Inhibition of Receptor Binding by High-Affinity RNA Ligands to Vascular Endothelial Growth Factor

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ABSTRACT: The proliferation of new blood vessels (angiogenesis) is a process that accompanies many pathological conditions including rheumatoid arthritis and solid tumor growth. Among angiogenic cytokines that have been identified to date, vascular endothelial growth factor (VEGF) is one of the most potent. We used SELEX [systematic evolution of ligands by exponential enrichment; Tuerk, C., & Gold, L. (1990) *Science 249*, 505–510] to identify RNA ligands that bind to VEGF in a specific manner with affinities in the low nanomolar range. Ligands were selected from a starting pool of about 10^{14} RNA molecules containing 30 randomized positions. Isolates from the affinity-enriched pool were grouped into six distinct families on the basis of primary and secondary structure similarities. Minimal sequence information required for high-afinity binding to VEGF is contained in 29–36-nucleotide motifs. Binding of truncated (minimal) high-affinity ligands to VEGF is competitive with that of other truncated ligands and heparin. Furthermore, truncated ligands from the six ligand families inhibit binding of [^{125}I]VEGF to its cell-surface receptors. Oligonucleotide ligands described here represent an initial set of lead compounds in our ongoing effort toward the development of potent and specific VEGF antagonists.

Neovascularization, or angiogenesis, is the process in which new blood vessels are formed from the existing endothelium in response to stimuli that signal inadequate blood supply. Although it is rare under normal physiological conditions, angiogenesis frequently accompanies certain pathological conditions such as psoriasis, rheumatoid arthritis, hemangioma, diabetic retinopathy, and solid tumor growth and metastasis (Folkman & Klagsbrun, 1987; Folkman, 1990). A number of growth factors that are capable of inducing angiogenesis *in vivo* have been identified to date including basic and acidic fibroblast growth factors, transforming growth factors α and β, platelet-derived growth factor, angiogenin, platelet-derived endothelial cell growth factor, interleukin-8, and vascular endothelial growth factor (VEGF,[1] also known as vascular permeability factor) (Klagsbrun & Soker, 1993).

Several recent observations have indicated that VEGF, as a secreted and specific mitogen for endothelial cells, may be one of the major angiogenesis inducers *in vivo*. For example, the expression of VEGF and its receptors accompanies angiogenesis associated with embryonic development (Breier et al., 1992) and hormonally regulated reproductive cycles (Shweiki et al., 1993). Further, in addition to promoting the growth of vascular endothelial cells and increasing vascular leakage, VEGF also induces several proteolytic enzymes including interstitial collagenase, urokinase-type plasminogen activator, and tissue-type plasminogen activator (Pepper et al., 1991). These observations are highly relevant in deliberating the angiogenic potency of VEGF, in view of the prominent role of these proteases and their regulators in angiogenesis-related extracellular matrix degradation.

One of the most serious disorders associated with pathological angiogenesis is tumor growth and metastasis (Folkman, 1990). Nutrient supply and removal of metabolic end products is often a limiting factor in the growth of aggressive solid tumors, and several recent reports have provided compelling evidence that VEGF may be one of the crucial tumor angiogenesis factors. The expression of VEGF and its receptor is dramatically upregulated in tumor cells adjacent to necrotic areas *in vivo*, suggesting that VEGF is involved in hypoxia-induced angiogenesis (Shweiki et al., 1992; Plate et al., 1992). It has been shown more recently that interference with the VEGF–VEGF receptor signaling, either with neutralizing antibodies to VEGF (Kim et al., 1993) or through expression of a dominant-negative VEGF receptor mutant (Millauer et al., 1994), inhibits the growth of human tumors in nude mice. These experiments suggest that antagonists of VEGF may be useful for controlling pathological angiogenesis. Specific inhibitors of VEGF are limited at present to monoclonal antibodies (Kim et al., 1993) and the soluble VEGF receptor (Kendall & Thomas, 1993).

Random oligonucleotide libraries represent assemblies of diverse molecular features from which rare sequences with unique functional properties can be isolated by selection–amplification (SELEX). It is becoming increasingly clear that SELEX is a powerful and highly versatile tool for discovering high-affinity oligonucleotide ligands to a wide range of molecular targets (Tuerk & Gold, 1990; Tuerk et al., 1992; Ellington & Szostak, 1990, 1992; Bock et al., 1992; Connell et al., 1993; Gold et al., 1993, Jellinek et al., 1993; Jenison et al., 1994). Starting with an initial pool of about 10^{14} RNA molecules randomized at 30 contiguous positions, we used SELEX to identify ligands that bind to VEGF with high affinity and specificity. We demonstrate that these ligands inhibit the initiating step of VEGF signaling:binding of the growth factor to specific cell-surface receptors.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human VEGF (165 amino acid form; M_r = 46 000) was a generous gift from Dr. Napoleone Ferrara (Genentech, S. San Francisco, CA). Low molecular weight heparin (M_r = 5100) was purchased from Calbiochem

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[1] Abbreviations: HSA, human serum albumin; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; SELEX, systematic evolution of ligands by exponential enrichment; VEGF, vascular endothelial growth factor.

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Starting RNA:
5'-GGGAGCUCAGAAUAAACGCUCAA[-30N-]UUCGACAUGAGGCCCGGAUCCGGC-3'

PCR Primer 1:
     Hind III
      ------
5'-CCGAAGCTTAATACGACTCACTATAGGGAGCTCAGAATAAACGCTCAA-3'
            T7 Promoter

PCR Primer 2:
      Bam H1
      ------
5'-GCCGGATCCGGGCCTCATGTCGAA-3'
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FIGURE 1: Starting RNA and PCR primers used in SELEX.

(La Jolla, CA). All other reagents and chemicals were of the highest purity available and were purchased from commercial sources.

SELEX. Essential features of the SELEX protocol have been described in detail (Tuerk & Gold, 1990; Schneider et al., 1992). Briefly, DNA templates for *in vitro* transcription (that contain a region of 30 randomized positions flanked by constant-sequence regions) and the corresponding PCR primers were prepared chemically using established solid-phase oligonucleotide synthesis protocols. The random region was generated by utilizing an equimolar mixture of the four nucleotides during oligonucleotide synthesis. The two constant regions were designed to contain PCR primer annealing sites, a primer annealing site for cDNA synthesis, a T7 RNA polymerase promoter region, and restriction enzyme sites that allow cloning into vectors (Figure 1). The initial pool of RNA molecules was prepared by *in vitro* transcription of approximately 600 pmol (4×10^{14} molecules) of the double-stranded DNA template utilizing T7 RNA polymerase (Milligan et al., 1987). Prior to selections, RNA was denatured at 90 °C for 2 min and cooled on ice. Affinity selections were done by incubating VEGF and RNA for 10–20 min at 37 °C in phosphate-buffered saline (PBS = 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and then separating the protein–RNA complexes from the unbound species by nitrocellulose filter partitioning (Tuerk & Gold,, 1990). The selected RNA (which typically amounted to 5–10% of the total input RNA) was then extracted from the filters and reverse transcribed into cDNA by avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). Reverse transcriptions were done at 48 °C (1 h) in 50 mM Tris buffer (pH 8.3) containing 60 mM NaCl, 6 mM $Mg(OAc)_2$, 10 mM DTT, 0.8 mM deoxynucleoside triphosphates, and 1 unit/μL reverse transcriptase. We amplified the cDNA by PCR as described (Tuerk & Gold, 1990) to produce double-stranded DNA template for the next round of *in vitro* transcription.

Nitrocellulose Filter Binding Assays. Oligonucleotides bound to proteins can be effectively separated from the unbound species by filtration through nitrocellulose membrane filters (Yarus & Berg, 1970; Lowary & Uhlenbeck, 1987; Tuerk & Gold, 1990). Nitrocellulose filters (0.2-μm pore size; Schleicher and Schuell, Keene, NH) were secured on a filter manifold and washed with 4–10 mL of buffer. Following incubation of internally radiolabeled RNA ([α-^{32}P]ATP) with serial dilutions of the protein for 10 min at 37 °C in buffer (PBS) containing 0.01% human serum albumin (HSA), the solutions were applied to the filters under gentle vacuum and washed with 5 mL of PBS. The filters were then dried under an infrared lamp, and the amount of radioactivity was determined by liquid scintillation counting.

Equilibrium Dissociation Constants. In the simplest case, equilibrium binding of RNA (R) to VEGF (P) can be described by eq 1,

$$R{\cdot}P \overset{K_d}{\rightleftharpoons} R + P \tag{1}$$

where K_d (=[R][P]/[R·P]) is the equilibrium dissociation constant. From the mass-balance equations, the fraction of bound RNA at equilibrium (q) can be expressed in terms of measurable quantities (eq 2),

$$q = (f/2R_t)\{P_t + R_t + K_d - [(P_t + R_t + K_d)^2 - 4P_tR_t]^{1/2}\} \tag{2}$$

where P_t and R_t are total protein and total RNA concentrations and f reflects the efficiency of retention of the protein–RNA complexes on nitrocellulose filters (Irvine et al., 1991; Jellinek et al., 1993). The average value of f for VEGF in our assays was 0.8.

Most RNA ligands exhibited biphasic binding to VEGF. For those ligands, binding of RNA to VEGF is described by a model in which the RNA is assumed to be partitioned between two noninterconverting components (R_1 and R_2) that bind to VEGF with different affinities (Jellinek et al., 1993):

$$R_1{\cdot}P \overset{K_{d1}}{\rightleftharpoons} R_1 + P \tag{3}$$

$$R_2{\cdot}P \overset{K_{d2}}{\rightleftharpoons} R_2 + P \tag{4}$$

In this case, the fraction of total bound RNA (q) is given by eq 5.

$$q = (f/2R_t)\{2P_t + R_t + K_{d1} + K_{d2} - [(P_t + \chi_1R_t + K_{d1})^2 - 4P_t\chi_1R_t]^{1/2} - [(P_t + \chi_2R_t + K_{d2})^2 - 4P_t\chi_2R_t]^{1/2}\} \tag{5}$$

where χ_1 and χ_2 (=$1 - \chi_1$) are the mole fractions of R_1 and R_2, and K_{d1} and K_{d2} are the corresponding dissociation constants.

Internally-labeled RNA ligands used for binding studies were prepared by *in vitro* transcription with T7 RNA polymerase (Milligan et al., 1987) and were purified on denaturing polyacrylamide gels to ensure size homogeneity. All RNA ligands were diluted to about 1 nM in PBS, denatured at 90 °C for 2 min, and then cooled on ice prior to further dilution and incubation with the protein. This denaturation/

renaturation cycle, performed at high dilution, is necessary to ensure that the RNA is essentially free from dimers and other higher order aggregates. Concentrations of the stock solutions of VEGF, from which other dilutions were made, were determined from the absorbance readings at 280 nm and the calculated value for ϵ_{280} of 46 600 M^{-1} cm^{-1} for the VEGF dimer (Gill & von Hippel, 1989). RNA concentrations were calculated from the absorbace readings at 260 nm (Sambrook et al., 1989) and were typically <50 pM in binding reactions. Data sets that define the binding curves were fit to either eq 2 or eq 5 by the nonlinear least squares method with the software package Kaleidagraph (Synergy Software, Reading, PA).

Minimal Sequence Determinations. High-affinity VEGF ligands were radiolabeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase or at the 3′-end with ^{32}pCp and T4 RNA ligase for the 3′- and 5′-boundary determinations, respectively. Radiolabeled RNA ligands were subjected to partial alkaline hydrolysis and then selectively bound in solution to VEGF at 5, 0.5, and 0.125 nM protein before being passed through nitrocellulose filters, and retained oligonucleotides were resolved on 8% denaturing polyacrylamide gels. Partial digests of the 5′- or 3′-labeled RNA ligands with RNAse T_1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used to mark the positions of labeled oligonucleotides ending with a guanosine.

Cloning and Sequencing. Individual members of the enriched pool were cloned and sequenced as described (Schneider et al., 1992).

Receptor Binding. VEGF was radioiodinated by the Iodogen method (Jakeman et al., 1992) to a specific activity of 2.4×10^4 cpm/ng. Human umbilical vein endothelial cells (HUVECs) were plated in 24-well plates at a density of 1–2 $\times 10^5$ cells/well and grown to confluence in EGM (Clonetics, San Diego, CA) for 24–48 h. At confluence, the cells were washed three times with PBS and incubated for 2 h at 4 °C in serum-free αMEM medium containing ^{125}I-labeled VEGF (20 ng/mL) in the presence or absence of unlabeled competitor (VEGF, epidermal growth factor, or RNA). For experiments done with RNA, we included 0.2 unit of placental ribonuclease inhibitor (Promega, Madison, WI) in the medium to ensure that the RNA ligands were not degraded during the course of the experiment. At the end of the 2-h incubation period, the supernatant was removed and the wells were washed two times with PBS. HUVECs were then lysed with 1% Triton X-100/1 M NaOH, and the amount of cell-associated [^{125}I]-VEGF was determined by γ counting.

RESULTS

SELEX. Random RNA used in the initial selection bound to VEGF with an affinity of approximately 0.2 μM. After 13 rounds of SELEX, the observed improvement in affinity of the evolved RNA pool was about 2 orders of magnitude (data not shown). We cloned and sequenced 64 isolates from this evolved pool and found 37 unique sequences (sequences that differed at only one or two positions were not considered unique). Most of the unique sequences (34 out of 37) could be classified into six families on the basis of sequence similarity in the evolved region (Figure 2).

Consensus Structures. In addition to allowing determination of the consensus primary structures, groups of similar sequences consisting of members that share a defined functional property often contain useful information for secondary structure analysis. Indeed, comparative analysis has proved to be one of the most powerful tools in elucidating

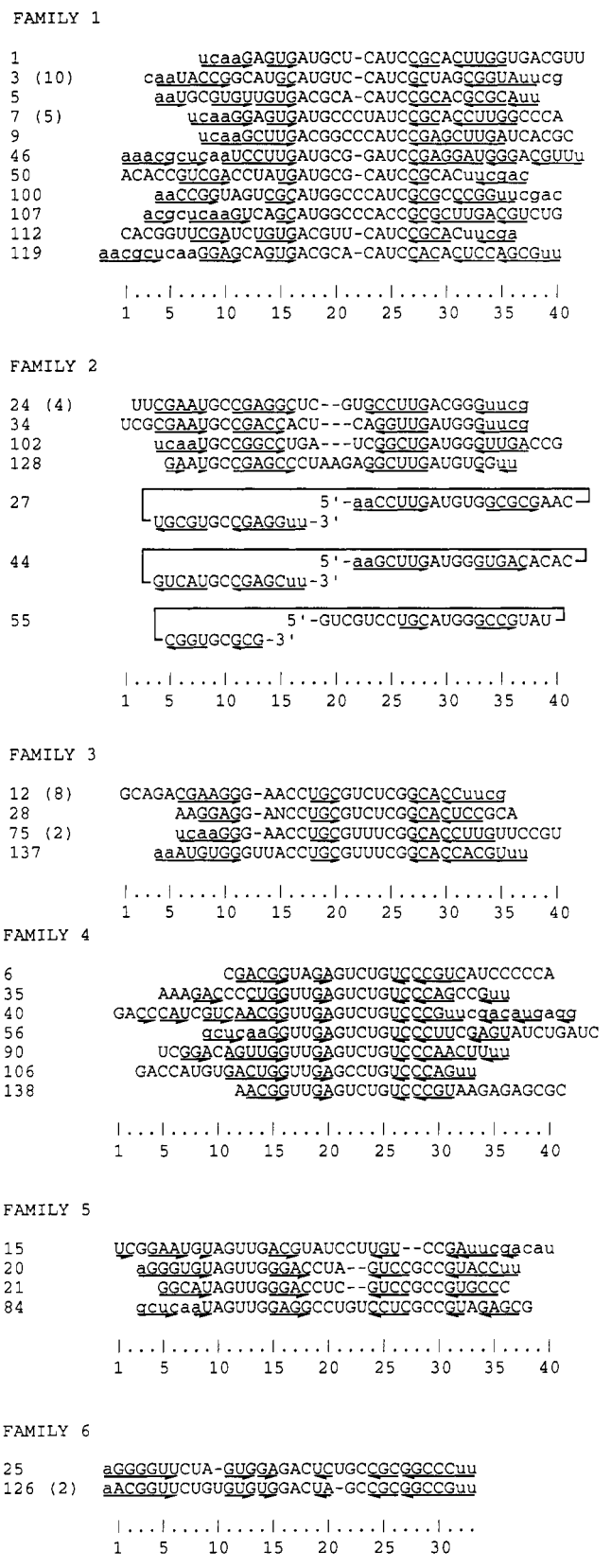

FIGURE 2: Six families of VEGF ligands showing aligned sequences and predicted secondary structures. Underline arrows indicate regions that are expected to be base paired. Lowercase and uppercase letters distinguish nucleotides in the constant and the evolved sequence regions, respectively. A number in parentheses following the clone number indicates the frequency (when greater than 1) with which that sequence was observed in the affinity-enriched pool. Nucleotide positions are numbered consecutively starting with the first uppercase (evolved region) nucleotide from the left in each aligned sequence set.

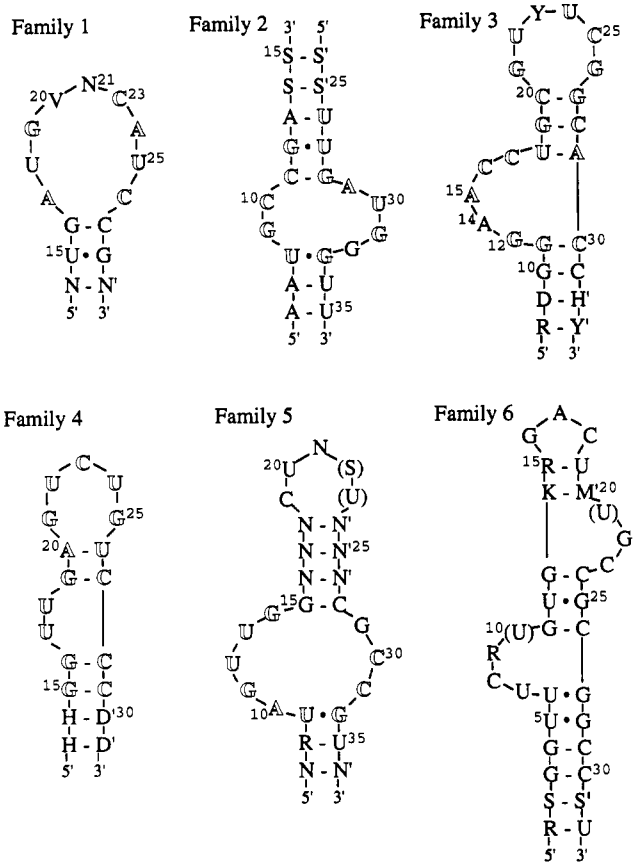

FIGURE 3: Consensus sequences and predicted secondary structures for the six families of VEGF ligand. Plain text designates positions that occur at >60% but <80% frequencies. For families 1–5, positions where individual nucleotides are strongly conserved (frequencies > 80%) are outlined (family 6 consists of only two sequences). A residue in parentheses occurs at that position at the same frequency as a gap. Nucleotide positions are numbered as they appear in Figure 2. In cases where numbering in the consensus structures is not consecutive (due to consensus gaps), the numbering of the flanking nucleotides is shown. R = A or G; Y = C or U; K = G or U; M = A or C; S = G or C; D = A, G, or U; H = A, U, or C; V = G, A, or C; N = any base; a prime (′) indicates a complementary base.

secondary structural elements of RNA (Fox & Woese, 1976; James et al., 1988; Gutell et al., 1992; Woese & Pace, 1993). The underlying assumption is that ligands with similar sequences are capable of adopting similar secondary structures in which the conserved residues are organized in unique, well-defined motifs. In this context, ligands with strong, unambiguous secondary structures can provide good structural leads for other sequences within a similar sequence set where consensus folding may be less obvious. Conserved elements of secondary structure, such as base pairing, may be detected through covariation analysis of aligned sequence sets (James et al., 1988; Gutell et al., 1992; Woese & Pace, 1993). The predicted consensus secondary structures for the six sequence families are shown in Figure 3.

The most highly conserved residues in the family 1 sequence set (A17, G19, and the CAUC sequence at positions 23–26) are accommodated in the 9–10-nucleotide loop. Base-pairing covariation between positions 16 and 27 (G-C occurs with a frequency of 8/11, or 8 out of 11 times, and C-G, with a frequency of 3/11), positions 15 and 28 (U-G, 7/11; G-C, 3/11; U-A, 1/11), and positions 14 and 29 (G-C, 5/11; U-A, 2/11; C-G, 1/11) supports the predicted secondary structure (Figure 2). It is worth noting that many ligands in this family have stable, extended stems that contain up to 15 base pairs.

In the family 2 sequence set, the strongly conserved UGCCG and UUGAUG(G/U)G sequences (positions 8–12 and 26–33) are circularly permuted (Figure 2), a feature observed previously in other SELEX experiments (Jenison et al., 1994; Tuerk et al., 1994). In the consensus secondary structure, the conserved nucleotides are found in an identical arrangement within or adjacent to the asymmetrical internal loop (Figure 3). This result strongly suggests that nucleotides outside of the consensus motif shown in Figure 3 are not important for binding (vide infra). Base-pairing covariation is noted between positions 5 and 36 (C-G, 2/7; G-C, 2/7; U-A, 1/7; G-U, 1/7), 6 and 35 (A-U, 4/7; C-G, 1/7; G-C, 1/7), 7 and 34 (A-U, 4/7; G/C, 1/7), 11 and 28 (C-G, 6/7; G-C, 1/7), 12 and 27 (G-U, 6/7; C-G, 1/7), 13 and 26 (A-U, 5/7; G-C, 1/7; G-U, 1/7), 14 and 25 (G-C, 4/7; C-G, 2/7), and 15 and 24 (C-G, 4/7; G-C, 2/7).

Family 3 and family 4 sequence sets are characterized by contiguous stretches of 21 (GGGAACCUGCGU(C/U)-UCGGCACC, positions 11–31) and 15 (GGUUGAGUCU-GUCCC, positions 15–29) highly conserved nucleotides arranged in bulged hairpin motifs (Figure 3). Base-pairing covariation is detected in family 3 between positions 8 and 33 (A-U, 2/4; G-C, 2/4), 9 and 32 (A-U, 2/4; U-A, 1/4; G-C, 1/4), and 10 and 31 (A-U, 1/4; G-C, 3/4) and in family 4 between positions 13 and 31 (A-U, 4/7; C-G, 2/7; U-A, 1/7) and 14 and 30 (C-G, 3/7; U-A, 3/7; A-U, 1/7).

The family 5 consensus secondary structure is an asymmetrical internal loop where the conserved UAGUUGG (positions 9–15) and CCG (positions 29–31) sequences are interrupted by less conserved nucleotides (Figure 3). Modest base-pairing covariation is found between positions 8 and 32 (A-U, 2/4; U-G, 1/4), 16 and 26 (G-C, 2/4; A-U, 1/4), 17 and 25 (A-U, 2/4; G-C, 1/4), and 18 and 24 (C-G, 2/4; G-C, 1/4).

Family 6 has only two sequences, and therefore the concept of consensus sequence or consensus secondary structure is less meaningful. Nevertheless, the two sequences are very similar (90% identity) and can be folded into a common bulged hairpin motif (Figure 3). Base-pairing covariation is found between positions 1 and 32 (A-U, 1/2; G-U, 1/2), 2 and 31 (C-G, 1/2; G-C, 1/2), 14 and 20 (U-A, 1/2; G-C, 1/2), and 15 and 19 (A-U, 1/2; G-U, 1/2).

Affinities. The affinity of all unique ligands for VEGF was first examined in a screening manner by determining the amount of RNA bound to VEGF at two protein concentrations (1 and 10 nM). Binding of the best ligands from each of the six sequence families was then analyzed over a range of protein concentrations (Figure 4). Dissociation constants were calculated by fitting the data points to either eq 2 (monophasic binding) or eq 5 (biphasic binding), and their values are shown in Table 1.

Minimal Ligands. In order to determine the minimal sequences necessary for high-affinity binding to VEGF, we performed deletion analyses with representative ligands from each of the six families. In these experiments, ligands are radiolabeled at either the 3′-end or the 5′-end (for the 5′- or 3′-boundary determinations, respectively), followed by limited alkaline hydrolysis, partitioning of the free and the protein-bound RNA, and analysis of the hydrolytic fragments that retained high affinity for VEGF on denaturing polyacrylamide gels (Tuerk et al., 1990). In each experiment, the smallest radiolabeled oligonucleotide bound by VEGF at the lowest protein concentration represents the information boundary. The combined results from the 3′- and 5′-boundary experiments define the minimal ligand (Figure 5). It is important to

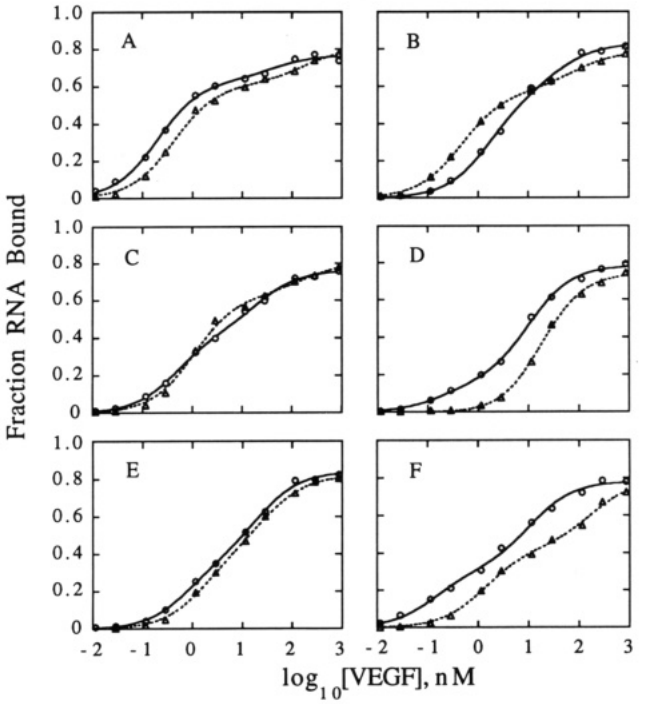

FIGURE 4: Binding curves for a representative set of high-affinity ligands to VEGF. Full-length (○) and truncated (△) ligands tested were 100 and 100t (family 1, panel A), 44 and 44t (family 2, panel B), 12 and 12t (family 3, panel C), 40 and 40t (family 4, panel D), 84 and 84t (family 5, panel E), and 126 and 126t (family 6, panel F). The fraction of ^{32}P-labeled RNA bound to nitrocellulose filters is plotted as a function of total protein concentration, and the lines represent the fit of the data points to eq 2 (ligand 40t) or to eq 5 (all other ligands). Each data point is an average of two independent determinations. RNA concentrations were determined from their absorbance reading at 260 nm (typically <50 pM). Binding reactions were done at 37 °C in PBS containing 0.01% human serum albumin.

Table 1: Dissociation Constants for a Representative Set of Full-Length and Truncated High-Affinity RNA Ligands for VEGF[a]

ligand	K_{d1}, nM[c]	χ_1[d]	K_{d2}, nM[e]
100	0.20 ± 0.02	0.82 ± 0.02	42 ± 30
100t	0.42 ± 0.04	0.76 ± 0.03	182 ± 94
44	1.7 ± 0.5	0.70 ± 0.11	38 ± 32
44t	0.48 ± 0.04	0.73 ± 0.01	82 ± 23
12	0.48 ± 0.07	0.56 ± 0.03	21 ± 5
12t	1.1 ± 0.2	0.78 ± 0.04	180 ± 160
40	0.19 ± 0.09	0.19 ± 0.04	10 ± 1
40t[f]	20 ± 1		
84	0.82 ± 0.2	0.45 ± 0.06	21 ± 5
84t	1.8 ± 0.4	0.53 ± 0.07	31 ± 10
126	0.14 ± 0.04	0.40 ± 0.04	11 ± 3
126t	1.4 ± 0.2	0.54 ± 0.03	181 ± 57

[a] Binding experiments were done as described in Experimental Procedures, and errors are given as standard deviations. [b] Full-length and truncated (t) ligands are listed in pairs and represent sequence families 1–6, in order. [c] Dissociation constant of the higher affinity binding component as defined in eq 5. [d] Mole fraction of the high-affinity binding component as defined in eq 5. [e] Dissociation constant of the lower affinity binding component as defined in eq 5. [f] Dissociation constant for ligand 40t was determined by fitting the data points to eq 2.

emphasize that the 3′- and 5′-boundaries are determined in separate experiments. Since the intact termini may provide additional contacts with the protein or participate in competing secondary structures, ligands truncated at both ends may have higher, lower, or equal affinities for the protein compared to their full-length or half-truncated equivalents (vide infra). The following truncated ligands whose sequences correspond to the experimentally determined boundaries were prepared by *in vitro* transcription from the synthetic DNA templates:

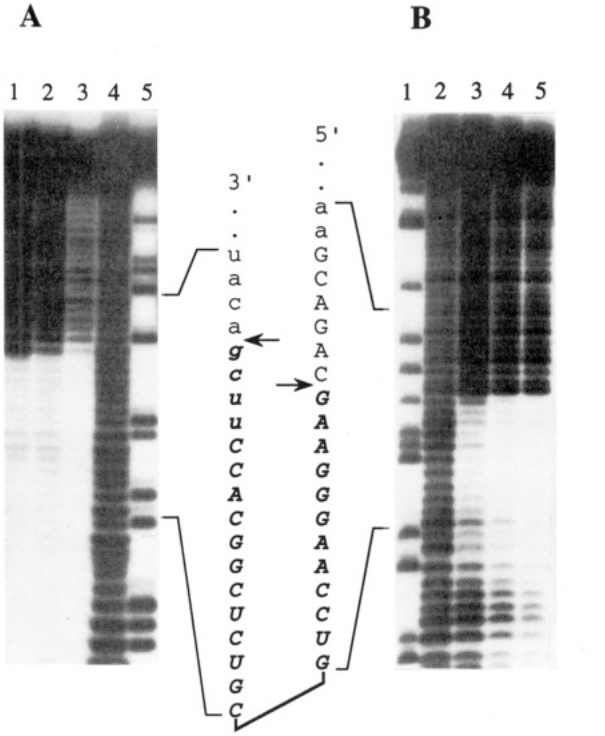

FIGURE 5: Determination of the 3′- and 5′-boundaries for a representative high-affinity VEGF ligand (ligand 12). The 3′-boundary determination (panel A) shows partially hydrolyzed 5′-end-labeled RNA (lane 4); hydrolytic fragments retained on nitrocellulose filters following incubation of the partially hydrolyzed RNA with VEGF at 5 nM (lane 1), 0.5 nM (lane 2), and 0.125 nM (lane 3); and a partial digest of the 5′-end-labeled RNA with RNAse T_1 (lane 5) resolved on an 8% denaturing polyacrylamide gel. The 5′-boundary (panel B) was determined in an identical manner except that RNA radiolabeled at the 3′-end was used. Shown are an RNase T1 digest (lane 1), a partial alkaline hydrolysis (lane 2), and hydrolytic fragments retained on nitrocellulose filters following incubation with VEGF at 5 (lane 3), 0.5 (lane 4), or 0.125 nM (lane 5). Arrows indicate the 3′- and the 5′-boundaries that define the minimal ligand (italicized). Lowercase and uppercase letters indicate nucleotides in the constant and evolved sequence regions, respectively.

100t (family 1), *GG*CCGGUAGUCGCAUGGCCCAUCGCGCCCGG; 44t (family 2), *GG*aaGCUUGAUGGGUGACACACGUCAUGCCGAGCu; 12t (family 3), *GG*AAGGGAACCUGCGUCUCGGCACCuucg; 40t (family 4), *GG*UCAACGGUUGAGUCUGUCCCGuucgac; 84t (family 5), *G*gcucaaUAGUUGGAGGCCUGUCCUCGCCGUAGAGC; and 126t (family 6), *GG*aACGGUUCUGUGUGUGGACUAGCCGCGGCCGuu (the letter t after the clone number designates a truncated sequence; italicized guanines are not present in the original sequences and were added to increase the transcriptional efficiency (Milligan et al., 1987); lowercase letters indicate nucleotides from the constant sequence region; nucleotides predicted to be base paired are underlined). Binding curves for these truncated ligands and their dissociation constants are shown alongside their parent (full length) ligands in Figure 4 and Table 1. Ligands 12t, 84t, and 100t bind to VEGF with affinities that are comparable to those of the corresponding full-length ligands. The affinities of ligands 40t and 126t, on the other hand, are diminished, while the affinity of ligand 44t is increased, compared to the respective full-length ligands. Binding of most truncated ligands remains biphasic.

We note that each of these minimal ligands contains its respective consensus motif. Clearly, the knowledge of information boundaries is in itself of substantial value for secondary structure determinations since many of the possible competing structures are eliminated. In this regard, we also

note that our consensus motifs are in excellent agreement with secondary structure predictions based on folding of the truncated ligands according to thermodynamic principles (Zucker, 1989).

Specificity. Binding of two truncated high-affinity ligands, 100t and 44t, to five other heparin binding proteins (basic fibroblast growth factor, platelet-derived growth factor, antithrombin III, thrombin, and plasminogen activator inhibitor I) was tested to address the question of specificity. Apparent dissociation constants were determined by nitrocellulose filter binding, and the following values were observed for ligands 100t and 44t, respectively: 1 and 0.6 μM with basic fibroblast growth factor, 0.6 and 0.6 μM with platelet-derived growth factor (AB isoform), 3 and 12 μM with antithrombin III, >10 and >10 μM with thrombin, and >10 and >10 μM with plasminogen activtor inhibitor I. Moreover, binding of these ligands to VEGF in a buffer that contains 10 mM dithiothreitol (reduced VEGF has no biological activity) is weaker by at least 4 orders of magnitude (data not shown).

Competition. Competition experiments (performed with ^{32}P-labeled ligands and unlabeled competitors by the nitrocellulose filter partitioning technique) revealed that binding of all possible pairwise combinations of truncated ligands representing each of the six families (100t, family 1; 44t, family 2; 12t, family 3; 40t, family 4; 84t, family 5; and 126t, family 6) was mutually exclusive. Furthermore, all of these ligands were displaced from the protein by low molecular weight heparin (data not shown). Truncated ligands and low molecular weight heparin were used in these studies in order to maximize the probability of observing noncompeting ligand pairs.

Receptor Binding. To ensure that [^{125}I]VEGF binds to specific sites on HUVECs, we confirmed that unlabeled VEGF, but not EGF, inhibits binding of [^{125}I]VEGF to HUVECs in a concentration-dependent manner. Furthermore, as previously described (Myoken et al., 1991), Scatchard analysis (Scatchard, 1949) of the binding data (corrected for nonspecific binding) revealed two classes of receptors on HUVECs that bind VEGF with dissociation constants of $\approx 5 \times 10^{-11}$ M (7000 receptors/cell) and $\approx 5 \times 10^{-10}$ M (20 000 receptors/cell) (data not shown).

A group of truncated RNA ligands representing each of the sequence families (100t, family 1; 44t, family 2; 12t, family 3; 40t, family 4; 84t, family 5; and 126t, family 6) as well as random RNA were tested for their ability to inhibit binding of VEGF to its cell-surface receptors. All high-affinity ligands, but not random RNA, inhibited VEGF–VEGF receptor interaction in a concentration-dependent manner with half-inhibition in the 20–40 nM range (Figure 6).

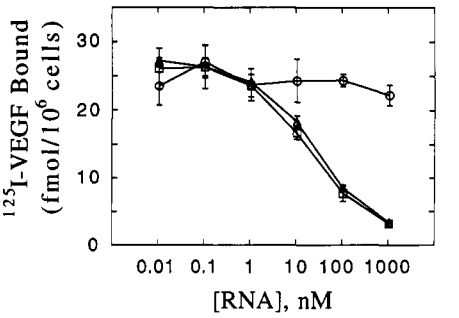

FIGURE 6: Effect of random RNA (O) and representative high-affinity RNA ligands 100t (family 1) (△) and 44t (family 2) (□) on binding of [^{125}I]VEGF to cell-surface receptors as a function of RNA concentration. Data points are averages of 2–4 determinations (error bars are standard deviations). The concentration-dependent inhibitory effect of high-affinity ligands representing other sequence families (12t, family 3; 40t, family 4; 84t, family 5; 126t, family 6) is virtually identical to that of ligands 100t and 44t.

DISCUSSION

Selection pressure of a SELEX experiment, which in this case demands high-affinity binding to VEGF, can concurrently favor discrete regions of sequence space. In other words, ligands with unrelated or distantly related sequences can have similar fitness in a defined competitive environment (Tuerk & Gold, 1990; Tuerk et al., 1992, 1994; Gold et al., 1993; Connell et al., 1993; Jellinek et al., 1993). In this SELEX experiment, ligands derived from the affinity-enriched pool could be classified into at least six different groups of similar sequences. Secondary structure predictions based on consensus, covariation, deletion analyses, and thermodynamically based folding algorithms indicate that these groups of ligands have distinct architectures. By extending the comparative analysis to the level of tertiary structures, we are exploring the possibility that ligands with different secondary structures can contact the protein with similarly shaped surfaces.

Although there are apparently multiple structural solutions to high-affinity binding to VEGF, functional properties of all examined ligands are consistent with their binding to the same general region of the protein. Binding of representative truncated RNA ligands is competitive not only with all other truncated ligands but also with heparin. Furthermore, all truncated ligands examined inhibit binding of VEGF to its cell-surface receptors. These and related observations from other SELEX experiments (Jellinek et al., 1993) suggest that many proteins may have sites that effectively represent "immunodominant" domains for nucleic acid binding. For heparin binding proteins, these sites appear to coincide with the heparin binding domains. As a caveat to these notions, we emphasize that even the minimal ligands described here are not small molecules (MW 10–13 kDa) and that cross-competition between ligands bound to distinct sites through partial overlap is possible. We also note that the existence of immunodominant sites does not preclude selection for ligands that bind to other sites, although modified selection strategies, such as saturation of the immunodominant sites with non-amplifiable ligands or selection by competitive displacement from other sites, may be necessary.

Biphasic binding is observed with the vast majority of the high-affinity ligands to VEGF. This type of binding has been observed previously with high-affinity RNA ligands from SELEX experiments targeting basic fibroblast growth factor (Jellinek et al., 1993) and also several other proteins (D. H. Parma, unpublished observations). A model that postulates two binding sites on the protein with different affinities for RNA can be dismissed since we use trace amounts of radiolabeled RNA in our binding experiments (<50 pM) and the protein is therefore present in large molar excess over RNA in most data points that define the binding curves (Figure 4). An RNA ligand would obviously not bind to a lower affinity site on the protein if a higher affinity site was available. We have previously suggested that biphasic binding could be adequately explained by a model in which at least two distinct species of RNA bind to their target with different affinities (Jellinek et al., 1993). Certainly, alternative conformers have been observed previously in RNA molecules of similar size (Heus et al., 1990; LeCuyer & Crothers, 1993). While it is tempting to speculate that the RNA ligands that exhibit biphasic binding represent assemblies of conformationally related species with slow interconversion rates, we emphasize

that the identities of these binding components are not known at present. It is notable in this regard that binding of the truncated ligands typically remains biphasic, although the number of possible competing structures is much smaller. The presence of two binding components in most high-affinity RNA ligands described here clearly complicates interpretation of both the competition and the receptor binding inhibition results. In the absence of further data, we prefer to confine our interpretation of these results to qualitative terms. Efforts to establish the identities of these distinct binding components are underway in our laboratories.

Biological actions of VEGF are mediated through specific tyrosine kinase receptors to which VEGF binds with dissociation constants between 10^{-10} and 10^{-12} M (Vaisman et al., 1990; de Vries et al., 1992; Myoken et al., 1991; Terman et al., 1991). All high-affinity RNA ligands tested bind to VEGF in a manner that antagonizes receptor binding. It is probable therefore that other high-affinity oligonucleotide ligands to VEGF will also be inhibitory. Recently, we have shown that specific, high-affinity oligonucleotide ligands to another potent angiogenic factor, basic fibroblast growth factor, inhibit binding of this growth factor to its cell-surface receptors (Jellinek et al., 1993). This observation is relevant in view of the recent finding that basic fibroblast growth factor and VEGF have a strong synergistic effect on the induction of angiogenesis *in vitro* (Pepper et al., 1992). Oligonucleotide-based compounds, or their mimetic analogs, clearly have the potential for becoming a new class of potent and specific inhibitors of pathological angiogenesis. Efforts aimed toward the development of therapeutic as well as diagnostic agents based on our findings are in progress.

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