

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 10⁶-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)¹ 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 exhibits 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 a K_d of 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 IC₅₀ values for cytotoxic ribonucleases are ≥10²-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 that Gly85 and Gly86 are 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 of the 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.

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Abbreviations: ANG, human angiogenin; bFGF, basic fibroblast growth factor; 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; SE, standard error; VEGF, vascular endothelial growth factor.

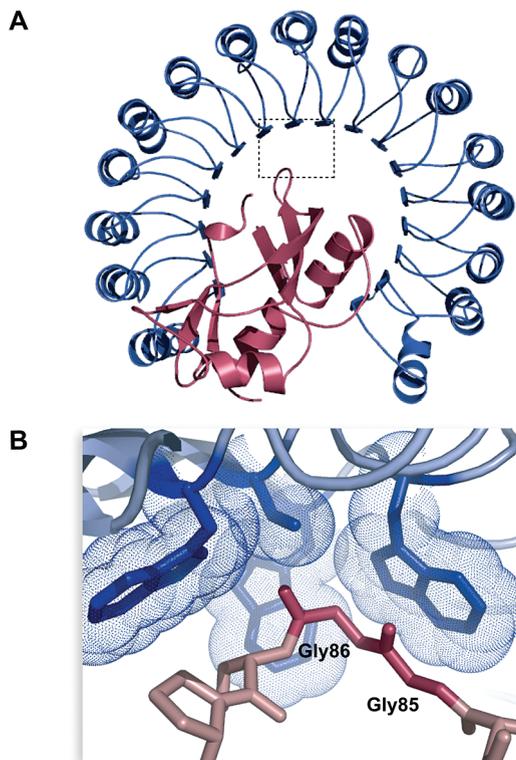


FIGURE 1: (A) Structure of the human RI-ANG complex [PDB entry 1a4y (16)]: RI in blue and ANG in red. (B) Contacts between RI and Gly85/Gly86 of ANG within the rectangle in panel A.

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	0.71×10^{-6c}
G85R/G86R ANG	73 ± 6	5 ± 1
wild-type RNase A	$(2.1 \pm 0.4) \times 10^7$	$(59 \pm 7) \times 10^{-6d}$
G88R RNase A	$(0.5 \pm 0.3) \times 10^7$	0.57 ± 0.05

^a Values of k_{cat}/K_M (\pm SE) are for catalysis of 6-FAM-dArUdAdA-6-TAMRA cleavage at pH 6.0 and 23 °C. ^b Values of K_d (\pm SE) are for the porcine RI complex at 23 °C. ^c Data from ref 17. ^d Data from ref 18.

The G85R and 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 than that of ANG (17) and 10-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 (Figure 2A,B). Histological

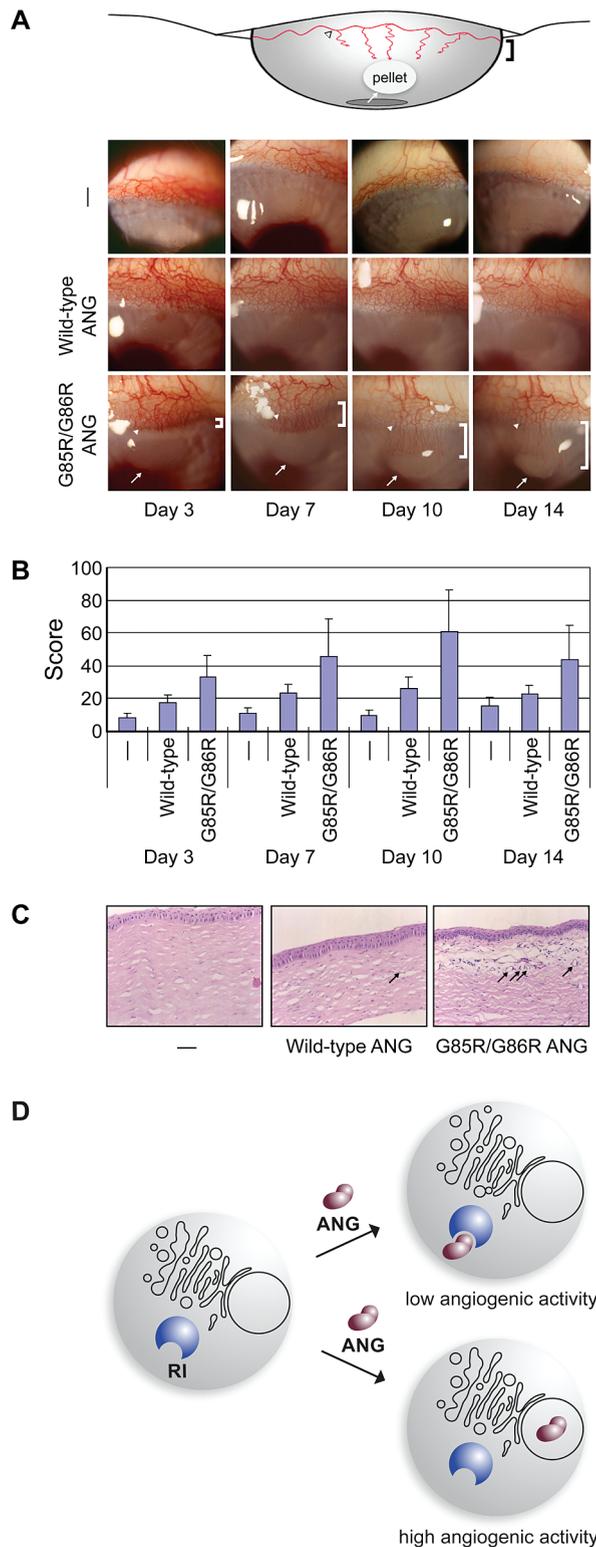


FIGURE 2: Induction of angiogenesis by wild-type ANG and its G85R/G86R variant in vivo. (A) Slit-lamp photographs of representative rabbit corneas 3, 7, 10, and 14 days after implantation of a hydrogel pellet containing vehicle, wild-type ANG (10 μ g), or its G85R/G86R variant (10 μ g); arrowheads, limbus; arrows, pellet; brackets, new blood vessels formed after treatment with the G85R/G86R variant. (B) Score for corneal neovascularization. Data are mean values (\pm SE) for eight scores (four eyes \times two observers). (C) Histological photographs (200 \times) of rabbit corneas 14 days after pellet implantation. Arrows denote new blood vessels. (D) Model for inhibition of the neovascularization activity of ANG by RI.

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 cytosolic concentration of RI [$4 \mu\text{M}$ (19)] greatly exceeds the value of K_d for the RI-G85R/G86R ANG complex [5 nM (Table 1)]. We suspect, therefore, that substitutions that lead to 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.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures and Figure S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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