Inhibition of Human Angiogenin by DNA Aptamers: Nuclear Colocalization of an Angiogenin−Inhibitor Complex†

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ABSTRACT: Specific ligands (aptamers) for angiogenin were selected from a 72-mer oligodeoxynucleotide library consisting of 28 randomized positions flanked by two constant regions of 22 residues each. From a starting pool of $\sim 10^{14}$ molecules, 19 angiogenin-binding ligands were obtained. Among them, two oligonucleotides showed significant inhibition of the ribonucleolytic activity of angiogenin with apparent K_is of 0.65 and 0.60 μM, respectively. One of them was shortened on the basis of its secondary structure to provide a 45-mer oligonucleotide that retained much of the inhibitory properties of the parent molecule. It inhibits both the angiogenic and cell proliferative activities of angiogenin but does not interfere with its nuclear translocation in human endothelial cells. Importantly, the inhibitor is cotranslocated to the nucleus with angiogenin in a ~1:1 stoichiometric ratio. These results demonstrate that the inhibition of angiogenin-induced cell proliferation and angiogenesis by the oligonucleotide is due to suppression of the ribonucleolytic activity of angiogenin, an event that occurs most likely within the cell nucleus.

Angiogenin is, in many ways, a remarkable protein since it belongs both to the ribonuclease superfamily (*1*) and to a class of direct inducers of angiogenesis (*2*). It was discovered as the result of studies designed to identify angiogenic molecules released into the growth medium by tumor cells in culture (*3*). The results of anti-angiogenin therapy experiments (*4, 5*) have clearly established that it is an *in vivo* angiogenic protein related to the process of angiogenesis and tumorigenesis. In addition, angiogenin mRNA and protein concentrations are elevated in tissues and cells of patients with a variety of tumors (*6*−*8*). Structure/function studies combining mutagenesis, X-ray crystallography, and *in vivo* angiogenesis assays on the chicken chorioallantoic membrane (CAM)[1] have demonstrated that the 123-residue protein contains a ribonucleolytic active site (*9*−*11*), a cell binding site (*12, 13*), and a nuclear localization sequence (NLS) (*14, 15*). Thus, angiogenin is a ribonuclease whose weak but characteristic ribonucleolytic activity (*16*) is essential for angiogenesis (*17*). It is pleiotropic toward its target cells: it binds to endothelial cells (*18*), interacts with a 170 kDa receptor (*19*) or a 42 kDa binding protein (*20*) on the cell surface, induces cell proliferation (19), activates cell-associated proteases (*21*), and stimulates cell migration and invasion (*22*). It also mediates cell adhesion (*23*) and promotes cell differentiation (*24*). All of these individual cellular events are necessary components of the process of angiogenesis. Further, angiogenin undergoes specific nuclear translocation in endothelial cells via receptor-mediated endocytosis (*14*) and NLS-assisted nuclear import (*15*). Increasing evidence points to RNA as the physiological substrate for angiogenin. The fact that exogenous angiogenin undergoes nuclear translocation, which is necessary for angiogenesis, strongly suggests that it exerts its ribonucleolytic activity once it accumulates in the nucleolus where 45S ribosomal RNA processing and ribosome biogenesis take place.

The multiple functions that it has toward its target cells as well as its three distinctive functional sites offer an ample array of regulatory mechanisms for controlling the activity of angiogenin. Indeed, both cytosolic ribonuclease inhibitor, which abolishes its ribonucleolytic activity, and its cell surface binding protein, actin, are potent antagonists of its angiogenic activity (*20, 25*). Angiogenin derivatives in which either the catalytic or the cell binding site has been mutated or otherwise altered lose angiogenic activity even though the other sites remain unchanged. Moreover, NLS variants of angiogenin fail to induce angiogenesis despite their intact cell binding ability and full ribonucleolytic activity. This evidence strongly suggests that molecules that bind to any one of the functional sites or interfere with any of its known cellular or enzymatic activities will prevent angiogenin-induced angiogenesis and have therapeutic value to angiogenesis-based diseases such as arthritis, diabetic retinopathy, psoriasis, tumor growth, and metastasis (*26*).

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[1] Abbreviations: BSA, bovine serum albumin; CAM, chorioallantoic membrane; FBS, fetal bovine serum; HE-SFM, human endothelial serum-free medium; HUVE, human umbilical vein endothelial; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; poly(C), polycytidylic acid; RNase A, bovine pancreatic ribonuclease A; SELEX, systematic evolution of ligands by exponential enrichment.

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Indeed, both anti-angiogenin monoclonal antibodies and the soluble angiogenin binding protein have been shown to suppress the establishment of human colon, lung, and fibroid tumor cells implanted subcutaneously into athymic mice (*4*, *5*).

As one of the multipronged approaches to understand the mechanism of action of angiogenin and to search for angiogenin inhibitors with therapeutic potential, we have employed the systematic evolution of ligands by exponential enrichment (SELEX) procedure (*27*) to isolate oligodeoxynucleotide aptamers for angiogenin. We report the isolation of an oligonucleotide that inhibits the ribonucleolytic, mitogenic, and angiogenic activity of angiogenin but does not affect the nuclear translocation of angiogenin in endothelial cells. Remarkably, the oligonucleotide ligand undergoes nuclear cotranslocation with angiogenin and accumulates in the nucleus in a stoichiometric ratio, suggesting that angiogenin exerts its ribonucleolytic activity in the cell nucleus and that inhibition of this activity within the nucleus is an effective means to abolish its angiogenic activity.

MATERIALS AND METHODS

Materials. Human angiogenin and its Q117G variant in which glutamine-117 has been replaced with glycine were prepared as recombinant proteins from an *Escherichia coli* expression system (*28*, *29*). *Pst*I, *Bam*HI, T4 polynucleotide kinase, Taq polymerase, pGEM-3Z, dNTP, avidin resin (avidin covalently coupled to methacrylate polymeric gel-filtration matrix), human fibronectin, and recombinant human basic fibroblast growth factor were from Promega; bovine pancreatic ribonuclease A (RNase A) and nuclease-free bovine serum albumin (BSA) were from Worthington; polycytidylic acid [poly(C)] was from Sigma; Iodo-beads iodination reagents and excellulose GF-5 desalting columns were from Pierce; Sephadex G-50 and CNBr-activated Sepharose 4B were from Pharmacia; complete protease inhibitor cocktail tablets were from Boehringer Mannheim; [γ-^{32}P]ATP (3000 Ci/mmol), and Na^{125}I (17.4 Ci/mg) were from Du Pont/New England Nuclear. ^{125}I-Labeled angiogenin and ^{32}P-labeled oligodeoxynucleotide were prepared with the use of Iodo-beads (*30*) and T4 kinase (*31*), respectively. Angiogenin−Sepharose resin was prepared by the procedure described for the preparation of RNase A−Sepharose (*32*). The oligodeoxynucleotide library was synthesized and gel-purified by Promega. Other individual oligonucleotides were synthesized either by Promega or by Amitof and were always annealed by heating at 65 °C for 3 min and cooling at room temperature for 15 min before being used for experiments.

SELEX. A 72-mer oligonucleotide library (5′-AACTG-CAGCCACTTAGTCTAAG [28 nt] CTGGATCCAGCGT-TCATTCTCA-3′) of 3×10^{15} unique DNA molecules, consisting of 28 randomized positions and two constant 5′ and 3′ regions of 22 nucleotides each, was synthesized chemically. The two 22-nucleotide constant regions were designed to contain annealing sites for PCR primers (22-mer) and restriction enzyme sites that allow cloning into the pGEM-3Z vector. An initial pool of mixed oligonucleotides was generated by large-scale polymerase chain reaction (PCR) amplification (2.5 mL combined total volume, 18 cycles of 1 min at 94 °C, 2 min at 66 °C, and 1 min at 72 °C) with 4 μg of the library ($\sim 10^{14}$ molecules) as the templates. The antisense PCR primer was biotinylated at the 5′ end so that one strand of the amplified DNA would be labeled. The biotinylated double-stranded DNA was applied to an avidin column equilibrated with 0.1 M Tris-HCl, pH 7.5, containing 0.1 M NaCl. The unbiotinylated strands were eluted with 0.2 M NaOH, neutralized with acetic acid, and precipitated with ethanol. The single-stranded DNA pool was dissolved in 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl, heated at 70 °C for 5 min, cooled at room temperature for 30 min, and passed through a Sepharose column to remove matrix-specific molecules. The flowthrough fractions of the Sepharose column were mixed with 0.5 mL of angiogenin−Sepharose and rotated at room temperature overnight. The resin was then packed into a column and washed with 5 mL of 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. The bound DNAs were eluted with 4 mL of 4 M guanidine thiocyanate in 50 mM citrate buffer, pH 7.4, containing 10 μg/mL glycogen and 1% β-mercaptoethanol over a period of 3 h, precipitated by ethanol, redissolved in 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, and subjected to further cycles of amplification by PCR and selection on angiogenin−Sepharose. An additional washing step with 2.5 mL of 0.2 M phosphate, pH 7.4, containing 0.4 M NaCl was introduced on the fourth round of selection to remove weakly bound species. The DNA molecules that eluted from the angiogenin affinity column at the seventh cycle were amplified with the use of unbiotinylated PCR primers and the resultant double-stranded DNA was precipitated. The DNA fragments digested with *Pst*I and *Bam*HI were purified by 4% agarose gel electrophoresis and cloned into the pGEM-3Z plasmid vector. A total of 19 clones were selected at random and their nucleotide sequences were determined.

Ribonucleolytic Assay. Ribonucleolytic activity of angiogenin was determined with poly(C) as the substrate. Angiogenin, 0.3 μM, or Q117G, 0.01−0.3 μM, was mixed with the oligonucleotides to be tested at concentrations ranging from 0.25 to 1.6 μM in 300 μL of 50 mM Hepes-HCl, pH 7.4, containing 0.1 M NaCl and 30 μg/mL BSA. Poly(C) was added to a final concentration of 0.15 mg/mL and the mixture was incubated at 37 °C for 0.5−50 h. Reactions were stopped by adding 0.7 mL of 3.4% ice-cold perchloric acid. The mixtures were vortexed, kept on ice for 10 min, and centrifuged at 15600*g* for 10 min at 4 °C. The absorbance of the supernatant was measured at 280 nm.

Angiogenesis Assay. The angiogenic activity of angiogenin or its mixture with oligonucleotide ligands was measured on the CAM by the method of Knighton et al. (*33*) as described (*3*). The oligonucleotide inhibitors were mixed with angiogenin 15 min prior to application onto the assay disks.

Cell Proliferation. The effect of the oligonucleotide on the mitogenic activity was determined with human umbilical vein endothelial (HUVE) cells as described (*19*). HUVE cells were purchased from Cell Systems Corp. as primary cultures and were maintained on fibronectin-coated dishes in human endothelial serum-free medium (HE-SFM) supplemented with 20 ng/mL basic fibroblast growth factor. Angiogenin was premixed with the oligonucleotide in phosphate-buffered saline (PBS) and incubated at room temperature for 15 min before addition to the cells.

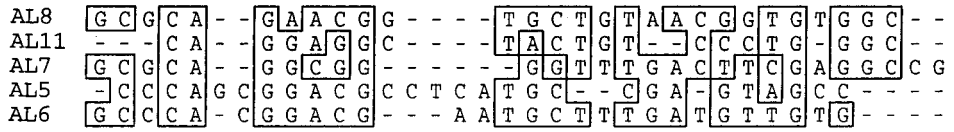

FIGURE 1: Nucleotide sequences of angiogenin-binding oligonucleotides. Sequences were aligned with the program PILEUP in the GCG package.

Nuclear Translocation. HUVE cells were seeded at $5 \times 10^3/cm^2$ and cultured in HE-SFM in the presence of 20 ng/mL basic fibroblast growth factor at 37 °C under a humidified atmosphere containing 5% CO_2 for 24 h. The cells were washed 3 times with prewarmed HE-SFM and incubated with ^{125}I-angiogenin (1 μg/mL) or with a mixture of ^{125}I-angiogenin and oligonucleotide in HE-SFM at 37 °C for 30 min. The plates were cooled at 4 °C for 10 min and the medium was aspirated. The cells were washed 3 times with cold PBS, detached by scraping with a rubber cell scraper, and centrifuged at 800*g* for 5 min. The cell pellet was washed once with cold PBS and lysed with 0.5% Triton X-100 in PBS containing 1× complete protease inhibitor cocktail at 4 °C for 30 min. The cell solubilizates were centrifuged at 1200*g* to pellet the nuclear fraction that was washed twice with PBS and the radioactivity was determined with a γ counter. In the nuclear cotranslocation experiment with ^{125}I-labeled angiogenin and ^{32}P-labeled oligonucleotide, half of the nuclear fraction was used to determine the radioactivity of ^{125}I with a γ counter and the other half was used to determine the radioactivity of ^{32}P by liquid scintillation counting.

RESULTS

Oligonucleotides That Bind to Angiogenin. Angiogenin-binding oligonucleotide ligands were obtained from a starting pool of approximately 10^{14} molecules by affinity chromatography on angiogenin−Sepharose. After seven rounds of selection and amplification no further enrichment could be achieved. The seventh round angiogenin-binding oligonucleotides were eluted from the affinity column, converted to double-stranded DNA, and cloned. A total of 19 clones were selected and the nucleotide sequences of the inserts were determined. Each of them has a distinct sequence, but five members (AL5, 6, 7, 8, and 11) share substantial sequence similarity in the evolved region (Figure 1).

Single-stranded oligonucleotides with similar sequences usually adopt similar secondary structures with the conserved residues organized in defined motifs. However, prediction of secondary structures with the FOLDRNA program in the GCG package (Genetic Computation Group, Inc.) showed that each of the five members with similar primary structures had a different secondary structure (data not shown).

Oligonucleotides That Inhibit the Ribonucleolytic Activity of Angiogenin. Inhibition of the ribonucleolytic activity of angiogenin (0.3 μM) by the oligonucleotides (1 μM) was measured with poly(C) as the substrate. Only two of the 19 oligonucleotides showed significant inhibition (Table 1). At an inhibitor/enzyme ratio of 3:1, AL5 and AL6 inhibited the ribonucleolytic activity of angiogenin by 55% and 53%, respectively.

Determination of K_i Values for AL5 and AL6. The concentration of poly(C) (0.15 mg/mL) in the ribonucleolytic assay was well below the K_m value of angiogenin. Under this condition, the IC_{50} value should closely resemble the K_i. However, owing to the high concentration of angiogenin required for this assay, the free inhibitor, $[I_f]$, differs significantly from its total concentration, $[I_t]$. This precludes the use of standard $1/v_0$ vs $[I_t]$ plots to determine a K_i value. An effort to lower the enzyme concentration to 0.1 μM by extending the incubation time to 6 days was unsuccessful owing to the high background generated by trace amounts of contaminant RNase. The angiogenin variant Q117G (*34*) was therefore used for this purpose since it has ~100-fold higher ribonucleolytic activity than angiogenin toward poly(C), and hence requires less enzyme or shorter incubation time. It gave the same inhibition data as were obtained with angiogenin. Table 1 shows that 1 μM AL5 and AL6 inhibited the ribonucleolytic activity of Q117G (0.3 μM) by 58% and 56%, respectively. These results indicate that AL5 and AL6 bind to Q117G in the same manner and with the same affinity as to angiogenin. Thus, Q117G was used to determine the K_i values of AL5 and AL6. The use of a low enzyme concentration (10 nM) and relatively high inhibitor concentrations (0.25 to 1.6 μM for AL5 and AL6, respectively) allowed more accurate determination of K_i values. A plot of $1/v_0$ vs $[I_t]$ gave K_i values of 0.65 and 0.60 μM for AL5 and AL6, respectively (Table 2). It is notable that the two previously best known synthetic inhibitors, 5′-diphosphoadenosine 2′-phosphate (*35*) and the C-terminal peptide (108−123) of angiogenin (*36*), have K_i values of 150 and 278 μM, which are 230- and 460-fold higher than that for AL6.

Table 1: Inhibition of the Ribonucleolytic Activity of Angiogenin and Q117G by AL5 and AL6[a]

oligonucleotides	% inhibition	
	angiogenin	Q117G
AL5	55	58
AL6	53	56

[a] Oligonucleotide inhibitors, 1 μM, were incubated with 0.3 μM angiogenin or Q117G for 50 and 0.5 h, respectively. Assays were performed as described under Materials and Methods.

Table 2: Apparent K_i Values of Angiogenin Inhibitors

inhibitors	apparent K_i (μM)
AL5	0.65
AL6	0.60
AL6-A	6.1
AL6-B	1.0
AL6-V	6.0
5′-diphosphoadenosine 2′-phosphate	150
C-terminal peptide (108−123) of angiogenin	278

[a] The K_i values for AL5, AL6, AL6-A, AL6-B, and AL6-V were determined by plots of $1/v_0$ vs $[I_t]$ from data collected by 17 h assays with 10 nM Q117G. The K_i values for 5′-diphosphoadenosine 2′-phosphate and the C-terminal peptide of angiogenin were from refs *35* and *36*, respectively.

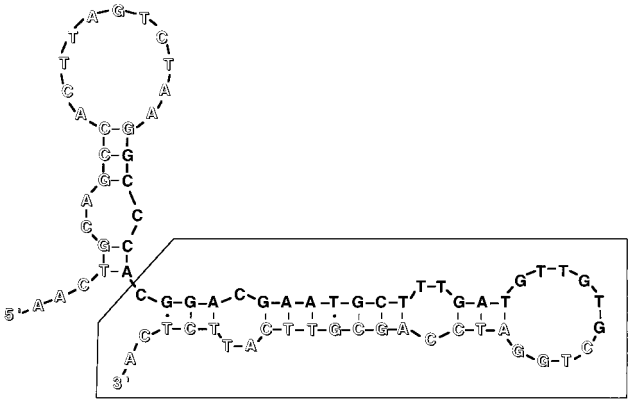

FIGURE 2: Secondary structure of AL6. The secondary structure was predicted with the program FOLDRNA in the GCG package. The evolved and constant regions are indicated by solid and open letters, respectively. AL6-B is indicated by the block.

Inhibitory Fragments of AL6. Among the 19 oligonucleotides selected by SELEX, AL6 is the most potent inhibitor of the ribonucleolytic activity of angiogenin. Its secondary structure (Figure 2) has two lobes, AL6-A and AL6-B, that comprise part of the variable region and one of the constant regions, respectively. The two lobes were synthesized separately and their inhibitory effects on the ribonucleolytic activity of angiogenin were tested. AL6-A is 10 times less effective, whereas AL6-B retains most of the activity of AL6 itself (Table 2). Attempts to shorten AL6-B were not successful since a few bases shorter at either end increased the K_i value markedly. The variable region of AL6 (28-mer, AL6-V) was also less inhibitory, probably owing to the different secondary structure it adopts. Further, no inhibition was observed when up to 3 μM of the antiparallel strand of AL6-B was used. These data indicate that formation of a correct secondary structure is critical for interaction with angiogenin. AL6-B is not an effective inhibitor of RNase A. No meaningful inhibition of the ribonucleolytic activity of RNase A toward poly(C) was observed at 1 μM AL6-B, and only 19% inhibition was obtained at 3 μM. Thus, AL6-B is highly specific for angiogenin.

Inhibition of Angiogenic Activity. The ability of AL6 and AL6-B to inhibit angiogenin-induced angiogenesis was examined by the CAM assay. Addition of 3 μg of AL6 to 10 ng of angiogenin decreases the percentage of positive eggs from 46% to 29%. The latter value does not differ significantly from the water control tested simultaneously (25%). AL6-B is a potent inhibitor of angiogenin: 1.5 μg of this aptamer completely abolishes the activity of 10 ng of angiogenin (Table 3). The same amount of the antiparallel strand of AL6-B (AL6-B-AP) is not inhibitory (55% positive eggs). These data demonstrate that angiogenin-induced formation of new blood vessels in the CAM is inhibited by the oligonucleotide ligands.

AL6-B Inhibits Angiogenin-Induced Cell Proliferation. Angiogenin induces cell proliferation of human endothelial cells under specific culture conditions (*19*). The effect of AL6-B on angiogenin-induced cell proliferation was examined with HUVE cells. Thus 50 000 cells were seeded in HE-SFM on attachment factor-coated 35 mm dishes and cultured at 37 °C. After 48 h, cell numbers were 51 300, 53 600, and 52 900 in the presence of 0, 1, and 5 μM AL6-B, respectively (Table 4). When 1 μg/mL angiogenin was present under the same conditions the number of cells increased to 61 700, 57 400, and 53 000, respectively, representing a 20%, 7%, and 0% increase in cell number over the corresponding controls. These data show that 1 μM AL6-B inhibits angiogenin-induced cell proliferation by 65% and 5 μM inhibits it completely.

Nuclear Translocation of ^{125}I-Angiogenin Is Not Altered by AL6-B. Nuclear translocation of angiogenin in endothelial cells is essential for angiogenin-induced angiogenesis and is a multistep process involving receptor-mediated endocytosis, vesicular transport through the cytoplasm, and NLS-assisted nuclear import (*14*, *15*). To investigate what the oligonucleotides may inhibit in addition to the ribonucleolytic activity and to gain insight into how AL6-B inhibits the mitogenic and angiogenic activities of angiogenin, we examined the effect of AL6-B on nuclear translocation of angiogenin in HUVE cells. As shown in Table 5, 1 μM AL6-B does not affect the accumulation of ^{125}I-angiogenin in the nucleus. Increasing the concentration of AL6-B to 5

Table 3: Effect of AL6-B on the CAM Activity of Angiogenin[a]

samples	no. of positive eggs/ no. of total eggs	% positive eggs	p
angiogenin (10 ng)	52/87	60	0.0001
angiogenin (10 ng) + AL6-B (1.5 μg)	14/51	27	0.45
angiogenin (10 ng) + AL6-B-AP (1.5 μg)	22/40	55	0.013

[a] Data were combined from multiple sets of experiments, each using between 7 and 23 eggs. The significance was calculated from χ^2 values, based on comparison with water control samples tested simultaneously, which produced a 20% positive response (17/83). Samples with $p < 0.05$ are considered positive.

Table 4: Effect of AL6-B on Angiogenin-Induced Cell Proliferation[a]

samples	cell numbers	%[b]
control	51 300 ± 900	100
angiogenin (1 μg/mL)	61 700 ± 1500	120
AL6-B (1 μM)	53 600 ± 1000	100
AL6-B (1 μM) + angiogenin (1 μg/mL)	57 400 ± 1000	107
AL6-B (5 μM)	52 900 ± 1400	100
AL6-B (5 μM) + angiogenin (1 μg/mL)	53 000 ± 1200	100

[a] HUVE cells at passage 5 were seeded in attachment factor- (Cell Systems) coated 35-mm dishes in HE-SFM at 5×10^3 cells/cm^2. Angiogenin, AL6-B, or a mixture of the two was added immediately after the cells were seeded. The cells were incubated at 37 °C under 5% CO_2 for 48 h. Cell numbers were determined with a Coulter counter. Numbers shown were from quadruplicates of each sample from a representative experiment. [b] Percent of cell number in angiogenin-treated samples versus the corresponding controls.

Table 5: Effect of AL6-B on Nuclear Translocation of Angiogenin in HUVE Cells[a]

AL6-B (μM)	nuclear angiogenin (cpm)	%
0	1090 ± 40	100
1	1070 ± 110	99
5	924 ± 100	85

[a] ^{125}I-Angiogenin (100 ng/mL, 1.4×10^5 cpm) was incubated with 5×10^4 cells in 35-mm dishes at 37 °C for 30 min. The nuclear fraction was isolated and the radioactivity was determined with a γ counter. Numbers shown were from quadruplicates of each sample from a representative experiment.

Table 6: Nuclear Cotranslocation of ^{125}I-Angiogenin and ^{32}P-AL6-B in HUVE Cells[a]

samples	nuclear ^{125}I-angiogenin		nuclear ^{32}P-AL6-B	
	cpm	nM	cpm	nM
^{125}I-angiogenin (1 μg)	1370 ± 60	0.41	0	0
^{32}P-AL6-B (5 μM)	0	0	0	0
^{125}I-angiogenin (1 μg) + ^{32}P-AL6-B (5 μM)	1790 ± 220	0.54	200 ± 30	0.75

[a] ^{125}I-Angiogenin (3.29×10^6 cpm/nmol), ^{32}P-AL6-B (2.66×10^5 cpm/nmol), or a mixture of the two was incubated with 5×10^4 cells in 35-mm dishes at 37 °C for 30 min. The nuclear fraction was isolated and the radioactivities of ^{125}I and ^{32}P were determined with a γ counter and by liquid scintillation counting, respectively. Numbers shown were from quadruplicates of each sample from a representative experiment.

μM reduces nuclear translocation by only 15%. However, at 1 μM, AL6-B already inhibited more than 65% of the proliferative activity (Table 4) and more than 50% of the ribonucleolytic activity of angiogenin (Tables 1 and 2). These results indicate that AL6-B does not substantially change the ability of angiogenin to bind to its cell surface receptors and to be endocytosed, transported across the cytoplasm, and subsequently imported into the nucleus.

AL6-B Is Cotranslocated to the Nucleus with Angiogenin. The lack of effect of AL6-B on nuclear translocation of angiogenin indicates that inhibition of the ribonucleolytic activity is the predominant cause of the inhibition of the mitogenic and angiogenic activity of angiogenin. To learn whether AL6-B is cotranslocated to the nucleus with angiogenin, AL6-B was labeled with ^{32}P and examined for nuclear translocation in the absence and presence of ^{125}I-angiogenin. Incubation of 5 μM ^{32}P-AL6-B and 1 μg/mL ^{125}I-angiogenin with HUVE cells at 37 °C for 30 min resulted in the accumulation of both angiogenin and AL6-B in the nuclear fraction at a 1:1.4 molar ratio (Table 6). No ^{32}P-labeled AL6-B was detected in the nucleus if it was incubated with cells under the same conditions but without angiogenin. The almost stoichiometric ratio of angiogenin to AL6-B in the nucleus suggests that they form a 1:1 complex that is translocated to the cell nucleus, presumably by the same machinery that the cells use for angiogenin alone. More importantly, this result suggests that AL6-B inhibits the ribonucleolytic activity of angiogenin within the cell nucleus, thereby inhibiting its angiogenic activity, and strongly implies that this is the location of the physiological substrate of angiogenin in endothelial cells.

DISCUSSION

Since angiogenin is responsible, at least under certain circumstances (*4*−*8*), for the formation of blood vessels, its modulation and control are critical for development, growth, maintenance, and repair of normal and abnormal tissues. Anti-angiogenin agents could play significant roles in the clinical treatment of angiogenesis-related diseases. Ever since the ribonucleolytic activity of angiogenin was found to be essential for angiogenesis, there has been a concentrated effort to identify inhibitors. Several, including human placental ribonuclease inhibitor (*37*) and the angiogenin C-terminal peptide (*36*), have been demonstrated to inhibit both the ribonucleolytic and angiogenic activities. Here, we used SELEX to identify oligonucleotide inhibitors for angiogenin.

SELEX is a combinatorial chemistry technique that allows the simultaneous screening of large random pools of oligonucleotides for a particular target (*38*, *39*). The underlying theory is that single-stranded oligonucleotides, like proteins, will fold into structural motifs with a high degree of molecular rigidity. When the starting pool is sufficiently complex, it is reasonable to anticipate that a few molecules will have a correct structure that allows them to bind to the target molecule with high affinity. Over 200 SELEX experiments have been carried out with a variety of target molecules including both large and small molecules such as proteins, amino acids, and nucleosides (*39*, *40*). Such experiments typically yield ligands with K_ds ranging from picomolar to nanomolar at a frequency of one ligand in 10^4−10^{14} different molecules. We used the SELEX procedure to obtain angiogenin aptamers from an oligodeoxynucleotide library randomized over 28 positions (to provide $4^{28} = 7.2 \times 10^{16}$ different sequence possibilities, of which $\sim 1 \times 10^{14}$ were tested).

From the starting pool, 19 molecules with distinct sequences were selected by affinity chromatography on angiogenin−Sepharose after seven rounds of iterative selection−amplification cycles. The estimated enrichment was approximately 10^7-fold, assuming a 10-fold enrichment per round of selection. Most SELEX procedures are designed to obtain truly high -affinity ligands rather than those with low k_{off} by limiting the contact time of an oligonucleotide pool with the target molecule to a short period of 5−10 min (*41*, *42*), or in some cases only 1 min (*43*). However, to isolate oligonucleotide ligands that are not only tight binders but also inhibitors of angiogenin, we employed an overnight incubation time based on the consideration that both mitogenic and angiogenic assays require several days. Under these conditions oligonucleotides that form stable complexes should antagonize the activity of angiogenin more effectively than those with low K_d but high k_{on} and k_{off}. Therefore, the oligonucleotides selected should represent ligands with slow dissociation rate constants rather than ones with tightest binding. It is also possible that ligands with even better inhibitory activity for angiogenin exist but were not detected since the initial pool of 10^{14} molecules was not large enough to include all possible sequences (7.2×10^{16}). However, considering that the number of antibodies a mouse can possibly generate is between 10^9 and 10^{11} (*44*), a pool of 10^{14} molecules is already incredibly complex. In any event, inhibition of the ribonucleolytic activity of angiogenin was used to screen the potential inhibitory ligands for angiogenin-binding oligonucleotides.

Some tight-binding ligands obtained by SELEX reportedly had nanomolar dissociation constants but failed to inhibit the biological activity of the target protein (*41*). Among the 19 oligonucleotides tested here, only two, AL5 and AL6, were found to be significant inhibitors of the ribonucleolytic activity of angiogenin. The other ligands showed only slight inhibition, presumably as a result of interaction with different

sites of angiogenin. Since the contact area between an oligonucleotide ligand and its protein target is about 300−400 Å^2 (*45*, *46*), oligonucleotides with affinity to other functional sites of angiogenin such as the cell-binding site or NLS may not disrupt catalytic activity. On the other hand, active -site-binding oligonucleotides may not affect the other activities of angiogenin. This is indeed the case. Thus, AL6-B inhibits the ribonucleolytic activity and thereby abolishes the mitogenic and angiogenic activity, but it does not interfere with the nuclear translocation of angiogenin in endothelial cells. More importantly, the oligonucleotide is cotranslocated to the nucleus with angiogenin. Although the detailed mechanism of angiogenin transport across the cytoplasm is unknown at present, receptor-mediated endocytosis and NLS-assisted nuclear import are believed to be the first and last steps in this process, respectively. The angiogenin−AL6-B complex must then be able to bind to the angiogenin receptor on the cell surface and remain intact in all subsequent steps required for internalization, transport across the cytoplasm, and import into the nucleus. Oligonucleotides are known to be taken up by living cells via cell surface DNA binding proteins (*46*, *47*). However, this requires 2−4 h and 50 h, respectively, for surface binding and cellular accumulation to reach saturation. AL6-B does not appear in the nuclear fraction of HUVE cells after 30 min of incubation in the absence of angiogenin but is detected at a 1:1 molar ratio to angiogenin when both are present. Thus, the nuclear accumulation of AL6-B observed here is not due to nonspecific cellular uptake but is the result of specific cotranslocation with angiogenin. Apparently, the angiogenin−AL6-B complex is capable of binding to the angiogenin receptor and using the cellular machinery for nuclear translocation. Therefore, it seems likely that the second messengers and other cellular activities that are stimulated by angiogenin are not affected by AL6-B. Since AL6-B completely abolishes both the mitogenic and angiogenic activities of angiogenin, it would seem that the ribonucleolytic activity of angiogenin is essential not only for angiogenesis but also for angiogenin-induced cell proliferation.

Extensive evidence points to RNA(s) or an RNA-like molecule(s) as the physiological substrate for angiogenin. However, the identity of this substrate(s) is not yet known. A search for identical or homologous sequences to that of AL6-B through the data bank at the National Center for Biotechnology Information revealed no significant and meaningful match. It is known that oligonucleotides obtained by SELEX usually do not resemble natural ones, presumably because SELEX does not demand biological fitness as is the case in genetics and evolution *in vivo*. It is unknown whether AL6-B competes with a natural DNA sequence located in the nucleolus that may bind angiogenin. In any event, the results show that the inhibition of angiogenin by AL6-B is mainly due to the specific binding of the ligand to the catalytic active site of the enzyme. The data provide yet additional strong evidence that inhibition of its ribonucleolytic activity is a feasible way to inhibit angiogenin-induced angiogenesis.

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