Potent 2′-Amino-2′-deoxypyrimidine RNA Inhibitors of Basic Fibroblast Growth Factor

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ABSTRACT: Screening of random oligonucleotide libraries with SELEX [systematic evolution of ligands by exponential enrichment; Tuerk, C., & Gold, L. (1990) *Science 249,* 505−510] has emerged as a powerful method for identifying high-affinity nucleic acid ligands for a wide range of molecular targets. Nuclease sensitivity of unmodified RNA and DNA, however, imposes considerable restrictions on their use as therapeutics or diagnostics. Modified RNA in which pyrimidine 2′-hydroxy groups have been substituted with 2′-amino groups (2′-aminopyrimidine RNA) is known to be substantially more resistant to serum nucleases. We report here on the use of SELEX to identify high-affinity 2′-aminopyrimidine RNA ligands to a potent angiogenic factor, basic fibroblast growth factor (bFGF). High-affinity ligands with the same consensus primary structure have been isolated from two independent libraries of approximately 6×10^{14} molecules containing 30 or 50 randomized positions. Compared to unmodified RNA with the same sequence, 2′-aminopyrimidine ligands are at least 1000-fold more stable in 90% human serum. The sequence information required for high-affinity binding to bFGF is contained within 24−26 nucleotides. The minimal ligand m21A (5′-GGUGUGUGGAAGACAGCGGGUGGUUC-3′; G = guanosine, A = adenosine, C = 2′-amino-2′-deoxycytidine, U = 2′-amino-2′-deoxyuridine, and *C* = 2′-amino-2′-deoxycytidine or deoxycytidine) binds to bFGF with an apparent dissociation constant (K_d) of $(3.5 \pm 0.3) \times 10^{-10}$ M at 37 °C in phosphate-buffered saline (pH 7.4). Dissociation of m21A from bFGF is adequately described with a first-order rate constant of $(1.96 \pm 0.08) \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 5.9 min). The calculated value for the association rate constant ($k_{on} = k_{off}/K_d$) was 5.6×10^6 M^{-1} s^{-1}. Highly specific binding of m21A to bFGF was observed: binding to denatured bFGF, five proteins from the FGF family (acidic FGF, FGF-4, FGF-5, FGF-6, and FGF-7), and four other heparin binding proteins is substantially weaker under the same conditions with $K_d^{bFGF}/K_d^{protein}$ values ranging from $(4.1 \pm 1.4) \times 10^{-2}$ to $>10^{-6}$. Heparin but not chondroitin sulfate competed for binding of m21A to bFGF. In cell culture, m21A inhibited [^{125}I]bFGF binding to both low-affinity sites ($ED_{50} \approx 1$ nM) and high-affinity sites ($ED_{50} \approx 3$ nM) on CHO cells expressing transfected FGF receptor-1. Basic FGF-dependent migration of bovine aortic endothelial cells as well as bFGF-induced proliferation of human umbilical vein endothelial cells was also inhibited by m21A in a concentration-dependent manner with ED_{50} values of 50−100 nM. The 2′-aminopyrimidine RNA ligand m21A therefore represents a useful lead compound in our efforts to develop potent oligonucleotide-based angiogenesis antagonists.

It has recently become clear that random oligonucleotide libraries represent collections of molecular entities that encompass a diverse spectrum of functional properties (Gold et al., 1995). Screening of these libraries with the SELEX[1] (*s*ystematic *e*volution of *l*igands by *ex*ponential enrichment) technology has emerged as a powerful and generic tool for identifying oligonucleotide sequences that bind to a variety of molecular targets with high affinity and specificity (Tuerk & Gold, 1990; Ellington & Szostak, 1990; Gold et al., 1993, 1995; Gold, 1995). Most SELEX experiments reported to date have utilized unmodified (natural) RNA and DNA libraries. Nuclease-resistant oligonucleotides would be more broadly applicable as diagnostics and therapeutics.

To generate nuclease-resistant random oligonucleotide libraries, we used modified RNA in which the 2′-hydroxy (-OH) group in the pyrimidine nucleotides was substituted with the amino (-NH_2) functionality. This modification is known to impart a substantial degree of mechanism-based protection against the majority of serum ribonucleases (Hobbs et al., 1973; Pieken et al., 1991). Importantly, the 2′-amino substitution is compatible with the enzymatic steps of SELEX: the 2′-amino-2′-deoxypyrimidine triphosphates are incorporated into RNA by T7 RNA polymerase (Aurup et al., 1992), and the modified (2′-aminopyrimidine) transcripts are substrates for avian myeloblastosis virus reverse transcriptase. Recently, SELEX was used to identify 2′-

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

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aminopyrimidine ligands to human neutrophil elastase (Lin et al., 1994). As a part of our ongoing efforts to develop oligonucleotide-based therapeutics, we describe in this report the use of SELEX with modified (2′-aminopyrimidine) RNA libraries in which we target an important angiogenic factor, basic fibroblast growth factor (bFGF).

Proliferation of new blood vessels from established endothelium, or angiogenesis, is a rare and tightly regulated process in healthy adults. Certain pathological conditions, however, including psoriasis, rheumatoid arthritis, diabetic retinopathy, and tumor growth are often associated with extensive neovascularization (Folkman & Klagsbrun, 1987). With the increased appreciation for the importance of angiogenesis in disease progression, interest has developed for exploring the utility of angiogenesis inhibitors as therapeutic agents (Folkman, 1972; Fidler & Ellis, 1994; Weinstat-Saslow & Steeg, 1994). Several inhibitors of angiogenesis have been described to date, and some have entered clinical trials (Maione & Sharpe, 1990; O'Reilly et al., 1994; Weinstat-Saslow & Steeg, 1994, and references cited therein). The mechanism of inhibition of angiogenesis by these agents, however, is generally not understood, nor have these inhibitors been discovered on the basis of knowledge of the molecular events of angiogenesis.

Considerable insight into the molecular basis of angiogenesis has been gained over the past decade with the identification and characterization of a number of angiogenic proteins (Folkman & Klagsbrun, 1987; Folkman & Shing, 1992). Basic FGF, a pleiotropic effector for many cells of mesenchymal and neuroectodermal origin, is one of the more potent angiogenic growth factors described to date (Basilico & Moscatelli, 1992). It belongs to the FGF family of proteins that now includes eight other members (Basilico & Moscatelli, 1992; Tanaka et al., 1992; Miyamoto et al., 1993). Basic FGF promotes angiogenesis directly by binding to two classes of binding sites on endothelial cell surfaces: heparan sulfate proteoglycans (low-affinity sites) and tyrosine kinase receptors (high-affinity sites) (Moscatelli, 1987). Receptor binding leads to stimulation of cellular responses, such as DNA synthesis, cell migration, and protease production, that are essential for angiogenesis (Klagsbrun & Shing, 1985; Moscatelli et al., 1986; Presta et al., 1986; Mignatti et al., 1989). There is now considerable evidence that bFGF may play an important role in a number of angiogenesis-dependent disease states (Hori et al., 1991; Takahashi et al., 1991; Nguyen et al., 1994; Reed et al., 1994; Li et al., 1994). Antagonists of bFGF therefore may find useful clinical applications. Several bFGF antagonists have been described to date including suramin (Moscatelli & Quarto, 1989; Takano et al., 1994) and suramin derivatives (Braddock et al., 1994), aromatic anionic compounds such as aurin tricarboxylic acid (Benezra et al., 1992), sulfated polysaccharides (Nakayama et al., 1993), heparin-derived oligosaccharides (Ishihara et al., 1993), phosphorothioate DNA (not antisense) (Guvakova et al., 1995), and antibodies (Hori et al., 1991; Takahashi et al., 1991). With the exception of antibodies, most of these compounds are known to interact with many other proteins, do not have affinities in the desirable range, or have unacceptable toxicities.

Recently, we used SELEX to identify unmodified RNA ligands that bind to bFGF with high affinity, high specificity, and in a manner that inhibits binding of bFGF to its receptors (Jellinek et al., 1993). In this report we show that the considerably more stable 2′-aminopyrimidine RNA ligands exhibit the same high-affinity binding and high specificity for native bFGF. We also show that a minimal 2′-aminopyrimidine ligand (26-mer), synthesized by the solid-phase phosphoramidite method, inhibits bFGF-induced migration and proliferation of endothelial cells by inhibiting binding of bFGF to both high-affinity and low-affinity cell surface sites.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human bFGF (154 amino acid isoform) purchased from Bachem California (Torrance, CA) was used for *in vitro* selections and initial affinity screenings. Most subsequent experiments were done with recombinant human bFGF (147 amino acid isoform) purchased from R&D Systems (Minneapolis, MN). Recombinant human acidic FGF, FGF-4, FGF-5, FGF-6, keratinocyte growth factor, and platelet-derived growth factor were purchased from R&D Systems. Human antithrombin III and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Low molecular weight heparin ($M_r \approx 5000$) was from Calbiochem (La Jolla, CA). Unfractionated porcine heparin (from intestinal mucosa, $M_r \approx 6\,000-30\,000$), shark chondroitin sulfate (from cartilage, $M_r \approx 50\,000$), and pooled human serum were from Sigma (St. Louis, MO). Rabbit polyclonal antibody to human bFGF was prepared as described previously (Sato & Rifkin, 1989).

Chemical Synthesis of Modified Nucleoside Triphosphates, Phosphoramidites, and Oligonucleotide Ligands. An improved synthesis of 2′-amino nucleosides has been reported recently (McGee et al., 1995). Synthesis of 2′-amino-2′-deoxypyrimidine 5′-triphosphates for *in vitro* transcription has been described in detail (McGee et al., 1995). The nucleoside phosphoramidites were prepared by standard methods (Sinha et al., 1984; Scaringe et al., 1990). The 2′-amino group was protected as the trifluoroacetyl derivative (Imazawa & Eckstein, 1979). Solid-phase phosphoramidite synthesis was performed on an Applied Biosystems Model 394 oligonucleotide synthesizer according to the manufacturer's protocols. The oligonucleotides were cleaved from the solid support and deprotected by published methods (Scaringe et al., 1990).

SELEX. A detailed description of the SELEX procedure has been presented previously (Tuerk & Gold, 1990; Jellinek et al., 1994). RNA used in initial selections was prepared by *in vitro* transcription from synthetic DNA templates (Milligan et al., 1987). *In vitro* transcriptions were typically done by incubating DNA templates (100−500 nM) with T7 RNA polymerase (5 units/μL) and ATP, GTP, 2′-NH_2-CTP, and 2′-NH_2-UTP (all at 1 mM) for 4 h at 37 °C in 40 mM Tris-Cl buffer (pH 8.0) containing 12 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 0.002% Triton X-100, and 4% polyethylene glycol. Substitution of 2′-NH_2-CTP and 2′-NH_2-UTP for CTP and UTP typically reduced the transcription yield by 2−5-fold, as expected (Aurup et al., 1992). After *in vitro* transcription the RNA was purified on 8% polyacrylamide gels containing 7 M urea, eluted from the gel by the crush and soak method, alcohol precipitated, and resuspended 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). Prior to incubation with bFGF, the RNA was heated at 90 °C for 2 min and cooled on ice. Affinity selections were done by incubating bFGF with RNA for at least 15 min at 37 °C in PBS containing 0.01% human serum

albumin and 1 mM dithiothreitol. Partitioning of free RNA from protein-bound RNA was done by nitrocellulose filtration as described (Jellinek et al., 1994). Reverse transcriptions were typically done in a total volume of 50 μL by incubating the selected RNA with 2 units/μL avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) and 0.8 mM deoxynucleoside triphosphates in 50 mM Tris-Cl buffer (pH 8.3) containing 60 mM NaCl, 6 mM $Mg(OAc)_2$, and 10 mM dithiothreitol at 37 °C for 30 min and then at 48 °C for an additional 30 min. Under these conditions, the yield of cDNA obtained with the 2′-aminopyrimidine RNA template is only slightly lower (15%) than that obtained with unmodified RNA template. PCR amplifications were done by subjecting the cDNA (<100 nM) to 12−20 rounds of thermal cycling (30 s at 93 °C, 10 s at 52 °C, and 60 s at 72 °C) in 10 mM Tris-Cl (pH 8.4) containing 50 mM KCl, 7.5 mM $MgCl_2$, 0.05 mg/mL bovine serum albumin, 1 mM deoxynucleoside triphosphates, 5 μM primers, and 0.1 unit/μL Taq polymerase.

Cloning and sequencing of the affinity-enriched pools, nitrocellulose filter binding assays, determination of equilibrium dissociation constants, and minimal sequence determinations have been described recently (Schneider et al., 1992; Jellinek et al., 1993, 1994).

Dissociation Constants and Dissociation Rate Determinations. All binding studies were done at 37 °C in PBS containing 0.01% human serum albumin and 1 mM dithiothreitol, as described (Jellinek et al., 1993, 1994). Oligonucleotides for all binding experiments were purified on denaturing polyacrylamide gels and subjected to a denaturation/renaturation cycle in binding buffer at high dilution (≈1 nM) prior to incubation with the protein (Jellinek et al., 1993, 1994). Binding mixtures were typically incubated at 37 °C for 15 min prior to nitrocellulose filter partitioning. Equilibrium dissociation constants were determined according to the formalism described previously (Jellinek et al., 1993, 1994). The dissociation rate constant for ligand m21A was determined by measuring the amount of ^{32}P 5′-end-labeled m21A bound to bFGF (5 nM) as a function of time following the addition of >1000-fold excess unlabeled m21A, using nitrocellulose filter binding as the partitioning method.

Heparin and Chondroitin Sulfate Competition. Sulfated polysaccharide competition was examined by adding the ^{32}P 5′-end-labeled ligand m21A (final concentration, 50 nM) to an equimolar amount of bFGF preincubated for 15 min with varying amounts of low molecular weight heparin ($M_r \approx 5000$), unfractionated heparin, or chondroitin sulfate. The amount of ligand bound to bFGF following a 30-min incubation at 37 °C was determined by nitrocellulose filter binding. PBS containing 0.01% HSA and 1 mM dithiothreitol was used in all incubation mixtures.

Receptor Binding Assay. The effect of RNA ligands on binding of [^{125}I]bFGF to cell surface receptors was examined as described (Moscatelli, 1987; Jellinek et al., 1993). Briefly, CHO cells that express transfected FGF receptor-1 or BHK-21 cells were incubated for 2 h at 4 °C with 10 ng/mL [^{125}I]-bFGF and varying concentrations of oligonucleotide ligands. In some cultures a 100-fold excess of unlabeled bFGF (1 μg/mL) or 10 μg/mL heparin was included. At the end of the incubation period [^{125}]bFGF was removed from the low-affinity binding sites (heparan sulfates) by two washes with 2 M NaCl in 20 mM HEPES, pH 7.5. Radiolabeled bFGF was then removed from the high-affinity binding sites (receptors) by two washes with 2 M NaCl in 20 mM acetate, pH 4.0. The amount of bound [^{125}I]bFGF was determined by γ-counting.

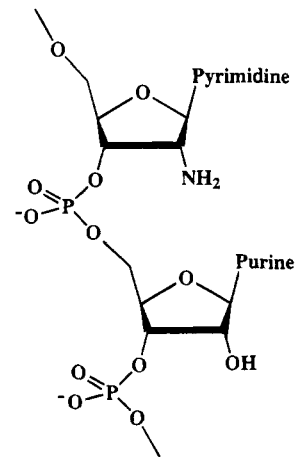

FIGURE 1: Structure of 2′-aminopyrimidine RNA.

Endothelial Cell Migration Assay. Confluent monolayers of bovine aortic endothelial (BAE) cells in 35-mm dishes were scraped with a razor blade to create a denuded area on the culture dish (Sato & Rifkin, 1989). The number of endothelial cells that moved from the edge of the wound into the denuded area in the presence of varying concentrations of oligonucleotide ligands was determined after 8 h. The assay was done in αMEM containing 0.1% bovine serum albumin.

DNA Synthesis Induction Assay. HUVECs (population doubling 5) were plated at 5000 cells/well on Collaborative Biomedical Products (Bedford, MA) fibronectin coated 96-well plates in M199 medium (90 μL) containing 1% fetal bovine serum and 4 mM HEPES (pH 7.4) (medium A) and supplemented with 10 μg/mL porcine heparin (Sigma, St. Louis, MO) for 90 min at 37 °C in an atmosphere containing 5% CO_2. At the end of this period, the cells were washed with serum-free M199, and medium A (without heparin) was added (90 μL/well). To the appropriate wells, bFGF in the presence of oligonucleotides was added in a total volume of 20 μL (PBS with 0.05% HSA) to final concentrations of 0.1 ng/mL bFGF and 0.1−1000 nM oligonucleotide. Control wells received 20 μL of buffer alone. After a 16-h incubation (37 °C, 5% CO_2), the cells were washed with serum-free M199 and incubated with spent medium A for an additional 9 h. [^3H]Thymidine (1 μCi/well, New England Nuclear, Boston, MA) was added along with unlabeled thymidine (final concentration, 0.01 μM) for the last 6 h. The cells were lysed in 0.2 N NaOH, and the DNA was precipitated with cold 10% trichloroacetic acid. The insoluble material was collected on glass-fiber filters and dried under an infrared lamp, and the amount of radioactivity was determined by scintillation counting.

RESULTS

We initiated two independent SELEX experiments (designated A and B) with 2′-aminopyrimidine RNA libraries (Figure 1) containing 30 and 50 contiguous randomized positions (Figure 2). The initial randomized pools consisted of approximately 6×10^{14} molecules (1 nmol). After 11 rounds of selection−amplification, we observed an improvement in overall affinity of the evolved ligand pools for bFGF of about 2 orders of magnitude (data not shown). Cloning

SELEX Experiment A

Starting RNA:
5'-GGGAGACAAGAAUAACGCUCAA[-30 N-]UUCGACAGGAGGCUCACAACAGGC-3'

PCR Primer 1:
5'-TAATACGACTCACTATAGGGAGACAAGAATAACGCTCAA-3'
T7 Promoter

PCR Primer 2:
5'-GCCTGTTGTGAGCCTCCTGTCGAA-3'

SELEX Experiment B:

Starting RNA:
5'-GGGAGGACGAUGCGG[-50 N-]CAGACGACUCGCCCGA-3'

PCR Primer 1:
5'-TAATACGACTCACTATAGGGAGGACGATGCGG-3'
T7 Promoter

PCR Primer 2:
5'-TCGGGCGAGTCGTCTG-3'

FIGURE 2: Starting RNA and PCR primers used in SELEX.

A

Clone	Evolved Sequence
14A	ACANGGAGUUGUGUGGAAGGCAGGGGGAGGuu
15A	UGUGUGGAAGGCAGUGGGAGGUUCAGUGGU
17A	AAAGUUGUGUGGAAGACAGUGGGAGGUGAA
21A	GUAGACUAAUGUGUGGAAGACAGCGGGUGGuu
29A	NNAGUUGUGUGGAAGACAGUGGGGGGUUGA
38A	GGUGUGUNGAAGACAGUGGGUNGUUUAGNC
49A	AUGGUGUGUGGAAGACAGUGGGUGGUUGCA
54A	ACUGUUGUGUGGAAGACAGCGGGUGGUUGA
60A	AAUGUAGGCUGUGUGGUAGACAGUGGGUGGuu
68A	GAUGUGUGGAGGGCAGUGGGGGGUACCAUA
74A	GGGGUCAAGGACAGUGGGUGGUGGUGGUGU
16B	UGCUGCGGUGCGCAUGUGUGGAAGACAGAGGGAGGUUAGAAUCAUGACGU
31B	ACAGACCGUGUGUGGAAGACAGUGGGAGGUUAUUAACGUAGUGAUGGCGC
38B	GCUGCGGUGCGCAUGUGUGGAAGACAGAGGGAGGUUAGGAAUCGUGCCGC
39B	GAAAACUACGGUGUGUGGAAGACAGUGGGAGGUUGGCAGUCUGUGUCCGU
62B	UCCAUCGUGGAAGACAGUGGGAGGUUAGAAUCAUGACGUCAGACGACUC
79B	UGUGAUUUGUGUGGAAGGCAGUGGGAGGUGUCGAUGUAGAUCUGGCGAUG
Consensus:	UGUGUGGAAGACAGUGGGDGGUU

B

Position:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Nucleotide:	U	G	U	G	U	G	G	A	A	G	A	C	A	G	U	G	G	G	D	G	G	U	U
Frequency:	15	16	15	17	17	15	16	16	15	17	13	17	17	17	12	17	17	17	-	16	17	17	13

FIGURE 3: Primary structure of a family of high affinity 2'-aminopyrimidine RNA ligands to bFGF. In panel A, aligned sequences isolated from the affinity-enriched 2'-aminopyrimidine RNA pools are shown, depicting nucleotides in the evolved region (nucleotides in the constant sequence region are only shown at positions where they agree with the consensus sequence and are distinguished by lowercase letters). Letters following the clone numbers designate the initial random library from which these ligands were isolated (A or B, Figure 2). The 2'-aminopyrimidines are depicted simply as C and U. For individual sequences, N is used to denote ambiguous positions on sequencing autoradiograms. In the consensus sequence, D denotes A, G, or U. In panel B, frequencies of consensus nucleotides are shown (out of the total of 17). The positions are numbered starting with the first conserved nucleotide.

and sequencing of 41 and 36 isolates from the affinity-enriched pools A and B resulted in the identification of several families of ligands on the basis of primary structure similarity in the evolved sequence region. The largest sequence family containing high-affinity ligands for bFGF is shown in Figure 3 (the 2'-amino-2'-deoxycytidine and 2'-amino-2'-deoxyuridine residues are depicted simply as C and U throughout this paper). Isolates from both SELEX experiments are represented in this family, suggesting a convergent solution to high-affinity binding to bFGF.

The stability of the 2'-aminopyrimidine-modified RNA ligands in pooled human serum (90%) is substantially increased compared to the unmodified RNA (Figure 4), as expected from previous studies (Pieken et al., 1991; Lin et al., 1994). Because very little degradation is detectable with the modified RNA ligand even after 2 days and the unmodified RNA with the same sequence is degraded extremely rapidly, we can only estimate that the increase in stability is at least 1000-fold.

Ligands that bind to bFGF with dissociation constants in the low nanomolar range were identified through affinity screening of all unique isolates from the affinity-enriched pools. Because our principal goal was to identify bFGF antagonists, we screened the best ligands for their ability to inhibit binding of bFGF to its cell surface receptors using baby hamster kidney (BHK-21) cells (data not shown). Ligand 21A (Figure 3) was identified as the most potent antagonist of receptor binding.

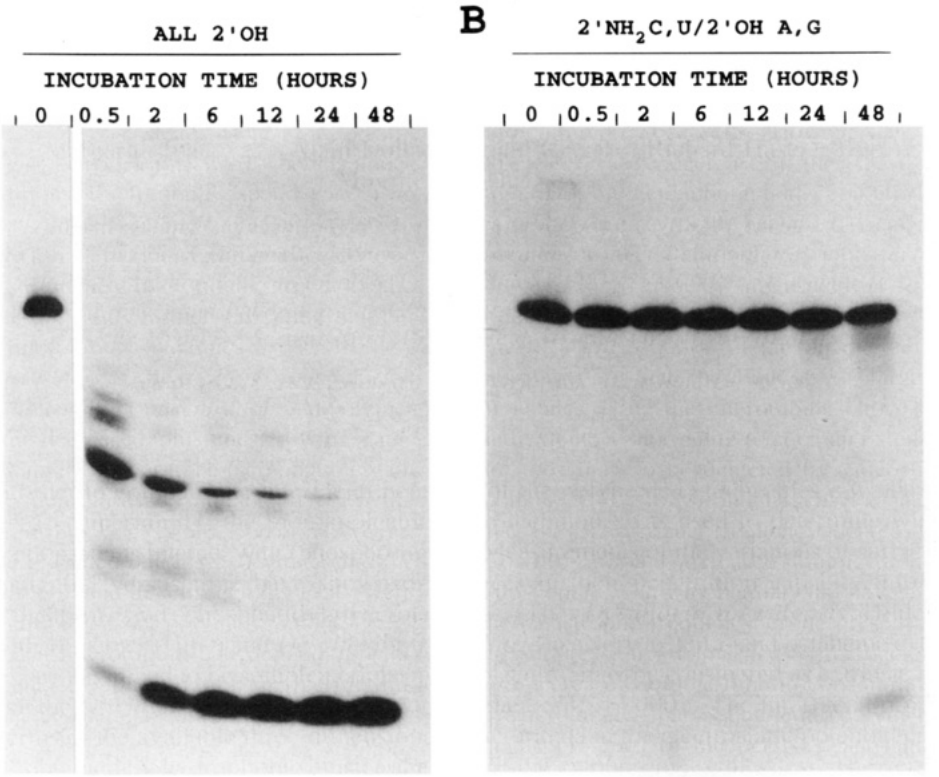

FIGURE 4: Comparison of stability of unmodified and 2′-aminopyrimidine-modified RNA in human serum. Unmodified and 2′-aminopyrimidine-modified RNAs corresponding in sequence to ligand 21A were prepared by *in vitro* transcription from the same synthetic DNA template. Both RNAs were internally radiolabeled ([α-^{32}P]ATP) and incubated at a concentration of 50 nM in 90% pooled human serum supplemented with 10 mM phosphate buffer (pH 7.4) at 37 °C. Aliquots from unmodified (panel A) and 2′-aminopyrimidine-modified RNA (panel B) incubation mixtures were withdrawn at indicated time intervals, frozen in dry ice until the end of the experiment, and loaded onto 20% denaturing polyacrylamide gels to resolve labeled degradation products.

The minimal sequence requirement for high-affinity binding was determined for ligand 21A by partial alkaline hydrolysis and reselection as described (Jellinek et al., 1994). These boundary experiments suggested that a core sequence of 24 nucleotides (5′-UGUGUGGAAGACAGCGGGUGG-UUC-3′) contained the high-affinity binding module (Figure 5). This minimal sequence represents essentially the entire consensus primary structure for this sequence family (Figure 3). It should be noted that the increased resistance to alkaline hydrolysis of the phosphodiester bonds adjacent (3′) to the 2′-aminopyrimidines becomes apparent upon resolution of the hydrolytic fragments on polyacrylamide gels where hydrolysis only 3′ to (ribo)purines is observed (Figure 5). As a consequence, these experiments allow us to establish the minimal sequence boundaries only to the nearest purine nucleotide.

We initially used *in vitro* transcription from synthetic DNA templates to test the suggested minimal ligand boundary. To increase the yield of *in vitro* transcription, we added two guanosines at the 5′ end of the minimal ligand (Milligan et al., 1987). This slightly longer ligand (5′-GGUGUGUG-GAAGACAGCGGGUGGUUC-3′, 26-mer) was found to bind to bFGF with severalfold higher affinity compared to the full-length ligand 21A (vide infra). Most subsequent binding and activity experiments were done with 2′-aminopyrimidine RNA ligands synthesized from phosphoramidite monomers on solid-phase support. Compared to *in vitro* transcription, synthesis of RNA by the solid-phase method has several advantages including independence from constraints imposed by the RNA polymerases, the ability to substitute a broad range of modified nucleotides at any position in the sequence, and the relative ease of scale-up. Chemically synthesized RNA ligands bind to bFGF with the same high affinity as those prepared by *in vitro* transcription. Solid-phase synthesis of 2′-aminopyrimidine RNA has allowed us to test several variants of the minimal sequence derived from ligand 21A. We found that the 26-mer bound to bFGF with severalfold higher affinity compared to the slightly smaller 24-mer (vide supra), confirming that the addition of two guanosines at the 5′ end confers a moderate binding advantage. In addition, we found that the 3′-terminal 2′-aminocytidine could be substituted with a 2′-deoxycytidine without a change in binding affinity. This 26-mer, 5′-GGUGUGUGGAAGACAGCGGGUGGUU*C*-3′, where *C* denotes either a 2′-aminocytidine or a 2′-deoxycytidine residue, will be referred to as ligand m21A. Substitution of all purines in m21A with 2′-deoxypurines or 2′-*O*-methylpurines, however, abrogates high-affinity binding (data not shown). The deoxyoligonucleotide analogue of this ligand, d(m21A) [5′-d(GGTGTGTGGAAGACAGCGGGTGGTTC)-3′], also exhibits very weak affinity for bFGF (Figure 6).

Binding of high-affinity minimal ligand m21A to bFGF is distinctly biphasic (Figure 6). All minimal ligands we tested, whether prepared by *in vitro* transcription or by chemical synthesis, exhibited this type of binding (data not shown). We interpret this result as evidence for the existence of at least two distinct species of RNA that have different affinities for the protein (Jellinek et al., 1993, 1994). The higher affinity fraction of m21A (71% of total RNA) binds to bFGF with a K_d of 0.35 ± 0.03 nM, whereas the minor fraction binds with substantially lower affinity (≈0.7 μM). The mole fraction of high-affinity species varies somewhat among preparations and is not affected by denaturation/renaturation cycles. Importantly, the high-affinity and low-

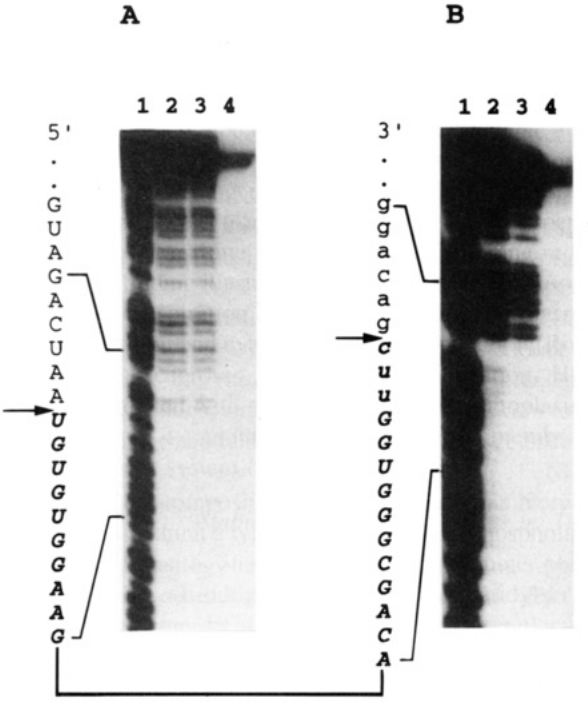

FIGURE 5: Determination of the minimal sequence requirement for high-affinity binding of ligand 21A to bFGF. Hydrolytic fragments resulting from partial alkaline hydrolysis of ligand 21A labeled at the 3′ (panel A) and the 5′ end (panel B) were resolved on 8% denaturing polyacrylamide gels. For the 5′-boundary determination (panel A), partially alkaline-hydrolyzed 3′-end-labeled RNA (lane 1) was reselected in the presence of 5 (lane 2), 0.5 (lane 3), or 0.125 nM bFGF (lane 4). As no hydrolysis occurs 3′ to the 2′-modified pyrimidines (note the missing bands), sequence assignment is straightforward without external markers. The 3′-boundary (panel B) was determined in an identical manner using 5′-end-labeled RNA (the lane assignments are the same as for panel A). Arrows indicate the 5′- and 3′-boundaries that define the minimal ligand (boldface italic letters). Lowercase and uppercase letters indicate nucleotides in the constant and evolved sequence regions, respectively.

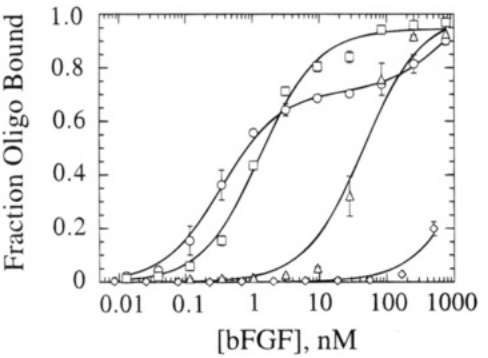

FIGURE 6: Binding of oligonucleotide ligands to bFGF. The fraction of ^{32}P-labeled oligonucleotide bound to varying concentrations of bFGF was determined by nitrocellulose filter partitioning. Oligonucleotides tested were ligand 21A (□), a random RNA library from which ligand 21A was selected (△), chemically synthesized minimal ligand m21A (○), and chemically synthesized d(m21A) (◇). Oligonucleotide concentrations in these experiments were <100 pM. The lines represent the fit of the data points to monophasic [ligand 21A, random library and ligand d(m21A)] or biphasic binding equations (ligand m21A) (Jellinek et al., 1993, 1994). Each data point represents the average of two determinations with standard deviations indicated. Binding reactions were done at 37 °C in PBS containing 1 mM dithiothreitol and 0.01% HSA as described (Jellinek et al., 1993).

affinity species can be separated on the basis of differential affinity for bFGF, confirming that biphasic binding is due to the existence of distinct species of RNA. This separation

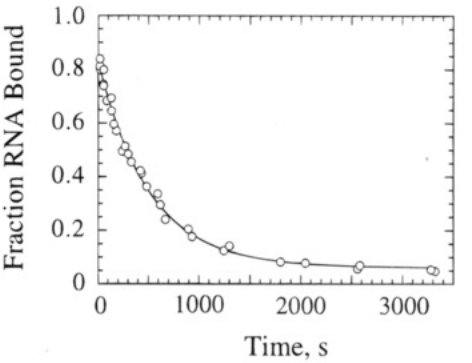

FIGURE 7: Dissociation of ligand m21A from bFGF. The amount of ^{32}P 5′-end-labeled ligand m21A bound to bFGF in PBS containing 0.01% HSA and 1 mM dithiothreitol at 37 °C was measured at the indicated time points by nitrocellulose filter binding following the addition of a >1000-fold excess of unlabeled m21A. The dissociation rate constant (k_{off}) was determined by fitting the data points collected in three separate experiments to the equation $A = A_o[\exp(-k_{off}t)] + b$, where A and A_o represent the normalized fractions of RNA bound to bFGF at any time (t) and at $t = 0$, respectively, and b represents binding of RNA to nitrocellulose filters in the absence of bFGF.

is most conveniently accomplished, on a small scale, by incubating the ligand with bFGF at low concentration (where only the high-affinity species binds) and separating the bound from the free RNA by nitrocellulose filter partitioning (data not shown). An effort aimed at characterizing the high- and low-affinity species is in progress.

Binding of the corresponding full-length ligand 21A is adequately described by a monophasic binding equation (Jellinek et al., 1993, 1994) with a K_d of 1.2 ± 0.2 nM. The random RNA library from which this ligand was identified binds to bFGF with an apparent affinity of 48 ± 12 nM (Figure 6).

The dissociation rate of the minimal ligand m21A from bFGF was determined by monitoring the decrease in the amount of bound radiolabeled ligand as a function of time following the addition of a large excess of unlabeled competitor. For this experiment we used the affinity-purified ligand m21A from which the low-affinity species had been removed. The value for the dissociation rate constant (k_{off}) of $(1.96 \pm 0.08) \times 10^{-3}$ s^{-1} ($t_{1/2}$ = 5.9 min) was calculated by fitting the data points to a first-order rate equation (Figure 7). The association rate constant was calculated to be 5.6×10^6 M^{-1} s^{-1}, assuming a simple bimolecular association model ($k_{on} = k_{off}/K_d$) and using a value of 0.35 nM for the K_d.

To examine specificity, we tested binding of m21A to heat-denatured bFGF, to five proteins from the FGF family of proteins [homology ranges from 30% to 55% over the core regions of these proteins (Basilico & Moscatelli, 1992)], and to four other heparin binding proteins (Table 1). In all cases, nitrocellulose filters were used to partition the free from the protein-bound oligonucleotide. Minimal bFGF ligand m21A exhibited high specificity for native bFGF. The only protein tested that showed a modest cross-reactivity was FGF-5, to which m21A bound with about 4% of the affinity for bFGF.

Both unfractionated heparin ($M_r \approx 6000-30\,000$) and low molecular weight heparin ($M_r \approx 5000$), at concentrations greater than 0.1 μg/mL, competed with m21A for binding to bFGF. Chondroitin sulfate, which does not bind to bFGF with appreciable affinity, was ineffective as a competitor up to 1500 μg/mL (Figure 8).

Table 1: Specificity of Ligand m21A for Native bFGF: Relative Binding Affinity ($K_d^{bFGF}/K_d^{protein}$) of Ligand m21A for Denatured bFGF, Five Proteins from the FGF Family, and Four Heparin Binding Proteins

protein[a]	$K_d^{bFGF}/K_d^{protein}$ [b]
bFGF (FGF-2)	1.0
denatured bFGF	$(8.2 \pm 1.4) \times 10^{-4}$
aFGF (FGF-1)	$(3.6 \pm 0.4) \times 10^{-4}$
K-FGF (FGF-4)	$(6.2 \pm 2.2) \times 10^{-4}$
FGF-5	$(4.1 \pm 1.4) \times 10^{-2}$
FGF-6	$(5.7 \pm 0.8) \times 10^{-4}$
KGF (FGF-7)	$(7.8 \pm 0.9) \times 10^{-4}$
VEGF	$(8.2 \pm 0.9) \times 10^{-4}$
PDGF AB	$(2.5 \pm 0.3) \times 10^{-3}$
antithrombin III	$(8.2 \pm 1.2) \times 10^{-6}$
thrombin	$(3.1 \pm 0.5) \times 10^{-5}$

[a] Abbreviations: aFGF, acidic FGF; KGF, keratinocyte growth factor; VEGF, vascular endothelial growth factor; PDGF AB, platelet-derived growth factor, AB isoform. [b] The K_d^{bFGF} value of $(3.5 \pm 0.3) \times 10^{-10}$ M for ligand m21A is used for computing the relative affinities. The ligand was prepared by *in vivo* transcription from synthetic DNA templates. Errors are computed as fractional uncertanties from independent K_d measurements (Taylor, 1982).

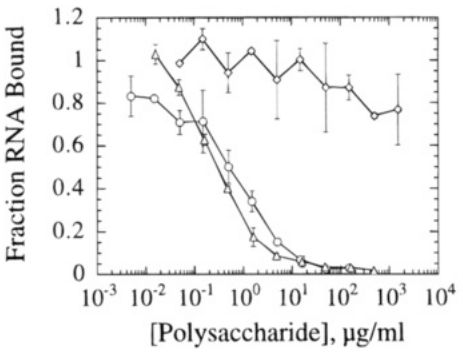

FIGURE 8: Competition by heparin and chondroitin sulfate for binding of RNA ligand m21A to bFGF. Binding of ^{32}P 5′-end-labeled RNA ligand m21A to bFGF (both at 50 nM) in the presence of varying amounts of low molecular weight heparin (○), unfractionated heparin (△), and chondroitin sulfate (◇) was determined by nitrocellulose filter binding and is expressed as the normalized fraction of RNA bound to bFGF. Data points represent the average values of two measurements with standard deviations shown.

Inhibition of [^{125}I]bFGF binding to cell surface receptors by the RNA ligand m21A is summarized in Figure 9. Ligand m21A inhibits binding to low-affinity sites on CHO cells expressing transfected FGF receptor-1 in a dose-dependent manner with an ED_{50} of 1 nM (Figure 9A). Binding to high-affinity sites is also inhibited with a slightly higher ED_{50} of about 3 nM (Figure 9B). The control DNA ligand, d(m21A) (DNA sequence equivalent of m21A), had no effect on [^{125}I]-bFGF binding to either class of binding sites at concentrations up to 1 μM. As expected, 10 μg/mL heparin competed efficiently for [^{125}I]bFGF binding to low-affinity sites but not for binding to high-affinity sites (Moscatelli, 1987). Unlabeled bFGF competed well for both low- and high-affinity [^{125}I]bFGF binding, and the inhibition was approximately the same as that observed with ligand m21A, indicating that the residual binding observed at high concentrations of the RNA antagonist was probably nonspecific (data not shown).

The effect of the RNA ligand m21A on endothelial cell motility was examined by measuring the migration of endothelial cells into a denuded area. The movement of bovine aortic endothelial cells (BAE) is dependent on endogenous bFGF and can be inhibited by addition of

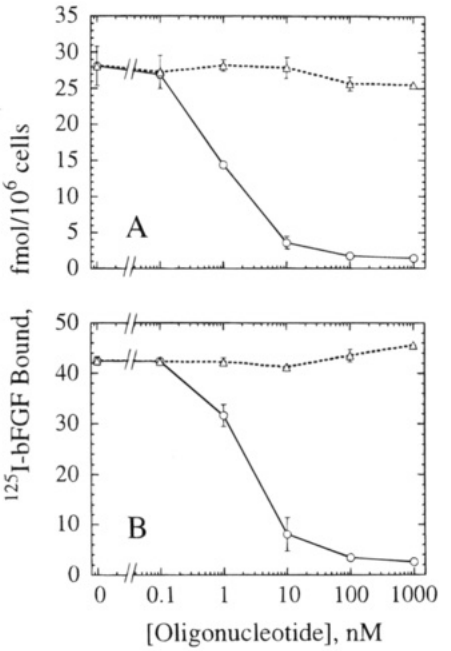

FIGURE 9: Effect of oligonucleotide ligands on binding of [^{125}I]-bFGF to CHO cells expressing transfected FGF receptor-1. Following incubation of transfected CHO cells with 10 ng/mL of [^{125}I]bFGF for 2 h at 4 °C with varying amounts of ligand m21A (○) and d(m21A) (△), the amount of radiolabeled bFGF bound to low-affinity sites (panel A) and high-affinity sites (panel B) was determined as described in Experimental Procedures. Each data point represents the average of two measurements with standard deviations shown.

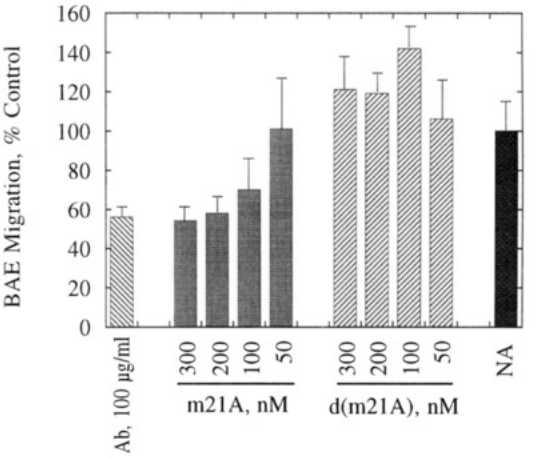

FIGURE 10: Effect of oligonucleotide ligands on BAE cell migration. Migration of BAE cells was determined by counting the number of cells that moved into a denuded area over a period of 8 h. Migration in the presence of varying amounts of ligand m21A, control DNA ligand d(m21A), or 100 μg/mL anti-bFGF antibody is expressed as the percent of untreated control (NA). The average values from two experiments are shown with standard errors.

neutralizing antibodies to bFGF (Sato & Rifkin, 1989). Ligand m21A inhibited BAE migration in a dose-dependent manner at concentrations greater than 50 nM (Figure 10). The control DNA ligand d(m21A) did not inhibit BAE migration at concentrations up to 300 nM. In fact a moderate stimulation of migration was observed. The extent of inhibition at high RNA ligand concentrations varied significantly between experiments ranging from almost 100% to <50% inhibition (data not shown). This is probably related in part to variable expression of other motility-inducing growth factors by BAE cells between experiments as well as subtle differences in the state of the cells at the time of

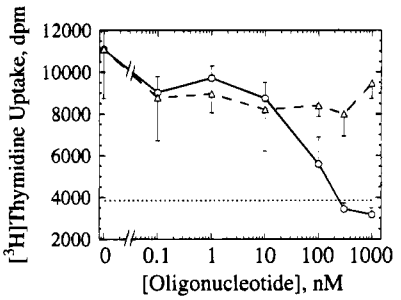

FIGURE 11: Effect of oligonucleotide ligands on induction of DNA synthesis by bFGF in HUVECs. HUVECs at 5000 cells/well were plated on fibronectin-coated 96-well plates in M199 medium containing 1% fetal bovine serum and 4 mM HEPES (pH 7.4), and were incubated for 16 h in the presence of 0.1 ng/mL bFGF and increasing concentrations of ligand m21A (○) or the DNA control ligand (△). In this experiment it was necessary to include a 3′ phosphorothioate cap in the control DNA ligand [5′-d(GGTGTGTGGAAGACAGCGGGTGGTTC)-P; P is the 3′ phosphorothioate cap sTsTsTsTsT-3′, where s indicates a phosphorothioate linkage] in order to prevent exonucleolytic degradation. The amount of [^{3}H]thymidine incorporated into DNA over a period of 6 h following the stimulation with bFGF was determined by lysing the cells with alkali and determining the amount of trichloroacetic acid-precipitable radioactivity by scintillation counting. Each point is the average of triplicate determinations with standard deviations shown. The dotted line represents the amount of [^{3}H]thymidine incorporated in the absence of bFGF.

wounding. Importantly, the total amount of motility that could be inhibited by m21A at high concentrations was comparable in all experiments to the effect of 100 μg/mL neutralizing bFGF antibody. This concentration of antibody is generally sufficient to inhibit all of the bFGF-dependent migration of endothelial cells.

Inhibition of bFGF-induced DNA synthesis was examined in cultures of human umbilical vein endothelial cells (HUVECs). Increase in the uptake of [^{3}H]thymidine was inhibited by ligand m21A in a concentration-dependent manner with an ED_{50} of about 50 nM (Figure 11). The control DNA ligand was not inhibitory at concentrations up to 1 μM.

Stabilities of the minimal RNA and DNA oligonucleotides under conditions of the cell culture assays were examined in separate experiments by denaturing polyacrylamide gel electrophoresis of 5′-[^{35}S]thiophosphate end-labeled ligands. In the bFGF receptor binding and the endothelial cell migration assays, neither the RNA ligand (m21A) nor the DNA control [d(m21A)] was significantly degraded over the duration of the experiments at oligonucleotide concentrations used in the assays. In the DNA synthesis induction assay, the extent of the RNA ligand degradation was negligible (<1%) over the 16-h incubation period at high ligand concentrations (300 nM) but was more significant at lower concentrations (1 nM) where approximately 20% of the ligand was degraded over the same period. The control DNA ligand, which in this experiment required a 3′-phosphorothioate cap to protect it from 3′ to 5′ exonucleolytic degradation, was degraded by <20% and <10% at 1 nM and 1 μM.

DISCUSSION

SELEX is a versatile technology that is not limited to the use of unmodified RNA or DNA libraries. The only requirement is that the modified nucleoside triphosphates used to generate random libraries are accepted as substrates by the relevant RNA or DNA polymerases. In RNA SELEX experiments, an additional requirement is that the modified transcripts can serve as templates for reverse transcription. Provided that these conditions are met, SELEX is open to a vast array of new chemistries that can expand the functional group repertoire of nucleic acid libraries or increase their resistance to nucleases (Lin et al., 1994; Latham et al., 1994; Eaton & Pieken, 1995). The use of nuclease-resistant random libraries may be particularly advantageous when SELEX is used as a tool for developing new therapeutic agents (Gold, 1995).

Comparison of SELEX experiments targeting the same molecule (bFGF) performed with unmodified RNA libraries (Jellinek et al., 1993) or the 2′-aminopyrimidine RNA libraries leads to several useful insights. Overall, the outcome of the two SELEX experiments is similar in many respects. The modified RNA ligands described in this report have the same high affinity and specificity as the unmodified RNA ligands identified previously. Furthermore, soluble heparin competes for binding of both the unmodified and the 2′-aminopyrimidine RNA ligands to bFGF. Importantly, the efficiency of SELEX for reproducibly identifying rare molecules with closely related sequences, previously demonstrated with unmodified RNA (Jellinek et al., 1993), is not compromised with the use of modified RNA libraries. We have shown here that high-affinity ligands to bFGF with the same consensus sequence have been identified in two independent SELEX experiments, within the context of 30 or 50 randomized positions (Figure 3). This is especially noteworthy in view of the relative scarcity of these molecules in the initial random pools. From its information content (Schneider et al., 1986), we can estimate that the consensus primary structure 5′-UGUGUGGAAGACAGUGGGDGGUU-3′ (D = A, G, or U) is expected to occur with a frequency of 3.4×10^{-13} or 1.2×10^{-12} in a window of 30 or 50 randomized positions. In a starting pool of approximately 6×10^{14} molecules, ligands that contain this consensus sequence are expected to occur only 200 or 720 times, respectively. In the SELEX experiments described here, extending the randomized region from 30 to 50 nucleotides does not appear to confer a selective advantage.

The 2′-amino substitution, on the other hand, clearly has a major effect on the primary structure of high-affinity ligands. Sequences of ligands derived from SELEX experiments targeting bFGF done with the modified (Figure 3) and unmodified RNA (Jellinek et al., 1993) are quite dissimilar. It is interesting to note that individual nucleotides occur at noticeably unequal frequencies in the evolved sequence regions of the affinity-enriched ligand pools (Figure 3) with guanosine being conspicuously overrepresented (43%), adenosine and uridine occurring at about equal frequencies (22% and 21%), and cytidine being underrepresented (14%). In essence, most of the ligands are G-rich and C-poor (this imbalance was not present in the random region of the synthetic DNA template). The overabundance of guanosine residues in particular has been observed in affinity-enriched pools in other SELEX experiments done with 2′-aminopyrimidines (Lin et al., 1994; Andrew Stephens, Torsten Wiegand, and David Parma, unpublished observations). It is uncertain at present whether the unequal nucleotide representation results from different efficiencies of nucleotide incorporation during enzymatic steps or is a reflection of the generally greater binding fitness of the G-rich sequences in the context of 2′-aminopyrimidine RNA.

A convincing consensus secondary structure in the group of sequences shown in Figure 3 is not apparent, although there are several possible (and typically mutually exclusive) consensus stems of three base pairs: UGU (positions 1−3 or 3−5) can hybridize with ACA (positions 11−13) or GUG (positions 14−16), and GAC (positions 10−12) or AGU (position 13−15) can hybridize with GUU (positions 21−23). We have not detected significant covariation between any of the positions in this sequence set, in part as a consequence of the highly conserved consensus primary structure. There is insufficient information in this data set to make a meaningful secondary structure prediction based on Watson−Crick base-pairing. It is relevant to note in this regard that although the amino-to-hydroxyl substitution at the 2′ position does not interfere directly with hydrogen bonding between complementary nucleic acids, it has been shown recently that 2′-aminouridine-containing oligonucleotides undergo helix-to-coil transitions at substantially lower temperatures (Miller at al., 1993; Aurup et al., 1994). The low propensity for duplex formation between poly(2′-aminouridine) and poly(riboadenosine) has also been observed previously (Hobbs et al., 1973). This is in accord with our preliminary observations that the T_m values of the 2′-aminopyrimidine-containing ligands are unusually low, often below 30 °C (Bruce Feistner and Stanley C. Gill, unpublished observations). It should be emphasized that all affinity selections and binding studies were done at 37 °C. Detailed examination of the thermal melting profiles of these ligands in the presence and absence of protein is currently underway.

Although the minimal ligand m21A contains four pairs of guanosine residues, the formation of an intramolecular G-quartet structure, suggested in other SELEX experiments (Macaya et al., 1993; Lin et al., 1994), is improbable because the two guanosine residues at the 5′ terminus are not required for high-affinity binding to bFGF (although their presence does improve binding severalfold). The formation of *inter*molecular G-quartet structures is also unlikely, as all binding experiments were done at high ligand dilutions (<100 pM). At these ligand concentrations no indication of higher-order structure formation is apparent on non-denaturing polyacrylamide gels (data not shown).

The pK_a of the 2′-amino group has been estimated to be about 6.2−6.5 (Verheyden et al., 1971; Gushelbauer & Jankowski, 1980), and therefore most of these groups are expected to be neutral. It is probable, however, that the 2′-amino groups in RNA molecules described here may cover a broader range of pK_a values in response to their local microenvironment, which in the bound state includes close proximity to the protein. The effect of the internal positively charged moieties on RNA structure remains to be explored. In view of the known importance of counterions in nucleic acid structure and function, this effect may be of considerable significance.

The 2′-aminopyrimidine ligand m21A effectively inhibits binding to bFGF to both low-affinity (heparan sulfate proteoglycans) and high-affinity sites (receptor tyrosine kinase) on CHO cells at low nanomolar concentrations. In this regard, the behavior of ligand m21A is similar to that of the unmodified RNA ligands we described previously (Jellinek et al., 1993). Effective inhibition of bFGF binding to both receptor classes distinguishes oligonucleotide ligands from other polyanions such as heparin, heparin-derived oligosaccharides, and dermatan sulfate that only inhibit binding of bFGF to low-affinity sites (Moscatelli, 1987; Ishihara et al., 1993). It is well established that the two classes of binding sites act cooperatively in binding of bFGF to cell surfaces (Yayon et al., 1991; Rapraeger et al., 1991; Moscatelli, 1992; Pantoliano et al., 1994; Roghani et al., 1994). Inhibition of bFGF binding to both receptor classes may be required for efficient neutralization of activity. It has been shown recently that bFGF is internalized via receptor-mediated as well as heparan sulfate-mediated pathways (Roghani & Moscatelli, 1992; Rusnati et al., 1993; Rieland & Rapraeger, 1993). Although the biological function of internalized bFGF remains to be elucidated, there is evidence that a fraction of internalized bFGF is translocated to the nucleus (Baldin et al., 1990) where it may regulate gene expression (Amalric et al., 1994).

It has been demonstrated recently that bFGF dissociates from the cell surface heparan sulfates with half-times on the order of several minutes, but much more slowly from the receptors, where dissociation half-times of about 2 h are observed (Moscatelli, 1992). The dissociation rate of bFGF from the high-affinity RNA ligand ($t_{1/2} \approx 6$ min) is thus comparable to the dissociation rate of bFGF from the low-affinity sites. The ability to compete effectively with binding to the tyrosine kinase receptors is likely to be an important determinant of inhibitor potency, since for many growth factors low fractional receptor occupancy appears to be sufficient to produce a maximal response (Carpenter & Cohen, 1979; Neufeld & Gospodarowicz, 1985; Massague & Like, 1985). In this regard, it may be advantageous to employ selection strategies that favor enrichment in ligands with slow dissociation rates as well as with low dissociation constants.

Compared to the ED_{50} value for inhibition of receptor binding, an approximately 30−100-fold higher dose of m21A ($ED_{50} \approx 100$ nM) is required to inhibit endothelial cell motility. In view of its high specificity, it is formally possible that m21A binds to bovine bFGF, which differs from human bFGF by only two amino acids (Abraham et al., 1986a,b), with lower affinity. Alternatively, as discussed above, low fractional receptor occupancy by endogenously produced bFGF may be sufficient to induce the maximal effect. In any event, all of the migration-inducing activity that could be inhibited by anti-bFGF antibody could also be inhibited at high concentrations of m21A. This is an important observation because the induction of endothelial cell migration is a crucial component of angiogenesis. Ligand m21A also inhibits bFGF-induced proliferation of endothelial cells with the ED_{50} value of about 50 nM. It should be noted that the inhibition by m21A of the bFGF-dependent increase in DNA synthesis can be overridden by high (10 ng/mL) concentrations of the mitogen (unpublished observations).

SELEX with modified oligonucleotides promises to be an exciting new direction in screening of combinatorial libraries. Recent work in our laboratories suggests that a wide variety of chemical functionalities can be incorporated into nucleic acids to create amplifiable libraries with novel physical and chemical properties [reviewed by Eaton and Pieken (1995)]. These developments are likely to further increase the utility of SELEX as a powerful tool for drug discovery.

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