-) only hydrolyzes cellular tRNA, protein synthesis was not restored by injecting RNasin™ and 5 or 18 and 28 S RNA (lanes 12 and 13, respectively). Angiogenin-(M-) does not appear to hydrolyze mRNAs, because the restored protein synthesis appeared identical to the proteins synthesized by uninjected oocytes as judged by SDS-polyacrylamide gel electrophoresis (Fig. 5B, lane 5).

Protein synthesis can also be restored to angiogenin-(M-) inactivated rabbit reticulocyte lysates by adding RNasin™ and total tRNA (Fig. 6A and B), thereby demonstrating that angiogenin-(M-) selectively hydrolyzes tRNA both in extracts (Fig. 6C) and in cells (Figs. 3–5). In contrast, RNase A hydrolyzes both ribosomal RNA and tRNA in oocytes (2) and in the reticulocyte lysates (Fig. 6C, lane D).

Angiogenin-(M-) appears to be the first known inhibitor of protein synthesis that functions by specifically hydrolyzing cellular tRNA (for comparison, see Table I). This tRNA-specific hydrolysis is distinct from previously described tRNA processing nucleases found in Escherichia coli (18).

Diphtheria toxin and ricin are cytotoxic because they specifically attack essential cellular targets required for protein synthesis (3, 4, 19). Consequently these toxins have been used pharmacologically as immunotoxins (see Refs. 20–22 for reviews). Recently RNases were tested for cytotoxicity following their introduction into mammalian cells. Prior et al. (23) demonstrated that a chimeric toxin composed of inactive
Angiogenin appears to be a secreted protein whose precise tRNase activity may occur within the angiogenin cellular tRNA. For example, α-sarcin enters virus permeabilization range required for blood vessel formation. However, responsible for angiogenin’s cellular tRNase specificity remains to be elucidated. Understanding the molecular basis for this phenomenon may even allow construction of new RNases that specifically hydrolyze certain viral RNAs, for example. It is likely that additional members of the RNase superfamily may also specifically hydrolyze cellular RNAs.

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### REFERENCES

### TABLE 1
**Summary of cellular RNAs hydrolyzed by the RNase A superfamily**

<table>
<thead>
<tr>
<th>Superfamily RNase</th>
<th>mRNA</th>
<th>tRNA</th>
<th>tRNA</th>
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<tr>
<td>Angiogenin</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>RNase A, B, C, S, and EDN</td>
<td>+</td>
<td>+</td>
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*pseudomonas exotoxin A and the bacterial ribonuclease barnase was cytotoxic, concluding that the cytotoxic effect was due to the ribonuclease activity of barnase molecules. Rybak et al. (24) demonstrated that mammalian ribonuclease A coupled to transferrin-specifically killed target cells via the transferrin receptor. Therefore, human RNases may represent an attractive alternative to conventional toxins such as ricin or diphtheria toxins in immunotoxin therapy.*