RNA ligands to human nerve growth factor

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ABSTRACT

High affinity RNA ligands to human nerve growth factor (NGF) were selected from pools of random RNA using SELEX [Tuerk, C. and Gold, L. (1990) *Science*, 249, 505–510]. Nerve growth factor, which is a protein required for the development of neurons, is not known to bind nucleic acids as part of its natural function. We describe two of the selected RNA molecules in detail. One of them is highly structured, folding into a pseudoknot with an additional hairpin-loop; this structure provides salt-resistant binding to NGF. The other is unstructured and elevated salt concentrations inhibit its binding. These molecules compete with each other for NGF binding. Our RNAs may furnish useful diagnostic tools for the study of an important neurotrophic protein; additionally, they illustrate another example of the potential for nucleic acids to take part in novel binding interactions.

INTRODUCTION

Since the original publication describing the SELEX procedure (1) that was used to isolate RNAs with high specificity and affinity for T4 DNA polymerase, there have been some forty other peptides and proteins targeted (for reviews see refs. 2,3). SELEX has become a widely used technique for identifying and characterizing interactions between RNA and proteins that are known to bind nucleic acids (4–8) as well as proteins and peptides lacking nucleic acid binding properties (9–13). In addition, this procedure has been used to study interactions between nucleic acids and other small molecules (14–18) and to investigate the catalytic power of RNA (19–21).

Using the relatively small, basic human nerve growth factor (NGF) protein, which has no known nucleic acid binding property, we set out to isolate specific binders from a random pool of molecules. NGF is a homodimeric protein consisting of monomers that are 13 kDa each (22). This protein is essential for growth, differentiation and maintenance of neurons (23,24).

Our results show that stringent binding conditions facilitate the selection of specific RNA ligands. Here, we describe the binding properties and determine the structures for two of the selected RNA molecules. One is highly structured, it forms a pseudoknot

(25) with an additional hairpin-loop; its binding to NGF is resistant to high ionic strength, suggesting it makes many non-ionic, base-specific contacts with the protein. The second is unstructured and its binding to the basic NGF protein appears to be chiefly ionic in nature. The two ligands compete with one another for binding. In addition, we demonstrate the specificity with which the selected ligands bind to NGF. Of the four proteins tested, our selected RNAs show high affinity binding to nerve growth factor only.

MATERIALS AND METHODS

Reagents

Human nerve growth factor was provided by Genentech. Molarities reported here are those for NGF monomers. Human thrombin was purchased from Enzyme Research Labs, Agouron Pharmaceuticals provided HIV-1 reverse transcriptase (RT) and calf thymus histone H1 was purchased from Boehringer Mannheim. AMV RT was purchased from Life Sciences Products, Inc., *Taq* DNA polymerase from Perkin Elmer Cetus and T7 RNA polymerase from US Biochemical. T4 DNA ligase, T4 polynucleotide kinase and *Bam*HI and *Hind*III restriction endonucleases were purchased from New England Biolabs.

All oligodeoxynucleotides used in this work were synthesized on Applied Biosystems DNA/RNA Synthesizers at the University of Colorado and at Genentech. Table 1 shows their sequences and describes their functions. An equimolar mixture of the four nucleoside 3'-phosphoramidites was used to synthesize the random portion of the 30N template. The synthetic oligoribonucleotide ligand to human thrombin, 27.33 (Fig. 1), was a gift from Dr Diane Tasset of NeXstar Pharmaceuticals, Inc.

Radio-labeled nucleotides were purchased from New England Nuclear Research Products. Dimethyl sulfate was purchased from Sigma and kethoxal (2-keto, 3-ethoxy-*n*-butyraldehyde), also used for chemical modification, was a gift from Dr Harry F. Noller of the University of California at Santa Cruz.

SELEX

Two separate SELEX experiments were performed. The primary difference between the two was the ionic strength of the binding buffers used during the selection steps: the buffers contained 50

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Figure 1. Sequences of the RNA molecules described in the text. (a) 30N-1, the SELEX starting material and 30N₀#4, an arbitrary sequence isolated out of the random 30N-1 DNA pool and used as a control in binding studies. Only the sequence derived from the 30 random bases are shown. (b) Some of the selected NGF ligands; the fraction of the final SELEX pools these molecules made up is indicated in parentheses. Single base variants are shown as single letters above the major sequence. The underlined sequences highlight the identity between RNA H1 and RNAs L2 and L6. (c) RNAs used in the specificity experiments. RNA H1-T is a truncated version of RNA H1; alterations introduced into the H1 sequence are highlighted in boldface type. 27.33 and RT Lig2 are SELEX ligands to human thrombin and HIV-1 reverse transcriptase respectively. They are described in detail elsewhere (12,38).

mM Tris acetate, pH 7.5 and either 50 mM (Buffer L) or 200 mM (Buffer H) NaCl.

To generate the random RNA pool for the experiment in Buffer L, 50 pmol of an oligonucleotide containing 30 random positions was amplified by PCR. Products were transcribed using T7 RNA polymerase (26). The selection steps were carried out essentially as described (1,6,27), except the selective binding reactions were incubated for 3 min at 37°C. The RNA was pre-filtered through nitrocellulose prior to the first, fourth, seventh, ninth and tenth rounds of selection and the cDNA was gel purified every third round. cDNA synthesis, PCR and RNA transcription steps were carried out as previously described (1). Sequence analysis of the tenth round RNA pool showed a non-random sequence distribution.

The random pool for the experiment in Buffer H was generated as above, except the PCR product was gel purified and the RNA was labeled with [α -³²P]UTP to a specific activity of ~1.6 Ci/mmol and gel purified. In the selective binding reactions, the labeled RNA was mixed with several different concentrations of NGF protein, incubated at 37°C for 3 min and vacuum filtered

through nitrocellulose. The filters were then washed with 5 ml of 50 mM Tris-acetate, pH 7.5. Radioactivity on the filters was measured by Cerenkov counting. RNA was eluted from these filters for the subsequent cDNA synthesis steps.

Isolation of specific sequences

DNA generated by the PCR from the tenth round of the selection in Buffer L and from the seventh round of the selection in Buffer H were gel purified and restricted with *Bam*HI and *Hind*III endonucleases. Using standard protocols and methods (29), these were subcloned into pUC18 and used to transform *E.coli* strain JM103. Both strands of plasmids from individual isolates were sequenced (6).

Computer sequence analysis

Sequences were grouped at the primary sequence level as described (29–31). Programs used for secondary structure analysis have also been described (32–34).

Table 1. Sequences and functions of oligodeoxynucleotide primers and templates used in this work

Sequence no.	Function(s)	Oligonucleotide sequence
1	Random template	5'-GCCGGATCCGGGCCTCATGTCGAA[N ₃₀]TT-GAGCGTTTATTCTGAGTCCC-3'
2	Upstream PCR primer with T7 promoter	5'-CCGAAGCTTAATACGACTCACTATAGGGA-GC TCAGAATAAACGCTCAA-3'
3	Downstream PCR primer, cDNA synthesis primer, sequence analysis primer	5'-GCCGGATCCGGGCCTCATGTCGAA-3'
4	Chemical modification primer	5'-GCCGGATCCGGGC-3'
5	T7 promoter, sequence analysis primer	5'-TAATACGACTCACTATA-3'
6	Template for transcription of H1-T RNA	5'-CTCATGTCGAATATGTAGCTATTCTGTTGGG-GAGCATGAGTTCTCCCTATAGTGAGTCGTATTA-3'
7	Template for transcription of HIV-RT Lig2 RNA	5'-AGTGCAATTTCCCGTTGCACTTCGGA-CCTATAGTGAGTCGTATTA-3'

Sequence 7 has been described elsewhere (38)

Nitrocellulose filter binding assays

Nitrocellulose filter binding assays were performed as described (25,26). Specific RNA sequences were transcribed *in vitro* by T7 RNA polymerase as described (26–35). RNAs were labeled with [α -³²P]UTP or -GTP to specific activities of ~300 Ci/mmol as described (36). Synthetic RNA ligand to human thrombin was phosphorylated using [γ -³²P]ATP to a specific activity of ~3000 Ci/mmol. RNA concentration in the binding reactions was ~10⁻¹⁰ M. In the salt titration, the NGF concentration was fixed at 10⁻⁶ M. For assays involving NGF, the binding buffers were the same as SELEX buffers L or H, described above (except in the salt titrations, where the NaCl concentration ranged from 0 to 500 mM). Binding reactions were incubated for 3 min at 37°C. The buffers and reaction conditions for binding assays involving HIV-1 reverse transcriptase and human thrombin were as described (11,37). The binding buffer for calf thymus histone H1 contained 100 mM potassium acetate, 1 mM dithiothreitol and 50 mM Tris acetate, pH 7.5; binding reactions were incubated for 5 min at 37°C. The reactions were filtered and the filters were dried and counted as described (35). Binding assays were repeated four to six times and the data were analyzed using the program described (35). Equilibrium competition experiments were performed in a fashion similar to previous studies of DNA–protein interactions (38). NGF protein (10⁻⁶M) was added to reaction mixtures containing 5 × 10⁻⁷ M labeled RNA and unlabeled competitor RNA (varying from 0 to 10⁻⁵ M). Incubation times and temperatures for these reactions are as described above.

Chemical modification assays

Chemical modification was carried out essentially as described (39,40). 30 pmol (0.75 μ g) of RNA was incubated at 45°C for 5 min in the modification buffer (80 mM potassium cacodylate, pH 7.2, 50 mM sodium chloride; reactions contained 20 mM magnesium chloride where indicated). Where NGF was present, 27 μ g (2 × 10⁻⁵ M final concentration) of protein was incubated with RNA for 15 min at 37°C. Modifying reagents dimethyl sulfate (2 μ l of a 1:25 dilution in 95% ethanol) and kethoxal (5 μ l of a 18 μ g/ml solution) were added to a final reaction volume of 60 μ l at 37°C for an additional 8.5 min. After this time, DMS

reactions were stopped by addition of 75 μ l stop buffer (1 M Tris-acetate, pH 7.5, 1.5 M sodium acetate, 0.1 M EDTA and 1 M 2-mercaptoethanol) and kethoxal reactions were stopped with the addition of potassium borate (pH 7.5) to a final concentration of 20 mM. Samples are precipitated using 3 vol of 95% ethanol. RNA was then phenol extracted twice and chloroform extracted once, precipitated with ethanol and resuspended in deionized, distilled water at 0.5 pmol/ μ l concentration. RNA was quick frozen and stored at -20°C. Kethoxal modified RNA samples were stored in 15 mM potassium borate. Subsequent reverse transcription and gel electrophoresis steps were performed as described (39), except that α -³²P labeled dCTP with a specific activity of 800 Ci/mmol was used.

RESULTS

SELEX

We performed SELEX experiments as described in the Materials and Methods section. The starting RNA contained 30 random bases flanked by fixed sequences for primer hybridization (30N-1 RNA, Fig. 1a). Random RNA binds to NGF with a micromolar K_d in low salt buffer. We performed two separate selection experiments, varying the stringency of the selective binding buffers: the buffers contained 50 mM Tris-acetate and either 50 mM (Buffer L) or 200 mM (Buffer H) NaCl. The experiments also differed in the relative amounts of RNA and protein in each selective binding step. For the 10 SELEX rounds in Buffer L, the RNA:NGF ratio was high (generally ranging from 10 to 2500) in order to increase competition among the RNA species for protein binding (41). In the seven rounds in Buffer H the ratio was ~1, but we measured the binding of RNA to NGF each round to ensure binding of 3–6% of the RNA pool.

Figure 1b shows the sequences and fractional abundances of several ligands isolated from the pools after 10 SELEX rounds in Buffer L and seven rounds in Buffer H. Fifteen of the 36 isolates from the pool selected in Buffer L have essentially the same sequence (RNA L1); we observe three other sequence families from this pool and the remaining seven sequences are unique. One sequence family from the pool selected in Buffer H, RNA H1, makes up eight of the nine isolates from that pool. This sequence

Figure 2. Assays of the RNA-NGF binding interactions. (a) The percent retention of the different RNA ligands on nitrocellulose filters incubated in the presence of varying amounts of NGF protein. Assays were carried out in Buffer L (see Materials and Methods). Data points shown are averaged from four to six separate experiments: *, RNA H1 (K_d for NGF is 2×10^{-7} M); X, RNA L1 ($K_d = 5 \times 10^{-7}$ M); □, RNA L2 ($K_d = 7 \times 10^{-7}$ M); ●, RNA L6 ($K_d = 5 \times 10^{-7}$ M); ○, RNA 30N0#4 ($K_d = 2 \times 10^{-6}$ M). (b) The percent retention of the ligands on nitrocellulose filters incubated in the presence of 10^{-6} M NGF protein and varying concentrations of NaCl. Symbols are the same as in (a). (c) Percent retention of radioactively labeled RNA L1 at 5×10^{-7} M, incubated in Buffer L in the presence of 10^{-6} M NGF protein and varying amounts of unlabeled competitor RNAs; symbols are as in (a). (d) Retention of labeled RNA H1 at 5×10^{-7} M incubated in Buffer L with 10^{-6} M NGF protein and varying amounts of unlabeled competitor RNAs; symbols are the same as in (a).

family is similar to two of the RNAs selected in Buffer L (RNA L2 and L6).

Binding properties of the NGF ligands

We used RNAs L1, L2, L6 and H1 (Fig. 1b) to characterize the RNA:NGF binding interaction. We were able to demonstrate NGF concentration dependent binding of these ligands (Fig. 2a). The different RNAs tested display a 10-fold range of equilibrium dissociation rate constants (K_d). RNA H1 exhibits the highest affinity for NGF, while the three RNAs selected in Buffer L bind to NGF with slightly lower affinities. An RNA isolated from the starting 30N population, 30N0#4, exhibits 3- to 10-fold lower affinity for NGF than the selected ligands (Fig. 2a).

The sensitivity of the selected binding interactions to ionic strength by measuring the percentage of RNA bound to NGF as a function of NaCl concentration was measured. As the NaCl concentration increases from 0 to 100 mM, the binding of all five

RNA decreases by ~50%; the affinity for NGF of RNA L1, which was selected in 50 mM NaCl and that of 30N0#4 RNA, continues to decrease at higher NaCl concentrations. In contrast, the binding of RNA H1 (the abundant species selected in 200 mM NaCl) remains constant from 100 to 500 mM NaCl. RNAs L2 and L6, which come from the lower salt concentration SELEX pool but have sequences and structures similar to RNA H1, also bind NGF at higher salt concentrations, although not as well as RNA H1 (Fig. 2b).

We examined the equilibrium competition for NGF binding between RNAs H1 and L1. Addition of increasing amounts of unlabeled RNA H1 to a reaction containing NGF and labeled RNA L1 effectively inhibits the binding of the labeled RNA to NGF while the control RNA from the parent pool fails to compete (Fig. 2c). Similarly, RNA L1 inhibits the binding of labeled RNA H1 (Fig. 2d). Addition of unlabeled 30N0#4 RNA up to 10^{-5} M does not significantly inhibit the binding of either selected ligand, which is consistent with the relative affinities (Fig. 2c and d).

Figure 3. Predicted structure of RNAs H1, L2 and L6. Numbers represent nucleotide positions from the 5' ends of each structure. Stems and loops labeled for L2 are the same for H1 and L6.

Structural analyses of the NGF ligands

We analyzed the selected ligands for potential secondary structure using computer programs (32–34) and inspected the resulting structures for higher-order interactions. This analysis yielded no significant secondary structure for RNA L1. In sharp contrast, RNAs H1, L2 and L6 are all able to form putative pseudoknot structures (25; Fig. 3). Interestingly, although different parts of the linear sequences are used for different structural features, all the elements are highly conserved.

The predicted structures for L1 and H1 were extensively tested with structure-specific base-modifying chemicals (40,41). The modifying reactions were carried out with and without NGF protein and with and without 20 mM magnesium chloride. RNA H1 gives two distinct protection patterns, depending on reaction conditions. In the absence of NGF protein and magnesium ion, the bases of the predicted Stems 1 and 2A are protected from modification. However, the bases in Stem 2 are all accessible to the probes (Fig. 4b, c and Fig. 5b). In the presence of either NGF protein or 20 mM magnesium chloride, all of the bases in the three predicted stems are protected from modification. It is reasonable to argue that the binding of NGF or the presence of 20 mM MgCl_2 stabilizes the pseudoknot structure. In addition to the proposed stems, positions A22 and A39 are also protected by either magnesium or NGF (Fig. 5b). These residues may become less accessible to the solvent as a result of pseudoknot formation (42–44). A37 shows magnesium-specific protection which suggests that at 20 mM MgCl_2 the structure of H1 is tightened and A37 is shielded from modification. Human nerve growth factor specifically protects several highly conserved bases in Loops 2 and 2A, as well as the universally conserved bulged residue A58, between Stems 2 and 2A. These positions may well make direct contacts with NGF.

The chemical probes modified nearly all of the bases we examined in RNA L1 (Figs 4a and 5a), suggesting that, as predicted by our computer model, the molecule forms no stable secondary structures derived from simple base-pairing. When

NGF is added to the modification reaction, certain positions are protected from modification while other positions show enhanced modification. This may reveal residues contacted directly by NGF during binding or result from structural perturbation caused by protein binding (45). We see no difference in the modification pattern upon addition of 20 mM MgCl_2 in the absence of NGF (data not shown).

We constructed a truncated version of RNA H1, called RNA H1-T (Fig. 1c). This molecule retains the predicted secondary structure of RNA H1, but lacks the single stranded sequences at the 5' and 3' ends and has a different sequence in Stem 1 to facilitate T7 transcription. Truncated H1-T binds to NGF with the same affinity as the intact parent (RNA H1). The dissociation constant is $\sim 10^{-7}$ M and shows similar resistance to high NaCl concentration (data not shown).

Binding specificity of SELEX ligands

We examined the binding specificities of RNA H1-T and of the RNA ligands that were selected to histone H1, human thrombin (11) and HIV-1 reverse transcriptase (37). Table 2 shows the dissociation constants between these ligands and each of these proteins. In each case, the affinity of a ligand for its cognate protein is greater than the affinity of 30N RNA for that protein; the affinity of a ligand for a non-cognate protein is less than or equal to the affinity of 30N RNA for that protein. In these experiments, histone was used as a non-specific nucleic acid binding control protein.

DISCUSSION

Specific RNA ligands to human nerve growth factor were isolated using SELEX. Binding properties and structures of two ligands were characterized. These RNA ligands and several other recently reported RNAs and DNAs, are examples of polynucleotide ligands selected *in vitro* to bind proteins that are not known to bind nucleic acids in nature (2). Our study demonstrates the affinity and specificity with which SELEX-generated ligands can bind their targets.

Figure 4. Autoradiographs of primer extension assays showing the reactivity to base modifying chemicals of the RNA ligands. The chemicals used were dimethyl sulfate (DMS) and kethoxal (40,41). Hash marks to the left of the autoradiograph mark every tenth base in the RNA sequence. (a) The reactivity to DMS of RNA L1. C and A residues in the RNA sequence are shown in the left lanes of the figure. Lane K is the extension of unmodified RNA. Lanes 1 and 2 are modified RNA without and with NGF protein respectively. Arrows point out the diminished reactivities of C45 and A50 upon addition of NGF and the enhanced reactivity of A48. (b) The reactivity to DMS of RNA H1. Labels are same as in (a). (c) The reactivity to kethoxal of RNA H1. Labeling is the same as in (a). (d) The change in reactivity to DMS of RNA H1 upon the addition to the reaction of $MgCl_2$. A and G residues in the RNA sequence are shown in the left lanes of the figure. Lanes 1 and 2 are extensions of modified RNA minus and plus magnesium chloride respectively.

Table 2. The binding specificity of SELEX ligands for their particular protein targets

Target protein		NGF ligand	Thrombin ligand	RT ligand	30N RNA
NGF	K_d (i):	1×10^{-7}	$>10^{-6}$	$>10^{-6}$	1×10^{-6}
	K_d (30N)/ K_d (i):	10	<1	<1	1
Thrombin	K_d (i):	$>>10^{-6}$	2×10^{-8}	$>10^{-6}$	3×10^{-6}
	K_d (30N)/ K_d (i):	<1	150	<1	1
HIV-1 RT	K_d (i):	$>>10^{-6}$	$>>10^{-6}$	4×10^{-9}	3×10^{-7}
	K_d (30N)/ K_d (i):	$<<1$	$<<1$	75	1
Histone H1	K_d (i):	2×10^{-8}	3×10^{-8}	2×10^{-7}	2×10^{-8}
	K_d (30N)/ K_d (i):	1	0.67	0.1	1

Equilibrium dissociation rate constants [$K_d(i)$] were measured for several RNAs–NGF ligand H1-T, human thrombin ligand 27.33 (12), HIV-1 reverse transcriptase ligand RT-Lig2 (38) and 30N RNA–binding to several proteins–human NGF, human thrombin, HIV-1 reverse transcriptase and bovine histone H1. The ratio $K_d(30N)/K_d(i)$ illustrates the affinity of a given ligand for a protein relative to the affinity of 30N RNA for the same protein.

The structure of our salt-resistant binders is rather interesting for a small RNA. These molecules form a pseudoknot with a hairpin loop in the loop 2. Because the entire structure requires a minimum of 48 nucleotides, it appears that many of the 30 nucleotides in the random region were selected based on their ability to base-pair with bases in the fixed region to form stems 1, 2 and 2A. Several of the unpaired bases in the structure might contact NGF directly. The highly conserved bases in loops 2 and 2A as well as the universally conserved bulged A between stems 2 and 2A are strongly protected from chemical attack upon addition of NGF.

The higher salt concentration selection yielded a single dominant molecular species in seven rounds of selection. This molecule,

RNA H1, binds to NGF with relatively high affinity, at NaCl concentrations as high as 0.5 M; its resistance to salt suggests that this molecule makes primarily base-specific, non-ionic contacts with NGF. The results from our chemical modification study argue that the pseudoknot structure is dependent on binding of NGF or $MgCl_2$. Stabilization of alternative RNA conformations upon protein binding has been observed in tRNA^{Gln} when it binds to its cognate synthetase (47) and in regions of 5S (48) and 16S (45,49) rRNAs upon binding ribosomal proteins. The ability of magnesium ion to stabilize RNA tertiary structures is well characterized (42–44,50). In addition, high concentrations of sodium ion stabilize higher-order RNA structures (48) and seem to mimic the stabilizing effects of magnesium (44). It is thus reasonable to

Figure 5. Graphic representations of the chemical modification data. The underscored sequences are the reverse transcription primer annealing sites; boldface type indicates sequence selected from the random portion of the 30N RNA. Up-side-down closed triangles, modified bases; ○, modified bases that are protected by addition of NGF. Up-side-down open triangle, modified base that is protected by addition of magnesium chloride to the reaction; ?, positions that are unobservable due to reverse transcriptase stops in control lanes. (a) Summary of the data for RNA L1. (b) Summary of the data for RNA H1. The upper portion of the figure shows the derived structure of the RNA in the absence of magnesium ion and NGF protein. Upon addition of either of these reagents, the data becomes consistent with the pseudoknotted structure shown in the lower portion of the figure.

argue that the pseudoknotted structure evident in RNA H1 dominated the high salt selection but not the low salt selection because the structure was more stable in the high salt buffer.

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