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Analysis of Protein–RNA Complexes Involving a RNA Recognition Motif Engineered To Bind Hairpins with Seven- and Eight-Nucleotide Loops

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Supporting Information

ABSTRACT: U1A binds U1hpII, a hairpin RNA with a 10-nucleotide loop. A U1A mutant (ΔK50ΔM51) binds U1hpII-derived hairpins with shorter loops, making it an interesting scaffold for engineering or evolving proteins that bind similarly sized disease-related hairpin RNAs. However, a more detailed understanding of complexes involving ΔK50ΔM51 is likely a prerequisite to generating such proteins. Toward this end, we measured mutational effects for complexes involving U1A ΔK50ΔM51 and U1hpII-derived hairpin RNAs with seven- or eight-nucleotide loops and identified contacts that are critical to the stabilization of these complexes. Our data provide valuable insight into sequence-selective recognition of seven- or eight-nucleotide loop hairpins by an engineered RNA binding protein.

A multitude of diverse RNA hairpins have been implicated in human disease.[1−3] These structurally and electronically complex targets can frustrate sequence-selective molecular recognition strategies centered on small molecules.[4] In contrast, Nature has evolved a suite of protein RNA recognition motifs (RRMs), capable of potent and sequence-specific binding to RNA targets.[5−7] These structural motifs are potentially promising starting points for engineered or evolved RRMs with enhanced affinity for disease-related RNAs, which may constitute a unique new class of protein therapeutics and basic research tools. A prototypical example of an RRM is found in the U1 small nuclear ribonucleoprotein [U1A (Figure 1A)], which binds U1 hairpin II RNA [U1hpII (Figure 1B)].[8,9] The U1A–U1hpII complex has attracted significant attention and frequently serves as a model for recognition of hairpin RNA by a protein.[10−14]

The native U1A–U1hpII RNA complex is stabilized by $\pi-\pi$ interactions involving loop nucleotides C5 and A6 and residues Tyr13 and Phe56 (Figure 1C).[8,15−17] In addition, a hydrogen bond network involving residues Asn15, Asn16, and Glu19 and nucleotides U2, G3, and G4 is implicated, at least in part, in sequence-selective recognition of U1hpII by U1A (Figure 1D).[8] Mutational studies have shown that these residues are important contributors to the high stability of the U1A–U1hpII complex.[16,18−21]

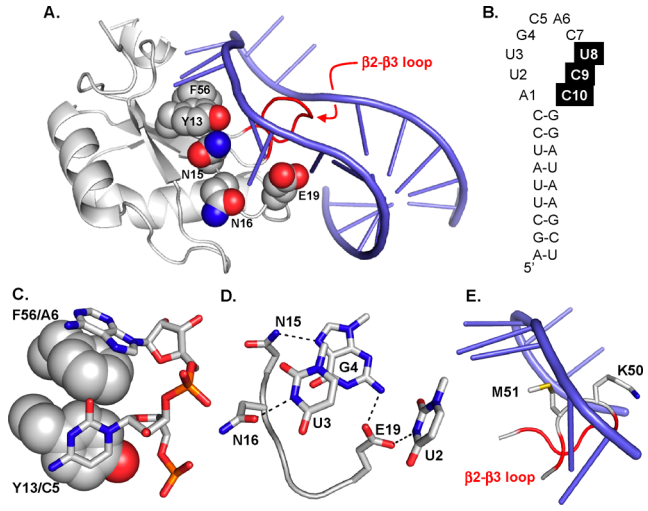

Figure 1. (A) U1A–U1hpII RNA interaction. (B) U1hpII RNA hairpin. (C) $\pi-\pi$ interactions between Tyr13 and Phe56 and C5 and A6, respectively. (D) Hydrogen bond network involving Asn15, Asn16, and Glu19 and nucleotides U2, U3, and G4. (E) $\beta_2-\beta_3$ loop residues Lys50 and Met51 are deleted in the ΔK50ΔM51 mutant used in this work.

While U1hpII contains a large 10-nucleotide loop, a number of disease-related RNA hairpins contain four- to eight-nucleotide loops. However, features of the U1A–U1hpII interaction suggest that U1A-derived proteins could be made to bind RNA hairpins with smaller loops. For example, U1A does not interact with loop nucleotides U9, C10, and C11, suggesting that smaller loops can be accommodated.[8,22] Similarly, residues Lys50 and Met51 in the $\beta_2-\beta_3$ loop of U1A that protrudes into the U1hpII RNA loop form no specific interactions with RNA (Figure 1E). While deletion of U1hpII loop nucleotides reduces U1A affinity by ∼3000-fold, a compensatory mutation in U1A that removes residues Lys50 and Met51 results in a protein that binds either hairpin with similar affinity.[23] These findings imply that the $\beta_2-\beta_3$ loop acts as a "steric ruler" and establishes lower size limits for the RNA

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Table 1. Binding Affinities for U1A ΔK50ΔM51 Mutants and Eight-Nucleotide Loop U1hpII-Derived RNAs

entry	U1A mutant	U1hpII mutant	K_D[a] (M)	ΔG (kcal/mol)[c]	$\Delta\Delta G$ (kcal/mol)[d]
1	ΔK50ΔM51	8 nt loop	$(4.0 \pm 0.4) \times 10^{-6}$	−7.35	–
2	Tyr13Gln[b]	8 nt loop	$(536.9 \pm 106.5) \times 10^{-6}$	−4.45	2.90
3	Phe56Ala	8 nt loop	$(414.9 \pm 81.0) \times 10^{-6}$	−4.61	2.74
4	Asn15Ala	8 nt loop	$(33.9 \pm 2.0) \times 10^{-6}$	−6.09	1.26
5	Asn16Ala	8 nt loop	$(15.7 \pm 1.0) \times 10^{-6}$	−6.54	0.81
6	Glu19Ala	8 nt loop	$(4.7 \pm 0.5) \times 10^{-6}$	−7.26	0.09
7	ΔK50ΔM51	G4A; 8 nt loop	$(11.8 \pm 2.5) \times 10^{-6}$	−6.71	0.64

[a]The error for the dissociation constant (K_D) is the standard deviation of three separate experiments. [b]All point mutants are derived from U1A ΔK50ΔM51. [c]ΔG is the free energy of the protein in complex with U1hpII-derived RNA calculated with the equation $\Delta G = -RT \ln K_D$. [d]$\Delta\Delta G$ is the difference in binding free energy between the complexes in entry 1 and indicated mutants thereof.

Table 2. Binding Affinities for U1A ΔK50ΔM51 Mutants and Seven-Nucleotide Loop U1hpII-Derived RNAs

entry	U1A mutant	U1hpII mutant	K_D[a] (M)	ΔG (kcal/mol)[c]	$\Delta\Delta G$ (kcal/mol)[d]
1	ΔK50ΔM51	7 nt loop	$(14.5 \pm 2.9) \times 10^{-6}$	−6.59	–
2	Tyr13Gln[b]	7 nt loop	negligible binding	–	–
3	Phe56Ala	7 nt loop	negligible binding	–	–
4	Asn15Ala	7 nt loop	$(34.8 \pm 2.7) \times 10^{-6}$	−6.07	0.52
5	Asn16Ala	7 nt loop	$(43.9 \pm 2.3) \times 10^{-6}$	−5.94	0.65
6	Glu19Ala	7 nt loop	$(17.7 \pm 3.5) \times 10^{-6}$	−6.47	0.12
7	ΔK50ΔM51	G4A; 7 nt loop	$(21.2 \pm 3.1) \times 10^{-6}$	−6.36	0.23

[a]The error for the dissociation constant (K_D) is the standard deviation of three separate experiments. [b]All point mutants are derived from ΔK50ΔM51. [c]ΔG is the free energy of the protein in complex with U1hpII-derived RNA calculated with the equation $\Delta G = -RT \ln K_D$. [d]$\Delta\Delta G$ is the difference in binding free energy between the complexes in entry 1 and indicated mutants thereof.

loop matching a given protein derivative. Because ΔK50ΔM51 is better suited to binding hairpin RNA with smaller loops, it represents a potentially interesting starting point for generating U1A-derived proteins with affinity for similarly sized disease-related RNA hairpins. However, a more detailed understanding of protein−RNA interactions involving ΔK50ΔM51 and U1hpII-derived RNAs with shorter loops is likely a prerequisite for the generation of such proteins.

Toward this end, we prepared a focused library of ΔK50ΔM51 mutants (Tyr13Gln, Asn15Ala, Asn16Ala, Glu19Ala, and Phe56Ala) and measured their affinity for U1hpII-derived RNAs containing seven- or eight-nucleotide loops. By analogy to identical mutational effects on the U1A−U1hpII interaction, our data implicate residues that stabilize complexes between ΔK50ΔM51 and U1hpII-derived RNAs with shortened loops and provide a basic framework upon which ΔK50ΔM51-derived proteins with altered sequence selectivity can be engineered or evolved.

As detemined by fluorescence polarization, ΔK50ΔM51 binds the eight-nucleotide loop U1hpII (U1hpII ΔU8ΔC9) with a dissociation constant (K_D) of 4.0 ± 0.4 μM (Table 1, entry 1). Tyr13Gln and Phe56Ala mutants bind the eight-nucleotide loop U1hpII-derived RNA with ∼134- and ∼104-fold lower affinities, respectively, than ΔK50ΔM51 (Table 1, entries 2 and 3, respectively), consistent with the established significance of these residues in stabilizing the wild-type complex.[16] The Ans15Ala and Asn16Ala variants bind ∼8.5- and ∼3.9-fold weaker, respectively, again consistent with the impact of these residues in the native complex, where the alanine mutation reduces affinity by ∼10- and ∼6-fold, respectively.[21] Together, these data establish that expected close contacts are maintained when the smaller hairpin is being bound.

Interestingly, while a Glu19Ala mutation in U1A significantly reduces the affinity for U1hpII in the native complex, an identical mutation in ΔK50ΔM51 did not appreciably alter the affinity for the eight-nucleotide loop U1hpII variant. As depicted in Figure 1D, U1A residues Asn15 and Glu19 engage in a bidentate hydrogen bond interaction with nucleotide G4. Baranger and co-workers have shown that mutating G4 to adenine lowers the U1A affinity by ∼10^5-fold, presumably in part because of the loss of a hydrogen bond between the carboxylate on Glu19 and the exocyclic amine on G4.[24] Given the apparent insignificance of Glu19 to the new complex, we wondered if a parallel indifference existed toward the G4 nucleotide. Indeed, the G4A ΔK50ΔM51 affinity was changed ∼2.9-fold (Table 1, entry 7). These data strongly suggest that the Glu19−G4 contact from the parent U1A−U1hpII complex is not important for the new interface.

Because deletion of two nucleotides from U1hpII diminished one of the known contacts with U1A, we next sought to explore the impact of an additional deletion using U1hpII ΔU8ΔC9-ΔU10. As seen in Table 2 (entry 1), ΔK50ΔM51 binds a seven-nucleotide loop U1hpII-derived RNA that lacks all three spacer nucleotides (U1hpII ΔU8ΔC9ΔC10) with good affinity (K_D = 14.5 ± 2.9 μM). As with the eight-nucleotide loop U1hpII variant, Tyr13Gln and Phe56Ala mutations drastically reduced the affinity for the seven-nucleotide U1hpII variant. These data suggest that Tyr13 and Phe56 are involved in binding the seven-nucleotide U1hpII variant, potentially through $\pi-\pi$ interactions similar to those found in the native complex. Similarly, ΔK50ΔM51 binds this seven-nucleotide loop RNA hairpin with ∼2.4- and ∼3-fold lower affinity following Asn15Ala and Asn16Ala mutations, respectively (Table 2, entries 4 and 5, respectively). These data suggest a binding mode that relies on Asn15 and Asn16 for stabilization of the protein−RNA complex. The stability of the complex involving the seven-nucleotide loop hairpin is given in Table 2 (entry 6). The Glu19Ala mutation (Table 2, entry 6) did not significantly lower the stability of the complex, and the G4A

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nucleotide variant (Table 2, entry 7) lowered it by only ∼1.5-fold. Thus, as with the eight-nucleotide complex, but in contrast to the parent one, these data suggest a less prominent role for the Glu19−G4A interaction.

Our findings not only improve our understanding of protein−RNA recognition by U1A variants but also may provide a foundation for evolving U1A ΔK50ΔM51 variants with altered binding specificity. We have reported the first solution phase dissociation constants for complexes involving ΔK50ΔM51, and variants thereof, and seven- or eight-nucleotide loop U1hpII RNAs, as well as the first mutational studies that provide insight into these protein−RNA complexes. We have shown that ΔK50ΔM51 binds both shortened-loop U1hpII variants with low micromolar dissociation constants; the eight-nucleotide loop hairpin is bound ∼3.6-fold tighter than the seven-nucleotide loop U1hpII variant. Like the native U1A−U1hpII interaction, residues Tyr13 and Phe53 on the solvent-exposed surface of the β-sheet are critical for protein−RNA complex stabilization. In addition, consistent with the native interaction, Asn15 and Asn16 play important roles in hairpin RNA recognition. However, unlike the native complex, which partially relies on Glu19 for sequence-selective recognition of loop nucleotide G4, Glu19 appears to play a much less important role in the ΔK50ΔM51 mutant. Taken together, these data provide valuable insight into the elements for recognition of seven- and eight-nucleotide loop RNA hairpins by an engineered RNA recognition motif and may be useful in developing U1A-derived proteins with affinity for disease-related hairpin RNAs with similarly sized loops.

■ ASSOCIATED CONTENT

Supporting Information

Materials, detailed experimental protocols, characterization of U1A mutants, fluorescence polarization data, and supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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