CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200800936

# RNA Aptamers That Reversibly Bind Photoresponsive Azobenzene-Containing Peptides

**Gosuke Hayashi, Masaki Hagihara, and Kazuhiko Nakatani*[a]**

**Abstract:** Modulation of biological networks assembled by diverse interactions among biologically active molecules has provided a platform for innovative biotechnologies. Here, we report RNA aptamers that bind to a photoresponsive peptide (KRAzR; Lys-Arg-azobenzene-Arg) containing azobenzene chromophore, which can change its structure by photoirradiation. Aptamers were identified after 10 cycles of an in vitro selection procedure starting with a DNA library containing a 70 nt random region. Surface plasmon resonance (SPR) analysis demonstrated that interactions between aptamers and KRAzR were fully controlled by appropriate photoirradiation to the SPR sensor chip. Upon irradiation of 360 nm on the KRAzR-immobilized surface, the binding of each aptamer to the surface was significantly decreased. Subsequent photoirradiation of the same surface with 430 nm restored the aptamer binding to the surface. We also observed that direct photoirradiation of the aptamer–peptide complex on a gold surface actively promoted dissociation of the complex. Furthermore, a doped reselection method was applied to acquire structural and sequence information of aptamer 66. From a data analysis of the conserved region and the mutation frequency, we were able to select a plausible secondary structure among three candidates predicted by computational folding simulation.

**Keywords:** aptamers · photoregulation · RNA secondary structure · RNA structures

## Introduction

A variety of interactions between RNAs and small organic molecules in living cells play a crucial role in the modulation of biological functions. Organic molecules which bind to ribosomal RNA act as antibiotics and inhibit protein biosynthesis in bacterial cells.[1–3] Riboswitches, structured elements that are generally positioned at the non-coding region of mRNAs, bind to the secondary metabolites and control gene expressions.[4,5] Small molecule effectors manipulate natural[6–8] and synthetic allosteric ribozymes.[9,10] In the living cell system, the change of the concentration of these small molecules resulted in a modulation of the biological function. Artificial control of the interaction between RNA and small molecular ligands would eventually enable us to turn on and off the protein expression by external stimuli. To modulate RNA–ligand interaction, functionalized molecules, the structure of which could be switched by external stimuli, have been investigated. Müller and co-workers studied on the redox-active flavin mononucleotide as a structure-controllable ligand for the allosteric ribozyme.[11]

Because light can be manipulated accurately in designated time and space, light-depending switches permit a precise spatiotemporal control of the concentration of the active form of the molecules in living cells or experimental systems.[12] Azobenzene is one of most well-studied photoresponsive chromophores that undergo an marked change in the geometry as well as a dipole moment upon isomerization of the central N=N double bond. Numerous applications of the photoinduced isomerization of the azobenzene chromophore have been reported for switching the biological function of the molecules. Cyclic peptides containing the azobenzene in the backbone have been constructed and used for the conformational switching of the peptide backbone.[13] Isacoff and Trauner et al. demonstrated the feasibility of the light-switching of a channel protein.[14] Komiyama and Asanuma et al. have synthesized the azobenzene-containing DNA and achieved the photochemical regulation of the dsDNA formation.[15,16] Our group has reported the re-

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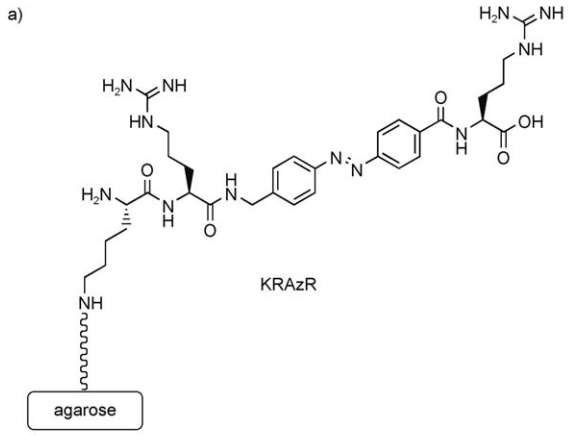
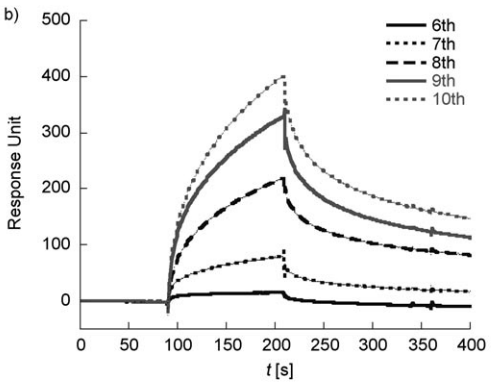
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.200800936.

versible hybridization of two natural unmodified DNA strands with the photoresponsive molecular glue for DNA.[17] These studies based on the modification of well-studied biomolecules and ligands with photoresponsive chromophores. The drawback of this approach was, however, the lack of small molecules that bind to RNA with high sequence and structural specificity.

We have reported an approach to find ligand–RNA pairs, in which the interactions could be modulated by appropriate photoirradiation.[18] Photochemical modulation of the ribozyme activity by light has also been recently reported by Sen and co-workers with a photoresponsive dihydropyrene molecule that has photochemically switchable, open and closed structural isomers.[19] In our preliminary communication, we reported a selection of RNA aptamers from a N70 random RNA library against photoresponsive peptide KRAzR containing azobenzene amino acid (Az) and investigated the response of the aptamers to the KRAzR that immobilized on to the gold surface upon photoirradiation. We here report an in vitro selection of new aptamers and the detailed analyses of their binding to KRAzR immobilized on the gold surface. The selected RNA aptamers showed reversible interaction with KRAzR immobilized on a gold surface in response to appropriate photoisomerization. A combination of aptamer sequence-binding relationship studies and a doped reselection strategy suggested the plausible secondary structures of an aptamer for the binding to (*E*)-KRAzR.




c)

| clone | | frequency |
|---|---|---|
| 56 | GUACAGAGUGCUAGUGCUAAGAGCCAUAAGAACCGAUCUAGCACCCUCCCAAUACGAGUUAUAUACCAAG | 7x |
| 57 | **A**UACAGAGUGCUAGUGCUAAGAGCCAUAAGAACCGAUCUAGCACCCUCCCAAUACGAGUUAUAUACCAAG | 1x |
| 54 | GUACAGAGUGCUAGUGCUAAGAGCCAUAAGAACCGAUCUAGCACCCUCCCAAUACGAGUUAUAUACCAA**A** | 1x |
| 58 | GUACAG**G**GUGCUAGUGCUAAGAGCCAUAAGAACCGAUCUAGCACCCUCCCAAUACGAGUUAUAUACCA-G | 1x |
| 66 | UCAACAACAAAAGGAUAAGCUAGGCGAGCCUUACCGAACGC | 2x |
| 74 | UCAACAACAAAAGGAUAAGCUAGGCGAGCCUUACCGA**G**C**A**C | 1x |
| 49 | GGGACAAAAUUAACAGAAAAACACGCUCGUGGGAAAACACGUUUUCCUCGGCUGCCCACUAGAUCCAUCG | 2x |
| 72 | ACUGAAGCAACCCGAACUAGGAUACAGUUAUUACCCAACGCGUGGGCCAACGCCGUGGCCCUAAAUACU | 2x |
| 73 | AGAAAGAUCCGAUAGAGAAAG | 1x |
| 51 | GCUACUACACAGCGAACAAACCGCUCUGCAAGGCAAAACGGCGCACACGAAACCCGAAUGGAGCCAAGCA | 1x |
| 64 | GGCAACCAAGAAAACCCAUGAACAGCAAGCGAGUCAACCUAACAUUCGCAAAACCGAACGAGUAGCAAAA | 1x |
| 77 | AUCCCCCGCACACACGUGAACGCUGCAAAGUGAGCAAAUUCCAAAAUACACUAAGCGAAAACCAAUUAGG | 1x |
| 52 | AGCCACCGAACAACCAUGAGGGGUCGGGGAGGCGCCCCGACCACACUAACAAUGCAGUCAGAUCCAACUC | 1x |
| 67 | AAACAAAACGCGAAGCUACCGACGAAAGCAACCGCACACGCCCACGUACGAGACAUAUGGCCACGCUCAA | 1x |
| 53 | CAAGGAUGAACUACAGGCGAAAAACCGUAUAGGAUUUCGACCGAGCAAGCCAACCGCACACCGCCCACCG | 1x |
| 50 | CCAGCCCACAACAAAGCCGCAAGCCAGGCACAGCAUGACAGAAGGCAAGCAAAAUCGUCAUGCCAUAGAC | 1x |
| 61 | ACACCCCACGGAGCCGACCGGCCCCCACUAAAACAACGCGAGUCUGACGCCGCAGACACAGUGAGUACCA | 1x |
| 55 | ACUCCGCGAAACCGCAGCUGCACAUCCACAGACAUGCGUAUUAACGGCAAGACAGAGGAAUUAACAGCAA | 1x |
| 59 | GACGGACCCGGAAACCAGCACUGCCGAGACAACGGCAUGAUCCGGUAGGACAAUGGAAGACAACAUACCA | 1x |
| 76 | AGUGGGCCCAACCGAUCCUGGCGAGCCAUACGACCUUGGCGAACGGAGCAUUUACAGCAAGCAAAAGCCU | 1x |
| 71 | GGAAACGCCACGGGCACAACACGCCACACGCGACUAGGCGAGUCGCGGCCCGCACCAUAAGCAUCCGCAG | 1x |
| 78 | GCCGAAUGUGCUACGAAUAUUAAGAUCGUCAGUCUGUAAACGCGUUACAGAGCACGCCGGAUACCUGCUG | 1x |

Figure 1. In vitro selection of KRAzR and obtained aptamers. a) Chemical structure of photoresponsive peptide KRAzR. KRAzR was immobilized to NHS-activated agarose through the ε-amino group of the lysine residue. b) SPR sensorgrams showing the binding of the RNA pool of each round to KRAzR immobilized on gold surface. The concentration of each RNA sample was adjusted to 1.0 μM. Sample solutions of the RNA pool were separately injected over the surface for periods of 120 s to monitor the association, and then running buffer was applied to monitor the dissociation. c) Sequences of KRAzR binding RNAs from cycle 10. Only the inserts corresponding to the randomized regions are shown. The sequences are listed with respect to their abundance. Base variations are indicated in bold face.

## Results and Discussion

**In vitro selection with KRAzR**: An in vitro selection of RNA aptamers that bind to agarose-immobilized KRAzR, consisting of Az and other three amino acids (Figure 1a) was previously performed,[18] and identified 26 aptamers through eight cycles of the selection. To drive the enrichment of aptamers, we have continued the selection process for additional two cycles. The RNA pool included 70 random nucleotides flanked by two defined primer-binding sites was used. Following the negative selection with a glycine-immobilized column, eluted RNA was applied to the agarose column of KRAzR for positive selection. Retained RNA fractions were recovered by competitive elution with 6 mM KRAzR. The degree of enrichment of RNA aptamers binding to (*E*)-KRAzR was confirmed by SPR measurements. As the selection cycle proceeded, the increase in the SPR response of each RNA pool to the KRAzR-immobilized surface was observed (Figure 1b). After 10 cycles, the reverse-transcribed DNA library was cloned into pUC19 vector and transformed into *Escherichia coli*. From 31 clones, 22 RNA aptamer sequences were obtained (Figure 1c). The variability of the isolated sequences was moderately converged compared with that of eight cycles; for example, aptamer 56 was found in seven times in 31 clones but other aptamers were found one or two times. The binding of identified aptamers was examined by SPR with the KRAzR sensor (Figure 2a). To evaluate the characteristics of the aptamer binding to the KRAzR-immobilized surface, the ratio ($RU_{400}/RU_{max}$) of the SPR signal at 200 s ($RU_{max}$), where the SPR signal reached to maximum, and that at 400 s ($RU_{400}$) was plotted against $RU_{max}$ (Figure 2b). In the plot of $RU_{400}/RU_{max}$ against $RU_{max}$ values, the affinity of the RNA aptamers to the surface is evaluated by the value of $RU_{max}$, whereas the $RU_{400}/RU_{max}$ ratio indicates the rate of dissociation ($k_d$). Aptamer 66 showed the highest affinity among seven aptamers with $RU_{max}$ of 1086 and the $RU_{400}/RU_{max}$ was 0.47. Aptamer 74, a minor sequence variant of aptamer 66 showed a decreased affinity ($RU_{max}$ 605) but had a similar dissociation rate. Aptamer 72 had a high affinity with $RU_{max}$ 893 to the surface and a very low $RU_{400}/RU_{max}$ of 0.20 indicating a relative high dissociation rate. In contrast, aptamers 56, 57, and 58 showed lower affinity but higher $RU_{400}/RU_{max}$ values than the other three aptamers. The $RU_{400}/RU_{max}$ was 0.57 for aptamer 56 and was reached to 0.72 for aptamer 57. These three aptamers have almost identical sequences, and therefore, they could be categorized to be one group. Detailed kinetic analyses of aptamer 66 and 58 to (*E*)-KRAzR-immobilized surface were performed by SPR experiments under the different aptamer concentrations with a curve fitting method (Figure S1). The rates of the binding ($k_{on}$) and the dissociation ($k_{off}$) for aptamer 66 were $3.7\times10^{3}\,M^{-1}\,s^{-1}$ and $2.9\times10^{-3}\,s^{-1}$, respectively, giving the dissociation constant ($K_D$) of $7.9\times10^{-7}$ M. The $k_{on}$ and $k_{off}$ for aptamer 58 were $1.5\times10^{3}$ and $3.4\times10^{-3}$, respectively, resulting in a $K_D$ value of $2.3\times10^{-6}$ M.

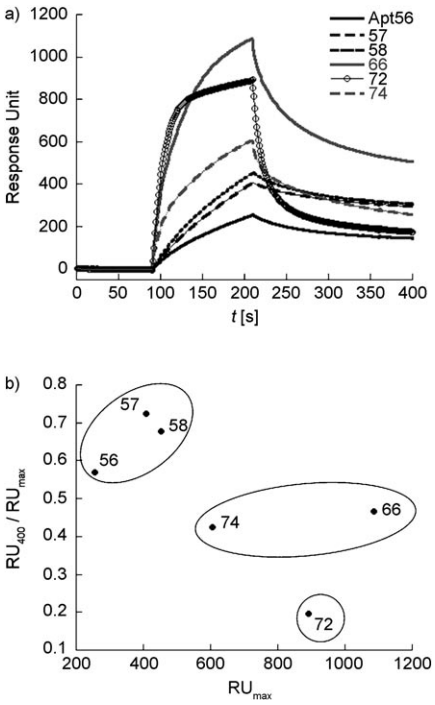



Figure 2. SPR analyses of selected RNA aptamers. a) SPR sensorgrams showing the binding of various RNA aptamers. Sample solutions (1.0 μM) of RNA aptamers were separately injected over the surface for periods of 120 s to monitor the association, and then running buffer was applied to monitor the dissociation. b) Scatter plots of $RU_{400}/RU_{max}$ against $RU_{max}$ for aptamer 56, 57, 58, 66, 72, and 74. RNA aptamers categorized into the same group in Figure 1c are circled with a solid line.

**Photoswitchable binding of selected RNA aptamers**: We investigated the photoswitchable binding of aptamer 58 as a representative of one group and 66 on the KRAzR-immobilized gold surface. Because irradiation of the sensor surface within the BIAcore instrument was not executable, the sensor chip was irradiated outside the instrument with a xenon lamp under coverage of buffer solution (Figure 3a). The sensor chip was then immediately set into the SPR instrument. Each aptamer solution was injected over the surface for 120 s to monitor the association. Then the running buffer was applied over the surface to monitor the dissociation of bound aptamers. Upon photoirradiation of the KRAzR-immobilized surface at 360 nm for five minutes, the binding of each aptamer to the surface was decreased by more than 85%, and subsequent photoirradiation of the same surface with 430 nm light restored the aptamer binding (Figure 3b–d).

To demonstrate that association and dissociation of aptamer–KRAzR complex are reversibly regulated by direct photoirradiation to the complex, we have investigated real-time monitoring of the ligand–aptamer interaction on a gold surface. For these studies, we used the SPR imaging instrument, where photoirradiation to the sensor was executable by using handmade chip holder through a window for photoirradiation.[20,21] KRAzR with polyethyleneoxide (PEO) linker at ε-amino group of the lysine residue was synthe-

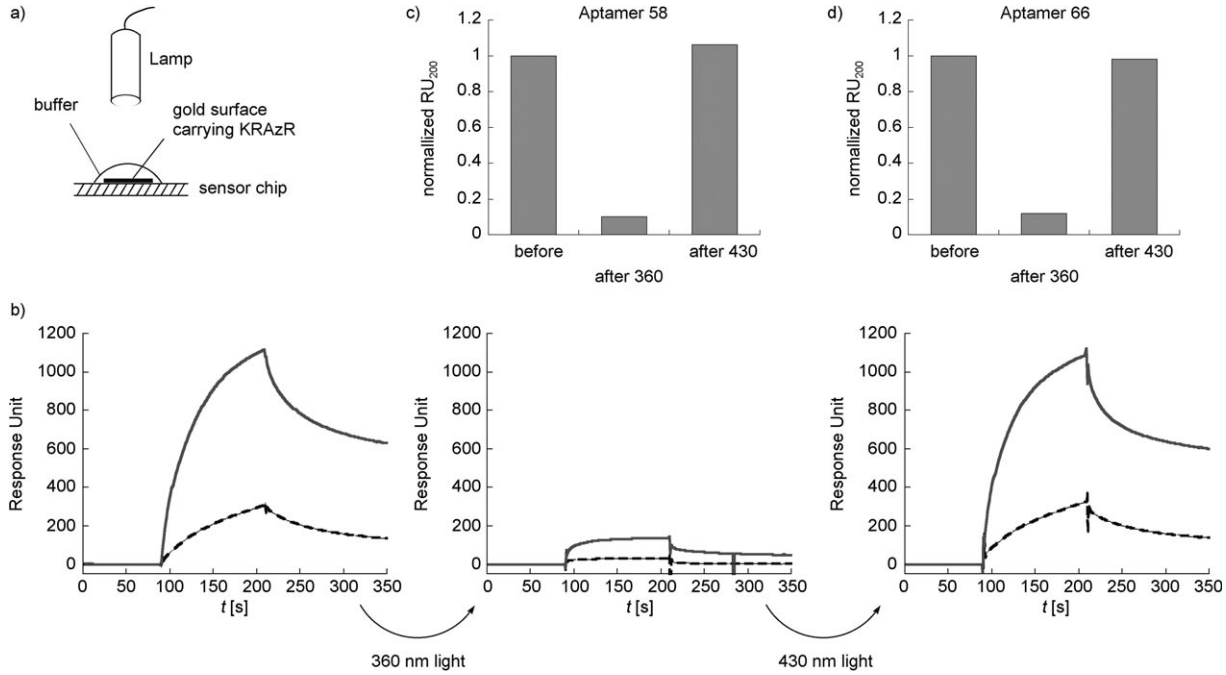



Figure 3. Photoswitchable changes in the affinity of aptamers on KRAzR-immobilized gold surface. a) Illustration of photoirradiation. The ligand-immobilized gold surface was covered with running buffer. After photoirradiation, the sensor chip was set into Biacore2000. b) SPR sensorgrams of aptamer 58 (black dotted line) and 66 (gray solid line) in the association and dissociation phase by injecting RNA over the surface for periods of 120 seconds. Measurements were done before photoirradiation, after photoirradiation at 360 nm for 5 min, and after subsequent irradiation at 430 nm for 5 min. c) and d) Normalized $RU_{200}$ (response unit at 200 s) before and after photoirradiation on SPR measurement.

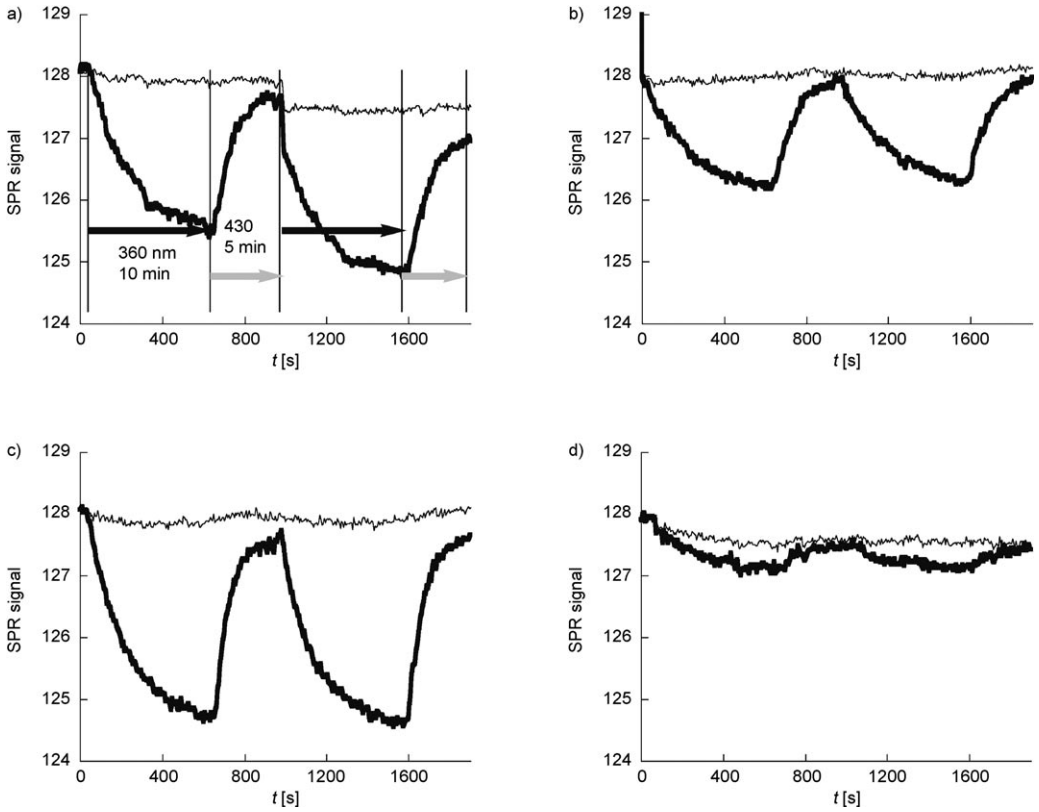



Figure 4. Real-time monitoring of photoresponsive binding of aptamers to KRAzR-immobilized surface. In each measurement, SPR signals were detected at two areas, with KRAzR (——) and without KRAzR (——). We analyzed the binding of aptamer 57 (a), 66 (b), and 72 (c). SPR response in the absence of RNA was shown in (d). In equilibrium between RNA and KRAzR, the flow was stopped and then photoirradiation started ($t=0$). The gold surface was exposed to 360 nm light for 10 min and 430 nm light for 5 min, alternately. The SPR signal change on KRAzR-immobilized surface and the background were described by the thick or thin lines, respectively.

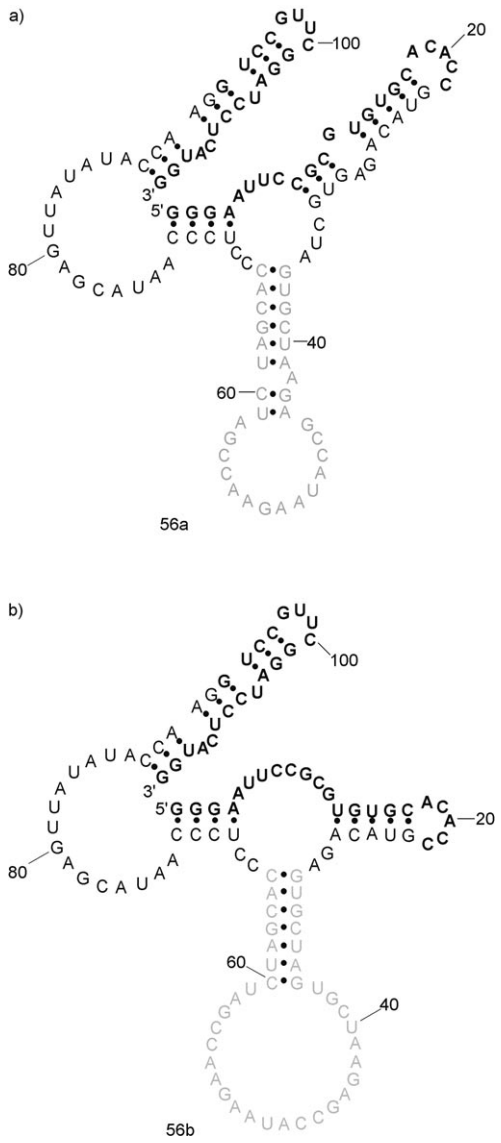



Figure 5. Simulated secondary structures for aptamer 56 by *mfold*. Forward and reverse primer sequences were shown in bold face. Stem-loop structures common to aptamers 56, 57, and 58 were depicted in gray. Hydrogen bonds were depicted by filled circles.

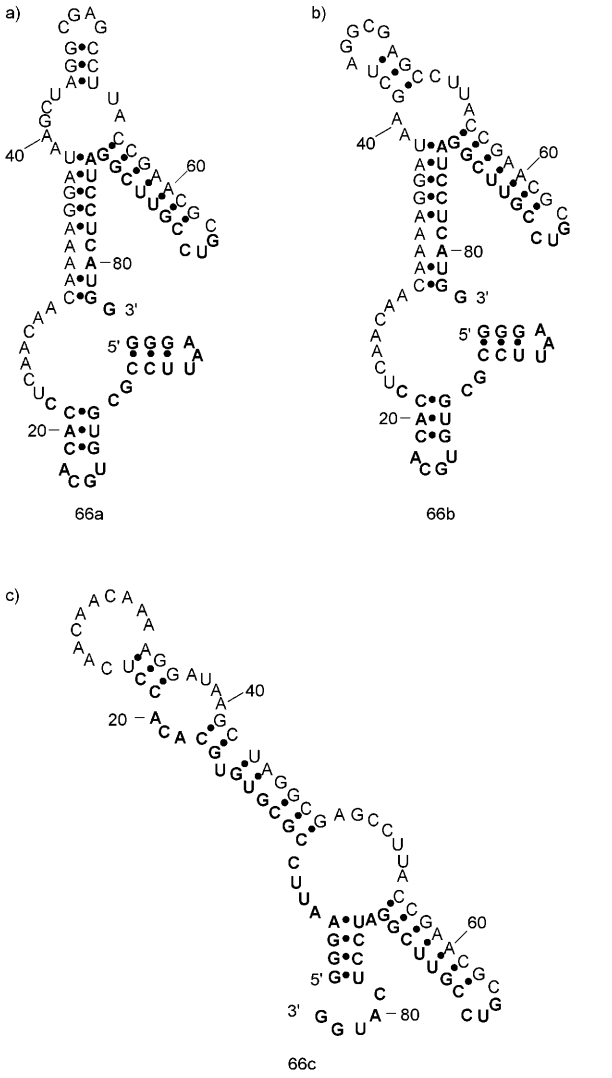



Figure 6. Simulated secondary structures 66a, 66b, and 66c of aptamer 66 by *mfold*. Forward and reverse primer sequences were indicated in bold face; ●: hydrogen bonds.

sized (Figure S2) and immobilized onto gold surface precoated by a carboxyl group. The PEO linker was introduced to increase the SPR signal by making KRAzR more flexible on the surface. Aptamer solution was injected to the surface, and then the flow was stopped to coat the sensor surface with the aptamer solution. Formation of complexes between (*E*)-KRAzR and aptamers 57, 66, or 72, on the surface was confirmed under the equilibrium state. Upon exposure the surface with 360 nm light for 10 minutes, significant decrease of the signal was observed (Figure 4). Subsequent photoirradiation of the surface at 430 nm for five minutes induced increase of SPR signals. The cycle of 360 nm and 430 nm photoirradiation could be repeated without significant loss of SPR signals. Without RNA aptamers, only a small change in the signal intensity was observed (Figure 4d). These results indicate that the significant SPR signal changes caused by alternate photoirradiation were in fact due to switching of the aptamer binding with response to the structural change of KRAzR isomer (*E* to *Z* or *Z* to *E*).

**Structure-binding relationship analysis**: Having confirmed the photoresponsive binding of aptamers selected from N70 random library against (*E*)-KRAzR, we then focused our attention on the investigation of structure-binding relationship of selected aptamers. Folding simulation with two groups of aptamers (aptamer56 and 66) was carried out by *mfold* program,[22] followed by a binding assay with truncated aptamer sequences by SPR. Each of aptamer 56, 57, and 58 provided two analogous secondary structures by the folding simulation, indicating that binding mode of these aptamers to KRAzR should resemble each other. The predicted structures of aptamer 56 (56a and 56b) were shown in Figure 5.

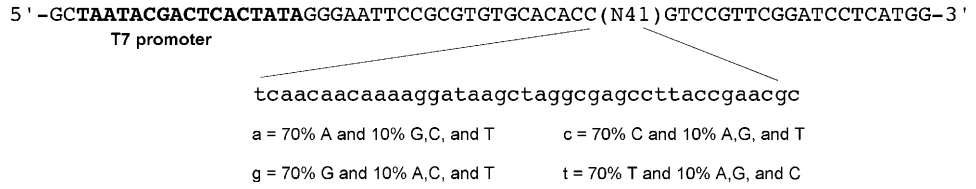



Figure 7. DNA library used for doped selection. The sequence of the randomized region consists of 41 nucleotides. Each nucleotide in the randomized region contains original sequence derived from aptamer 66 with probability of 70%.

The stem-loop structure consisting of nucleotides 36–66 in 56a and that of nucleotides 30–66 in 56b were found in all of aptamer 56, 57, and 58 (Figure 5).

RNA sequences of these stem-loop structures were separately prepared and analyzed on the KRAzR-immobilized surface by SPR. The SPR data, however, showed that both stem-loops were not responsive to the binding of aptamer 56 to (*E*)-KRAzR (Figure S3). Three secondary structures 66a, 66b, and 66c were obtained for aptamer 66 by *mfold* program (Figure 6). The structures of 66a and 66b were identical except for one stem-loop structure consisting of nucleotides

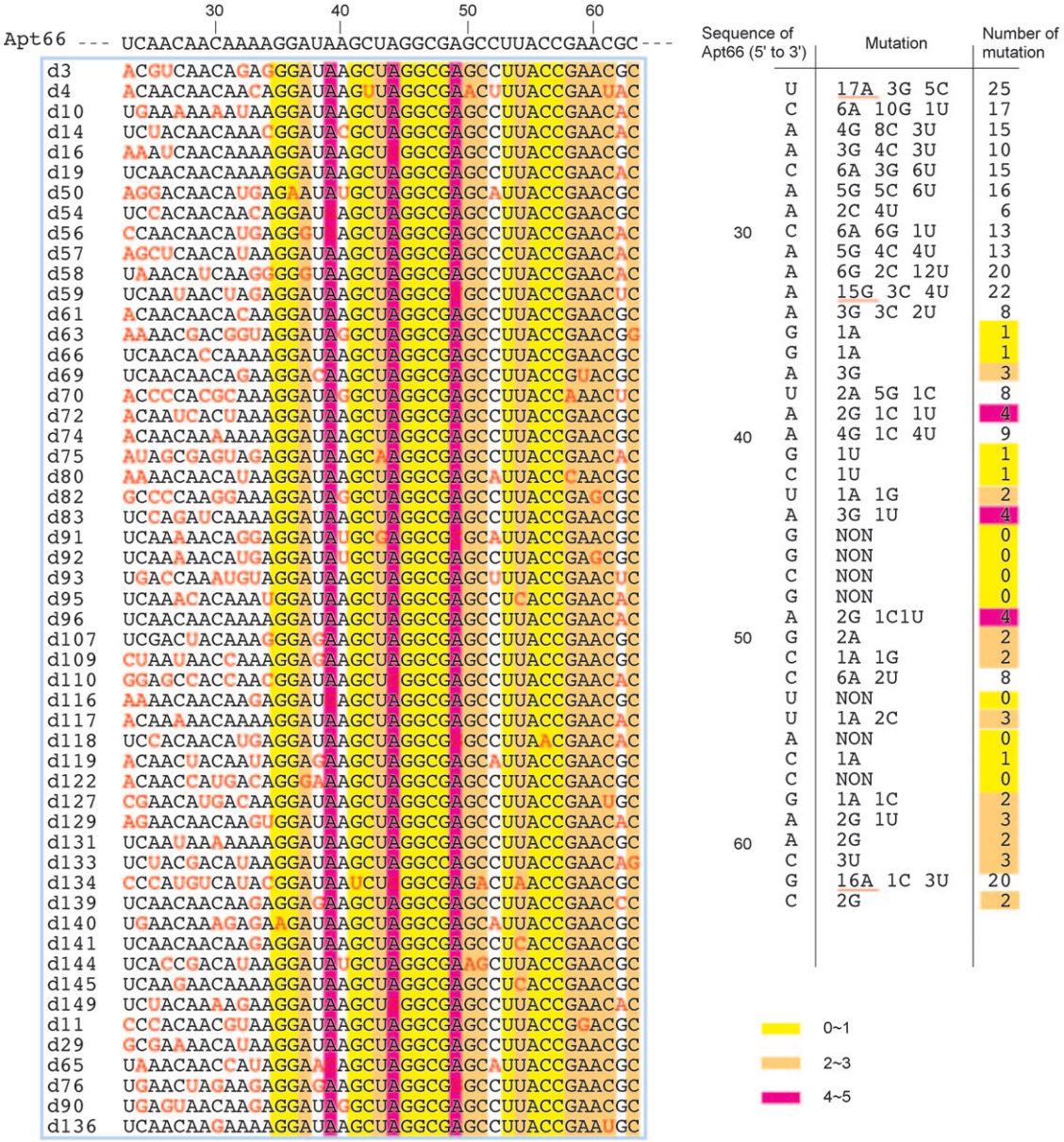



Figure 8. Resulting aptamer sequences from the doped selection. a) Sequences of 53 RNAs obtained from the round 4. Sequences corresponding to the randomized regions are shown. Mutations from the original sequence are indicated in red. b) A table showing mutation profiles. Conserved nucleotides were classified into three groups by the mutation frequency; 0–1 (yellow), 2–3 (orange), and 4–5 (purple). Dominant mutations found at positions 23, 33, and 62 were underlined in red.

around 40–55. The structure of 66c has quite different constitution from 66a and 66b. A structural feature of 66c is that forward and reverse primer region hybridized each other to form a circular conformation. To probe essential region for binding to KRAzR, we tested interaction between truncated 66a, 66b and 66c and (*E*)-KRAzR by SPR, but found that none of these showed the affinity to the KRAzR surface (Figure S4). The series of experiments suggest that interaction between these aptamers and KRAzR might be achieved by using not only the partial sequence of the randomized region but also the whole sequence including forward and reverse primer region.

**Doped selection**: In order to gain deeper insight into the sequence and secondary structures responsible to the binding of aptamer 66 to KRAzR, we further investigated aptamer 66 by doped reselection strategy,[23,24] a kind of in vitro selection, in which essential regions in the aptamer sequence for binding to ligand can be surveyed. A doped sequence pool was generated on the basis of the original sequence of aptamer 66 having 41 nt randomized region in total 83 nt length (Figure 7). Each nucleotide base in the original sequence was mutagenized to contain 70% wild-type nucleotides and 30% non-wild-type nucleotides. For example, if the wild-type base is A, the mutagenized base is composed of 70% of A and 10% each of G, C, and T. By means of the affinity separation with agarose derivatized with KRAzR, the RNA sequences binding to KRAzR was enriched. After four rounds of selection, the enriched RNA pool was reverse-transcribed and PCR-amplified. The subsequent cloning and sequencing gave 53 RNA sequences. The mutation profiles of these clones compared with the original aptamer 66 were shown in Figure 8. Doped-reselection method enabled us to distinguish conserved regions from nonconserved regions by frequency of mutations at the particular nucleotide position. Among 41 nucleotides, seven nucleotides, G45, G46, C47, G48, U53, A55, and C57 were completely conserved. Other five nucleotides G35, G36, G41, C42, and C56 were mutated only one time in 53 clones. If certain nucleotides do not contribute to the interaction with KRAzR, the frequency of mutation at the position should be about 16 (30% of 53) because the degree of the mutation frequency of the initial DNA library is 30%. The mutation frequencies of the nucleotides in the position 35–63 were considerably smaller than the original value of 16, indicating that the original sequence of aptamer 66 in this region was most likely responsible for the binding to KRAzR. In marked contrast, the nucleotides in the position of 23–33 retained the original mutation frequency, suggesting that the sequence variability in this region was not likely responsible for the aptamer binding to KRAzR.

The secondary structure model 66c was supported by the results of the doped reselection. The distribution of the mutation frequency obtained in Figure 8 was in good agreement with secondary structure of 66c, whereas not to 66a and 66b. Conserved nucleotides were mainly distributed to the stem regions formed by hybridization with the primer sequences, and seemed to play an important role in keeping the secondary structure of 66c (Figure 9). The nucleotides positioned at the end of the stem showed low frequencies of the mutation, whereas nucleotides forming loop structures exhibited relatively high mutation frequencies. The non-conserved region (23–33) obtained in the doped reselection was distributed to the loop structure in 66c. This suggests that this loop structure would not participate in the interaction with KRAzR. Although the stem structure formed by forward and reverse primer sequence seem to serve as a clip for holding the conformation of 66c, the truncated variants of aptamer 66, tApt66 that lacked a part of primer sequences actually did not bind to KRAzR on SPR measurements (Figure S4), indicating that both of primer sequences were essential for the binding to KRAzR. Considering all of these results, we could guess that secondary structure 66c is the most plausible structure of the three.

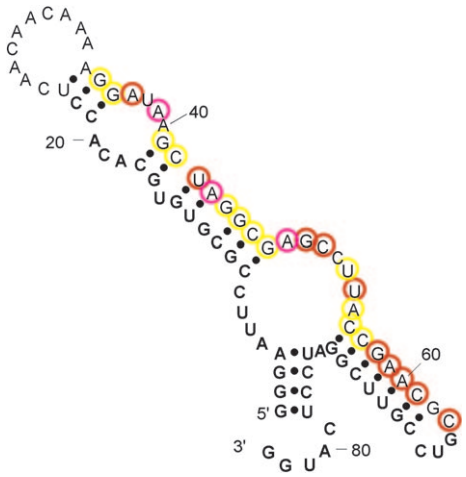



Figure 9. Proposed secondary structure of aptamer 66 necessary for binding to KRAzR. A structure consistent with the result of the doped reselection and additional SPR experiments is depicted. Conserved nucleotides in the doped reselection were circled, and the color is corresponding to that in Figure 8. Forward and reverse primer sequences were depicted in bold face; ●: hydrogen bonds.

## Conclusion

In this work, we reported characterization of several RNA aptamers obtained by in vitro selection with photoresponsive peptide, KRAzR. Each aptamer selectively bound to *E* isomer of KRAzR immobilized on SPR gold surface, and not to *Z* isomer. We could also confirm the direct photoinduced dissociation of aptamer–peptide complex on SPR measurements. The changes of the SPR sensorgrams were fully matched to the photoirradiation to the surface. The doped reselection method revealed conserved region of aptamer sequence. Combining doped reselection with secondary structure obtained by RNA-folding program, we could determine the plausible secondary structure of aptamer 66. These studies provided us important insights into the molecular design of photoresponsive peptides and the library

design of RNA pools that will be useful for the improved photoregulation of small molecule-RNA interaction with regard to the efficiency and reversibility.

## Experimental Section

**In vitro selection**: For the starting selection on KRAzR, a 131-mer synthetic oligonucleotide (5′-GC*TAATACGACTCACTATA*GG**GAATTC**-CGCGTGTGCACA CC($N_{70}$)GTCCGTTC**GGATCC**TCATGG-3′) that contained a T7 RNA polymerase promoter (underlined), a 70-nt random region and recognition sites of restriction enzymes, *Eco*RI and *Bam*HI, respectively (shown in a bold face), was purchased from Japan Bio-Service Inc. (Saitama, Japan). An amount of 70 μg of DNA ($10^{15}$ molecules) was amplified by PCR in a total volume of 8.0 mL in 80 tubes containing 100 μL of PCR mix (1 μM of forward and reverse primers; dNTP mix; Taq polymerase) for 4 cycles (temperature cycle conditions: 98 °C, 10 s; 55 °C, 30 s; 72 °C, 30 s). After isopropanol precipitation, 170 μg of amplified DNA were used in a 2.7 mL transcription reaction in 27 tubes, each containing 100 μL of transcription mix (AmpliScribe T7 high yield transcription kits) and transcribed at 37 °C for 6 h. DNase I was added and incubation was continued for 30 min. The RNA was purified by gel filtration by NAP 10 column (Amersham Biosciences).

KRAzR was coupled to Affi-gel 10 (Bio-Rad) according to the manufacturer's instructions. The resin was prepared with 5.0 μM ligand per milliliter of agarose (5.0 mM). For preselection, a glycine-derivatized agarose (10.0 mM) was used. To minimize the enrichment of RNA molecules that bind to the parts of agarose and spacer region, the pool was pre-selected on glycine-derivatized agarose. Agarose for the selection was equilibrated with several column volumes of binding buffer (250 mM NaCl; 50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$). A 500 μL sample of the RNA pool (550 μg) in the binding buffer was loaded onto the pre-selection column. The pre-selection column was rinsed with 1.0 mL of binding buffer, and the eluting RNA was directly applied onto the selection column. This column was rinsed with 500 μL and then with 4 mL of a binding buffer. The flow through was discarded. Elution of the aptamers was performed with 3.0 mL of a 6.0 mM solution of KRAzR in a binding buffer. The eluted RNA was purified by isopropanol precipitation and reverse-transcribed at 42 °C for 30 min by reverse transcriptase. The resulting cDNA was PCR-amplified by Taq polymerase, purified, and transcribed. The purified RNA was used as the input for the next selection cycle.

Sample solutions of RNA pool (1 μM) were separately injected over KRAzR-immobilized surface (see below) for periods of 120 s to monitor the association by SPR, and then running buffer was applied to monitor the dissociation.

**Binding study of aptamers to KRAzR by SPR assay**: SPR measurements were carried out at 25 °C using BIAcore 2000 (GE Healthcare). Photoresponsive peptide KRAzR was immobilized onto the CM5 sensor chip (GE Healthcare) covered with NHS-activated carboxylic acid. After purification by PAGE (8% acryl amide, 200 V, 30 min), transcribed RNA samples were dissolved in a running buffer (250 mM NaCl; 50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$) and analyzed by SPR at concentration with 1.0 μM with Kinject command defining an association time of 120 s and a dissociation time of 120 s. In each measurement, bound RNA was washed out by 10 mM NaOH for regeneration of the surface. Photoisomerization of KRAzR on gold surface was done by irradiation of 360 or 430 nm light to the peptide surface through the droplet of a running buffer.

**Synthesis of KRAzR-PEO**: Colorless oil of polyethyleneoxide (PEO) linker (7.8 mg, 16.5 μmol) was dissolved in 500 μL of MeOH. To 1 mL of 5 mM KRAzR in reaction buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl) was added 200 μL of the PEO linker solution and stirred at room temperature for 5 h. The reaction was monitored by HPLC. The reaction mixture was purified by HPLC (water/acetonitrile containing 0.1% TFA). The isolated compound (KRAzR-PEO-Boc) was identified by ESI-TOF MS. Aqueous hydrochloric acid (4 M, 500 μL) was added to the eluent for deprotection of Boc group. After the reaction had been completed, the sample was evaporated. The residue was dissolved in water and concentration of the solution was determined by UV absorbance.

**Direct photoirradiation to the aptamer–peptide complex on SPR imaging sensor**: A gold-coated chip (TOYOBO, SPR-200) for SPR-imaging analysis was purchased from TOYOBO (JAPAN). A 5 mL of 1 mM PEG6-COOH alkanethiol (TOYOBO, SPT-0012 A) in ethanol was added onto the gold surface and reacted for more than 7 h. After washed with ethanol and distilled water, the COOH-modified surface was reacted for 30 minutes with 200 μL of EDCI/NHS solution to create a NHS-activated surface. The 10 nL drops of 1 or 0.5 M KRAzR-PEO in reaction buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl) were delivered automatically on the NHS-activated COOH surface using an automated spotter (TOYOBO). The amide coupling was left reacted for 5 h. A 200 μL of 100 mM ethanolamine was further reacted on the array surface for 1 h to block the remained NHS group. The array surface was rinsed with 500 mM KCl, 10 mM NaOH and water.

The KRAzR-PEO immobilized SPR array was placed in the SPR imaging instrument MultiSPRinter (SPR-101, TOYOBO, Japan). After washing the surface with 50 mM NaOH for 5 min, running buffer (binding buffer used in in vitro selection) was applied onto the array for 1 min. RNA aptamer (10 μM, 200 μL) in the running buffer were applied with a flow speed of 100 μL $min^{-1}$. The flow was stopped after the whole sample was injected. Then 360 nm light was directly irradiated to the surface for 10 min and continuously 430 nm light was irradiated for 5 min. For control experiments, a series of irradiation were performed in the presence of RNA pool of 1 st round and in the absence of RNA aptamer. All SPR experiments were performed at room temperature. The SPR array was reused after washing with 50 mM NaOH for 3 min. The SPR sensorgrams were collected with MultiSPRinter Analysis program (TOYOBO).

**Doped reselection**: For preparing doped RNA pool, a 83 mer synthetic ssDNA (5′-GC*TAATACGACTCACTATA*GG**GAATTC**CGCGTGTGCA-CACC(tcaacaacaaaaggataagctaggcgagccttaccgaacgc)GTCCGTTC-**GGATCC**TCATGG-3′) that contained a T7 RNA polymerase promoter (underlined), a 70-nt random region and recognition sites of restriction enzymes, *Eco*RI and *Bam*HI, respectively (shown in a bold face) was purchased from Japan Bio-Service Inc. The initial sequence of RNA pool was based on that of aptamer 66. 41 positions t42–c82 were doped to 30%; 10% of each mutant nucleotide. 11.5 nmol ($10^{15}$ molecules) of ssDNA was used for the first PCR for doped selection. Production of RNA pool and selection procedure was as for KRAzR selection.

## Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (S) (18105006) for K.N. and by a Grant-in-Aid for JSPS Fellows (18-3285) for G.H. from Japan Society for the Promotion of Science (JSPS). Competing interests statement: The authors declare that they have no competing financial interests.

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Received: May 16, 2008
Revised: August 4, 2008
Published online: November 26, 2008

  