Specific RNA Binding by a Single C$_2$H$_2$ Zinc Finger*

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Zinc finger proteins with high affinity for human immunodeficiency virus Rev responsive element stem loop IIB (RRE-IIIB) were previously isolated from a phage display zinc finger library. Zinc fingers from one of these proteins, RR1, were expressed individually and assayed for RRE-IIIB affinity. The C-terminal zinc finger retained much of the binding affinity of the two-finger parent and was disrupted by mutations predicted to narrow the RRE-IIIB major groove and which disrupt Rev binding. In contrast, the N-terminal zinc finger has a calculated affinity at least 1000-fold lower. Despite the high affinity and specificity of RR1 for RRE-IIIB, binding affinity for a 234-nucleotide human immunodeficiency virus Rev responsive element (RRE$_{234}$) was significantly lower. Therefore, zinc finger proteins that bind specifically to RRE$_{234}$ were constructed using an in vitro selection and recombination approach. These zinc fingers bound RRE$_{234}$ with subnanomolar dissociation constants and bound the isolated RRE-IIIB stem loop with an affinity 2 orders of magnitude lower but similar to the affinity of an arginine-rich peptide derived from Rev. These data show that single C$_2$H$_2$ zinc fingers can bind RNA specifically and suggest that their binding to stem loop IIB is similar to that of Rev peptide. However, binding to RRE$_{234}$ is either different from stem loop IIB binding or the tertiary structure of stem loop IIB is changed within the Rev responsive element.

C$_2$H$_2$ zinc finger proteins comprise a diverse family of DNA- and RNA-binding proteins. NMR and crystallographic data show clearly that zinc fingers bind DNA through $\alpha$-helical contacts in a slightly enlarged DNA major groove (1, 2). Similarly, current evidence suggests that zinc finger proteins require specific amino acids in $\alpha$-helices for high affinity RNA binding (3–5). For example, TFIIIA, a nine-zinc finger protein, binds 5 S rRNA through four central zinc fingers (4–7) spanning 50–60 nucleotides (5–9). Mutation of amino acids within the $\alpha$-helices of this group of zinc fingers significantly reduces RNA affinity. In vitro selection techniques, particularly phage display, have been used to exploit the modular nature of zinc finger binding to DNA to design proteins with affinity for new DNA targets (10–15). In these cases, amino acids within the $\alpha$-helices of a known DNA-binding zinc finger protein are changed to make appropriate base contacts with the new DNA target. To explore the potential for designed RNA-binding proteins, we previously reported the isolation of zinc finger proteins with high affinity for HIV RRE-IIIB and 5 S rRNA through a combination of phage display and gene shuffling (16). These zinc fingers most likely make critical contacts through amino acids in $\alpha$-helices because these amino acids were randomized in the displayed zinc finger library.

The 234-nucleotide HIV RRE (RRE$_{234}$) found within the env gene was chosen as a zinc finger target because of the potential for designing a protein that competitively inhibits Rev binding and HIV propagation (17). Rev is an essential HIV protein that induces the accumulation of unspliced and partially spliced viral RNA in the cytoplasm of infected cells. Rev activity is dependent upon binding to stem loop IIB of the RRE and subsequent multimerization (18–20). Rev multimerization is cooperative and results in a high apparent binding affinity for RRE (21, 22). Thus, an effective zinc finger-based inhibitor that functions in the absence of multimer formation could be based on a mixture of zinc fingers that bind to multiple sites on the RRE in addition to the primary Rev binding site in stem loop IIB.

Here we report that RR1, a zinc finger protein previously selected for binding to RRE-IIIB, and a single zinc finger derived from it bind RRE-IIIB with similar RNA sequence and structural requirements as the arginine-rich Rev peptide (amino acids 34–50). In contrast to the high affinity binding to stem loop IIB, RR1 has lower affinity for the full-length RRE$_{234}$. In light of these results, we constructed high affinity zinc fingers that bind RRE$_{234}$ by phage display and in vitro recombination of $\alpha$-helix randomized zinc fingers.

MATERIALS AND METHODS

Phage Display Selection—Selections for RNA-binding proteins were done with an $\alpha$-helix randomized two-zinc finger library as described previously (16). Briefly, the phage library was incubated with biotinylated target RNA for 1 h, and RNA-phage complexes were immobilized on streptavidin-coated microtiter plates. Phage were eluted from the plates after extensive washing and amplified in Escherichia coli K91 cells for additional rounds of selection. Each selection included a 1000-fold molar excess of yeast tRNA over biotinylated target RNA. Between selection rounds, zinc finger cDNA was amplified, recombined (shuffled), and cloned back into the phage vector (fd.tet.7000), and electroeluted into K91 cells (>10$^9$ plaque-forming units) for phage production.

Peptide Purification, Gel Shift Analysis, and KD Determination—Zinc finger cDNA was amplified by polymerase chain reaction and cloned into pET28b(+) for expression of N-terminal (His)$_6$-tagged fusions. All plasmid constructs were confirmed by automated DNA sequencing. Zinc fingers were expressed in E. coli BL21 cells, purified by nickel affinity chromatography, folded by dialysis, and cleaved from (His)$_6$ tags as described previously (16). Gel shift analysis was performed as described previously, except for the case of full-length RRE, in which electrophoresis was done for 5 h at room temperature with 8 mA constant current. $K_D$ was determined by plotting the fraction of RNA bound against protein concentration and curve fitting to a variation of $K_D = \frac{\text{fraction of RNA bound}}{\text{protein concentration}}$. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* The abbreviations used are: TFIIIA, transcription factor IIIA; RRE-IIIB, Rev responsive element stem loop IIB; HIV, human immunodeficiency virus; RRE, Rev responsive element.
the Hill equation using SYSTAT (5).

**RNA Synthesis**—Overlapping oligonucleotides corresponding to RRE IIB RNA with or without the G71A and G46:C74 to C46:G74 alterations were synthesized, annealed, and filled in with the Klenow fragment of DNA polymerase I. The filled in product included a T7 promoter and a 5′ EcoRI site and a 3′ SmaI site, which were used for subcloning into pUC19. Before RNA production, the resultant plasmids were digested with SmaI. The RRE IIB RNA synthesized from these templates included 5′ GGG and 3′ CCC extensions (Fig. 2A). The DNA template for production of RRE34 RNA was made by polymerase chain reaction amplification of the RRE from the plasmid pDM128 (23) with specific primers. The polymerase chain reaction product, which included a 5′ T7 promoter, was purified on a QiaGen polymerase chain reaction purification column and used directly for RNA production. All RNAs were synthesized by *in vitro* transcription with T7 RNA polymerase using an Ambion MegaShort Kit according to the manufacturer’s instructions.

**RESULTS**

A Single Zinc Finger Is Sufficient for RRE-IIB Interaction—Previously, we reported the selection of zinc finger peptides with high affinity and specificity for both RRE-IIB and 5 S RNA (16). The library used for these selections consisted of two zinc fingers, both with limited randomization in α-helix positions −1 through +10 (for sequence examples, see Fig. 5B). Comparison of zinc finger sequences from selected phage showed that the C-terminal zinc finger was consistently basic, whereas the N-terminal zinc finger was frequently acidic. For example, the α-helix (−2 to +10, see Fig. 5) of the N-terminal zinc finger of RR1 has a calculated pI of 4.2, whereas the α-helix of the C-terminal zinc finger has a pI of 12.2. We sought to determine the contribution of individual zinc fingers to RNA affinity because many RNA-binding proteins, including the α-helix from Rev (REV34−50), have basic RNA binding domains. The N-terminal (zf1) and C-terminal (zf2) zinc fingers from RR1 were expressed individually, and their affinity for RRE-IIB RNA was measured by electrophoretic mobility shift assay. RR1zf1 did not bind to 32P-labeled RRE-IIB RNA at protein concentrations as high as 1500 nM (Fig. 1A). In contrast, RR1zf2 bound RRE-IIB RNA with an apparent $K_D$ of 19.7 ± 7.9 nm, which is 2–3-fold lower than the affinity of RR1 ($K_D$ 7.9 nm). RR1zf2-RRE-IIB complexes were not competed with a 1000-fold molar excess of tRNA or poly(rA) (data not shown). If RR1 zinc fingers function independently, the C-terminal zinc finger contributes over 95% of the binding energy. Given this assumption, the calculated affinity of the N-terminal zinc finger is −300 nm.

**RRE-IIB Mutations Affect Single-finger Binding**—The dominant contribution by the basic zinc finger in RR1 suggested that RRE-IIB-selected zinc fingers could bind RRE-IIB in a manner similar to that of the arginine-rich Rev34−50 peptide, which makes α-helix-RNA major groove contacts (24). To test this hypothesis, two mutants of RRE-IIB RNA, G71A and G46:C74 to C46:G74 (Fig. 2A), which greatly reduce the affinity of the RNA for Rev34−50, were used as substrates (25). The G71A mutation disrupts a purine-purine base pair responsible for widening the RNA major groove and thereby excludes the Rev34−50 α-helix. Mutation of G46:C74 to C46:G74 is predicted not to alter the structure of the RNA but instead to change the sequence of bases that make critical Rev34−50 contacts. Affinity of the two-finger protein RR1 was reduced at least 100-fold by the RRE-IIB mutations (Fig. 2B). In gel shift assays with purified RR1zf2, C46:G74 and G71A both failed to form a discrete shifted species, indicative of either greater nonspecific binding or significantly decreased stability during electrophoresis (Fig. 3C). Based on the amount of unshifted RNA, RR1 zinc finger binding is more severely reduced by the G71A mutation. Thus, it is probable that selected zinc fingers require distortion of the RNA helix to allow the zinc finger α-helix access to the rich hydrogen bonding potential within the major groove.

**Phage Display Selection of RRE-binding Peptides**—RR1 and other zinc fingers selected to the stem loop IIB region bind a more extensive 234-nucleotide region of the RRE with at least 100-fold lower affinity than RRE-IIB (Fig. 3). The zinc finger protein SFR1 was isolated to the full-length RRE (below) and is shown in Fig. 3 for comparison.) Furthermore, in some cases (RR1 and RR31), the zinc fingers failed to form discrete shifted species, suggesting nonspecific RNA affinity or lower stability during electrophoresis. To isolate zinc fingers that bind RRE34 and to determine whether other sites on full-length RRE other than stem loop IIB are conducive for zinc finger α-helix binding, we conducted phage display selection using immobilized
Five rounds of phage display selection were executed in the presence of a 1000-fold molar excess of tRNA. The percentage of phage retained on RRE234 RNA increased over five rounds of selection, whereas the percentage of phage retained in negative control experiments without RNA remained below 0.1% (Fig. 4A). To improve the affinity of selected proteins, zinc finger DNA from selection round 4 was recombined between further rounds of selection (26, 27). In addition, DNA used for recombination was doped with unselected randomized oligonucleotides used to make the library. This provides additional diversity by introducing new zinc finger sequences in tandem with preselected zinc fingers. DNA from round 4 of the initial selec-

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tion series was amplified, digested, and recombined and then subcloned into fd.tet.7000 for production of phage. Between rounds 5 and 6, zinc finger DNA was recombined along with 10% zinc finger library DNA, and after round 6, DNA was recombined again. Finally, three rounds of phage display selection without recombination were done (Fig. 4B).

Clones were randomly selected and sequenced after the initial five rounds of selection and again after the final round of selection and recombination (Fig. 5B). With the exception of positions +8 and +10, the C-terminal zinc fingers of phage recovered from the final round of selection (SFR series) were identical. Phage sequenced after the fifth round of the initial selection were more diverse, with only one phage (FR2) containing a second zinc finger identical to the majority of shuffled clones. As observed with RRE-IIB- and 5S rRNA-selected zinc fingers, the C-terminal α-helix of the RRE-selected clones was conserved and basic (pI = 12.19 for clone SFR8), whereas the α-helix of the first zinc finger was less well conserved and acidic (pI = 6.26). It is likely that, as in the case of RR1, the second zinc finger of these peptides contributes most or all of the energy for RNA binding. Interestingly, lysine is frequently selected over arginine between the zinc coordinating histidines.

Affinity of RRE-binding Peptides—Three clones from the final round of selection and recombination, SFR1, SFR4, and SFR12, were recombinantly produced, and their affinity for RRE RNA was determined by gel shift analysis using protein titration (Fig. 6A). All three peptides bound RRE RNA with a \( K_D \) of \( 0.1 \) nM (Fig. 6B). Rev binds to RRE RNA with a \( K_D \) of 1–10 nM. These three RRE RNA-selected proteins had lower affinity for RRE-IIB RNA (\( K_D \) of around 250 nM) (Fig. 6C), suggesting that these zinc finger peptides make significant contacts outside the stem loop IIB region of RRE RNA.

DISCUSSION

The zinc finger motif has proven to be an excellent framework for construction of novel specific RNA-binding proteins (16, 28). We have shown that a combination of phage display and gene shuffling of a zinc finger library can be used to construct zinc finger proteins with specific affinity for RRE-IIB, 5S rRNA, and RRE234. The RRE-IIB-selected peptide RR1 requires only the second finger for high affinity binding, and it is likely that all zinc finger peptides thus far selected exclusively use the C-terminal zinc finger for RNA binding because this finger is consistently more conserved and basic than the N-terminal finger. The dominant role for this zinc finger is surprising because it is closer to the phage surface during
specific RNA binding by a single $C_2H_2$ zinc finger

Fig. 6. RRE$_{234}$ and RRE-IIB RNA binding of selected and recombined zinc finger proteins. A, electrophoretic mobility shift assay. Radiolabeled RRE$_{234}$ RNA (0.01 nM) was incubated with the indicated nanomolar concentrations of zinc finger protein, and RNA-protein complexes were separated by nondenaturing polyacrylamide gel electrophoresis. F, RNA incubated without protein. $K_D$ is reported in nanomolar units ± S.E.M. B, determination of equilibrium dissociation constants. $K_D$ was calculated as described in the Fig. 1 legend and is reported in A. SFR4, SRF12, SRF1, ○, Error bars, S.E. of the mean for at least three determinations. C, electrophoretic mobility shift assay. RRE$_{234}$-selected zinc finger proteins were used in gel shift analysis with $^{32}$P-labeled RRE-IIB as outlined in the Fig. 1 legend. F, RNA incubated without protein.

Mutation of RR1zf2 at the C-terminal Arginine and two nonessential Arginines.

**Fig. 5B:** The expression of RR1zf2 in vivo and the increase in affinity for peptide binding are closely related. RR1zf2 was fused to a wild-type Rev sequence and a C-terminal Arginine. Alignment of RR1zf2 with ZF2-Rev using zinc coordinating histidines as a reference point (Fig. 5B) reveals that the amino acid position critical for Rev binding, only a-helix position +1 (arginine) is identical. However, rotating the RR1zf2 helix with respect to ZF2 in a helical wheel plot aligns four identical amino acids including asparagine 22, suggesting that the RR1zf2 a-helix and ZF2 could make similar RNA contacts. The rotation is equivalent to aligning the N terminus of RR1zf2 with the C-terminal half of ZF2-Rev and may suggest that the helix of RR1 does not penetrate as deeply into the major groove of RRE-IIB. Determination of the structure of RR1zf2 bound to RRE-IIB RNA will reveal the exact nature of the peptide-RNA contacts and provide the foundation to further increase affinity using structure-based design.

The recent measurements of the binding of Rev to RRE and a number of derivatives using surface plasmon resonance suggest affinities 1–2 orders of magnitude higher than previous estimates by gel shift or filter binding assays (22). The $K_D$ for the high affinity site in RRE-IIB is between 10 and 100 pM. Lower affinity sites for three additional molecules of Rev are in the $K_D$ range of 40–200 nM. Clearly, potential inhibitors of Rev function that are not based on dominant negative Rev mutations (33) and do not multimerize must achieve affinities for RRE-IIB in the picomolar range or alternatively target other sites of Rev interaction on the RRE. Isolation of zinc fingers that bind RRE$_{234}$ up to 250 times better than stem loop IIB suggests that zinc fingers can be isolated against regions other than IIB. Conversely, zinc fingers that bind to RRE-IIB with
significantly higher affinity than RRE234 illustrate that zinc finger binding is sensitive to RNA tertiary structure.

Zinc finger phage display coupled with structure-based design offers a unique opportunity to study protein-RNA interaction as well as the potential for construction of high affinity RNA-binding peptides for possible therapeutic use. Our demonstration that a single zinc finger can bind specifically to an RNA opens the way to a comprehensive screen of zinc finger sequence space that was not possible for two-zinc finger proteins.

REFERENCES


*Specific RNA Binding by a Single C2H2 Zinc Finger* 1973