# Ribonuclease Inhibitor Regulates Neovascularization by Human Angiogenin

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# Ribonuclease Inhibitor Regulates Neovascularization by Human Angiogenin†

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## Abstract

Human angiogenin (ANG) is a homologue of bovine pancreatic ribonuclease (RNase A) that induces neovascularization. ANG is the only human angiogenic factor that possesses ribonucleolytic activity. To stimulate blood-vessel growth, ANG must be transported to the nucleus and must retain its catalytic activity. Like other mammalian homologues of RNase A, ANG forms a femtomolar complex with the cytosolic ribonuclease inhibitor protein (RI). To determine whether RI affects ANG-induced angiogenesis, we created G85R/G86R ANG, which possesses $10^6$-fold lower affinity for RI but retains wild-type ribonucleolytic activity. The neovascularization of rabbit corneas by G85R/G86R ANG was more pronounced and more rapid than by wild-type ANG. These findings provide the first direct evidence that RI serves to regulate the biological activity of ANG *in vivo*.

Angiogenin (ANG) is a potent inducer of blood vessel growth (1) and has been implicated in the establishment, growth, and metastasis of tumors (2,3). A homologue of bovine pancreatic ribonuclease (RNase A (4-6); EC 3.1.27.5), ANG is the only human angiogenic factor that displays ribonucleolytic activity. ANG was first isolated from the conditioned medium of human adenocarcinoma cells (1), and is present in normal human plasma (7) as well as numerous other tissues and organs (8). After receptor-mediated endocytosis (9), a nuclear localization sequence (NLS) directs ANG to the nucleus (10). The receptor-binding, nuclear localization, and ribonucleolytic activity of ANG are all required for angiogenic activity (9-11). In endothelial and smooth muscle cells, ANG induces a wide range of cellular responses, including transcriptional activation (12), differentiation (13), cell migration and invasion (14), and tube formation (13).

The ribonuclease inhibitor (RI (15)), a cytosolic protein found in all mammalian tissues analyzed to date, binds to mammalian ribonucleases with extraordinary affinity. The RI·ANG complex (Figure 1A) is among the tightest of known protein-protein interactions with $K_d$ =

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0.71 fM (17). Binding to RI blocks the active site of the enzyme and abolishes ribonucleolytic activity.

A known role for RI is to protect cellular RNA from invading ribonucleases (19,20). ANG, however, possesses <1% of the ribonucleolytic activity of its homologues with cytotoxic activity (21). Moreover, the $IC_{50}$ values for cytotoxic ribonucleases are $\geq 10^2$-fold greater than the concentration of ANG required to induce endothelial cell proliferation *in vitro*. Thus, a major role for RI as an antagonist of the cytotoxic activity of ANG is unlikely.

Does RI play a role in ANG-induced angiogenesis? The exogenous addition of extracellular RI is known to antagonize angiogenesis (22,23). That experiment, however, puts RI in a nonnative location. We sought to determine whether endogenous, intracellular RI regulates ANG-induced neovascularization. We reasoned that we could do so by using a variant of ANG that evades RI.

To disrupt its interaction with RI, we introduced arginine residues at positions 85 and 86 of ANG (Figure 1B). As residues 85 and 86 are distal from the enzymic active site, we suspected that substitutions there would not affect the catalytic activity of ANG. Indeed, a thorough genetic selection did not reveal Gly85 and Gly86 as being residues critical to the enzymatic activity of ANG (24). These two residues are also distal from the loop implicated in binding to a cell-surface receptor (9) and from the NLS (10).

Ribonucleases were produced in *Escherichia coli* with yields of ≥40 mg of purified enzyme per liter of culture. Both wild-type ANG and its G85R/G86R variant migrated as a single band during zymogram electrophoresis (Figure S1 in Supporting Information), indicating that these enzymes are free from contaminating ribonucleolytic activity (21). The $k_{cat}/K_M$ values for the two ribonucleases were indistinguishable (Table 1), as anticipated.

The G85R/G86R substitutions lead to a marked decrease in affinity for RI. The $K_d$ for the complex of RI with G85R/G86R ANG is 5 nM (Table 1), which is $10^7$-fold higher that of ANG (17) and $10^1$-fold higher than that of G88R RNase A, an analogous variant (18). Hence, G85R/G86R ANG is an ideal probe for detecting a role for RI in angiogenesis. Its affinity for RI is reduced dramatically by the two substitutions, but its catalytic activity is not affected (Table 1). Thus, any observed increase in the angiogenic activity of G85R/G86R ANG can be attributed to its diminished affinity for RI.

The assay used most often to assess ANG-induced angiogenesis enlists the chick chorioallantoic membrane (CAM) (25). Although ANG effectively stimulates neovascularization in the CAM assay, avian species do not have a homologue of RI. As a result, the CAM assay cannot reveal a role for RI in ANG-induced angiogenesis. Instead, we assayed angiogenic activity in a mammalian tissue—rabbit cornea—that is otherwise avascular (26).

Rabbit corneas implanted with a hydrogel pellet containing G85R/G86R ANG not only generated more blood vessels, but also demonstrated more rapid blood-vessel growth than did corneas treated with wild-type ANG (Figures 2A and 2B). Histological examination revealed many typical capillaries as well as edema in the area between the limbus and pellet of corneas treated with the G85R/G86R variant (Figure 2C). In contrast, few new vessels were observed in corneas treated with wild-type ANG.

Thus, we report the first evidence that native RI regulates the angiogenic activity of ANG *in vivo* (Figure 2D). Although G85R/G86R ANG induced angiogenesis more effectively than did the wild-type enzyme, the cystosolic concentration of RI (4 μM (19)) greatly exceeds the value of $K_d$ for the RI·G85R/G86R ANG complex (5 nM; Table 1). We suspect, therefore, that substitutions endowing additional RI evasion could enhance angiogenic activity even further.

Finally, we note that our finding could have medicinal implications. The promotion of neovascularization has the potential to alleviate coronary artery disease and promote wound healing (27,28). Such regenerative therapies could employ VEGF and bFGF, as well as a "hyperangiogenic" protein such as G85R/G86R ANG. Recently, however, a unique medicinal role for ANG has become apparent. Loss-of-function mutations in the angiogenin gene have been found in patients with amyotrophic lateral sclerosis, a fatal neurodegenerative disorder (29-31). Hyperangiogenic variants of ANG or small-molecule antagonists (32) of the RI-ANG interaction could serve as the basis of a chemotherapeutic regimen for such patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

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## Abbreviations

ANG, human angiogenin
bFGF, basic fibroblast growth factor
BS-RNase, bovine seminal ribonuclease
CAM, chorioallantoic membrane
NLS, nuclear localization sequence
ONC, Onconase® (a registered trademark of Alfacell, Inc.)
PDB, Protein Data Bank
RI, ribonuclease inhibitor protein
RNase A, bovine pancreatic ribonuclease
RNase 1, human pancreatic ribonuclease
VEGF, vascular endothelial growth factor

## REFERENCES

1. Fett JW, Strydom DJ, Lobb RR, Alderman EM, Bethune JL, Riordan JF, Vallee BL. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry 1985;24:5480–5486. [PubMed: 4074709]
2. Olson KA, Byers HR, Key ME, Fett JW. Prevention of human prostate tumor metastasis in athymic mice by antisense targeting of human angiogenin. Clin. Cancer Res 2001;7:3598–3605. [PubMed: 11705882]
3. Olson KA, Byers HR, Key ME, Fett JW. Inhibition of prostate carcinoma establishment and metastatic growth in mice by an antiangiogenin monoclonal antibody. Int. J. Cancer 2002;98:923–929. [PubMed: 11948474]
4. Raines RT. Ribonuclease A. Chem. Rev 1998;98:1045–1065. [PubMed: 11848924]
5. Pizzo E, D'Alessio G. The success of the RNase scaffold in the advance of biosciences and in evolution. Gene 2007;406:8–12. [PubMed: 17616268]
6. Marshall GR, Feng JA, Kuster DJ. Back to the future: Ribonuclease A. Biopolymers 2008;90:259–277. [PubMed: 17868092]
7. Bläser J, Triebel S, Kopp C, Tschesche H. A highly sensitive immunoenzymometric assay for the determination of angiogenin. Eur. J. Clin. Chem. Clin. Biochem 1993;31:513–516. [PubMed: 8218584]
8. Moenner M, Gusse M, Hatzi E, Badet J. The widespread expression of angiogenin in different human cells suggests a biological function not only related to angiogenesis. Eur. J. Biochem 1994;226:483–490. [PubMed: 7528139]

9. Hu GF, Riordan JF, Vallee BL. A putative angiogenin receptor in angiogenin-responsive human endothelial cells. Proc. Natl. Acad. Sci. USA 1997;94:2204–2209. [PubMed: 9122172]
10. Moroianu J, Riordan JF. Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity. Proc. Natl. Acad. Sci. U.S.A 1994;91:1677–1681. [PubMed: 8127865]
11. Shapiro R, Fox EA, Riordan JF. Role of lysines in human angiogenin: Chemical modification and site-directed mutagenesis. Biochemistry 1989;28:1726–1732. [PubMed: 2497770]
12. Xu ZP, Tsuji T, Riordan JF, Hu GF. The nuclear function of angiogenin in endothelial cells is related to rRNA production. Biochem. Biophys. Res. Commun 2002;294:287–292. [PubMed: 12051708]
13. Jimi S, Ito K, Kohno K, Ono M, Kuwano M, Itagaki Y, Ishikawa H. Modulation by bovine angiogenin of tubular morphogenesis and expression of plasminogen activator in bovine endothelial cells. Biochem. Biophys. Res. Commun 1995;211:476–483. [PubMed: 7540839]
14. Hu G, Riordan JF, Vallee BL. Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities. Proc. Natl. Acad. Sci. USA 1994;91:12096–12100. [PubMed: 7991590]
15. Dickson KA, Haigis MC, Raines RT. Ribonuclease inhibitor: Structure and function. Prog. Nucleic Acid Res. Mol. Biol 2005;80:349–374. [PubMed: 16164979]
16. Papageorgiou A, Shapiro R, Acharya K. Molecular recognition of human angiogenin by placental ribonuclease inhibitor—an X-ray crystallographic study at 2.0 Å resolution. EMBO J 1997;16:5162–5177. [PubMed: 9311977]
17. Lee FS, Shapiro R, Vallee BL. Tight-binding inhibition of angiogenin and ribonuclease A by placental ribonuclease inhibitor. Biochemistry 1989;28:225–230. [PubMed: 2706246]
18. Vicentini AM, Kieffer B, Matthies R, Meyhack B, Hemmings BA, Stone SR, Hofsteenge J. Protein chemical and kinetic characterization of recombinant porcine ribonuclease inhibitor expressed in *Saccharomyces cerevisiae*. Biochemistry 1990;29:8827–8834. [PubMed: 2271559]
19. Haigis MC, Kurten EL, Raines RT. Ribonuclease inhibitor as an intracellular sentry. Nucleic Acids Res 2003;31:1024–1032. [PubMed: 12560499]
20. Rutkoski TJ, Raines RT. Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity. Curr. Pharm. Biotechnol 2008;9:185–189. [PubMed: 18673284]
21. Leland PA, Staniszewski KE, Park C, Kelemen BR, Raines RT. The ribonucleolytic activity of angiogenin. Biochemistry 2002;41:1343–1350. [PubMed: 11802736]
22. Shapiro R, Vallee BL. Human placental ribonuclease inhibitor abolishes both angiogenic and ribonucleolytic activities of angiogenin. Proc. Natl. Acad. Sci. U.S.A 1987;84:2238–2241. [PubMed: 3470787]
23. Polakowski IJ, Lewis MK, Muthukkaruppan V, Erdman B, Kubai L, Auerbach R. A ribonuclease inhibitor expresses anti-angiogenic properties and leads to reduced tumor growth in mice. Am. J. Pathol 1993;143:507–517. [PubMed: 7688185]
24. Smith BD, Raines RT. Genetic selection for critical residues in ribonucleases. J. Mol. Biol 2006;362:459–478. [PubMed: 16920150]
25. Riordan JF. Angiogenin. Methods Enzymol 2001;341:263–273. [PubMed: 11582782]
26. Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: A critical overview. Clin. Chem 2003;49:32–40. [PubMed: 12507958]
27. Lutter G, Quaden R, Cremer J. Biological bypass in cardiovascular surgery. Thorac. Cardiovasc. Surg 2004;52:237–248. [PubMed: 15293163]
28. Tello-Montoliu A, Patel JV, Lip GYH. Angiogenin: A review of the pathophysiology and potential clinical applications. J. Thromb. Haemost 2006;4:1864–1874. [PubMed: 16961595]
29. Greenway MJ, Andersen PM, Russ C, Ennis S, Cashman S, Donaghy C, Patterson V, Swingler R, Kieran D, Prehn J, Morrison KE, Green A, Acharya KR, Brown RH Jr. Hardiman O. ANG mutations segregate with familial and ‘sporadic’ amyotrophic lateral sclerosis. Nat. Genet 2006;38:411–413. [PubMed: 16501576]
30. Crabtree B, Thiyagarajan N, Prior SH, Wilson P, Iyer S, Ferns T, Shapiro R, Brew K, Subramanian V, Acharya KR. Characterization of human angiogenin variants implicated in amyotrophic lateral sclerosis. Biochemistry 2007;46:11810–11818. [PubMed: 17900154]

31. Kishikawa H, Wu D, Hu G.-f. Targeting angiogenin in therapy of amyotropic lateral sclerosis. Expert Opin. Ther. Targets 2008;12:1229–1242. [PubMed: 18781822]

32. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. Nature 2007;450:1001–1009. [PubMed: 18075579]

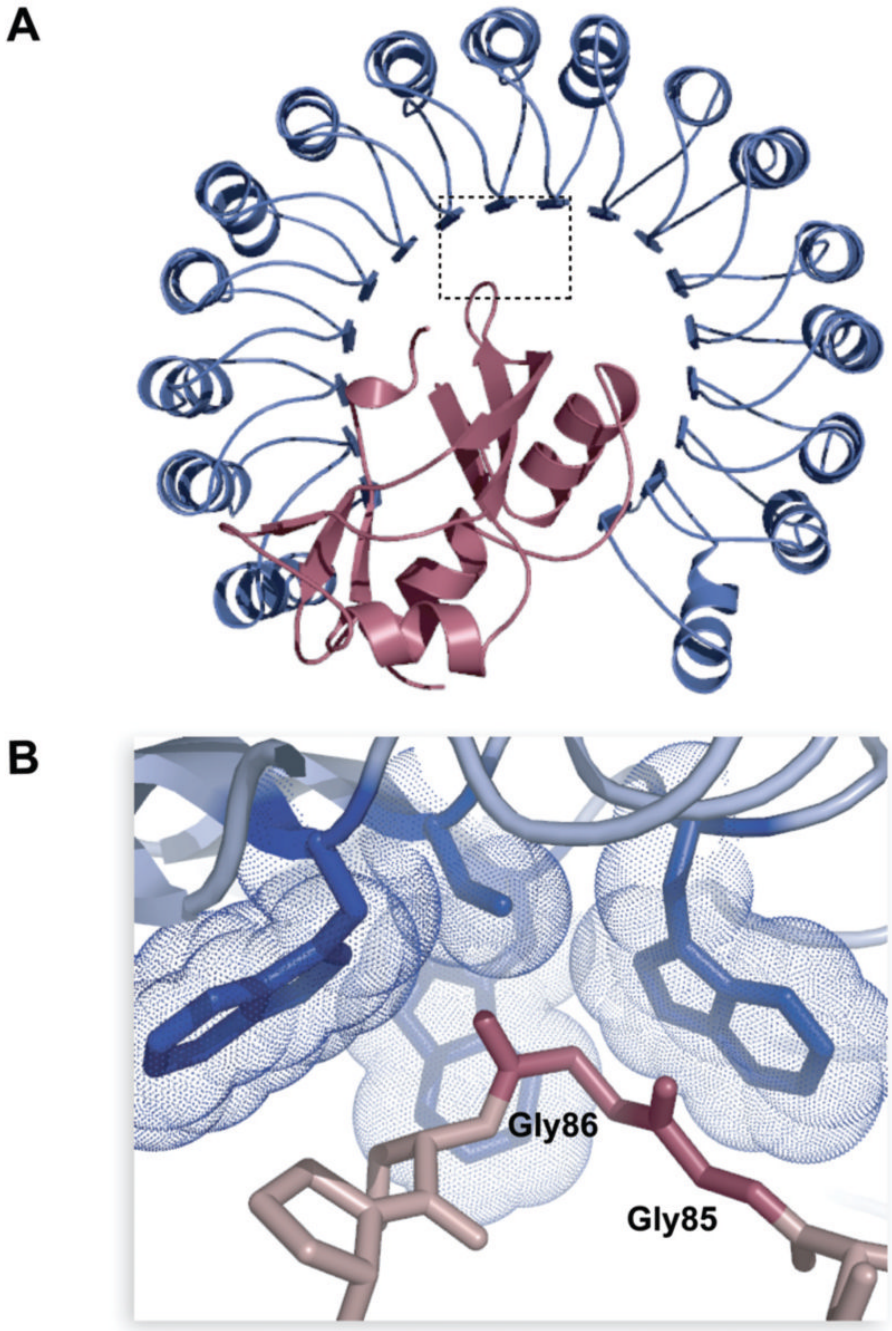


**Figure 1.**
(A) Structure of the human RI·ANG complex (PDB entry 1a4y (16)). RI, blue; ANG, red. (B) Contacts between RI and Gly85/Gly86 of ANG within the rectangle of panel A.

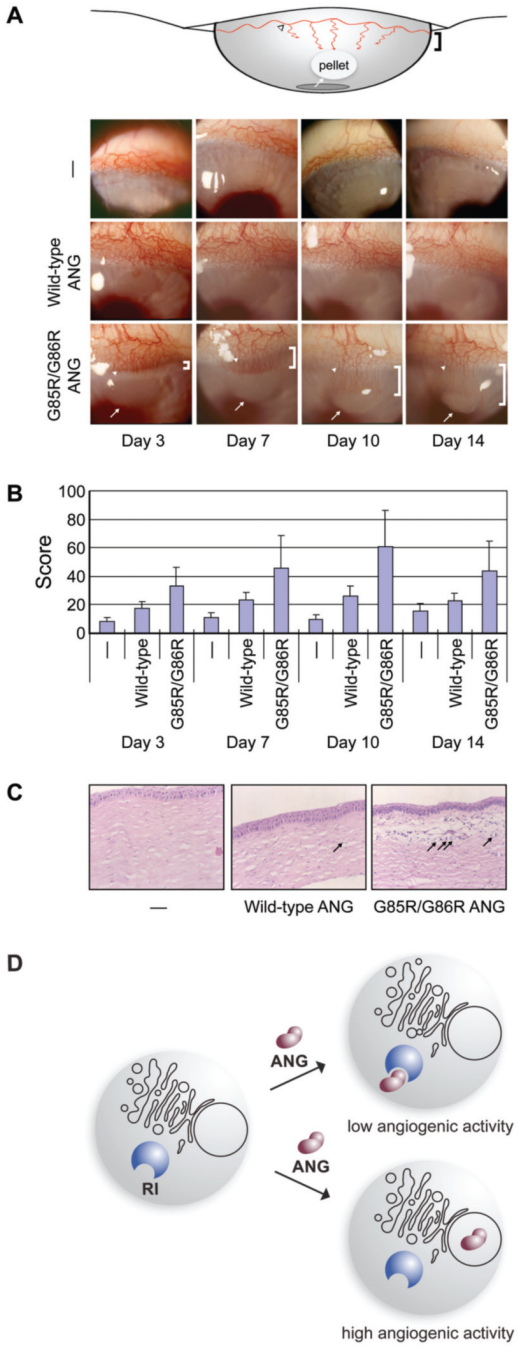


**Figure 2.**

Induction of angiogenesis by wild-type ANG and its G85R/G86R variant *in vivo*. (A) Slit-lamp photographs of representative rabbit corneas 3, 7, 10, and 14 days after implantation of a hydrogel pellet containing vehicle, wild-type ANG (10 μg), or its G85R/G86R variant (10 μg). Arrowheads, limbus; arrows, pellet; brackets, new blood vessels formed after treatment with the G85R/G86R variant. (B) Score for corneal neovascularization. Data are mean values (±SE) for 8 scores (4 eyes × 2 observers). (C) Histological photographs (×200) of rabbit corneas 14 days after pellet implantation. Arrows indicate new blood vessels. (D) Model for inhibition of the neovascularization activity of ANG by RI.

**Table 1**

Properties of ANG, RNase A, and Their Variants

| Ribonuclease | $k_{cat}/K_M$ ($M^{-1}s^{-1}$)[a] | $K_d$ (nM)[b] |
|---|---|---|
| Wild-type ANG | 78 ± 12 | $0.71 \times 10^{-6}$ [c] |
| G85R/G86R ANG | 73 ± 6 | 5 ± 1 |
| Wild-type RNase A | $(2.1 \pm 0.4) \times 10^7$ | $(59\pm7) \times 10^{-6}$ [d] |
| G88R RNase A | $(0.5 \pm 0.3) \times 10^7$ | 0.57 ± 0.05 |

[a] Values of $k_{cat}/K_M$ (±SE) are for catalysis of 6-FAM-dArUdAdA-6-TAMRA cleavage at pH 6.0 and 23 °C.

[b] Values of $K_d$ (±SE) are for the porcine RI complex at 23 °C.

[c] Data from ref 17.

[d] Data from ref 18.