Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 6, pp 1115–1122, 1997

PII: S0968-0896(97)00047-3
0968-0896/97 $17.00 + 0.00

RNA Aptamers that Specifically Bind to a K *Ras*-Derived Farnesylated Peptide

Bryant A. Gilbert, Ma Sha, Scott T. Wathen and Robert R. Rando*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, U.S.A.

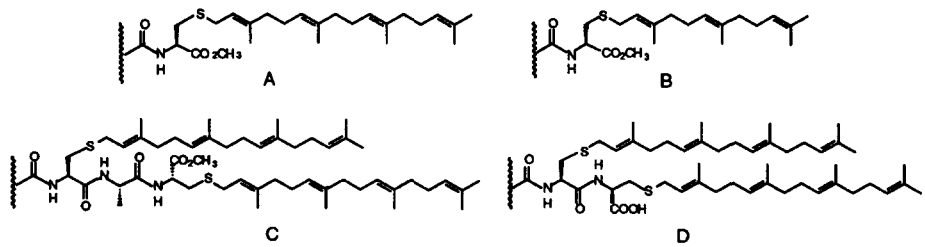
Abstract—RNA aptamers were selected against an affinity column containing a farnesylated peptide modeled after the carboxyl terminus of K *ras*, the major oncogenic form of this small G protein family. After 10-rounds of selection, 25% of the RNA applied to the column could be specifically eluted. Sequence analysis of the binding RNA aptamers revealed two consensus sequences—GGGUGGG and GGGAGG. Quantitative fluorescence binding studies on two of the high-affinity aptamers, showed a binding affinities of 139 nM and 0.93 μM, respectively for the farnesylated peptide. Binding to the nonfarnesylated peptide was at least 10-fold weaker, showing that the aptamers can recognize the hydrophobic farnesyl moiety. High affinity aptamers could be useful in specifically interfering with oncogenic ras function in particular, and G proteins in general. © 1997 Elsevier Science Ltd.

Introduction

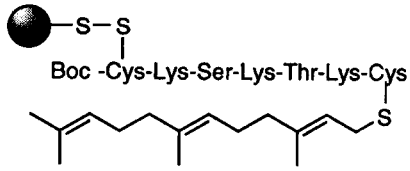
Heterotrimeric and small G proteins are hydrophobically post-translationally modified at their carboxyl terminal cysteine residues (γ subunit in the case of heterotrimeric G proteins) by isoprenylation/methylation (Scheme 1).[1–5] These modifications enable the modified proteins to associate with membranes and be functional in signal transduction processes.[6] In the absence of isoprenylation G proteins are inactive.[6–8] The *ras* family of small G proteins, which are singly farnesylated/methylated (Scheme 1-B), are important and well-studied members of the G protein signal transducing family.[9–12] The *ras* proteins are essential for regulating growth control in cells, and mutants in *ras* that are permanently activated are oncogenic.[13] Since farnesylation of *ras* is essential for its activity,[7] inhibitors of the farnesyl transferase enzymes which catalyze *ras* farnesylation are of substantial interest as potential anti-cancer agents.[14–16] An alternate approach to inhibiting *ras* function would be to prevent its access to cell membranes by designing molecules able to specifically complex with the farnesylated cysteine moiety. Since G proteins exhibit little, if any, sequence homology adjacent to the isoprenylated cysteine residue, molecules able to recognize the isoprenylated cysteine residue and part of the adjacent peptide sequence would be expected to be specific for a particular G protein.

RNA diversity libraries[17–19] represent a possible approach to arriving at molecules able to specifically bind to isoprenylated cysteine containing proteins. In this article, we explore the use of selected RNA molecules to bind to a farnesylated peptide with a sequence taken from the carboxyl terminal sequence of K *ras* (SKTKC-farn), which is the major oncogenic form of *ras*.[13]

In vitro selected RNA molecules (aptamers) have been readily selected against a variety of small molecules and macromolecules.[17–21] Aptamers directed against small molecules can specifically bind to these molecules with mM–nM dissociation constants.[17–21] Often the small molecules that allow for the selection of specific, high affinity aptamers are themselves highly polar and charged, such as ATP,[22] cyanocobalamin,[23] and aminoglycosides.[21] The efficiency of selections in these cases is probably due to electrostatic and hydrogen bond interactions between the ligands and the RNA molecules.[24] The selection of RNA molecules directed against hydrophobic molecules would perhaps be expected to be a more problematic enterprise. In this article, it is shown that tight binding aptamers can be selected that recognize the farnesyl unit and the peptide of the K *ras*-derived peptide (Scheme 2), showing that



Scheme 1. The Biochemical scope of isoprenylation/methylation. (A) Singly geranylgeranylated/methylated protein; (B) Singly farnesylated/methylated protein; (C) Doubly geranylgeranylated/methylated CAC containing protein; (D) Doubly geranylgeranylated CC containing protein.



Scheme 2. Structure of affinity ligand used for selections.

RNA molecules possess capabilities of recognizing and binding to hydrophobic moieties.

Results

Selection of RNA molecules that bind to immobilized *Ras* peptide

Selection experiments were performed starting with a DNA molecule containing a random 60-mer region as described in the Methods section.[21] The synthetic DNA was amplified by PCR, transcribed into RNA, and the RNA was applied to a Sepharose column containing the affinity ligand shown in Scheme 2. As the cost of synthesizing the ligand is substantial, and its aqueous solubility is limited, it was not possible to utilize the farnesylated peptide as an affinity eluant. Consequently, the ligand was removed from the disulfide column by elution with dithiothreitol (DTT). As described in the Methods section and as shown in Figure 1, after 10 selections approx. 25% of the RNA was specifically bound to the column. RNA eluted after the last round of selection was reverse-transcribed and cloned. Forty-five clones were randomly picked and sequenced. Twenty-eight out of the total of 45 clones were found to contain unique sequences, as shown in Table 1. Sequence G2 was found six times, G18 was found twice, G32 was found twice, G37 was found three times, and G40 was found twice (two clones of the total of 45 did not have inserts). Moreover, several of the unique sequences are virtually identical (i.e., G18 and G26, G19 and G25, and G4 and G23). Among the unique sequences, a seven base consensus sequence, GGGUGGG was discovered which appeared 15 times. A second consensus sequence GGGAGG was also found and appeared 16 times. In addition, many other near consensus sequences were found which differed from the indicated consensus sequences by a single nucleotide substitution.

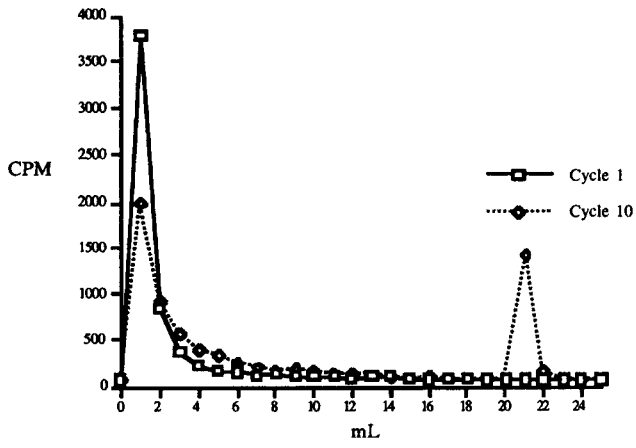


Figure 1. The elution profile for cycle 1 and cycle 10 of the RAS RNA aptamer selection. After the RAS affinity column was washed with 20 column volumes of selection buffer, the bound aptamers were specifically removed with 20 mM DTT in the selection buffer.

Screening of sequences by column chromatography for binding affinities

Eleven out of the 28 unique sequences were randomly chosen and were screened for approximate affinities to the farnesylated *ras* peptide affinity column. The dissociation constants were approximated by the procedure indicated in the Methods section and these are listed in Table 2. The binding could be categorized into three groups. The high affinity binding group included aptamer clones G4, G26, G5, G30 and G27 which have approximate K_d values of less than 4 μM. The intermediate group includes clones G15, and G22 which have K_d values of less than 13 μM. The weak binding group includes clones G2, G13, G14 and G21 which did not have a clearly measurable affinity under the experimental conditions used for the measurements (see Methods section).

Fluorescence binding assay

In order to obtain more accurate dissociation constants of RNA aptamer binding to the *ras*-based peptide, a quantitative fluorescence binding assay was used. Previously, we had developed fluorescence quenching and depolarization assays to accurately measure RNA-ligand affinities.[21] In the current study, a tryptophan labeled K-*ras*-based peptide, shown in Scheme 3 was prepared, along with its nonfarnesylated counterpart. Aptamer G4 and G26 were selected for further study for the accurate determination of binding as the column affinity measurements suggested that the aptamers derived from these clones would show high-affinity binding. In the presence of aptamer G4, strong fluorescence enhancement of the farnesylated *ras* peptide (Fig. 2) was observed while fluorescence quenching was observed with the nonfarnesylated *ras* peptide. The coexistence of fluorescence enhancement and fluorescence quenching has been observed elsewhere.[25] Despite the difference in the direction of the fluorescence signal change, the same Eadie–Hofstee plot (ΔF vs ΔF/[RNA]), can be used to calculate the binding constants.[25] In Figure 3 are shown data for the fluorescence quenching of the peptide induced by the G4 aptamer. In the case of G26, fluorescence quenching of the farnesylated probe by the RNA occurred. The data could be plotted as with G4 to determine binding affinities (Fig. 4). Table 3 summarizes the G4 and G26 aptamer binding to the farnesylated and nonfarnesylated *ras* peptides. The K_d of the farnesylated *ras* peptide for the G4 aptamer is 139 ± 12 nM, while the K_d for nonfarnesylated *ras* peptide with the same aptamer is 1.34 ± 0.08 μM. Thus, there is an

Table 1. Nucleotide sequences of aptamers. The sequence of the random region of 28 clones with unique sequences is shown. A total of 45 clones were sequenced. The total sequence is: 5′-GGGAGAAU UCCGACCAGAAGCCU-random region-CAUAUGUGCGUC UACAUGGAUCCU-CA-3′. Consensus sequences are highlighted. Overlap between consensus sequences is indicated by the symbols [], () and //

G2
5′...GUGAUUGCUGGAUCUCUAGGCAAACGAGGAGUA**GGGAGG** UCUA UAGGGUGUUGCUGUUGG...3′
G3
5′...GAUCGUGUAAGUCUCGUAUGGUUUUGGUUGAUGGAGUA**GGGAGG**UA UAACUGC...3′
G4
5′...GCUCAG**GGGUGGG** UCGUAUAAUUAGCGUA**GGGAGG** UAGUGGAUGA GUAAGCCUGGGAUGU...3′
G5
5′...G**GGGAGG** A**[GGGUG(GG]GAGG)** UGGUUAGUAGUUUAAGUCCGGA AUCAGAUUCAUGGGAACGC...3′
G6
5′...AGUAAUGCUGUUGUCAGUCUCUUCCGUACGAACAGGGGUCGUCAUCAGGCGUAUGGCUAGAU...3′
G10
5′...AGGCCAGUCGA**[GGGU(GGG]A/GG)GAGG/** UAUAUGGCCGAGUCUA UCUGACCAUCGGAAGC...3′
G13
5′...GCGAACGUGUUGCAUGAUUGUCAGUUGGUUGUUUCCAGCCAACUUGGGAGAAGGGAGUUA...3′
G14
5′...UAGUACCAUUGUUGUGAAGCGUGCGGUUAUGGAGGA**GGGUGGG** UAC CGCACUACGGAUGG...3′
G15
5′...GAGGGUGGAGGUGGGUGGAAUAAAAGCUGGUUCGGCAUGACUCAGGAGACUAUUCGUAUG...3′
G17
5′...GGGAGUA**GGGAGG** CCUAGUAUUGGCGGCUCCGGUUUGACUCUUCUUG GGGUACCUUGUGUG...3′
G18
5′...GAGAUGUAUCGUUGCCGGGGAU**GGGUGGG** UGGUGUGAAGGCGAUCGU CAUCAGUCGAGC...3′
G19
5′...CUAGCAA**GGGAGG** ACAUGA**GGGUGGG** GUGCUAGUCGCUUAGGCUU UGGGCUUCUAGCAG...3′
G21
5′...GAGGAGUAGAGUGUGGGCACAUGUCGGUUGGCAAUAAUGGAGGAGGAGGGUUUUGCUAGC...3′
G22
5′...GGAUUGGUUGUGGGAUGGGUA**GGGUGGG** UGGUGUGGCCGAUCCGUCC UUGAGCUAAUGGC...3′
G23
5′...GCUCAG**GGGUGGG** UCGUAUAAUUAGCGU**GGGAGG** UAGUGGAUGAG UAAGCCUGGGAUGU...3′
G24
5′...GA**GGGAGG** CUGUGUGGGUGGUUUGUGUACGGUUUGGAAUUUUCCACG ACCCUGUUGCGUG...3′
G25
5′...CUAGAA**GGGAGG** ACAUGA**GGGUGGG** GUGCUAGUCGCUUAGCGUUU GGGCCUUCUCAGCAG...3′
G26
5′...GAGAUGUAUCGUUGCCGGGGAU**GGGUGGG** UGGUGUGAAGGCGAUCGU CAUCAGUUCGAGC...3′
G27
5′...GAGAUGUAUCGUUGCCGGGGAU**GGGUGGG** UGGGUGUGAAGGCGAUCG UCAUCAGUCGGGC...3′
G30
5′...GACGGGUGA**GGGAGG** GUGGAUGUGUGGAAUAACUGUGAGCUUUUGU CGUAGUUGCCGUGC...3′
G31
5′...CAGCUGUGGCCAGGUGGGC**GGGAGG** GAGUGAAGCUGGCCAUGCAGUUG GUUUUCUCUAAG...3′
G32
5′...GGUUGUGGAUAUCCGUUGUGUGGAGGGAUGAGGAGCAAACGGAUCUCUAGGUCGUUAGUG...3′
G33
5′...GGUUGUGGAUAUCCGUUGUGUGGAGGGAUGAGGAGCAAACGGAUCUCUAGGUCGUUAGUG...3′
G34
5′...CGCAAGGGUACCUUAGCUAAGGCUGAAUUUGAUGAUUGGUGGAG**[GGGU(GGG]AGG)** AGGGGC...3′
G35
5′...GCCUGGCGACAACGCACUGCAUCCAACCUAUCUACUUCAAUGUAACUUCGUACAGUGUAC...3′
G37
5′...CGAGCUUGACUACUGCUAGCGGAAGUGUGGU**GGGUGGG** UAGGGGCCG UUGCUAUGUAGAG...3′
G40
5′...UGUAGGGUCCGAAUGAGUAGGCGAUGGAGGGAUGCGACUAAUAUGCU**GGGUGGG** GACUCG...3′
G41
5′...GUGCUAAGGCCUGUCGAUUUGUUGCCUGAAGGU**[GGGUG(GG]GU/GG)GAG G/**CUAGAGUAAGAU...3′

approximately 10-fold discrimination in binding due to the farnesyl group. The binding of the farnesylated peptide to G26 was weaker than with G4. Moreover, in the case of G26, the nonfarnesylated peptide appeared to have no affinity for the aptamer.

Discussion

The farnesylated carboxyl terminus of the *ras* family of G proteins is required for membrane association and biological function.[7,8] Inhibitors of *ras* farnesyl transfer-

Table 2. Approximate dissociation constants of aptamers as measured by the column binding assay

RNA Aptamer	K_D (approximate)
G4	<1.0 μM
G26	3.7 μM
G5	3.9 μM
G30	3.9 μM
G27	2.0 μM
G15	2.5 μM
G22	112.6 μM
G2	n.b.[a]
G13	n.b.
G14	n.b.
G21	n.b.

[a]n.b. = no binding.

ase appear to block membrane association of oncogenic *ras* and interfere with *ras* function.[14–16] These molecules may be useful as anti-cancer drugs. An alternative way of preventing *ras* function is to design molecules capable of binding to the farnesylated carboxyl terminus of this protein, thus disabling its ability to associate with membranes. Whether or not *ras* binds to membranes via hydrophobic interactions or by means of protein–ligand interactions is unimportant for this approach. Along these lines, it is interesting to note that studies on the inhibition of *ras* function with small molecule farnesyl cysteine analogues suggest the possibility that ligand–protein interactions may mediate interactions between the farnesylated carboxyl terminus of *ras* and the membrane.[26] In this article we demonstrate that RNA aptamers can be selected which specifically bind to a carboxyl terminal farnesylated peptide designed based on K *ras*, the major oncogenic form of *ras*.[13] Moreover, full binding affinity was dependent on the presence of the farnesyl moiety.

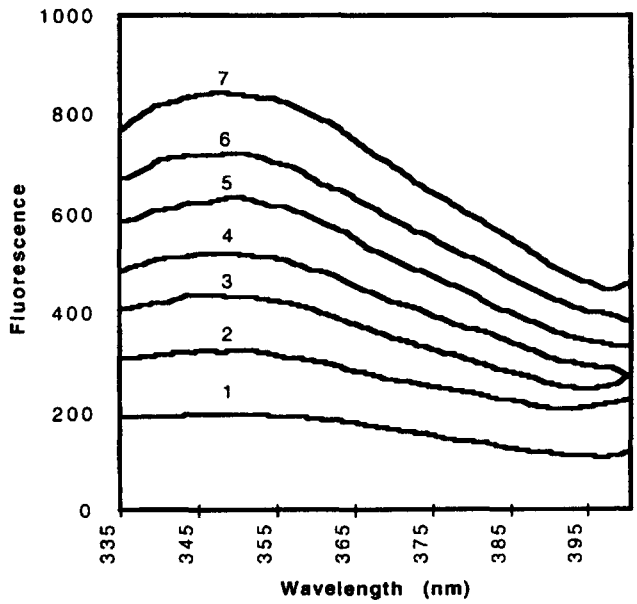


Figure 2. Fluorescence emission spectra of the *ras* peptide solution in the presence of varying concentrations of G4 RNA. This interaction is revealed as fluorescence enhancement. [*Ras* peptide] = 100 nM. [G4 RNA]: 1, 0; 2, 0.14 μM; 3, 0.18 μM; 4, 0.24 μM; 5, 0.4 μM; 6, 0.5 μM; 7, 0.8 μM.

In any aptamer studies it is important to develop quantitative tools to measure binding affinities. In the present studies fluorescence measurements were made. It is noteworthy that aptamer binding to fluorescent peptides either led to enhancement or quenching of tryptophan fluorescence. G4 enhanced the fluorescence of the farnesylated peptide, while G26 quenched it. It is not possible at this point to attribute these differences in fluorescence behavior to structural features of the aptamers because their structures are not known. However, in a general way tryptophan fluorescence can be quite sensitive to local environment with respect to such features as hydrophobicity, potential stacking interactions, and polarity.

Having a quantitative assay to measure binding affinities of farnesylated peptides to the aptamers was important for uncovering RNA molecules of interest.

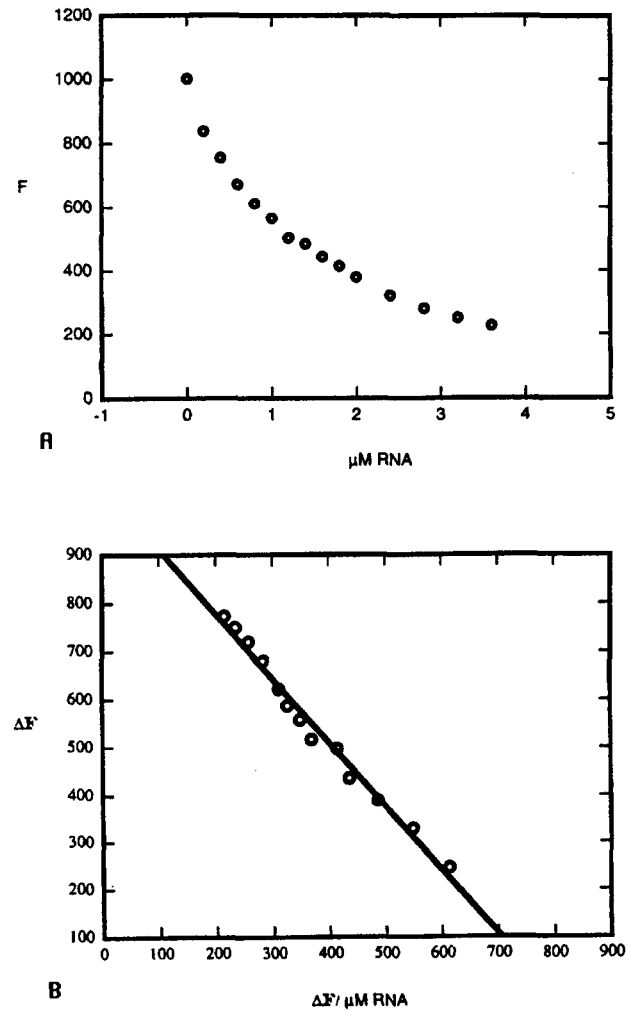


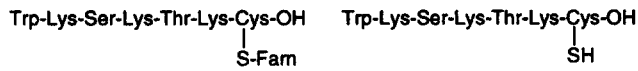
Figure 3. Fluorescence quenching of the non-farnesylated *ras* peptide upon adding of G4 RNA (A) [*Ras* peptide] = 1 μM. (B) Eadie–Hofstee plot of the quenching data, the K_D was obtained from the slope as 1.34 ± 0.08 μM.

Table 3. Fluorescence binding summary of aptamer RNAs with farnesylated and non-farnesylated *ras* peptides

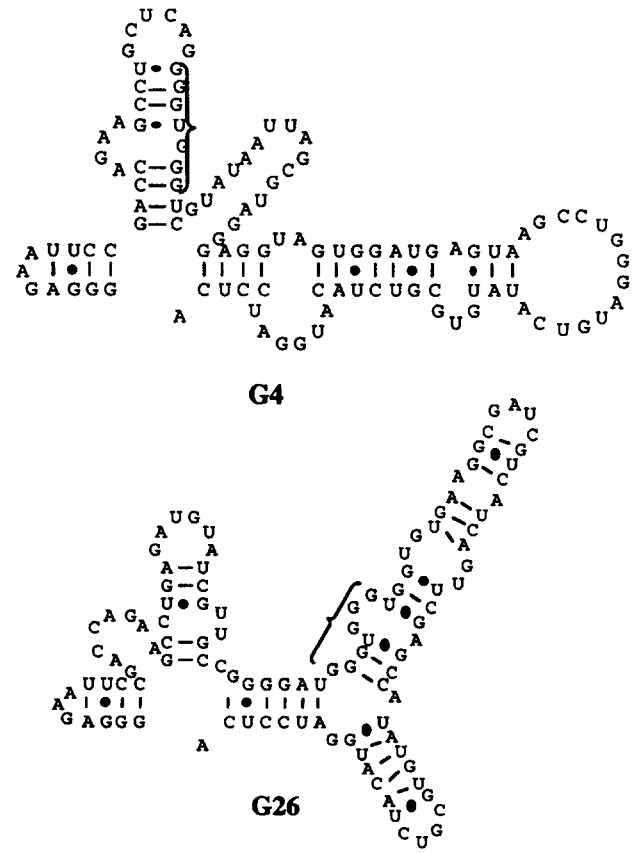
Aptamer RNA	Farnesylated *Ras* peptide	*Ras* peptide
G4	K_D =139 ± 12 nM	K_D = 1.34 ± 0.08 μM
G26	K_D = 0.93 ± 0.05 mM	No binding

From 45 randomly selected RNA sequences that were eluted from the affinity column we were able to find 28 unique sequences. Two consensus sequences (GGGUGGG and GGGAGG) were prominently displayed (Table 1). The sequences of 11 aptamers that were screened by the column binding assay are listed in Table 2. The sequences are shown in three groups. The high affinity binding group, including all RNA molecules with K_d<4 μM all had the GGGUGGG sequence. The highest affinity aptamer G4 had both the GGGUGGG and GGGAGG consensus sequences. The intermediate binding group had one clone with GGGUGGG and one without. In the weak or nonbinding group, three out of four sequences did not have the GGGUGGG sequence. Overall, the consensus sequence GGGUGGG was found in most of the binding sequences (5 out of 7), but was not found in most of the non-binding sequences (3 out of 4 not found). One sequence in the nonbinding RNA group with the GGGUGGG consensus sequence might represent a mutation in the last round of PCR out side the consensus sequence range which distorted the RNA's secondary structure. This suggests that the consensus sequence GGGUGGG might be important for *ras* peptide binding, but that this sequence alone is not sufficient for high-affinity binding.

We have no experimental information yet on how the consensus sequence(s) might be folded in order to serve as part of the binding site for the *ras* peptide. The G4 and G26 sequences were, however, folded (Scheme 4) into a secondary structure using M-fold[27] to generate structural hypotheses. The folding patterns suggested that GGGUGGG may be part of a bulge structure. Bulge or stem-loop structures would be considered interesting as possible binding-sites for the farnesylated peptide. However, further experimental evidence will be required before the minimum binding-site of the aptamers can be defined. The M-fold data can serve as a working hypothesis for the structures of these aptamers at this point.



Scheme 3. Fluorescent ligands used to measure binding affinities.



Scheme 4. M-fold secondary structure predictions for G4 and G26. Indicated regions show the GGGUGGG consensus regions.

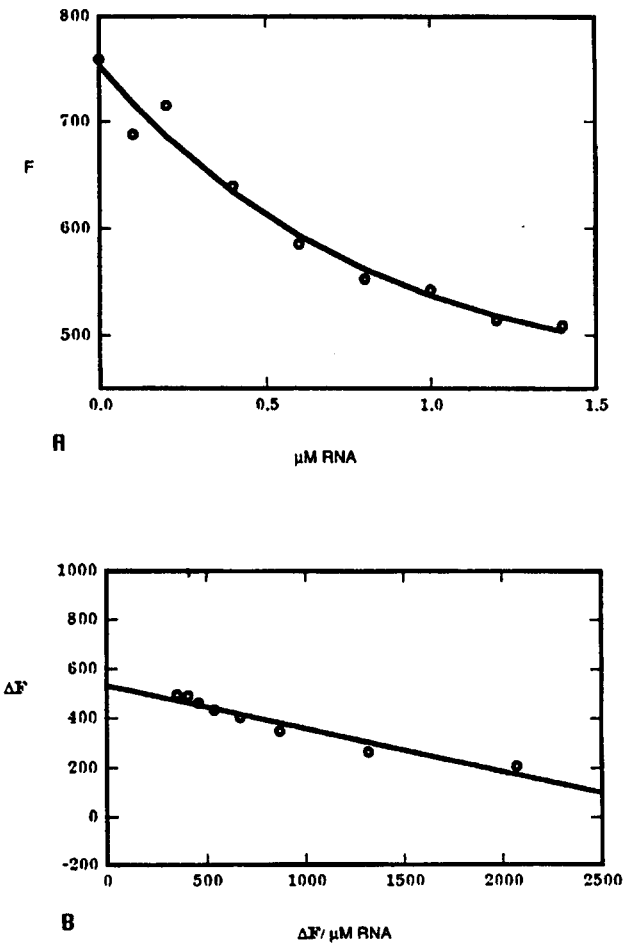


Figure 4. Fluorescence quenching of the nonfarnesylated *ras* peptide upon adding of G26 RNA (A) [*Ras* peptide] = 1 μM. (B) Eadie–Hofstee plot of the quenching data, the K_D was obtained from the slope as 0.93 ± 0.05 μM.

Fluorescence spectroscopy is useful for measuring RNA-ligand affinities.[21,28] In this article, we have also used a fluorescence technique to measure affinities for to high-affinity aptamers. Both aptamers (G4 and G26) discriminated between the farnesylated and non-farnesylated peptides, showing that hydrophobic recognition occurred. While RNA-mediated hydrophobic recognition is not unheard of,[20] it is quite rare. It will be interesting to determine if RNA aptamers can be selected to discriminate between farnesyl and other hydrophobic moieties as well. Higher stringency selections are possible in order to generate higher affinity aptamers with a great deal of specificity directed against the farnesyl moiety. The affinities measured between the farnesylated *ras* peptide and some of the aptamers are already significant; G4 bound with a K_D of 139 nM. It should be possible to generate even tighter binding RNA aptamers to the *ras* constructs. It will be of interest to determine if these molecules can interfere with *ras* function.

Materials and Methods

Materials

Affi-Gel 10 was purchased from Bio Rad Inc. Sephadex G-50 and thiopropyl Sepharose 6B were from Pharmacia α-^{32}P-dATP and α-^{32}S-dATP were purchased from New England Nuclear. The GeneAmp PCR kit, GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit and Amplicycle Sequencing kit were from Perkin–Elmer. The two primers used and the 109-mer random DNA pool were purchased from Oligos Etc. Restriction enzymes *Eco*RI and *Bam*HI were from Boehringer Mannheim. pBluescript II SK (–) phagemid, XL1-Blue MRF', *E. coli* host cells and KS primer were from Stratagene. The Wizard Miniprep DNA Purification System was from Promega. The tryptophan labeled *ras* peptide (WKSKTKC) was from Bio-Synthesis. All other chemicals were from Aldrich or Fluka and were of the highest purity available. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy were recorded on a Varian VRX 500S Spectrometer operating at a proton frequency of at 499.843 MHz. Dimethylsulfoxide (DMSO-d_6) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. HPLC solvents were from J. T. Baker.

Methods

Syntheses.

Synthesis of the fluorescent farnesylated *ras* peptide (WKSKTKC[Farn]). The fluorescent peptide (WKSKTKC, corresponding to the carboxyl terminus of K *ras* with an extra tryptophan residue) was farnesylated on the cysteine residue according to the published procedure.[29] The yield of the farnesylated product was approximately 30%. The desired product was isolated by HPLC on a C18 column. A linear gradient starting with 55% H_2O/45% acetonitrile and ending with 5% H_2O/95% acetonitrile (including 10 mM TFA) was used. The starting peptide had a retention time of 11 min and the farnesylated peptide had a retention time of 26 min. The molecular mass of farnesylated peptide was confirmed by electrospray ionization mass spectrometry at the Harvard Microchemistry Facility $(M+H)^+$ = 1084; Calcd Mass = 1083.

Synthesis of the affinity ligand BOC-Cys(SH)-Lys-Ser-Lys-Thr-Lys-Cys(SFarn)-OMe. The peptide was synthesized according to previously published protocols.[30] The peptide was purified by reverse-phase flash chromatography (acetonitrile:water:TFA. 75:25:0.01) to provide the farnesylated peptide as a white solid: TLC (silica) R_f = 0.30 (MeOH:CH_2Cl_2, 80:20). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.25 (2H, brs), 6.65 (2H, brs), 5.15 (1H, t, J = 7Hz), 5.04 (2H, t, J = 5Hz), 4.22 (1H, d, J = 8Hz), 4.16 (1H, dd, J = 8, 21.5Hz), 4.12 (1H, d, J = 6.5Hz), 3.91 (1H, t, J = 9Hz), 3.89 (1H, brs), 3.13 (2H, m), 2.54 (1H, dd, J = 9, 13.5Hz), 2.43 (1H, dd, J = 7, 13.5Hz), 2.35 (3H, m), 1.95 (3H, s), 2.07-1.88 (10H, m), 1.76 (1H, m), 1.61 (6H, s), 1.53 (6H, s), 1.41 (1H, m), 1.35 (9H, s), 1.08 (1H, m), 0.84 (3H, d, J = 6.5Hz), 0.80 (9H, m).

Preparation of the thiopropyl sepharose 6B *ras* affinity chromatography support. Thiopropyl sepharose 6B was washed, activated, and coupled to the mercapto peptide following the manufacturer's protocol. The coupling of the ligand to the gel was achieved via a disulfide exchange reaction of the gel's 2-mercaptopyridine with the free thiol of the amino terminal Boc cysteine. The concentration of ligand bound to the gel was determined by spectroscopically measuring the concentration of liberated 2-mercaptopyridine. Using this protocol, the concentration of bound ligand was estimated at 15 mM. The RNA selections were performed in 1.0 mL column volumes with a buffer (selection buffer) containing 140 mM NaCl, 5 mM KCL, 1 mM $CaCl_2$, and 20 mM Tris acetate at pH 7.4. The RNA bound ligands were eluted from the support (elution buffer) with the selection buffer containing 20 mM DTT.

Preparation of the nucleic acid pools. The original double-stranded DNA pool was constructed by large scale PCR amplification of the synthesized 109-mer containing 60 random nucleotides.[20,21] An amount of 50 μg of a chemically synthesized 109-mer, including primer regions identical to those previously described, was used for a 10-mL scale PCR reaction. The original RNA pool used in the selections was constructed by reverse transcription of 120 μg double-stranded DNA (which contains a maximum of $\sim 10^{15}$ individual sequences). ^{32}P-labeled RNA was used to follow all selections. RNA, purified by Sephadex G-50 column chromatography to remove the unincorporated nucleotides, was heated at 75 °C for 5 min, and then cooled to room temperature.

Methods for RNA aptamer selection. The buffer used for loading the RNA molecules onto the column consisted of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM Tris, pH 7.4. The elution buffer was the same with the addition of 20 mM DTT. RNA transcription and RT-PCR reactions were carried out as previously published.[21]

The selection procedure used was as follows. The RNA solution was heated to 75 °C, then allowed to slowly cool to room temperature. An amount of 5 μL of the sample was saved for counting, and 100 μL added to 1.0 mL of the *ras* affinity gel. The RNA was then applied directly onto the *ras* affinity column with 1.0 mL of selection buffer. The RNA was equilibrated on the affinity column for 10 min, then the column was washed with 20-column volumes of selection buffer. The RNA binding ligands were removed from the support with 4-column volumes of selection buffer containing 20 mM of DTT. The RNA was precipitated with ethanol and glycogen as a carrier. RNA reverse transcription and DNA amplification were performed in a single tube, using the GeneAmp Thermostable rTth Reverse Transcriptase kit from Perkin–Elmer. The PCR reaction was done for only 4–8 cycles to ensure high quality PCR products. Cloning of PCR DNA products was conducted using a pBluescript II SK (–) phagemid cloning vector and an XL-1 MRF' *E. coli* as the host bacteria.[21]

The selection proceeded as indicated below:

Summary of *Ras*-RNA selection

Cycle #	% CPM retained
1	0.004
2	0.05
3	0.2
4	0.04
5	1.2
6	7.8
7	12
8	17
9	17
	(with 20 mM threitol prewash)
10	25
	(with 20 μM peptide prewash)

After 10 cycles the selection rose to 25%. To increase the stringency of the selection, in cycle 9, 20 mM of threitol was added to the loading buffer in column volume washes. Threitol is structurally analogous to DTT, but the dithiol moieties are replaced with hydroxyl groups. To ensure the aptamer pool was binding to both the farnesylated cysteine and poly-lysine portions of the ligand, in cycle 10, 20 mM of peptide (Ac-Lys-Ser-Thr-Lys amide) was added to the loading buffer in column volume washes. The DNA in of cycle 10 was cloned and 45 colonies were sequenced. Eleven sequences were randomly selected and the corresponding RNA generated. The K_D values for each aptamer was estimated by gel filtration chromatography. Aptamers that appeared to bind to the ligand tightly by this estimate were selected for further study by quantitative fluorescence methods.

Determination of aptamer dissociation constants.

(A) Column binding assay. The dissociation constant K_d of selected RNA molecules to the farnesylated *ras* peptide on the selection column was estimated according to the method of Majerfeld et al.,[31] using the following equation:

$$K_d = L_c(V_n/(V_e - V_n)$$

where L_c is the concentration of affinity ligand within the bed, V_e is the median elution volume in the absence of ligand, and V_n is the median elution volume in the absence of affinity. Approximately 10 μg of [^{32}P]RNA in the selection buffer was applied to a 250 μL selection column with farnesylated *ras* peptide as ligand on the resin. The column was washed with the selection buffer without ligand to determine the $V_e{\cdot}L_c$ was 140 μM, V_n was taken as 1.5 × the bed volume, 375 μL. The principle assumptions made when using this equation are that (1) chromatography is conducted near equilibrium, and (2) that the column is not near saturation with RNA. But still, the K_d was calculated as an estimate to compare the affinities of different selected RNA to the ligand. More accurate binding measurements were performed by fluorescence method described below.

(B) Fluorescence measurements to determine affinities. Steady-state fluorescence measurements were performed on a Perkin–Elmer model 512 double beam fluorescence spectrometer. The excitation wavelength was 280 nm. All measurements were done at 23 °C in the selection buffer contains 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM Tris, pH 7.4. The K_d values of the RNA aptamers for the *ras* peptide were calculated by constructing an Eadie–Hofstee plot as described elsewhere.[25]

Acknowledgements

This work was supported by U.S. Public Health Service National Institute of Health Grant EY-03624.

References

1. Casey, P. J. *Science.* **1995**, *268*, 221.
2. Maltese, W. A. *FASEB J.* **1990**, *4*, 3319.
3. Sinensky, M.; Lutz, R.J. *BioEssays* **1992**, *14*, 25.
4. Casey, P. J. *Curr. Biol.* **1994**, *6*, 219.
5. Glomset, J. A.; Farnsworth, C. C. *Annu. Rev. Cell Biol.* **1994**, *10*, 181.
6. Parish, C. A.; Rando, R. R. *Biochemistry* **1996**, *35*, 8473.
7. Hancock, J. F.; Magee, A. I.; Childs, J. E.; Marshall, C. J. *Cell* 1989, *57*, 1167.

8. Fukada, Y.; Takao, T.; Ohguro, H.; Yoshizawa, T.; Akino, T.; Shimonishi, Y. *Nature (London)* **1991**, *346*, 658.

9. Downward, J. *BioEssays* **1992**, *14*, 177.

10. Leevers, S. J.; Paterson, H. F.; Marshall, C. J. *Nature (London)* **1994**, *369*, 411.

11. Kato, K.; Cox, A. D.; Hisaka, M. M.; Graham, S. M.; Buss, J. E.; Der, C. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6403.

12. Barbacid, M. *Annu. Rev. Biochem* **1987**, *56*, 779.

13. Barbacid, M. *Eur. J. Clin. Invest.* **1990**, *20*, 225.

14. Gibbs, J. B. *Cell* **1991**, *65*, 1.

15. James, G.; Goldstein, J. L.; Brown, M. S. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4454.

16. Nagasu, T.; Yoshimatsu, K., Rowell, C.; Lewis, M. D.; Garcia, A. M. *Cancer Res.* **1995**, *55*, 5310.

17. Ellington, A. D.; Szostak, J. W. *Nature (London)* **1990**, *346*, 818.

18. Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505.

19. Beaudry, A.; Joyce, G.F. *Science* **1992**, *257*, 635.

20. Famulok, M.; Szostak, J. W. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 979.

21. Wang, Y.; Rando, R. R. *Chemistry & Biology* **1995**, *2*, 281.

22. Sassanfar, M.; Szostak, J. W. *Nature (London)* **1993**, *364*, 550.

23. Lorsch, J. R.; Szostak, J. W. *Biochemistry* **1994**, *33*, 973.

24. Jiang, F.; Kumar, R. A.; Jones, R. A.; Patel, D. J. *Nature (London)* **1996**, *382*, 183.

25. Sha, M.; Ferre-D'Amare, A. R.; Burley, S. K.; Goss, D. J. *J. Biol. Chem.* **1995**, *270*, 19325; Sha, M.; Balasta, M. L.; Goss, D. J. *J. Biol. Chem.* **1994**, *269*, 14872.

26. Marom, M.; Haklai, R.; Ben-Baruch, G.; Marciano, D.; Egozi, Y.; Kloog, Y. *J. Biol. Chem.* **1995**, *270*, 22263.

27. Jaeger, J. A.; Turner, D. H.; Zuker, M. *Methods Enzymol.* **1989**, *183*, 281.

28. Wang, Y.; Killian, J.; Hamasaki, K.; Rando, R. R. *Biochemistry* **1996**, *35*, 12338.

29. Xue, C. B.; Becker, J. M.; Naider, F. *Tetrahedron Lett.* **1992**, *33*, 1435.

30. Ma, Y.-T.; Gilbert, B. A.; Rando, R. R. *Biochemistry.* **1993**, *32*, 2386.

31. Majerfeld, I.; Yarus, M. *Nature Struct. Biol.* **1994**, *1*, 287.

(*Received in U.S.A.* 2 *September* 1996; *accepted* 18 *February* 1997)