Contribution of Individual Amino Acids to the 5S RNA Binding Activity of the *Xenopus* Zinc Finger Protein p43[†]

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ABSTRACT: *Xenopus* zinc finger protein p43 binds to 5S RNA in immature oocytes to form a 42S ribonucleoprotein storage particle. To determine the role of individual zinc fingers of the protein in this RNA binding activity, a series of deletion and substitution mutants of p43 were constructed. The effects of the various mutations on the RNA binding activity of p43 were determined using a quantitative equilibrium binding assay. The results indicate that zinc fingers 1 and 4 of p43 are essential for the binding of the protein to 5S RNA. In the case of finger 1, four amino acids key to RNA binding are found on the same face of the α-helix, while in the case of finger 4, two key residues are clustered at the start of the α-helix. The similarities and differences in the mechanisms by which fingers 1 and 4 of p43 interact with 5S RNA are compared to the interaction of the zinc fingers of *Xenopus* transcription factor IIIA with 5S RNA.

In the early stages of oogenesis in *Xenopus*, two major ribonucleoprotein particles (RNP) accumulate in the cytoplasm, providing a mechanism to store 5S RNA prior to ribosome assembly later in oocyte development (*1–3*). The 7S RNP is a simple bimolecular complex consisting of 5S RNA bound by a molecule of transcription factor IIIA (TFIIIA) (*4*). In comparison, the 42S RNP is a tetrameric complex of 15S subunits consisting of p48 protein, a p43 protein, tRNA and 5S RNA in the molar ratio of 2:1:3:1 (*1, 2, 5*). The p48 protein is an EF-1α-type protein that interacts with a variety of aminoacyl-tRNAs in the 42S RNP (*6–8*) while the p43 protein binds to the 5S RNA. Sequence analysis of the cDNAs of TFIIIA and p43 has shown that each contains 9 tandem zinc fingers (*9–11*). Although both proteins bind to 5S RNA, there is only a 33% amino acid sequence identity between their zinc fingers, much of which is accounted for by the conserved residues required to form the zinc finger structure. In addition to this difference in primary amino acid sequence, there is also a functional difference between the two proteins. TFIIIA binds to both 5S RNA and the 5S RNA gene, while p43 is only capable of binding to the 5S RNA (*4, 9, 12*). The interactions of TFIIIA with 5S RNA and the 5S RNA gene have been studied extensively by biochemical approaches (*13–23*). The information from those studies combined with the information from three-dimensional structures for the complexes formed by zinc fingers 1−6 of TFIIIA with DNA (*24*) and zinc fingers 4−6 with 5S RNA (*25*) has provided a detailed understanding of how TFIIIA forms specific interactions with two structurally distinct nucleic acid ligands. Although the mechanism by which p43 binds to 5S RNA is less well understood, the evidence from a small number of biochemical studies has demonstrated that it differs from the interaction of TFIIIA with 5S RNA in several ways. Darby and colleagues demonstrated using truncation mutants that the first four zinc fingers of p43 are essential for binding of the protein to 5S RNA, while it is zinc fingers 4−7 of TFIIIA that interact with the RNA (*17*). Comparison of the effects of a large panel of site directed mutants of the 5S RNA ligand on the binding of TFIIIA and p43 clearly demonstrated that the two proteins rely upon different sequence/structural elements of the RNA for formation of their respective ribonucleoprotein particles (*26*). The most compelling amino acid sequence identity found between TFIIIA and p43 is a threonine-tryptophan-threonine (TWT) motif at the start of the α-helix of zinc finger 6. These amino acids play a key role in the formation of specific protein−RNA contacts in the complex formed by zinc fingers 4−6 of TFIIIA and a truncated 5S RNA ligand (*25*). Mutagenesis studies have shown that substitution of these amino acids in TFIIIA results in a loss of RNA binding activity, but the same substitution in p43 has no effect on 5S RNA binding (*27*).

To gain a better understanding of how p43 forms a specific complex with 5S RNA, and how that differs from the formation of the TFIIIA−5S RNA complex, we have created and assayed a series of site-directed mutants of p43. The results of this study have identified amino acid residues in zinc fingers 1 and 4 of p43 that are essential for binding to 5S RNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors. The plasmid vector pET30a was used to express recombinant wild type and mutant p43 proteins in *Escherichia coli* strain BL21 DE3 using methods that have been described elsewhere (*26, 28*).

Construction of Mutant p43 Expression Vectors. All mutants used in this study were constructed by the poly-

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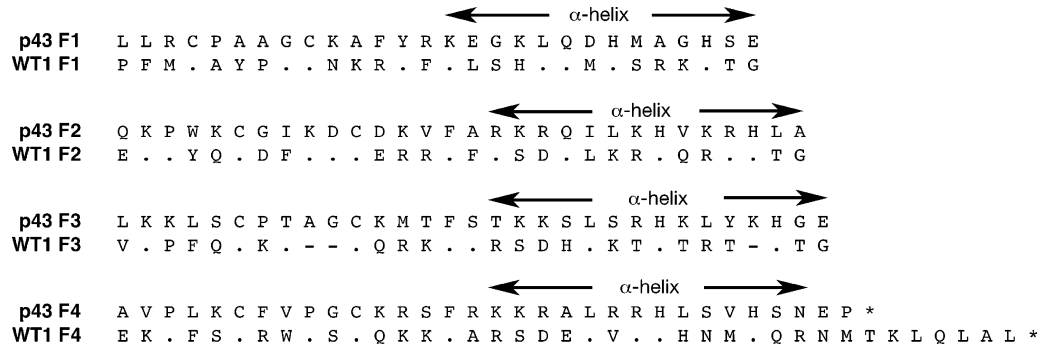

FIGURE 1: Comparison of the amino acid sequence of the first four zinc fingers of p43 with those of WT1 used to create finger swap and scanning mutations in p43. The boundaries of the α-helical region of each finger are indicated by the arrows.

merase chain reaction (PCR). Amino and carboxy terminal truncation mutants of p43 were constructed using upstream primers containing a recognition sequence for the restriction enzyme *Nco*I, and downstream primers containing a recognition sequence for the restriction enzyme *Eco*RI. PCR products were digested with *Nco*I and *Eco*RI and ligated into plasmid pET30a that had been digested with the same restriction enzymes. Ligation reactions were used to transform *E. coli* strain DH5α, and putative constructs were identified by colony PCR using primers specific to pET30a sequences flanking the multiple cloning site. Plasmids were isolated from appropriate colonies and the correctness of the plasmid constructs was confirmed by DNA sequencing before protein expression and purification.

Substitution mutants of p43 peptides were constructed by overlap extension PCR (*29*). For the finger swap mutants, the region of the WT1 cDNA encoding the zinc finger domain was used as a donor of zinc finger encoding cassettes and primers were designed that contained base sequences that encoded the junction between the donor WT1 finger and the acceptor p43 finger. Figure 1 shows a comparison of the sequence of the WT1 zinc fingers compared to the first four zinc fingers of p43. Scanning point mutants were created with primers encoding the desired codon change. Final PCR products were digested with *Nco*I and *Eco*RI then ligated into pET30a as described above.

Expression and Purification of Recombinant Wild Type and Mutant Proteins. Preparation of wild type p43 protein was carried out as described previously (*22, 26*). Mutant p43 proteins were expressed as his-tagged fusions from pET30a and purified as described previously (*30*). Protein purity was confirmed by polyacrylamide gel electrophoresis, and the concentration of each protein preparation was determined by the method of Bradford (*31*).

Equilibrium Binding of 5S RNA to Mutant p43 Proteins. The apparent association constants for the binding of radiolabeled nucleic acids to wild type and mutant p43 proteins were determined using a double filter binding assay described elsewhere (*26, 32*). The binding buffer consisted of 20 mM Tris-HCl pH 7.5 (20 °C), 5 mM $MgCl_2$, 100 mM KCl, 10 μM $ZnCl_2$, 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride, 100 μg/mL BSA and 1 μg/mL poly[dI-dC]. The affinity of each mutant protein for 5S RNA was determined using three or more independent assays in which the affinity of wild type p43 for the RNA was measured in parallel as a control. Apparent dissociation constants (K_d) for the binding of the mutant and wild type proteins to 5S RNA were calculated by fitting the data to a simple bimolecular equilibrium model using the general curve fitting function of Kaleidagraph software (Synergy Software, Reading PA) and the following equation:

$$\frac{[\text{RNA-protein}]}{[\text{RNA}]_{\text{total}}} = \frac{[\text{protein}]_{\text{total}}}{[\text{protein}]_{\text{total}} + K_d} \quad (1)$$

where $[\text{RNA}]_{\text{total}} \ll K_d$ and $[\text{RNA-protein}]/[\text{RNA}]_{\text{total}}$ is reported as the fraction of RNA bound. Association constant (K_a) values are reported as the reciprocal of the measured K_d values. Relative K_a values for the binding of mutant proteins to 5S RNA were calculated by dividing the K_a values determined for the mutant proteins by the K_a values determined in parallel for the wild type protein.

For some of the mutant proteins, the apparent affinity for 5S RNA was significantly decreased. The standard filter binding protocol relies upon the assumption that 100% of the protein is in an active form, and therefore for these mutant proteins it was necessary to confirm that this assumption was valid. In these cases the filter binding assay was modified so that the wild type or mutant protein was held at a constant concentration and incubated with a series of 5S RNA concentrations ranging from 1.69×10^{-10} M to 1.69×10^{-7} M. The determination of apparent association constants using this assay does not rely upon the concentration of active protein. All other conditions of this assay are identical to the standard filter binding assay. After a 90 min incubation at 20 °C, each sample was filtered through the double nitrocellulose/nylon filter stack to provide a determination of protein-bound 5S RNA (nitrocellulose filter) and free 5S RNA (nylon filter). Apparent dissociation constants were calculated by fitting the data using the appropriate equation and the general curve fitting function of Kaleidagraph software (Synergy Software, Reading PA) to a simple bimolecular equilibrium model using the following equation:

$$\frac{[\text{RNA-protein}]}{[\text{protein}]_{\text{total}}} = \frac{[\text{RNA}]_{\text{free}}}{[\text{RNA}]_{\text{free}} + K_d} \quad (2)$$

where $[\text{RNA-protein}]/[\text{protein}]_{\text{total}}$ is measured as the ratio of $[\text{RNA}]_{\text{bound}}$ to $[\text{protein}]_{\text{total}}$. For all the mutant proteins tested the results from the two different assays were identical within experimental error.

RESULTS

Previous studies comparing the 5S RNA binding properties of TFIIIA and p43 demonstrated that fingers 4−7 of TFIIIA are critical for RNA binding, while fingers 1−4 of p43 are essential for RNA binding (*16, 17, 19, 30, 33*). Consistent

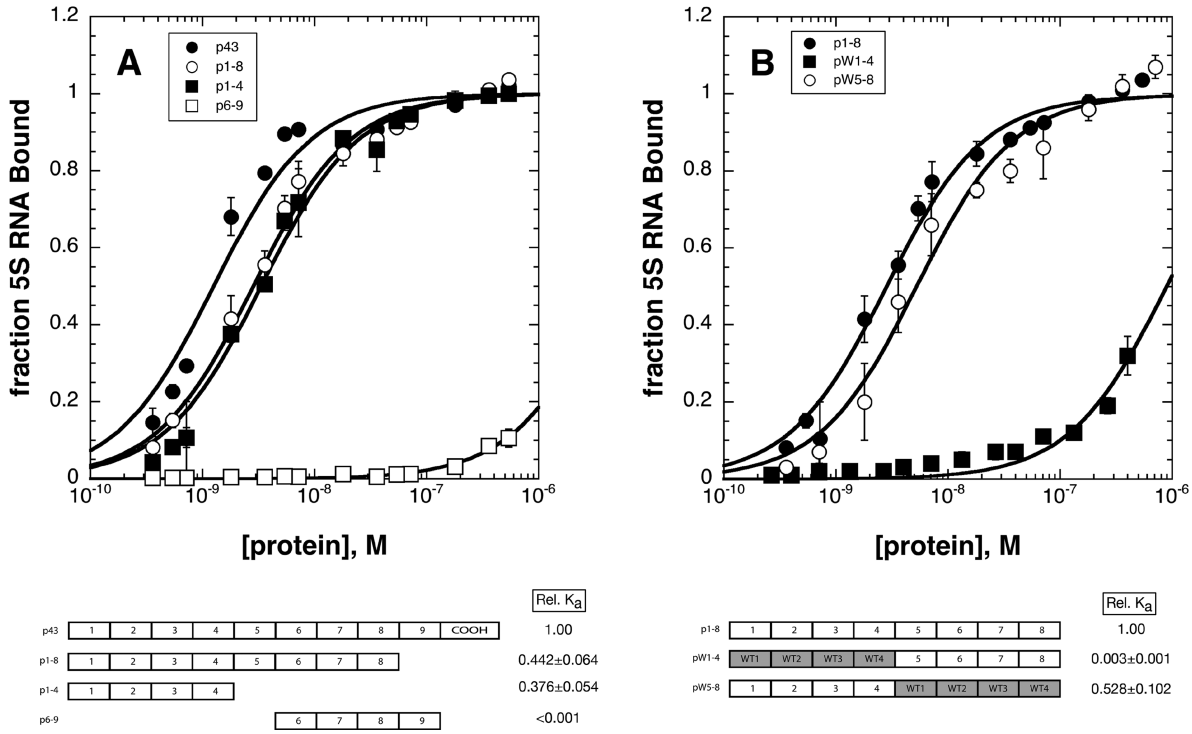

FIGURE 2: (A) Equilibrium binding of wild type p43, p1−8, p1−4 and p6−9 to 5S RNA. (B) Equilibrium binding of p1−8, pW5−8 and pW1−4 to 5S RNA. Each data point is the mean value from three independent experiments, with the standard deviation for each point represented by the error bars. Best-fit curves to a simple bimolecular equilibrium are shown for each protein. A schematic representation of each protein and its binding affinity relative to either p43 (panel A) or p1−8 (panel B) are shown below each graph.

with the results reported previously by Darby and colleagues (*17*), peptides containing fingers 1−4 or 1−8 of p43 bind to 5S RNA with affinities close to that of full length p43, while a peptide containing fingers 6−9 of p43 has no appreciable affinity for 5S RNA (Figure 2A). The key role of the first four fingers of p43 within the context of the p1−8 peptide was demonstrated by the loss of 5S RNA binding activity when fingers 1−4 were replaced by the four zinc fingers of the WT1 protein (Figure 2B). In comparison, replacing the last four fingers of the p1−8 peptide with those of WT1 has very little effect on the affinity of the protein for 5S RNA.

In order to determine the role of each of the first four fingers of p43 in binding to 5S RNA, a series of single finger swap mutants were constructed in the p1−4 peptide (Figure 3). As the results in Figure 3A show, each single finger substitution in p1−4 resulted in a decrease in affinity for 5S RNA. Relatively small effects were observed for substitution of finger 2 (8× decrease) and finger 3 (18× decrease), in comparison to the much larger decreases observed for substitution of finger 1 (200× decrease) and finger 4 (>1000× decrease). Most of the decrease observed for the full finger swaps of finger 1 or finger 4 appear to be the result of amino acid substitutions in the α-helical portion of each zinc finger (Figure 3B).

In order to gain further insight into the interaction of zinc fingers 1 and 4 of p43 with 5S RNA, we first constructed a series of scanning mutants throughout the α-helical region of each finger as a means to identify specific regions of the α-helix for investigation by point mutagenesis. Using donor sequences from the α-helix of finger 1 of WT1, five scanning mutants were constructed in finger 1 of p1−4 (Figure 4). Replacement of the entire α-helix of p43 finger 1 with the corresponding α-helix from finger 1 of WT1 resulted in a 40× decrease in binding to 5S RNA. However, K28 and Q33 are identical in the finger 1 α-helices of p43 and WT1 and were not tested directly in that mutant. Therefore, when the scanning mutants were created, alanine substitutions were incorporated at these two positions in order to test for the putative importance of all of the α-helical residues. As the filter binding data in Table 1 show, scanning mutant pW1(G30S,K31H) decreased binding of the protein to 5S RNA by a factor of 22×, while scanning mutant pW1(S40T,-E41G) decreased binding of the protein to 5S RNA by a factor of 33×. Scanning mutants pW1(K28A,E29L),-pW1(Q33A,D34M) and pW1(M36S,A37R,G38K) each decreased the binding of p1−4 to 5S RNA by at least a factor of 1000×. With the exception of the K28A and Q33A substitutions, all of the other amino acid replacements found in these three scanning mutants are also found in the pW1α mutant, which only had a 40× reduction in affinity for 5S RNA. The magnitude of the K28A substitution (see below) cannot account for the large effect of the pW1(K28A,E29L) scanning mutant on 5S RNA binding compared to pW1α. Neither can the magnitude of Q33A substitution account for the magnitude of the effect of the pW1(Q33A,D34M) scanning mutant compared to pW1α. All of the substitutions in the pW1(M36S,A37R,G38K) mutant are found in pW1α. Thus for these three scanning mutants, the effects on 5S RNA binding that are observed cannot be accounted for simply by the amino acid replacements and most likely indicate that the context of each multiple substitution plays a role in the magnitude of the effect observed.

In order to identify individual amino acids of finger 1 which make significant contributions to 5S RNA binding, a series of alanine point mutants were constructed within the α-helix. The results in Table 1 show that alanine point substitutions at residues K28, K31, and D34 decrease the affinity of p1−4 for 5S RNA by 7 to 20×. These point

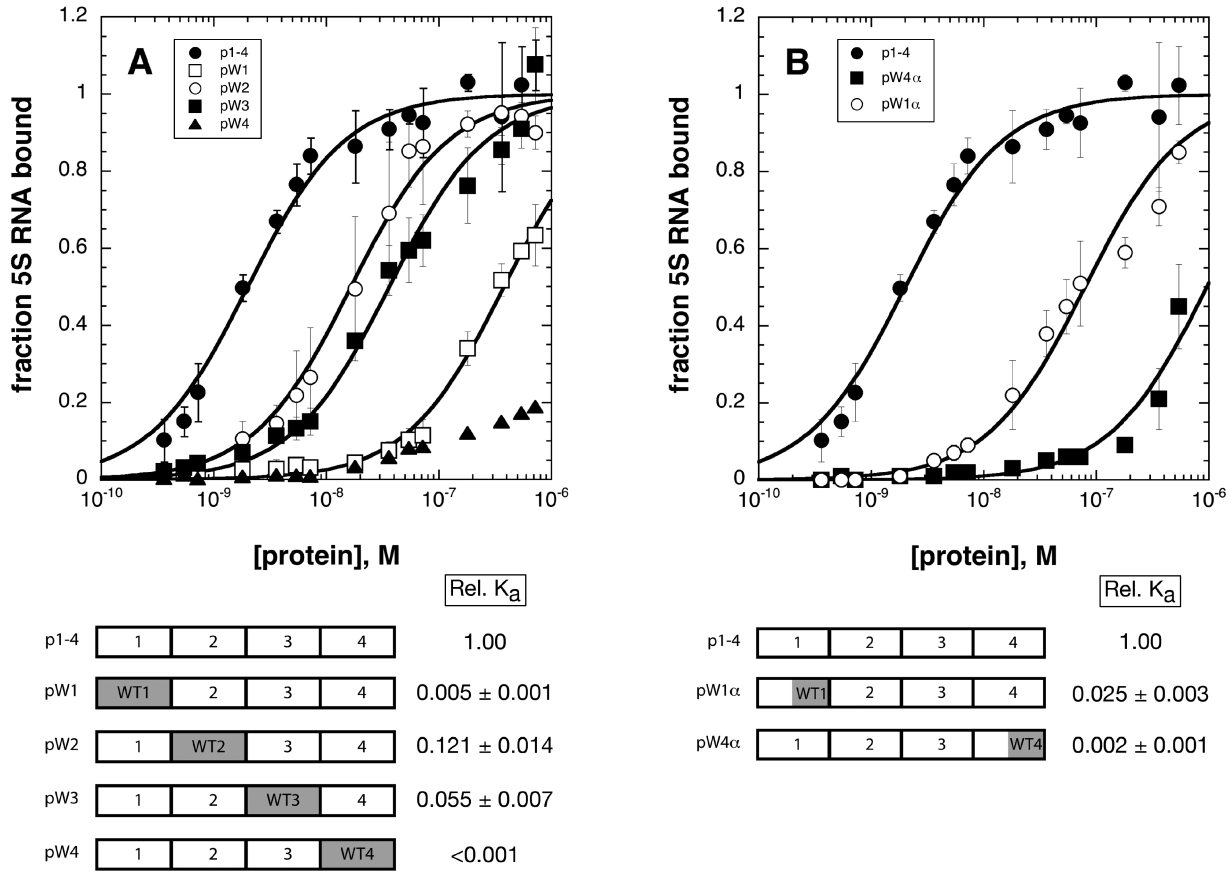

FIGURE 3: (A) Equilibrium binding of p1−4, pW1, pW2, pW3 and pW4 to 5S RNA. (B) Equilibrium binding of p1−4, pW1α and pW4α to 5S RNA. Each data point is the mean value from three independent experiments, with the standard deviation for each point represented by the error bars. Best-fit curves to a simple bimolecular equilibrium are shown for each protein. A schematic representation of each protein and its binding affinity relative to p1−4 are shown below each graph.

	15 20 25 30 35 40	Rel. K_a
p1-4	L L R C P A A G C K A F Y R K E G K L Q D H M A G H S E	
pW1α	- - - - - - - - - - - - - - - - K L S H L Q M H S R K H T G	0.025 ± 0.003
pW1(K28A,E29L)	- - - - - - - - - - - - - - - - A L - - - - - - - - - - - -	<0.001
pW1(G30S,K31H)	- - - - - - - - - - - - - - - - S H - - - - - - - - - - -	0.045 ± 0.007
pW1(Q33A,D34M)	- A M - - - - - - -	<0.001
pW1(M36S,A37R,G38K)	- S R K - - -	<0.001
pW1(S40T,E41G)	- T G	0.030 ± 0.004
pW1(K28A)	- - - - - - - - - - - - - - - - A - - - - - - - - - - - - -	0.152 ± 0.014
pW1(E29A)	- - - - - - - - - - - - - - - - - A - - - - - - - - - - - -	0.457 ± 0.042
pW1(G30A)	- - - - - - - - - - - - - - - - - - A - - - - - - - - - - -	0.378 ± 0.044
pW1(K31A)	- - - - - - - - - - - - - - - - - - - A - - - - - - - - - -	0.082 ± 0.018
pW1(Q33A)	- A - - - - - - - -	0.574 ± 0.070
pW1(D34A)	- A - - - - - - -	0.057 ± 0.008
pW1(M36A)	- A - - - - -	0.898 ± 0.166

FIGURE 4: Amino acid sequences of wild type finger 1 of p1−4 and the constructed scanning and point mutants. The binding affinities of each protein relative to p1−4 are indicated.

mutants reduce the free energy of binding from 1.10 to 1.67 kcal·mol^{-1} (Table 1); together K28, K31 and D34 contribute 37% of the total free energy of complex formation.

A similar approach was taken to investigate the role of α-helical residues of finger 4 in 5S RNA binding. Scanning mutants in finger 4 were created using donor sequences from finger 4 of WT1, with the exception of K119 and R125 which were replaced with alanine because of residue identity between the two proteins at those positions (Figure 5). The scanning mutations had varying effects on the binding of p1−4 to 5S RNA (Table 2). Mutants pW4(R124V,R125A) and pW4(L127H,S128N,V129M) each reduced the affinity of p1−4 for 5S RNA by a factor of 33×. In comparison, the two scanning mutations at the start of the α-helix,

Table 1: Effects of Scanning and Point Mutations in Finger 1 on the Binding of p1−4 to 5S RNA[a]

protein	K_a (M^{-1})	ΔG (kcal·mol^{-1})	$\Delta\Delta G$ (kcal·mol^{-1})
p1−4	$4.98 \pm 0.44 \times 10^8$	-11.66 ± 0.86	
pW1	$2.49 \pm 0.50 \times 10^6$	-8.57 ± 1.72	3.08
pW1α	$1.25 \pm 0.15 \times 10^7$	-9.51 ± 1.13	2.14
pW1(K28A,E29L)	$<2.50 \times 10^5$	>7.24	>4.42
pW1(Q33A,D34M)	$<2.50 \times 10^5$	>7.24	>4.42
pW1(M36S,A37R,G38K)	$<2.50 \times 10^5$	>7.24	>4.42
pW1(S40T,E41G)	$1.48 \pm 0.14 \times 10^7$	-9.61 ± 0.91	2.04
pW1(G30S,K31H)	$2.23 \pm 0.31 \times 10^7$	-9.85 ± 1.36	1.81
pW1(K28A)	$7.56 \pm 0.18 \times 10^7$	-10.56 ± 0.25	1.10
pW1(E29A)	$2.27 \pm 0.06 \times 10^8$	-11.20 ± 0.29	0.46
pW1(G30A)	$1.88 \pm 0.14 \times 10^8$	-11.09 ± 0.83	0.57
pW1(K31A)	$4.08 \pm 0.80 \times 10^7$	-10.20 ± 2.00	1.46
pW1(Q33A)	$2.86 \pm 0.24 \times 10^8$	-11.34 ± 0.97	0.32
pW1(D34A)	$2.84 \pm 0.33 \times 10^7$	-9.99 ± 1.15	1.67
pW1(M36A)	$4.47 \pm 0.73 \times 10^8$	-11.60 ± 1.88	0.06

[a] Apparent association constants determined by the nitrocellulose filter binding assay. Each value reported represents the mean of 3 or more independent determinations with the associated standard deviations. K_a values of less than 2.50×10^5 M^{-1} could not be measured reliably. Relative affinities were arrived at by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild type protein determined in parallel. The errors for relative affinities are given by the expression $\sigma = \{(\sigma 1/M1)^2 + (\sigma 2/M2)^2\}^{1/2} \times M2/M1$, where M1 and M2 are the respective association constants for wild type and mutant protein and the σ values are the corresponding standard deviations for these determinations.

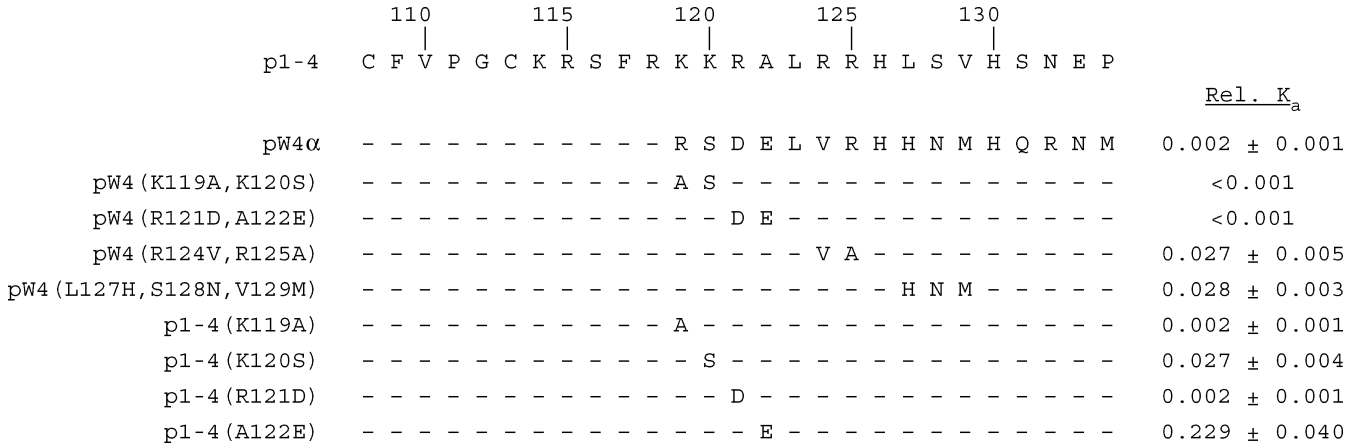

FIGURE 5: Amino acid sequences of wild type finger 4 of p1−4 and the constructed scanning and point mutants. The binding affinities of each protein relative to p1−4 are indicated.

Table 2: Effects of Scanning and Point Mutations in Finger 4 on the Binding of p1−4 and p1−8 to 5S RNA[a]

protein	K_a (M^{-1})	ΔG (kcal·mol^{-1})	$\Delta\Delta G$ (kcal·mol^{-1})
p1−4	$4.98 \pm 0.44 \times 10^8$	-11.66 ± 0.86	
pW4	$<2.50 \times 10^5$	>7.24	>4.42
pW4α	$9.96 \pm 0.01 \times 10^5$	-8.04 ± 0.01	3.62
pW4(K119A,K120S)	$<2.50 \times 10^5$	>7.24	>4.42
pW4(R121D,A122E)	$<2.50 \times 10^5$	>7.24	>4.42
pW4(R124V,R125A)	$1.33 \pm 0.19 \times 10^7$	-9.55 ± 1.39	2.11
pW4(L127H,S128N,V129M)	$1.38 \pm 0.09 \times 10^7$	-9.57 ± 0.66	2.09
p1−4(K119A)	$9.90 \pm 0.01 \times 10^5$	-8.04 ± 0.01	3.62
p1−4(K120S)	$1.33 \pm 0.19 \times 10^7$	-9.55 ± 1.36	2.11
p1−4(R121D)	$9.90 \pm 0.01 \times 10^5$	-8.04 ± 0.01	3.62
pW4(A122E)	$1.14 \pm 0.17 \times 10^8$	-10.80 ± 1.65	0.86
p1−8	$3.51 \pm 0.28 \times 10^8$	-11.46 ± 0.92	
p1−8(K119A)	$1.00 \pm 0.01 \times 10^6$	-8.04 ± 0.08	3.41
p1−8(K120S)	$1.00 \pm 0.01 \times 10^6$	-8.04 ± 0.08	3.41
p1−8(R121D)	$1.46 \pm 0.25 \times 10^8$	-10.94 ± 1.88	0.51

[a] Apparent association constants determined by the nitrocellulose filter binding assay. Each value reported represents the mean of 3 or more independent determinations with the associated standard deviations. K_a values of less than 2.50×10^5 M^{-1} could not be measured reliably. Relative affinities were arrived at by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild type protein determined in parallel. The errors for relative affinities are given by the expression $\sigma = \{(\sigma 1/M1)^2 + (\sigma 2/M2)^2\}^{1/2} \times M2/M1$, where M1 and M2 are the respective association constants for wild type and mutant protein and the σ values are the corresponding standard deviations for these determinations.

pW4(K119A,K120S) and pW4(R121D,A122E), each reduced the affinity of p1−4 for 5S RNA by more than 1000×. The role of the first four amino acids of the α-helix of finger 4 was investigated further by point mutagenesis (Figure 5). Since one of the residues to be tested was A122, rather than make alanine substitutions each point mutation corresponded to the substitution found in the relevant scanning mutant. As the results in Table 2 show, point mutagenesis of the three basic residues had a significant effect on the binding of p1−4 to 5S RNA, decreasing the affinity of the protein

for the RNA by as much as 500×. Reductions in the free energy of binding ranged from 2.11 to 3.62 kcal·mol^{-1}, consistent with the loss of one or more direct bonding contacts between these amino acids and the RNA. In comparison, substitution of the nonpolar alanine residue with a negatively charged glutamic acid had little effect on the affinity of p1−4 for 5S RNA and the free energy of binding.

In the full length p43 protein, finger 4 is an internal zinc finger and not a terminal zinc finger as it is in the p1−4 peptide. To ensure that the effects observed in the p1−4 peptide of the point mutations of K119, K120 and R121 are reflective of the role of these residues in 5S RNA binding by p43, the point mutants were reconstructed in the p1−8 peptide. As the data in Table 2 show, the K119A and K120S mutations have the same effects on 5S RNA binding regardless of whether they are in the p1−4 or p1−8 context. Each of these individual substitutions reduces the free energy of the binding of p1−8 to 5S RNA by 30%. In comparison, the R121D mutation has a much smaller effect on the binding of p1−8 to 5S RNA compared to its effect on the binding of p1−4.

DISCUSSION

Zinc finger proteins carry out diverse functions in the cell, generally involving specific interactions with DNA, RNA or protein ligands. Some members of this class of proteins are multifunctional, which requires that the zinc finger domains in these proteins interact with more than one class of ligand. For example, the Wilms tumor suppressor protein WT1 acts as a transcriptional regulator by binding to specific sites in DNA (*34, 35*). It is also believed to be involved in downstream transcript processing or regulation by virtue of a putative RNA binding activity located in the zinc finger domain and the presence of the protein in poly-A+ RNPs (*36, 37*). Further complexity in the function of the WT1 zinc finger domain has been implied by the observation that this domain interacts with a number of protein partners (*38–40*).

The first zinc finger protein to be characterized was TFIIIA (*10, 11*). This protein is also multifunctional: TFIIIA acts as a specific transcription factor involved in expression of the 5S RNA gene, and in amphibia and teleosts the protein also forms a 7S RNP with the 5S RNA transcript that is stored in the cytoplasm of immature oocytes (*4*). In addition to the 7S RNP storage particle, the 5S RNA is also stored in a 42S RNP that includes the zinc finger protein p43 (*1, 2*). Although both TFIIIA and p43 each consist of nine tandem zinc finger motifs, there is little sequence homology between the two proteins except for the conserved residues required to coordinate zinc and fold each motif into a zinc finger (*9*).

Given the similarities and differences between these two zinc finger proteins, it has been of some interest to compare their interactions with the same 5S RNA ligand. A great deal of biochemical investigation into the interaction of TFIIIA with 5S RNA indicated that zinc fingers 4 to 6 were critical for binding of the protein to RNA (*16, 17, 19, 41*). Subsequently, determination of a three-dimensional structure for a complex formed between a peptide consisting of zinc fingers 4 to 6 of TFIIIA and a truncated version of 5S RNA provided a detailed picture of the interaction of the protein with RNA that was consistent with the biochemical data (*25*). For example, in the three-dimensional structure of the complex, the TWT residues of finger 6 interact directly with the CACA bases of loop A of the 5S RNA. Replacement of the CACA bases of the 5S RNA or substitution of the TWT amino acids in finger 6 of TFIIIA abrogates complex formation (*20, 27*).

Previous biochemical comparisons of the interaction of TFIIIA and p43 with 5S RNA have identified an intriguing mix of similarities and differences (*13, 20, 21, 26, 42*). Both proteins bind to 5S RNA with very similar affinity constants. Complex formation with TFIIIA is favored by both enthalpy and entropy, whereas complex formation with p43 has an unfavorable enthalpy but large favorable entropy term. Although there is an overlap in the RNA binding determinants recognized by both proteins, each also relies on unique features of the 5S RNA in forming a specific complex (*26*). The effects on RNA binding of truncation mutants of TFIIIA and p43 demonstrated that zinc fingers 4 to 6 are essential for the TFIIIA−5S RNA interaction while for p43 zinc fingers 1 to 4 are critical (*16, 17*). Particularly noteworthy is the observation that the TWT amino acid motif in zinc finger 6 of both proteins is critical for the binding of TFIIIA to 5S RNA, but not for the interaction of p43 with the RNA (*27*).

In order to gain further understanding into the mechanism of the binding of p43 to 5S RNA, and to compare it to the mechanism of binding of TFIIIA to 5S RNA, we undertook a biochemical analysis of the role of specific zinc finger residues of p43 in RNA binding. The results demonstrate that zinc fingers 1−4 of p43 are essential for high affinity binding to 5S RNA. Although replacement of any one of the first four zinc fingers results in a decreased affinity for the RNA, fingers 1 and 4 appear to be particularly crucial to formation of the protein−RNA complex. Further investigation by scanning mutagenesis identified α-helical residues K28, K31 and D34 in finger 1 as being potential RNA-contacting residues while there appears to be a requirement for a small aliphatic residue at position 37 or 38. All of these key residues would be oriented on the same face of the α-helix of zinc finger 1. Similar analysis of zinc finger 4 identified K119 and K120 at the start of the α-helix as being critical for high affinity binding of p43 to 5S RNA.

The X-ray crystallographic analysis of the complex formed between zinc fingers 4−6 of TFIIIA and a truncated version of 5S RNA shows two distinct modes of zinc finger interaction with RNA (*25*). In the case of zinc fingers 4 and 6, the amino terminal end of the α-helix is tipped toward the RNA, allowing three to four consecutive residues to form specific bonding interactions to the bases and backbone. In comparison, the α-helix of zinc finger 5 is oriented parallel to the backbone of a double helical section of the RNA. In this orientation, amino acids distributed throughout the α-helix form contacts to the structural backbone of the RNA. The residues involved are not restricted to a single face of the α-helix of zinc finger 5.

The biochemical data in this paper indicate that zinc finger 4 of p43 likely tips its α-helix toward specific bases of the 5S RNA, analogous to the manner in which zinc fingers 4 and 6 of TFIIIA interact with the RNA. In comparison, zinc finger 1 of p43 apparently uses a method to interact with the 5S RNA that is different from the two modes of interaction identified for the zinc fingers of TFIIIA and is

analogous to the manner by which zinc fingers interact with DNA. In the interaction of zinc finger proteins with DNA, the α-helix of an interacting finger lies in the major groove and residues on one face of the helix form specific bonding interactions with the base pairs of the DNA (*43, 44*). This mode of interaction is far less likely to occur when a zinc finger protein binds to RNA, primarily because the RNA lacks a regular periodic structure and the major groove within base paired regions of RNA is generally too narrow to accommodate an α-helix. Studies of the binding of 5S RNA mutants to p43 and TFIIIA indicate that p43-specific binding determinants include helix II and hairpin loop C (*26*). If either of these areas is the binding subsite for zinc finger 1, perhaps local structure allows for an orientation of the α-helix such that RNA−protein contacts are formed on one face.

Zinc finger proteins often carry out multiple functions in the cell, involving their interaction with more than one type of macromolecular ligand. TFIIIA acts as a 5S RNA gene-specific transcription factor and also forms a 7S RNP storage particle by binding to the 5S RNA. Both of these ligand binding activities involve the zinc fingers of the protein. In the case of p43, the only known biological function is the formation of the 42S RNP particle for storage of 5S RNA. Only the first four zinc fingers of p43 are required for binding to the RNA, which raises the question of what function the remaining zinc fingers of p43 have. One possible function would be forming protein−protein interactions in the assembly of the 42S RNP. An additional interesting question concerns the potential role of the conserved TWT sequence motif in zinc finger 6 of p43. In TFIIIA these amino acids are critical for binding to 5S RNA, which is not the case for p43. Does this motif have some other function in p43, and if so does the motif carry out a similar function in TFIIIA? Questions such as these suggest that there is more to learn about the function of the zinc fingers in TFIIIA and p43.

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