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Photocrosslinking of RNA and PhotoMet-Containing Amphiphilic α-Helical Peptides

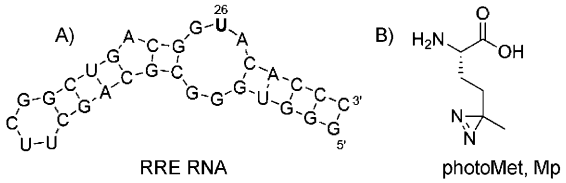
Soonsil Hyun, Areum Han, and Jaehoon Yu*[a]

RNAs play a central role in biological systems. The functions of RNAs are intimately connected with RNA-binding proteins (RBPs) and, as a result, their roles cannot be considered in the absence of the complementary proteins. During the period of their existence in cells, RNAs must interact with proteins in order to function.[1] Known RBPs comprise a significant portion of the total cellular proteome and their number is likely much larger than is currently known.[2] Therefore, among biological macromolecules, RNA–protein interactions are only exceeded in frequency by protein–protein interactions.

Photocrosslinking methods have been powerful tools for exploring the nature of RNA–protein interactions.[3,4] Recently, the cross-linking and immunoprecipitation (CLIP) method has been used to elucidate unknown RNA sequences that interact with RBPs.[5] However, this method leads indiscriminately to cross-linked adducts between almost all bases and many amino acids. In order to obtain greater selectivity, special psoralen-tagged amino acid(s) have been inserted into the respective RNA and protein backbones.[6,7] Unfortunately, these photosensitive groups may themselves cause non-natural interactions to take place.

In recent applications of photocrosslinking, substances that do not alter natural interactions between biological systems and that serve as reactants for the in situ photogeneration of carbenes have been widely used in place of large photosensitive molecules.[8,9] Covalent adducts arising by irradiation of photolabile diazirines have been used to characterize many protein–protein,[10] protein–carbohydrate,[11] protein–peptide,[12] and even protein–small molecule interactions.[13] In theory, a diazirine moiety can be incorporated into a specific base of RNA so that irradiation would promote formation of covalent adducts between the interacting RNAs and RBPs.[14,15] To obtain information from endogenous RNA sequences, however, the diazirine moiety must be located in an opposite part of RNA, even though this combination makes the formation of covalent adducts less likely to occur, due to the lack of strong nucleophiles in RNA. To the best of our knowledge, this report presents the formation of covalent adducts between RNA and diazirine-containing peptides for the first time.

We have studied RNA-specific, amphiphilic peptides with nanomolar affinities against various hairpin RNA targets.[16] We hypothesized that the nanomolar binding affinities of these peptides would enable the formation of covalent adducts through reactions of photogenerated carbenes with even relatively poor nucleophiles in RNA. It is convenient to insert a photolabile diazirine into peptides since the modified peptide photoMet can be readily synthesized.[11] Below, we describe the synthesis of photoMet-containing amphiphilic peptides, which upon irradiation form covalent adducts with the typical hairpin RNA, Rev responsive element (RRE) of HIV-1.[17] Formation of covalent adducts takes place specifically at sites where a nucleophile is available near to a photogenerated carbene.



As an alternative sequence to the original de novo designed Lys- and Leu-rich peptides,[18] an amphiphilic Arg-rich peptide (ARRP in Table 1) would have stronger contacts with RNA. ARRP has an affinity comparable to that of the natural Rev peptide against RRE RNA.[21] One of these amino acids can be substituted by a photoactive diazirine-bearing Met analogue (photoMet) without greatly diminishing its interactions with RNA.

Table 1. K_d values for the binding of photoMet-labeled peptides with their RRE RNA target.[a]

Peptide	Peptide sequences	K_d [nM] against RRE RNA
ARRP	Ac-LRRLLRLLRRLLRLAG	2.2
L1Mp	Ac-MpRRLLRLLRRLLRLAG	4.5
R2Mp	Ac-LMpRLLRLLRRLLRLAG	14
R3Mp	Ac-LRMpLLRLLRRLLRLAG	1.1
L4Mp	Ac-LRRMpLRLLRRLLRLAG	4.2
L5Mp	Ac-LRRLMpRLLRRLLRLAG	2.8
R6Mp	Ac-LRRLLMpLLRRLLRLAG	1.6
L7Mp	Ac-LRRLLRMpLRRLLRLAG	0.70
L8Mp	Ac-LRRLLRLMpRRLLRLAG	4.8
R9Mp	Ac-LRRLLRLLMpRLLRLAG	5.2
R10Mp	Ac-LRRLLRLLRMpLLRLAG	0.29
L11Mp	Ac-LRRLLRLLRRMpLRLAG	3.1
L12Mp	Ac-LRRLLRLLRRLMpRLAG	2.6
R13Mp	Ac-LRRLLRLLRRLLMpLAG	4.0
L14Mp	Ac-LRRLLRLLRRLLRMpAG	0.92
A15Mp	Ac-LRRLLRLLRRLLRLMpG	0.48
G16Mp	Ac-LRRLLRLLRRLLRLAMp	0.80

[a] Affinities were measured at 20 °C using a fluorescence anisotropic technique and rhodamine-Rev peptide as a probe. Each value indicates the average of more than three independent experiments.[19]

[a] S. Hyun, A. Han, Prof. J. Yu
Department of Chemistry and Education, Seoul National University
Seoul 151-742 (Korea)
Fax: (+82)2-880-7761
E-mail: jhoonyu@snu.ac.kr

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Six Arg residues in the hydrophilic sphere and eight Leu residues in the hydrophobic sphere of ARRP were sequentially replaced by photoMet.[19] Binding affinities of the peptides against RRE RNA (Table 1) were found to vary in the range of 0.29 nM to 14 nM. Weaker binding was seen for peptides with photoMet mutations in hydrophilic spheres (average K_d = 4.4 nM) as compared to those with mutations in hydrophobic spheres (K_d = 2.7 nM). This trend indicates that Arg residues in hydrophilic spheres contribute more greatly to binding with RNA than do those in hydrophobic spheres. As a result, the hydrophilic sphere has a higher probability of providing nucleophiles for photocrosslinking.

Next, we probed conditions that would cause efficient photocrosslinking. Initially, mixtures containing variable concentrations of each peptide (1:1→1:100 RNA/peptide) in the library and low concentrations of RNA (>10 nM) were irradiated. Under these conditions no covalent adducts were produced. In contrast, irradiation of mixtures containing micromolar ranges of RNA (1:1→1:40 RNA/peptide) led to formation of covalent adducts (Figure 1).[19] Interestingly, the amounts of covalent adducts generated in these reactions depends greatly on the position of the photoMet residue. Only R10Mp, which has the strongest binding affinity, formed a crosslink adduct (~8%) with RRE RNA at the optimized conditions.[20]

Footprinting experiments were carried out to determine the nature of the RNA nucleophiles that participate in photoreactions with the most selective R10Mp mutant. The isolated covalent adducts, in which both ends of RRE RNA are labeled with ^{32}P, were subjected to alkaline hydrolysis.[19] Autoradiograms of the hydrolysis products of both 5′- and 3′-end-labeled RRE-peptide covalent adducts after electrophoresis displayed discreet ladders, while those of the intact RNA treated in the same manner did not (Figure 2). This analysis showed that a nucleophile in uridine 26 is responsible for covalent adduct formation. The region of RRE RNA that contains uridine 26 is a bulge, a typical binding site of the Rev peptide[21] and ARRP. Exposure of the key uridine moiety to the surface of RNA might increase the probability for covalent adduct formation. The electron rich carbon-carbon double bond in this base is likely responsible for the covalent bond-forming reaction with the carbene.[22]

In summary, a library of photoMet-labeled peptides was designed and synthesized using an Arg- and Leu-rich α-helical amphiphilic peptide. Irradiation of mixtures of these peptides and RRE hairpin RNA promoted formation of covalent adducts. The efficiency for formation of the covalent adducts was found to depend on the position of the photoMet mutation in the peptide. Analysis of the covalent adduct generated efficiently by irradiation of a mixture of RRE RNA and R10Mp peptide showed that uridine 26 in the bulged stem is responsible for covalent bond formation with the carbene intermediate. These covalent adducts between RNA and peptides can be generally used for the elucidation of RNA–peptide (or RNA–protein) interactions.

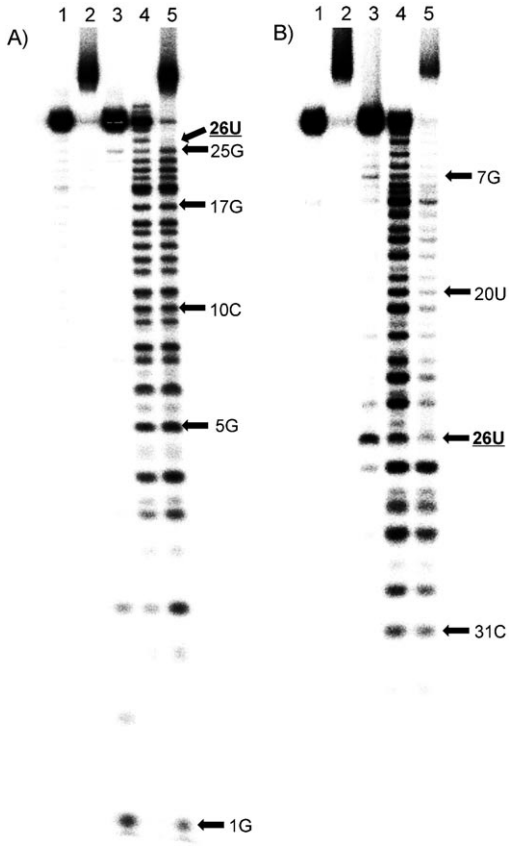

Figure 2. Analysis of crosslinked position. A) autodiagram of 5′-^{32}P end-labeled RRE. B) autodiagram of 3′-^{32}P-end-labeled RRE. Lane 1, intact RRE RNA; lane 2, intact covalent adducts w/R10Mp; lane 3 RNase T1 marker; lane 4, alkaline hydrolysis for RRE RNA; lane 5, alkaline hydrolysis for covalent adducts w/R10Mp.

Experimental Section

Syntheses of peptides: Peptides were synthesized on Rink amide methylbenzhydrylamine (MBHA) resins using a standard solid phase protocol and purified using HPLC, and identified by MS.[19]

Photocrosslinking: Initial photocrosslinking experiments were performed in ten replicates on a 30 μL scale in a cross-linking buffer containing Tris (50 mM), $MgCl_2$ (1 mM), NaCl (150 mM), DTT (1 mM) at pH 7.5. Briefly, mixtures of a peptide and preannealed ^{32}P-labeled RRE RNA in described concentrations were incubated for 10 min on ice using 150 μL PCR tubes. The reaction mixtures were irradiated at 302 nm by a UV transilluminator (SL-20 High Performance DNA Image Visualizer™, SeouLin Bioscience. Co., Ltd, Seoul, Korea) for 10 min and incubated for 10 min on ice again. Cross-linking with various concentrations of R10Mp was performed to observe EC_{50} values (4.3 μM, Figure 3) of RRE with the mutant peptide. To

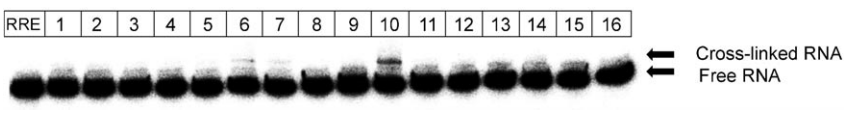

Figure 1. Sequence selectivity of the photocrosslinking with 5′ ^{32}P-end labeled RRE RNA. RRE, intact RRE RNA 5 μM; lane 1~16, each Mp mutant of ARRP irradiated with RRE RNA 5 μM in order.

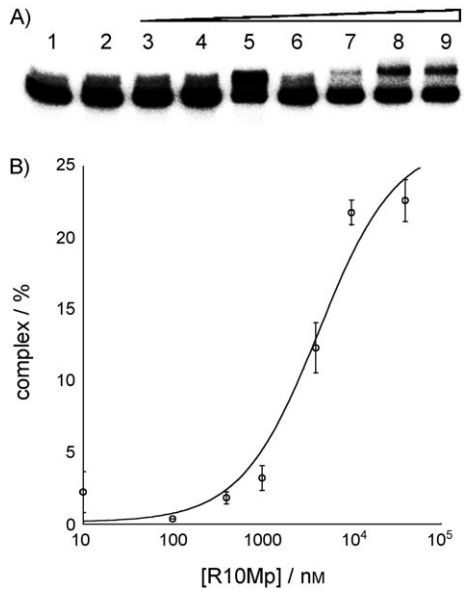

Figure 3. Cross-linking efficiency of R10Mp. A) Representative autoradiogram of photocrosslinking reaction of R10Mp with RRE RNA. Lane 1, intact RRE RNA 1 μM; lane 2 ~9, irradiated under previously described condition; lane 2, reaction w/o R10MP; lane 3 ~9, RRE RNA 1 μM reaction w/10, 100, 400, 1000, 4000, 10000, 40000 nM of R10Mp, respectively. The value indicates average and one standard deviation of individual two experiments. B) Fitting curve for cross-linking efficiency. $EC_{50} = 4.3 \pm 1.9$ μM.

scale up cross-linked adducts for footprinting experiments, radiolabeled RRE (50 nM) was irradiated in the presence of R10Mp (50 μM) in a final volume of 300 μL.

Fluorescence anisotropy (FA): FA measurements were performed on a PerkinElmer LS55 equipped with a thermocontrolled water circulator as described previously.[16]

Labeling of RRE RNA: The RRE probe (~80 pmoles) was radioactively labeled at the 5′-end by using of [γ-^{32} P] ATP (500 μCi, New England Biolabs) and polynucleotide kinase (50 units, New England Biolabs), after the hydrolysis of 5′-phosphate by alkaline phosphatase (20 units, CIP, New England Biolabs). The same amount of RRE RNA was 3′-end-labeled using cytidine 3′,5′-bis(phosphate) (Perkin–Elmer) catalyzed by T4 RNA ligase 1 (New England Biolabs) following the manufacturer's protocol. Labeled probe was separated from small nucleotides by passage through G-25 Sephadex beads (Sigma).

RNA Footprinting Assay: A labeled solution of RRE RNA (~10 nM) was heated to 65 °C for 5 min and slowly cooled to room temperature in a buffer containing 4-(2-hydroxyethyl) piperazine-1-ethansulfonic acid (HEPES, 20 mM), $MgCl_2$ (1 mM), KCl (5 mM), and NaCl (140 mM) at pH 7.4. RNase T1 (0.2 units or 0.1 unit for 5′-end-labeled or 3′-end-labeled RRE, respectively, Ambion, Austin, TX, USA) was added to the resulting solution, and it was incubated for 15 min at room temperature. Alkaline hydrolysis was performed at 95 °C for 15 min in the presence of 4 μg of yeast RNA using alkaline hydrolysis buffer (Ambion). Then, the fragmented RNA was obtained by ethanol precipitation and dried out. Resulting RNA was electrophoresed by using a polyacrylamide (20%)–urea (7 M) gel at 1000 V for 8 h. The gel was dried over 40 min at 80 °C. The gel was exposed to a phosphorimager screen and individual bands were quantified on a FLA-3000 and analyzed with Multi Gauge Ver. 3.0 software (Fuji Photo).

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Keywords: carbenes · helical peptides · peptides · photocrosslinking · RNA

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