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Replacing a Surface Loop Endows Ribonuclease A with Angiogenic Activity*

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Angiogenin (ANG) promotes the formation of blood vessels in animals. This hormone is a small, monomeric protein that is homologous to bovine pancreatic ribonuclease A (RNase). ANG is a poor ribonuclease but its ribonucleolytic activity is essential for its angiogenic activity. RNase is not angiogenic. A hybrid protein was produced in which 13 residues of a divergent surface loop of ANG were substituted for the analogous 15 residues of RNase. The value of k_{cat}/K_m for the cleavage of uridylyl(3′→5′)adenosine by this hybrid protein was 20-fold less than that of RNase but 10^5-fold greater than that of ANG. The thermal stability of the hybrid protein was also less than that of RNase. Nevertheless, the RNase/ANG hybrid protein promotes angiogenesis in mice at least as extensively as does authentic ANG. Thus we present a protein endowed with a noncognate biological activity simply by replacing a single element of secondary structure. In addition, a 13-residue peptide corresponding to the surface loop of ANG inhibits endogenous angiogenesis in mice. These results support a model in which both a surface loop and a catalytic site are necessary for the promotion of blood vessel formation by ANG or RNase. The dissection of structure/function elements in ANG reveals a unique opportunity to develop new molecules that modulate neovascularization.

Surface loops tend to be the most divergent regions in homologous proteins (Kimura, 1982) and appear to play only a minor role in dictating protein structure (Brunet *et al.*, 1993). Despite the apparent plasticity of surface loops, their deletion (Kuipers *et al.*, 1989; Pompliano *et al.*, 1990; Baldisseri *et al.*, 1991), insertion (Toma *et al.*, 1991; Wolfson *et al.*, 1991; Braxton and Wells, 1992; Eijsink *et al.*, 1993; Vuilleumier and Fersht, 1994), or substitution (Harper and Vallee, 1989; Hynes *et al.*, 1989; Allemann *et al.*, 1991; Hedstrom *et al.*, 1992) can have a dramatic effect on protein function. Unfortunately, this effect is usually deleterious, with any enhanced activities being simple cognates of those of the wild-type protein.

Angiogenesis refers to the formation of new blood vessels or neovascularization. In the last decade, a few proteins that promote angiogenesis have been identified (Folkman and Klagsbrun, 1987; Folkman and Shing, 1992; Maragoudakis *et al.*, 1992; Auerbach and Auerbach, 1994; Folkman, 1995). Perhaps the most intriguing of these proteins is angiogenin (ANG)[1] (Fett *et al.*, 1985). ANG is a potent inducer of neovascularization in animals. The human hormone is a monomeric protein of 123 amino acid residues that is homologous to bovine pancreatic ribonuclease A (RNase), a paradigmatical enzyme that catalyzes the degradation of RNA. Although the two proteins are 33% identical in amino acid sequence, ANG is a poor ribonuclease and RNase is not angiogenic (Fett *et al.*, 1985).

The mechanism of action of ANG is unknown, but the properties of ANG mutants provide some clues. Amino acid residues have been mutated in the region of ANG that corresponds to the catalytic active site of RNase. The resulting proteins are neither ribonucleolytic nor angiogenic (Shapiro *et al.*, 1989; Shapiro and Vallee, 1989). Thus, the ribonucleolytic activity of ANG, albeit poor, seems to be essential for its angiogenic activity. In contrast to active-site mutations, mutations in a particular surface loop, which is the most divergent part of the two proteins, eliminate the angiogenic but not the ribonucleolytic activity of ANG (Harper and Vallee, 1989).

The angiogenic activity of ANG appears to require two protein sites, one for cleaving RNA and another for binding to a cellular receptor (Hallahan *et al.*, 1991). These two functions, both of which are essential for angiogenesis, are likely to originate from the two parts of the protein highlighted in Fig. 1. We have tested the two-site model of ANG action by assessing the properties of the surface loop, both when substituted for the corresponding region in RNase and as an isolated peptide.

EXPERIMENTAL PROCEDURES

Materials

Human ANG was from R&D Systems (Minneapolis, MN). The material from R&D Systems contains added human serum albumin (HSA) to stabilize ANG. The same HSA used by R&D Systems was obtained from Miles (Kankakee, IL). Peptides (58–70)ANG and (59–73)RNase were synthesized by Operon (Alameda, CA). UpA was synthesized by J. E. Thompson using the methods of Ogilvie *et al.* (1978) and Beaucage and Caruthers (1981).

Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA), except for acetonitrile, which was from Baxter Healthcare

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[1] The abbreviations used are: ANG, human angiogenin; HSA, human serum albumin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PBS, Dulbecco's phosphate-buffered saline; RNase, bovine pancreatic ribonuclease A (EC 3.1.27.5); RNasin, cytosolic ribonuclease inhibitor; UpA, uridylyl(3′→5′)adenosine.

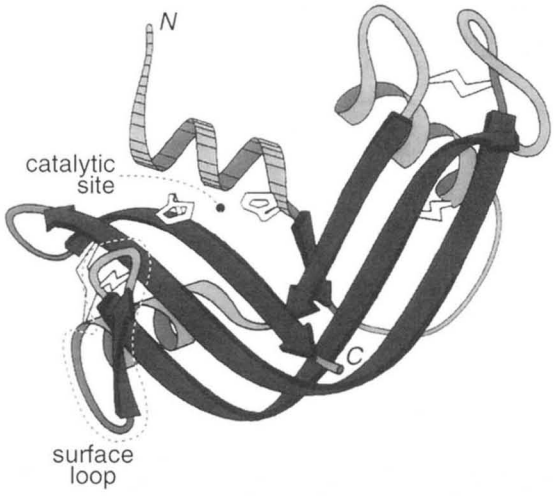

FIG. 1. **Three-dimensional structure of RNase (Wlodawer and Sjölin, 1983).** The three-dimensional structure of ANG is similar, except for one surface loop (Acharya *et al.*, 1994). This divergent surface loop and the conserved catalytic site are indicated.

(McGaw Park, IL). Expression vector pET22B(+) and *Escherichia coli* strain BL21(DE3) were from Novagen (Madison, WI). Enzymes for the manipulation of DNA were from Promega (Madison, WI). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Methods

DNA oligonucleotides were synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer by using the β-cyano phosphoramidite method (Sinha *et al.*, 1984). DNA sequences were determined with the Sequenase Version 2.0 kit from U. S. Biochemical Corp. (Cleveland, OH). Manipulations of DNA were performed as described by Ausubel *et al.* (1989).

Ultraviolet absorbance measurements were made on a Cary model 3 spectrophotometer (Varian, Palo Alto, CA) equipped with a Cary temperature controller. Concentrations of UpA were determined by ultraviolet absorption using $\epsilon_{260} = 24{,}600\ \text{M}^{-1}\ \text{cm}^{-1}$ at pH 7.0 (Warshaw and Tinoco, 1966). Concentrations of protein were determined by ultraviolet absorption using RNase: ${\epsilon_1}^{0.1\%}_{\text{cm}} = 0.72$ at 277.5 nm, M_r 13,700; ANG: $\epsilon^{0.1\%}_{1\ \text{cm}} = 0.85$ at 278 nm, M_r 14,400; RNase/ANG: $\epsilon^{0.1\%}_{1\ \text{cm}} = 0.70$ at 278 nm; M_r 13,500. The extinction coefficients for ANG and the RNase/ANG hybrid were calculated from that of RNase (Sela *et al.*, 1957) with the method of Gill and von Hippel (1989).

Production of RNase/ANG Hybrid Protein—Plasmid pBXR directs the production of RNase in *E. coli* (Raines and Rutter, 1989; delCardayré *et al.*, 1995). This plasmid was constructed by inserting the cDNA that codes for RNase between the *Msc*I and *Sal*I sites in expression vector pET22B(+). Oligonucleotide-mediated site-directed mutagenesis (Kunkel *et al.*, 1987) of plasmid pBXR was used to replace the cDNA that codes for residues 59–73 of RNase with DNA that codes for residues 58–70 of ANG. The oligonucleotide used was AGTAGCTCTGTCTCAAGTTTTCTCTGTGTGGGTTACCGTTCTTGTTTTCGCACACGGC, where the reverse complement of the underlined bases codes for residues 58–70 of ANG.

The RNase/ANG hybrid protein was produced and purified as had been other mutants of RNase (Thompson and Raines, 1994; delCardayré and Raines, 1994; 1995; Messmore *et al.*, 1995; delCardayré *et al.*, 1995). Briefly, *E. coli* strain BL21(DE3) carrying the mutated plasmid and induced with isopropyl-1-thio-β-D-galactopyranoside produced the RNase/ANG hybrid fused to the pelB signal sequence. The RNase/ANG hybrid protein was solubilized from inclusion bodies, folded/oxidized *in vitro*, and purified by fast protein liquid chromatography on a Mono S column (Pharmacia Biotech Inc.). SDS-polyacrylamide gel electrophoresis and zymogram electrophoresis of the pure protein indicated that the pelB signal sequence was removed by endogenous *E. coli* proteases, as had been observed with other mutants of RNase.

Endotoxin Titer—Proteins produced in *E. coli* can be contaminated with endogenous endotoxins that possess biological activities. Accordingly, gel clot assays for endotoxin titer in the preparations of RNase/ANG hybrid and RNase A were performed at Associates of Cape Cod (Woods Hole, MA). Results were compared to reference standard endotoxin EC-5 (FDA, CBER) using lot no. 42-134-576 from Pyrotell®.

Thermal Stability—As RNase is denatured, its 6 tyrosine residues become exposed to solvent and its extinction coefficient at 287 nm decreases significantly (Hermans and Scheraga, 1961). The thermal stabilities of RNase and the RNase/ANG hybrid (which has 5 tyrosine residues) were assessed by monitoring the change in A_{278} with temperature, as described by Pace *et al.* (1989). Briefly, the temperature of a solution of protein (0.1–0.5 mg/mL) in Dulbecco's phosphate-buffered saline (PBS, which contained (in 1 liter) KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g), and $Na_2HPO_4{\cdot}7H_2O$ (2.16 g)) was increased (unfolding) or decreased (folding) in 0.2 °C increments between 15 and 80 °C, and A_{278} was recorded after 1 min of equilibration at each new temperature. The data were fit to a two-state model for denaturation, and used to calculate T_m, the midpoint in the thermal unfolding curve.

Ribonucleolytic Activity—The cleavage of UpA was monitored by an adenosine deaminase coupled assay (Ipata and Felicioli, 1968) using $\Delta\epsilon_{265} = -6000\ \text{M}^{-1}\ \text{cm}^{-1}$. Assays were performed at 25 °C in 0.1 M Mes-HCl buffer, pH 6.0, containing NaCl (0.1 M), substrate (0.10–0.80 mM), and enzyme (5.5 nM). The values of k_{cat}, K_m, and k_{cat}/K_m were determined from initial velocity data with the program HYPERO (Cleland, 1979).

Angiogenic Activity—Angiogenesis was assayed using the disc angiogenesis assay (Fajardo *et al.*, 1988; Polakowski *et al.*, 1993).[2] In the disc assay, a test substance (1.0 nmol test protein; 10 nmol test peptide) in PBS was absorbed into the central core excised from a polyvinyl alcohol sponge disc. The core was then coated with ethylene vinyl copolymer (ELVAX) to produce a slow-release formulation, and the core was then reinserted into its polyvinyl sponge disc. The entire disc was then covered on its two flat surfaces with Whatman No. 1 filter paper made impermeable by filling the pores with a plexiglass/ethylene dichloride mixture. Assembled discs were implanted subcutaneously into the flanks of adult mice (strain BALB/c). Ten discs were implanted for each test substance. Each mouse carried an experimental disc in one flank and a control disc (usually RNase) in the other flank. After 10 days *in vivo*, the discs were recovered, fixed, and embedded in paraffin. Medial plane sections (7–10 μm thick) of the discs were prepared and stained with hematoxylin/eosin to permit visualization of cells that had penetrated laterally into the disc. The fraction of a particular section into which cells had penetrated was determined by an image analysis system using the program OPTIMAS (Jandel Scientific, San Rafael, CA). All analyses were performed blindly; operators of OPTIMAS did not know which test substance was on a disc.

RESULTS

Production of RNase/ANG Hybrid—The elaboration of structure/function relationships in proteins can be facilitated by using the techniques of recombinant DNA to produce mutant proteins (Knowles, 1987). Application of these techniques to RNase has been limited because RNase is notoriously difficult to produce in heterologous systems, presumably because it is cytotoxic. We have overcome this barrier by developing an efficient system to produce RNase in *E. coli* (delCardayré *et al.*, 1995). This system allows us to isolate in one chromatographic step enough pure protein (50 mg/liter of culture) for virtually any type of biological or biophysical experiment.

The experiments described herein were based on a hybrid protein in which 13 residues of the surface loop of ANG (residues 58–70; Fig. 2) were substituted for the analogous 15-residue sequence of RNase (residues 59–73). Oligonucleotide-mediated site-directed mutagenesis was used to replace the cDNA that codes for the loop of RNase with DNA that codes for the loop of ANG. The resulting RNase/ANG hybrid protein was produced with our existing system for the production of RNase. The RNase/ANG hybrid protein was >95% pure according to SDS-polyacrylamide gel electrophoresis as well as zymogram electrophoresis, which is an extremely sensitive method for detecting ribonucleolytic activity (data not shown). Gel clot assays showed that endotoxin contributed <1 ppm to either the

[2] We also attempted to use the chick embryo chorioallantoic membrane assay for angiogenesis (Knighton *et al.*, 1977), but found the results to be less reproducible than those from the disc angiogenesis assay.

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                10          20          30          40
ANG      --qdnsR YTHFL TQHYD A-kpq-G rddRY CESIM RRRGL ts-pCK
RNase    ke---tA AAKFE RQHMD SstsaaS s-sNY CNQMM KSRNL tkdrCK
                9          21          30          41

                50          60          70          80
ANG      DINTF IHgNK RSIKA ICenk ngnph r----en-lr iskss FQVTT
RNase    PVNTF VHeSL ADVQA VCs-- -qknv ackngqtncy qsyst MSITD
                51          59          73          83

                90          100         110         120
ANG      CKLHg gspW-p pcqYR ATAGF RNVVV ACEn--g lPVHL Dqsif rrp
RNase    CRETg sskYpn -caYK TTQAN KHIIV ACEgnpy vPVHF Da-sv
                94          103         115         124
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FIG. 2. **Sequence alignment of ANG and RNase based on structural superposition (Acharya *et al.*, 1994).** Residues with C_α positions within 1.2 Å are shown in *uppercase letters*. The residues of a divergent loop are shown in a *box*. Cys-65 and Cys-72, which form a disulfide bond in native RNase (Fig. 1), are absent from ANG.

purified RNase/ANG hybrid or the purified RNase A. Three properties of the purified RNase/ANG hybrid protein were examined: thermal stability, ribonucleolytic activity, and angiogenic activity.

Thermal Stability—The thermal stability of the hybrid protein was assessed because the protein was to be exposed to high temperature (38.6 °C) for up to 14 days during *in vivo* assays for angiogenesis. RNase was found to have T_m = 66 ± 1 °C in PBS. In contrast, the hybrid protein was found to have T_m = 38 ± 2 °C in PBS. The denaturation of both proteins was reversible with only minimal hysteresis (data not shown).

Ribonucleolytic Activity—The ability of the RNase/ANG hybrid to catalyze the cleavage of RNA was evaluated because the ribonucleolytic activity of ANG is essential for its angiogenic activity (Shapiro and Vallee, 1989). As shown in Table I, the RNase/ANG hybrid is a potent ribonuclease, having suffered only an 8-fold decrease in the value of k_{cat} and a 3-fold increase in the value of K_m upon mutation. The value of k_{cat}/K_m for the hybrid was 10^5-fold greater than that of authentic ANG.

Angiogenic Activity—The ability of the RNase/ANG hybrid to promote neovascularization was evaluated by using a disc angiogenesis assay. In this assay, porous discs were imbued with a slow-release formulation of a test substance in PBS. The discs were implanted subcutaneously into mice, recovered after 10 days, and examined for the penetration of endothelial cells. These results are shown in Fig. 3. The variation in the penetration of cells into similar discs implanted into different mice was small, indicating that mice varied little in their capacity to support angiogenesis.

The results of the disc angiogenesis assay of proteins are shown in Fig. 3*A*. Discs containing RNase had 36.4 ± 1.1% cellular penetration. This value did not differ significantly from that of discs containing only HSA or PBS (data not shown). In contrast, discs imbued with the RNase/ANG hybrid protein had 45.3 ± 1.8% penetration. These data indicate that the RNase/ANG hybrid protein stimulates angiogenesis in mice, promoting 24% more penetration than did RNase and 4% more than did authentic ANG. To verify the angiogenic activity of the RNase/ANG to promote angiogenesis, we also used the mouse cornea as described (Muthukkaruppan and Auerbach, 1979; Polakowski *et al.*, 1993). The RNase/ANG hybrid elicited neovascularization in all six mice tested.[3]

The results of the disc angiogenesis assay of peptides are shown in Fig. 3*B*. Discs containing the (59–73)RNase peptide and (58–70)ANG peptide had 38.1 ± 1.9% and 30.1 ± 2.8% penetration, respectively. These data indicate that (58–70)ANG inhibits angiogenesis, promoting 21% less penetration than did (59–73)RNase. Finally, the results in Fig. 3*B* show

[3] R. T. Raines, M. P. Toscano, V. R. Muthukkaruppan, and R. Auerbach, unpublished results.

TABLE I

Steady-state kinetic parameters for catalysis of UpA cleavage by RNase, RNase/ANG hybrid, and ANG

All 3 proteins were produced in *E. coli.*

Protein	k_{cat}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{rel}$
	s^{-1}	*mM*	$10^6\ M^{-1}\ s^{-1}$	%
RNase	1630 ± 90	0.55 ± 0.06	3.0 ± 0.4	100
RNase/ANG hybrid	200 ± 40	1.5 ± 0.3	0.13 ± 0.04	4.3
ANG[a]	ND[b]	ND	0.0000011	0.000037

[a] Data from Harper and Vallee (1989).
[b] ND, not determined.

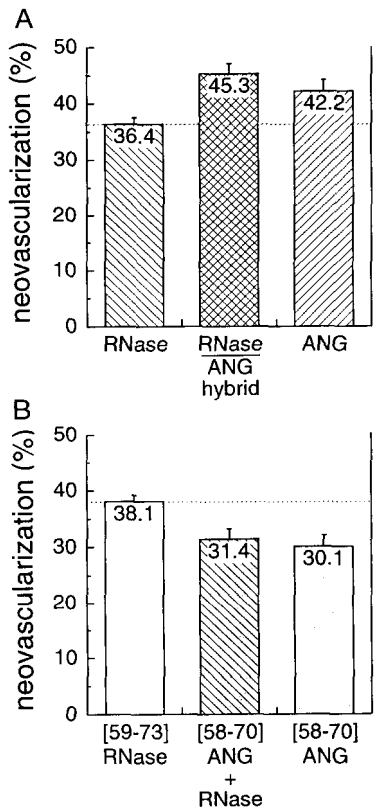

FIG. 3. **Comparison of cellular infiltration into discs imbued with a test protein or peptide.** Values were obtained by averaging the penetration for each disc containing a test substance, which was either a protein (*A*, 1.0 nmol) or a peptide (*B*, 10 nmol). The value in *B* for (58–70)ANG + RNase was from discs containing both substances.

that (58–70)ANG peptide and RNase do not work in trans (that is, in an intermolecular manner) to stimulate angiogenesis.

DISCUSSION

Angiogenesis has been likened to blood coagulation, in that it must remain poised but quiescent (Folkman and Klagsbrun, 1987). Rampant angiogenesis is associated with a wide variety of pathological conditions. For example, tumor growth and metastasis rely on angiogenesis, and approximately 50% of a solid tumor consists of blood vessels and interstitial space (Weinstat-Saslow and Steeg, 1994). In addition to supplying nutrients to the tumor, these vessels provide a pathway by which tumor cells enter the circulatory system and metastasize to remote sites. Angiogenesis also plays a key role in normal processes that involve tissue growth and development. For example, the generation of blood vessels is a primary event in the establishment of the placenta, and is an important feature of the mammary gland changes associated with lactation (Reynolds *et al.*, 1992). The healing of wounds and fractures and the success of limb and organ transplants also require neovascularization.

The goal of our work is to understand the molecular basis of the angiogenesis induced by the protein hormone, ANG. We are guided by the two-site model of ANG action (Hallahan *et al.*, 1991). In this model, angiogenesis is induced because one site in ANG interacts with a putative cell-surface receptor, which directs cells to internalize ANG. The other site catalyzes the cleavage of RNA, perhaps in the nucleolus (Moroianu and Riordan, 1994). The catalytic site of ANG is conserved in its homolog, RNase (Figs. 1 and 2). In contrast, the surface loop of ANG that may bind to a putative cell-surface receptor has diverged from that in RNase and all other members of the RNase superfamily (Beintema, 1987). The sequence of this loop is conserved, however, in mammalian angiogenins (Bond *et al.*, 1993).

We have tested the two-site model of ANG action by replacing the surface loop in RNase (residues 59–73) with the corresponding region of ANG (residues 58–70). The resulting hybrid protein is designed to be a molecule that is both an efficient catalyst of RNA degradation and a strong promoter of neovascularization. Realizing that mutant proteins like the RNase/ANG hybrid can suffer unintended and subtle changes in structure or stability (Knowles, 1987), we used enzymatic activity, which requires the precise alignment of many nonsequential residues, as a probe of protein structure. The replacement of residues 59–73 of RNase with residues 58–70 of ANG decreases the specific activity of RNase by only 8-fold (Table I). This modest effect indicates that the three-dimensional arrangement of the amino acid residues in the active site of RNase/ANG is probably similar to that in the active site of RNase at 25 °C.

The deletion, insertion, or substitution of surface loops is not likely to have a dramatic effect on the tertiary structure of a protein (Brunet *et al.*, 1993). Still, such changes can affect protein stability. The thermal stability of the RNase/ANG protein was tested, and found to be significantly lower than that of RNase. Since mice have a body temperature close to the T_m of the hybrid protein, much of the hybrid protein imbued in a disc may be denatured during *in vivo* assays of angiogenesis. Only the native form of our hybrid protein possesses ribonucleolytic and (presumably) angiogenic activity. Thus, the low thermal stability of the RNase/ANG hybrid protein suggests that our *in vivo* assays underestimate the angiogenic potency that can result from replacement of residues 59–73 of RNase with residues 58–70 of ANG.

The substitution of a surface loop in RNase with the analogous loop of ANG endows RNase with a distinct, new activity, the ability to promote angiogenesis (Fig. 3*A*). Our hybrid protein is the exact complement of that of Harper and Vallee (1989), who replaced residues 58–70 of ANG with residues 59–73 of RNase. These workers found that their hybrid protein was unable to induce angiogenesis on the chorioallantoic membrane of chick embryos. Our strategy is similar to that of Benner and co-workers, who found that replacing residues 63–74 of RNase with residues 62–71 of ANG resulted in a 60-fold decrease in catalytic activity (Allemann *et al.*, 1991). Benner and co-workers did not assess the angiogenic activity of their mutant protein. Our results, along with those of Harper and Vallee (1989) and Allemann *et al.* (1991), strongly support the two-site model of ANG action. In addition, our work provides a rare example of a protein endowed with a noncognate biological activity simply by replacing a single element of secondary structure.

The peptide (58–70)ANG is an inhibitor of endogenous angiogenesis (Fig. 3*B*). This result is remarkable considering the inherent conformational entropy of peptides and their susceptibility to degradation by *in vivo* proteases. This result portends the existence of an ANG receptor that can bind to residues 58–70 of ANG but not to residues 59–73 of RNase. A peptide corresponding to another region of ANG, (108–123)ANG, has been reported to decrease significantly the neovascularization elicited by ANG in the chicken chorioallantoic membrane assay (Rybak *et al.*, 1989). The similarity of the sequences of (58–70)ANG and (108–123)ANG (Fig. 4) suggests that these two peptides may act by binding to the same cellular receptor. Nevertheless, residues 108–123 of ANG are similar to residues 108–123 of RNase (Fig. 2). This region in intact ANG is therefore unlikely to be involved in receptor binding. Other known peptide/protein inhibitors of *in vivo* angiogenesis appear to be unrelated to (58–70)ANG (Ingber and Folkman, 1988; Maione *et al.*, 1990; Moses *et al.*, 1990; Polakowski *et al.*, 1993).

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[58-70]ANG   ENKNGNP-HREN--LR
              ||  | | | ::  :|
[108-123]ANG EN--GLPVHLDQSIFRRP
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FIG. 4. **Comparison of residues 58–70 and 108–123 of human ANG.** *Vertical line*, identical residues; colon, similar residues.

Vallee and co-workers have proposed that actin is the *in vivo* receptor for ANG (Hu *et al.*, 1993). Actins are abundant intracellular proteins with an excess of acidic residues (Elzinga *et al.*, 1973). ANG (pI > 9.5) (Fett *et al.*, 1985), like RNase (pI = 9.3) (Ui, 1971), has an excess of basic residues (Fig. 2). The affinity of ANG and RNase for actin is therefore not surprising. Indeed, RNase can compete with ANG for binding to actin (Hu *et al.*, 1991). Although the affinity of ANG for actin is quite high ($K_d = 5 \times 10^{-10}$ M) (Hu *et al.*, 1991), the affinity of ANG for cytosolic ribonuclease inhibitor is much higher ($K_d = 7 \times 10^{-16}$ M) (Lee *et al.*, 1989). What then is the significance of the ANG-actin interaction? Recently, we provided evidence that bovine seminal ribonuclease, a homolog of ANG with potent cytotoxic activity, has evolved an unusual quaternary structure for the sole purpose of eluding ribonuclease inhibitor (Kim *et al.*, 1995). Similarly, a high concentration of actin, as is found in an endothelial cell, could be effective in shielding ANG (or our RNase/ANG hybrid) from a low concentration of inhibitor.

The existence in ANG of a distinct site for each of two functions provides a unique opportunity to modulate angiogenesis. For example, the results in Fig. 3*B* suggest that angiogenesis can be inhibited by interfering with the interaction of ANG and its receptor. Since tumors require blood vessels to grow, inhibition of blood vessel development provides a strategy for both preventing the growth of primary tumors and inhibiting tumor metastasis (Folkman, 1971, 1972; Gimbrone *et al.*, 1972). Inhibitors of neovascularization would also be useful in the treatment of rheumatoid arthritis, psoriasis, scleroderma, hemangiomas, and diabetic retinopathy (Auerbach and Auerbach, 1994).

The results in Fig. 3*A* suggest that a molecule in which the ANG surface loop was attached to a ribonuclease (or perhaps, to another cytotoxin) may be able to promote angiogenesis.[4] (Auerbach and Auerbach, 1994). Thus, understanding the molecular basis for neovascularization by ANG could lead to a new class of pharmaceutics that promote blood vessel development. Such drugs would aid in the treatment of cartilaginous trauma, decubitis ulcers, wounds, fractures, and transplants.

An additional application of biochemical research on angiogenesis has recently become apparent. When a coronary artery

[4] Angiogenesis factors could be acting by derepressing rather than promoting neovascularization. Fewer than 0.01% of vascular endothelial cells are in the cell cycle. The relative quiescence of these cells may be due to a homeostatic mechanism in which endogenous inhibitors (or RNA secondary structure) may suppress cell proliferation. ANG and the RNase/ANG hybrid protein may act by eliminating this inhibition.

becomes partially occluded by an atherosclerotic plaque, the heart muscle normally supplied by that artery becomes starved for oxygen and other nutrients. In response, the body grows small collateral arteries to supply viable tissue. In essence, this response is nature's own version of coronary artery bypass surgery. Unfortunately, the new collateral arteries are often unable to compensate for the partial occlusion, and the risk of a heart attack from the complete occlusion of a main coronary artery remains high. Unger *et al.* (1994) have reported that dog cardiac muscle treated with basic fibroblast growth factor, a protein hormone that induces angiogenesis, experienced a marked increase in both collateral artery formation and transmural blood flow. Although these results portend an exciting treatment for heart-muscle ischemia, protein hormones are notorious for their side effects. For example, basic fibroblast growth factor also stimulates the proliferation of cartilage cells, fibroblasts, and smooth muscle cells. The identification of the precise regions in protein hormones that are responsible for neovascularization may allow biological chemists to create molecules that promote this process, but not others.

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