J|A|C|S
COMMUNICATIONS

Published on Web 03/23/2007

α-Helical Peptide Containing *N*,*N*-Dimethyl Lysine Residues Displays Low-Nanomolar and Highly Specific Binding to RRE RNA

Soonsil Hyun,[†] Hyun Jin Kim,[†] Nam Ju Lee,[†] Kyung Hyun Lee,[†,‡] Yeongran Lee,[†] Dae Ro Ahn,[‡] Keysun Kim,[‡] Sunjoo Jeong,[§] and Jaehoon Yu*[,†]

Department of Chemistry and Education, Seoul National University, Seoul 151-742, Korea, Life Science Division, Korea Institute of Science and Technology, P.O. Box 131 Cheongryang, Seoul 130-650, Korea, and Department of Molecular Biology, Dankook University, Seoul 140-714, Korea

Received November 18, 2006; E-mail: jhoonyu@snu.ac.kr

Recently, endogenous enzyme mediated methylation and demethylation processes have been extensively explored owing to their involvement in post-translational modifications that control gene expressions.[1] Earlier work has shown that enzyme catalyzed methylations are associated with RNA-binding peptides and proteins,[2] even though no evidence exists for the direct binding of the enzymes to RNA. These peptides and proteins have rigid structural motifs, such as in α-helices and zinc fingers.[3] In addition, the RNA-binding peptides that possess α-helical structures contain numerous basic amino acid residues,[3c] a clue that their strong binding is a consequence of electrostatic interactions of their ammonium groups with phosphate backbones of the RNAs. A recent investigation has shown that a methylated Lys ammonium residue, located in a β-turn region, interacts with a purine/pyrimidine group in nucleic acids[4] through a novel cation-π interaction.[5] Accumulating observations suggest that methylated Lys and Arg residues in RNA-binding proteins might be responsible for the specificity and selectivity.[6]

Although it has been reported that synthetic peptides containing Arg or Lys residues bind to RNA,[7] a systematic study aimed at uncovering the effect(s) of Lys and/or Arg methylation on RNA binding has not been conducted. Below, we describe studies of *N*,*N*-dimethyl-Lys containing α-helical peptides which show that one of member of this family has a low-nanomolar binding affinity to RRE RNA and a greater specificity than natural Rev peptide against Rev response element (RRE) RNA.

Peptide-**a**, adapted from an amphiphilic peptide originally aimed at calmodulin,[8] has a predicted structure in which its Lys and Leu residues are orientated on opposite sides of an α-helix. In one sense, peptide-**a** structurally resembles aminoglycosides in that several primary amine groups are located in close proximity to one another. A BLAST search of this peptide showed high homology not only with antimicrobial peptides[9] but also with RNA helicase and a TPR repeat,[10] all of which are typical RNA-binding proteins.[11]

N-Methylation of amino groups in Lys containing peptides generally leads to increased basicity and hydrophobicity, and it enhances ammonium ion formation at physiological pH. To explore the effects of these changes on RNA binding affinities, one (peptide-**b**−**d**) and two Lys amine groups (peptide-**e**−**g**) in peptide-**a** were separately replaced by the *N*,*N*-dimethylamine groups. In addition, a tris-*N*,*N*-dimethyl-Lys analogue (peptide-**h**), containing the maximum number of replacements possible without distorting the peptide's rigid conformation,[12] was prepared.[13] Analysis of the circular dichroism (CD) spectra of the peptides both in pH 7.4 phosphate buffer and in 50% 2,2,2-trifluoroethanol (TFE) showed that all of the methylated peptides have similar α-helical structures irrespective of number(s) or position(s) of *N*,*N*-dimethyl-Lys group(s) (Table 1).[14] Nonmodified peptide-**a** has the highest α-helicity content that is compatible with conformational rigidity of Rev peptide.[15]

Table 1. Sequences of the Synthesized Peptides and Their α-Helicities and Binding Affinities against RRE RNA

peptide	sequences (position(s) of K*)[a]	helicity (%)[b]	K_d (nM)[c]
a	LKKLLKLLKKLLKLKG	26/57	22
b	LKKLLKLLKKLLK*LKG (13)	5/45	79
c	LKKLLKLLK*KLLKLKG (9)	9/48	74
d	LKK*LLKLLKKLLKLKG (3)	8/50	75
e	LKKLLKLLK*KLLK*LKG (9, 13)	7/49	30
f	LKK*LLKLLKKLLK*LKG (3, 13)	6/43	69
g	LKK*LLKLLK*KLLKLKG (3, 9)	8/52	9.1
h	LKK*LLKLLK*KLLK*LKG (3, 9, 13)	6/47	87
Rev[d]	TRQARRNRRRRWRERQRAAAAR	33/73	8.5

[a] K* = N^{ϵ},N^{ϵ}-dimethyl Lys. [b] In 10 mM H_3PO_4 /50% TFE in 10 mM H_3PO_4 at pH 7.4.[18] [c] Affinities were measured at 20 °C using fluorescence anisotropy technique.[18] [d] The N-terminus of the peptide is succinylated.[16]

RRE RNA from HIV-1 was chosen as an initial target RNA because it has Rev peptide as a natural binding ligand. Binding affinities of the synthetic peptides were determined by using a fluorescence anisotropy technique with rhodamine-labeled peptide-**a** as a probe molecule.[17] A Job's plot confirmed 1:1 binding stoichiometry between RRE RNA and peptide-**g** as well as Rev peptide using fluorescence intensity changes of 2-aminopurine labeled RNA.[18] Binding affinities of the synthetic peptides were in the mid- to low-nanomolar ranges (Table 1). An order of magnitude differences in affinities, however, is surprising since the peptides have nearly the same α-helical contents and only differ by small changes in the positions of the *N*,*N*-dimethyl-Lys residues. Strongest binding, compatible with that of Rev peptide (K_d = 8.5 nM),[19] is displayed by peptide-**g** (K_d = 9.1 nM) that contains two *N*,*N*-dimethyl-Lys at positions 3 and 9. In contrast, the two other peptides with two *N*,*N*-dimethyl-Lys groups (peptide-**e** and -**f**) do not bind to RRE as strongly as peptide-**g**. Peptide-**h** with a maximum three *N*,*N*-dimethyl-Lys groups showed one of the worst binding affinity. The data suggest that the position of the N,N-dimethylation has a significant effect on the RNA binding affinities of the peptides, whereas the degree of this modification and α-helical content do not play significant roles in governing binding.

Three additional peptides, peptides-**g1**, -**g2**, and -**g3** with *N*,*N*-dimethyl-Lys at positions 2, 9; 3, 10; and 2, 10, respectively, were prepared in an attempt to optimize RNA binding. Affinities (K_d = 31, 33, and 43 nM) of peptides-**g1**, -**g2**, and -**g3** are about 4 times weaker than that of peptide-**g**, even though they have nearly the same α-helical contents (54, 51, 49%, respectively in 50% TFE)

[†] Seoul National University.
[‡] Korea Institute of Science & Technology.
[§] Dankook University.

10.1021/ja068265m CCC: $37.00

Table 2. Affinity (K_d) Comparison of Peptide-**a**,**g** and Rev against Various RNAs[a]

peptide	RRE RNA	$tRNA^{mix}$	TAR RNA
a	22	55 (2.5)	62 (2.8)
g	9.1	42 (4.6)	53 (5.8)
Rev	8.5	20 (2.4)	21 (2.5)

[a] Values are in nM. Discrimination ratios (K_d against the other RNAs/K_d against RRE) are calculated in parenthesis. Same conditions as was performed for Table 1.

as does peptide-**g** (52%). The observations once again show that the positions of the *N*,*N*-dimethyl-Lys residues in the peptide are most significant in governing interactions.

To evaluate specificity, peptide-**g** binding affinities to other RNAs[18] were measured and compared with those of peptide-**a** and Rev (Table 2). Dissociation constants for peptide-**g** binding to $tRNA^{mix}$ and TAR are relatively weak, affording high discrimination ratios (4.6/5.8). In contrast, conformationally restricted peptide-**a** binds to other RNAs similarly, giving low discrimination ratios (2.5/2.8). The result suggests that N-methylations on Lys residue contribute increasing affinity and improving specificity of peptides against RNA as well. Even Rev, a natural peptide against RRE, binds strongly to other RNA, affording lower discrimination ratios (2.4/2.5) than peptide-**g**.[20]

One possible reason for the specificity displayed by peptide-**g** is that it undergoes a RNA selective and induced conformational change. To test this proposal, conformational changes of peptide-**g** in complex with RRE RNA were monitored by CD.[18] A dose-dependent decrease of molar ellipticity at 222 nm is observed upon the addition of peptide-**g**. An α-helicity increase of peptide-**g** and (or) a conformational change of RRE could be possible reasons for this. The maximum ellipticity change of RRE in the presence of neomycin B was obtained by the addition of 20 equiv of the drug.[18] The ellipticity change of RRE caused by peptide-**g** (2 equiv) at 222 nm is even larger than that promoted by saturated neomycin B, suggesting that the increase of α-helicity of peptide-**g** is a contributor to this change. α-Helicity of the peptide increases from its original value of 7.5% to 11% and 18% when 0.1 and 0.3 equiv of RRE (relative to peptide-**g**) are present.[18] This observation indicates that the peptide undergoes an induced fit associated with a significant conformational change when it binds to RRE.[21] RNA footprinting experiment was carried out for mapping a binding-site of the peptide on RRE RNA.[18] The autoradiogram by RNAse V1 showed that the binding site is similar to that of Rev, which is known to bind to the stem or internal loop region.[22]

The *N*,*N*-dimethyl-Lys groups have increased basicity and their existence as ammonium ions causes adjacent non-methylated-Lys amine groups to have lower basicities. Therefore, positively charged and neutral positions in the synthetic peptides are governed by the sites of N,N-dimethylation. As a result, the positioning of positively charged ammonium and neutral amine moieties might be the source of the specificity of binding of *N*,*N*-dimetyl-Lys containing peptides to RRE RNA. Thus, it is possible that negatively charged phosphates in RRE RNA are oriented properly to electrostatically interact best with the ammonium groups in peptide-**g**. Another possible rationale for the observed binding selectivities is that ammonium ions of *N*,*N*-dimethyl-Lys residues in peptide-**g** are ideally positioned to interact in a π-cation manner with purine/pyrimidine bases in the RRE RNA backbone.[4]

Increasing knowledge gained from studies of N-methylation/N-demethylation enzymes and methylated Lys and Arg containing proteins points to the important role that these substances play as inducers of epigenetic changes.[23] In addition, Lys and Arg methylation processes are proposed as key events in RNA-mediated signal transduction, nuclear transport, and modulations of nucleic acid interactions.[24] Owing to this, the results emanating from the current study provide strong support for the proposal that N-methylation of Lys residues promotes specific RNA-protein interactions and could serve as a potent control element in gene expression. Structural studies of peptide-**g** with complex with RRE RNA and biological tests of these RNA specific peptides are in progress.

Acknowledgment. Financial support was provided by 21C Frontier Program Center for Biological Modulators and KOSEF.

Supporting Information Available: Complete lists of authors for refs 10 and 11; experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Metzger, E.; Wissmann, M.; Yin, N.; Müller, J. M.; Schneider, R.; Peters, A. H.; Günther, T.; Buettner, R.; Schüle, R. *Nature* **2005**, *437*, 436−439.
(2) McBride, A. E.; Silver, P. A. *Cell* **2001**, *106*, 5−8.
(3) (a) Das, C.; Frankel, A. D. *Biopolymers* **2003**, *70*, 80−85. (b) Friesen, W. J.; Darby, M. K. *Nat. Struct. Biol.* **1998**, *5*, 543−546. (c) Mills, N. L.; Daugherty, M. D.; Frankel, A. D.; Guy, R. K. *J. Am. Chem. Soc.* **2006**, *128*, 3496−3497.
(4) (a) Hughes, R. M.; Waters, M. L. *J. Am. Chem. Soc.* **2006**, *128*, 13586−13591. (b) Hughes, R. M.; Waters, M. L. *J. Am. Chem. Soc.* **2006**, *128*, 12735−12742. (c) Hughes, R. M.; Waters, M. L. *J. Am. Chem. Soc.* **2005**, *127*, 6518−6519. (d) Sathyapriya, R.; Vishveshwara, S. *Nucleic Acids Res.* **2004**, *32*, 4109−4118.
(5) Gallivan, J. P.; Dougherty, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9459−9464.
(6) (a) Tan, R.; Frankel, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4247−4252. (b) Liu, Q.; Dreyfuss, G. *Mol. Cell Biol.* **1995**, *15*, 2800−2808.
(7) (a) Weeks, K. M.; Ampe, C.; Schultz, S. C.; Steitz, T. A.; Crothers, D. M. *Science* **1990**, *249*, 1281−1285. (b) Takahashi, T.; Hamasaki, K.; Ueno, A.; Mihara, H. *Bioorg. Med. Chem.* **2001**, *9*, 991−1000.
(8) (a) Cox, J. A.; Comte, M.; Fitton, J. E.; DeGrado, W. F. *J. Biol. Chem.* **1985**, *260*, 2527−2534. (b) Usui, K.; Ojima, T.; Takahashi, M.; Nokihara, K.; Mihara, H. *Biopolymers* **2004**, *76*, 129−139.
(9) Haynie, S. L.; Crum, G. A.; Doele, B. A. *Antimicrob. Agents Chemother.* **1995**, *39*, 301−307.
(10) Pain, A.; et al. *Science* **2005**, *309*, 131−133.
(11) Hendrickson, E. L.; et al. *J. Bacteriol.* **2004**, *186*, 6956−6969.
(12) Kirk, S. R.; Tor, Y. *Bioorg. Med. Chem.* **1999**, *7*, 1979−1983.
(13) The peptides were synthesized on solid-supported beads by using a standard Fmoc protocol and were purified by HPLC and characterized by MALDI-TOF mass spectrometry.
(14) Scholtz, J. M.; Qian, H.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Biopolymers* **1991**, *31*, 1463−1470.
(15) Tan, R.; Frankel, A. D. *Biochemistry* **1994**, *33*, 14579−14585.
(16) Succinylated Rev_{34-50} peptide was custom made from Peptron (Daejeon, Korea). MS: $(M+H)^+$ 2977.0, calcd; 2977.0, obsd.
(17) (a) Ahn, D. R.; Yu, J. *Bioorg. Med. Chem.* **2005**, *13*, 1177−1183. (b) Lee, J.; Kwon, M.; Lee, K. H.; Jeong, S.; Hyun, S.; Shin, K. J.; Yu, J. *J. Am. Chem. Soc.* **2004**, *126*, 1956−1957.
(18) See Supporting Information.
(19) Luedtke, N. W.; Liu, Q.; Tor, Y. *Biochemistry* **2003**, *42*, 11391−11403. The value is a little larger than the reported one, probably because a short RRE RNA (32 nt for this study vs. 66 nt for the reported value) was used.
(20) An electrophoretic gel mobility shift assay[19] was tried to confirm specificity of peptide-**g**. Complex bands of peptide-**g** and RRE RNA, however, disappeared accompanying an increase of the precipitated material remaining in the injection well.[18]
(21) Calabro, V.; Daugherty, M. D.; Frankel, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 6849−6854.
(22) Ippolito, J. A.; Steitz, T. A. *J. Mol. Biol.* **2000**, *295*, 711−717.
(23) Bannister, A. J.; Kouzarides, T. *Nature* **2005**, *436*, 1103−1106.
(24) Gary, J. D.; Clarke, S. *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *61*, 65−131.

JA068265M