J|A|C|S
COMMUNICATIONS

Published on Web 12/21/2005

Targeted Cleavage of HIV Rev Response Element RNA by Metallopeptide Complexes

Yan Jin and J. A. Cowan*

Evans Laboratory of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

Received August 3, 2005; E-mail: cowan@chemistry.ohio-state.edu

Viruses mediate a variety of infectious diseases; however, an effective cure remains elusive. In the case of AIDS, frequent mutation of the viral genome makes treatment even more challenging. RNA is the genetic material for retroviruses. Since there is no cellular RNA repair system, RNA has been seen as a viable drug target for more than a decade. Anti-sense recognition is a widely employed RNA targeting strategy, and efforts have been made to elaborate RNA interference (based on antisense targeting) into a pharmaceutical reality.[1] However, the 2′-hydroxyl group protection required in RNA synthesis, the instability of RNA, and the challenge of cellular delivery have limited the practical applications of this technique. Peptide nucleic acid (PNA) derivatives have found increasing use in antisense recognition strategies[2,3] but also face synthetic and solubility difficulties. In addition, as a consequence of the complex secondary and tertiary structures of the anti-sense and target molecules, recognition may not occur as expected.

A distinct RNA targeting strategy is based on the structural recognition of a viral RNA motif and cognate protein. Usually, this kind of recognition is specific and essential for the life cycle of the virus and is likely to be of significant pharmaceutical potential. The interaction between the HIV Rev response element (RRE) mRNA and the virus-encoded Rev protein is a well-studied example. Rev binds to, and regulates the export of, unspliced or single-spliced mRNA from the nucleus to the cytoplasm.[4,5] A small peptide domain of Rev (~17 amino acids) appears to convey specificity of binding to the RRE RNA and mimics many of the characteristics of Rev protein binding to RRE RNA.[6,7] The RNA−peptide complex has been structurally characterized by NMR,[6] showing the α-helical arginine-rich Rev peptide to bind to the major groove of RNA near a purine-rich internal loop that is broadened by a G-G base pair with an unusual structure (Figure 1, right). Efforts have been made to develop clinical applications by interfering with the interaction between Rev and RRE RNA;[8] however, the decoys have a limited half-life and single turn-over behavior, and so the dosage is usually high with the likelihood of severe side effects.

Herein, we report the results of RNA cleavage studies that have been carried out to develop anti-viral drugs that mediate catalytic irreversible degradation of the target. Multiturnover performance, which further decreases drug dosage and potential side effects, is also sought. Prior designs of RNA cleavage moieties include modified ribozymes[9] and traditional coordination complexes.[10−16] Since these metal complexes are relatively small molecules, their binding affinity and selectivity toward an RNA target is relatively poor.

In this paper we describe a strategy where a specific RNA recognition sequence and catalytic metal ions are combined within a common peptide ligand. Scheme 1 illustrates the use of an N-terminal metal binding ATCUN (amino-terminal copper and nickel binding) motif[17] that is extended to include the RRE RNA recognition peptide. Prior studies have demonstrated effective DNA cleavage and/or linearization by Cu−ATCUN or other metallopeptide complexes,[18−20] while selective nucleobase damage of $tRNA^{Phe}$ and HIV TAR RNA has been studied with Ni−ATCUN complexes.[14] The peptide ligand is readily prepared by automated synthesis and demonstrates outstanding metal binding and solubility characteristics, while the Rev peptide sequence is known to promote efficient cellular uptake.[21]

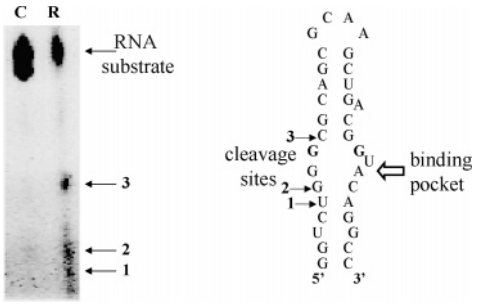

Figure 1. (Left) Cleavage reactions analyzed by 8 M urea denaturing 20% polyacrylamide gel electrophoresis: lane C, results from a control reaction with 10 μM [RRE] and 100 μM ascorbate; lane R, results from a cleavage reaction with 10 μM [RRE], 10 μM [copper−Rev1 complex], and 100 μM ascorbate in 20 mM HEPES with 100 mM NaCl at 37 °C for 3 h. (Right) Schematic illustration of the stem loop structure adopted by RRE RNA IIB,[6] showing the cleavage sites identified by mass spectrometry studies.

Scheme 1. Rev1 Metallopeptide Design Showing Metal Binding and RRE RNA Targeting (Underlined) Sequences

GGH-TRQARRNRRRRWRERQR

metal binding motif RNA targeting motif

RRE RNA cleavage by the copper−Rev1 peptide complex was studied in vitro using 5′-fluorescein end-labeled RNA in the presence and absence of mild reducing agents such as ascorbate. RRE RNA was incubated with the Cu^{2+}−peptide complex under physiological conditions, and the products of cleavage were separated on denaturing 15% or 20% polyacrylamide gels (Figure 1, left). Cleavage of RRE RNA was effective under stoichiometric conditions (1:1 ratio of RRE RNA and Cu−peptide complex) in the presence of ascorbate. Three product bands were observed.

Cleavage sites were assigned by mass spectrometric studies (Table 1). In a control reaction sample, only the substrate peak was recognized with an accurate mass of 11 023 Da (calcd 11 022.7 Da). After reaction, the intensity of multiple charged peaks corresponding to RRE RNA substrate decreased, while new *m*/*z* ratio peaks appeared (Table 1 and Supporting Information). The size of the product species identified by mass spectrometry matched the product sizes judged by PAGE. Combining both the PAGE and mass spectrometry data, RRE cleavage by the Cu−Rev1 peptide complex is not random, but rather three specific cleavage sites were observed within the likely the binding pocket for the metallopeptide complex (Figure 1, right). The accurate masses obtained for the

10.1021/ja055272m CCC: $33.50

Table 1. ESI-MS Analysis of the Cleavage Products following Treatment of RRE by the Cu^{2+}−Rev1 Complex with Ascorbate[a]

product band	sequence assignment	*m/z* found	*m/z* predicted hydrolytic cleavage	*m/z* predicted oxidative cleavage
1	5′-GGUC-Pi-X[b]-3′	1425.3[c]	1318.7[c]	1425.7[c,e]
2	5′-GGUCU-Pi-X[b]-3′	1718.6[c]	1624.9[c]	1720.9 [c,f]
3	5′-GGUCUGGG-Pi-X[b]-3′	1428.8[d]	1329.8[d]	1428.4 [d,g]

[a] Predicted mass values are based on atomic weight as $(M + H)^-$ unless specially stated. Mass spectra of cleavage products and possible reaction pathways leading to their formation are presented in Supporting Information (Figure SM7 and Scheme SM1). [b] X stands for the partial ribose left after cleavage. In the case of hydrolytic cleavage the mass of X is zero. [c] $z = -1$. [d] $z = -2$. [e] Cleavage product $(M + Na)^-$ following C-1′H abstraction with $X = CH_2CH_2COCHO$. [f] Cleavage product $(M + K)^-$ following C-4′H abstraction (O_2 mediated pathway) with $X = CH_2COO^-$. [g] Cleavage product $(M + 2Na + K)^{2-}$ following C-4′H abstraction (H_2O mediated pathway) with $X = CH_2COCH_2CH(OH)$ CHO.

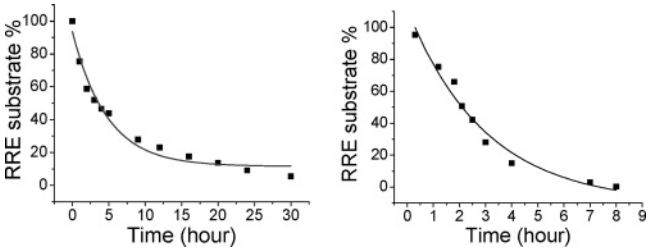

Figure 2. Evaluation of observed rate constants for RNA cleavage. Both reactions were carried out with 1 mM ascorbate in 20 mM HEPES, 100 mM NaCl at 37 °C. RRE RNA cleavage was monitored by following the loss of RRE RNA substrate by gel electrophoresis. (Left) [RRE] = 5 μM and [copper−Rev1 complex]:[RRE] ratio of 1:1, $k_{obs} \approx 0.21$ h^{-1} ($R^2 = 0.98$). (Right) [RRE] = 5 μM and [Cu^{2+}−Rev1 complex]:[RRE] = 20:1, $k_{obs} = 0.34$ h^{-1} ($R^2 = 0.97$).

cleavage products are different from the calculated mass values expected for products from hydrolysis but consistent with those expected following strand scission by a C-1′H or C-4′H oxidative cleavage path in the presence of ascorbate. Control reactions with metal-free Rev1 peptide, nontargeting Cu^{2+}−ATCUN peptides, or Cu^{2+}(aq) were also carried out and analyzed by PAGE. No reaction was promoted by the Rev1 peptide or by the $[GGH-Cu]^+$ or $[KGHK-Cu]^+$ ATCUN peptides. In the case of free Cu^{2+}(aq), RNA products were smeared on the gel as a result of random cleavage, and so no specific product was observed. The different cleavage behavior and distinct reaction pathways for Cu^{2+}−ATCUN complex and Cu^{2+}(aq) have already been discussed in our previous work.[20]

The time dependence of cleavage under oxidative conditions yielded $k_{obs} \approx 0.21$ h^{-1} (or $k_2 \approx 700$ M^{-1} min^{-1}). In the absence of ascorbate no activity was observed, relative to control experiments, and so no hydrolytic cleavage of RRE RNA occurs. Increasing the ratio of metallopeptide:RRE to 3:1 did not significantly change the reaction (presumably the unique high-affinity site is populated). However, at 20:1 the reaction proceeded more quickly ($k_{obs} \approx 0.34$ h^{-1}) as a result of nonspecific cleavage most likely from secondary binding sites (Figure 2).

The binding affinities of the Rev1 and Cu−Rev1 peptides to the labeled RRE RNA were determined to be ∼30 and 35 nM, respectively. These numbers are consistent with published data for binding of the native Rev peptide lacking the ATCUN motif,[22] and so addition of the N-terminal GGH and bound metal does not appear to influence Rev targeting.

The activities of other metal complexes of Rev1 were examined (Fe^{3+}, Ni^{2+}, and Co^{2+}). In the presence of ascorbate, Cu^{2+}−Rev1 complex had the highest RRE cleavage activity, while Fe^{3+}−Rev1 shows modest cleavage activity under hydrolytic conditions. Neither of Ni^{2+} nor Co^{2+} derivatives showed cleavage activity under hydrolytic or oxidative conditions. Cleavage results were observed to be similar in the presence of various concentrations of divalent magnesium, which often regulates RNA folding, consistent with current knowledge concerning Rev binding to RRE RNA. The observed cleavage specificity is consistent with a copper-bound nondiffusible reactive oxygen species as the reactive intermediate[20] and imposes certain stereoelectronic preferences for optimal activity. Such requirements are consistent with prior RNA cleavage work reported by us[15,23] and help to explain reactivity patterns observed in published work.[10,11,14]

In conclusion, while site-specific or shape-specific cleavage of structured RNA has previously been reported for metal complexes under oxidative conditions,[10−15,23] this report defines the first complex that *stoichiometrically* targets HIV RRE RNA for site-specific cleavage with a biological co-reactant (such as ascorbate) under physiological conditions.

Acknowledgment. This work was supported by the National Institutes of Health (GM063740). Mass spectrometry was carried out by the OSU Mass Spectrometry and Proteomics Facility.

Supporting Information Available: Synthetic procedures, binding assays for RRE RNA complex formation with the Rev1 peptide or copper derivative, protocols for PAGE gels, and mass spectrometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Henry, C. M. *Chem. Eng. News.* **2003**, *81* (51), 32−35.
(2) Cook, P. D. *Anticancer Drug Des.* **1991**, *6*, 585−607.
(3) Riguet, E.; Tripathi, S.; Chaubey, B.; Desire, J.; Pandey, V. N.; Decout, J.-L. *J. Med. Chem.* **2004**, *47*, 4806−4809.
(4) Kjems, J.; Brown, M.; Chang, D. D.; Sharp, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 683−687.
(5) Tiley, L. S.; Malim, M. H.; Tewary, H. K.; Stockley, P. C.; Cullen, B. R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 758−762.
(6) Battiste, J. L.; Mao, H.; Rao, N. S.; Tan, R.; Muhandiram, D. R.; Kay, L. E.; Frankel, A. D.; Williamson, J. R. *Science* **1996**, *273*, 1547−1551.
(7) Smith, C. A.; Chen, L.; Frankel, A. D. *Methods Enzymol.* **2000**, *318*, 423−438.
(8) Sullenger, B. A.; Gilboa, E. *Nature* **2002**, *418*, 252−258.
(9) DeRose, V. J. *Chem. Biol.* **2002**, *9*, 961−969.
(10) Murakawa, G. J.; Chen, C. H.; Kuwabara, M. D.; Nierlich, D. P.; Sigman, D. S. *Nucleic Acids Res.* **1989**, *17*, 5361−5375.
(11) Chow, C. S.; Barton, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 2839−2841.
(12) Carter, B. J.; Vroom, E.; Long, E. C.; Marel, G. A.; Boom, J. H.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9373−9377.
(13) Muller, J. G.; Zheng, P.; Rokita, S. E.; Burrows, C. J. *J. Am. Chem. Soc.* **1996**, *118*, 2320−2325.
(14) Brittain, I. J.; Huang, X.; Long, E. C. *Biochemistry* **1998**, *37*, 12113−12120.
(15) Sreedhara, A.; Cowan, J. A. *J. Biol. Inorg. Chem.* **2001**, *6*, 166−172.
(16) Iranzo, O.; Elmer, T.; Richard, J. P.; Morrow, J. R. *Inorg. Chem.* **2003**, *42*, 7737−7746.
(17) Lau, S.-J.; Kruck, T. P. A.; Sarkar, B. *J. Biol. Chem.* **1974**, *249*, 5878−5884.
(18) Mack, D. P.; Dervan, P. B. *Biochemistry* **1992**, *31*, 9399−9405.
(19) Nagaoka, M.; Hagihara, M.; Kuwahara, J.; Sugiura, Y. *J. Am. Chem. Soc.* **1994**, *116*, 4085−4086.
(20) Jin, Y.; Cowan, J. A. *J. Am. Chem. Soc.* **2005**, *127*, 8408−8415.
(21) Hariton-Gazal, E.; Feder, R.; Mor, A.; Graessmann, A.; Brack-Werner, R.; Jans, D.; Gilon, C.; Loyter, A. *Biochemistry* **2002**, *41*, 9208−9214.
(22) Harada, K.; Martin, S. S.; Tan, R.; Frankel, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11887−11892.
(23) Sreedhara, A.; Patwardhan, A.; Cowan, J. A. *Chem. Commun.* **1999**, 1147−1148.

JA055272M