Selective RNA Binding by a Single CCCH Zinc-Binding Domain from Nup475 (Tristetraprolin)†

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ABSTRACT: Regulation of gene expression takes place at several different levels and involves specific domains involved in specific protein−nucleic acid interactions. The protein Nup475 (also known as Tristetraprolin and TS11) binds to AU-rich sequence elements in certain mRNA molecules and favors the degradation of these mRNAs. The nucleic acid binding domain of Nup475 consists of two CCCH zinc-binding domains. A 36-amino acid peptide corresponding to the first of these CCCH domains has been synthesized and characterized. This peptide binds metal ions such as zinc(II) and cobalt(II) with affinities comparable to those of other authenticated zinc-binding domains. The zinc(II) complex of this peptide binds the RNA oligonucleotide UUUAUUU labeled with fluorescein on the 3′-end with an affinity of approximately 5 μM and discriminates against other sequences lacking the central A or the flanking U residues. These results demonstrate for the first time that a single CCCH domain is capable of binding single-stranded RNA with considerable affinity and selectivity. The combination of this well-behaved domain and the fluorescence-based binding assay sets the stage for more detailed structure−activity studies.

Nup475 (*1*) [also known as tristetraprolin (*2*) or TS11 (*3*)] is a member of a family of proteins that contain zinc-binding domains of the form Cys-X_8-Cys-X_5-Cys-X_3-His (hereafter termed CCCH domains). This protein was first identified as the product of an immediate early gene, produced in response to serum, insulin, and other growth factors in murine fibroblasts (*1*−*3*). Characterization of Nup475-deficient mice led to the discovery that this protein functions as a sequence-selective RNA-binding protein (*4*−*6*). Specifically, mice homozygous for a disruption of the gene that encodes Nup475 exhibited a phenotype that included cachexia, arthritis, and conjunctivitis, all hallmarks of the overproduction of the cytokine tumor necrosis factor α (TNF)[1] (*4*). These symptoms could be prevented by treatment with anti-TNF antibodies (*4*). Studies on macrophages from these mice revealed that TNF overproduction occurred due to an increase in the half-life of TNF mRNA (*5*). On the basis of this observation, it was proposed that Nup475 interacts with TNF mRNA and regulates its half-life (*5*). TNF mRNA includes AU-rich elements (AREs) in the 3′-untranslated region (3′-UTR) that had previously been associated with short half-life mRNAs (*7*, *8*). Cotransfection studies revealed that Nup475 regulates TNF production by binding to the AREs of its mRNA (*5*, *6*) and promoting mRNA degradation. More recently, it has been shown that Nup475 regulates other cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), by analogous mechanisms (*9*).

Nup475 includes two CCCH domains separated by a linker of 18 amino acids (*1*). The AREs in the TNF mRNA have arrays of the sequence 5′-UUAUUUAUU-3′. The optimal binding site for Nup475 has been revealed to be 5′-UUAUUUAUU-3′ through the use of SELEX experiments (*10*). A series of gel shift experiments with wild-type Nup475 and a series of mutants with the ARE from TNF mRNA have been reported. One important finding was that the metal binding cysteines and histidines from **both** CCCH domains were required for RNA binding that could be detected by these methods (*6*). This led to the conclusion that both domains were required for RNA binding.

As part of a program directed toward the structural basis of function for CCCH domain-containing proteins, we have previously demonstrated that these domains bind cobalt(II) and, by inference, zinc(II), in a tetrahedral fashion (*11*). Furthermore, we have determined the three-dimensional structure of the amino-terminal CCCH domain from murine Nup475 (*12*). This structure was determined in the context of a two-domain fragment that included a mutation in the second domain that increased solubility, but disrupted domain folding. This structure determination did allow more precise definition of the domain boundaries. This structure (Figure 1) revealed that the protein folded into a disklike arrangement almost entirely devoid of regular secondary structural elements. The presence of two CCCH domains in the RNA-binding portion of Nup475 and the occurrence of repeating UUAUU sequences in the Nup475 binding site suggest that

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[1] Abbreviations: ARE, AU-rich element; F, fluorescein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-perfomance liquid chromatography; K_d, dissociation constant; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight (mass spectrometry); NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; *r*, anisotropy; SELEX, systematic evolution of ligands by exponential enrichment; TFA, trifluoroacetic acid; TNF, tumor necrosis factor α; TTP, tristetraprolin.

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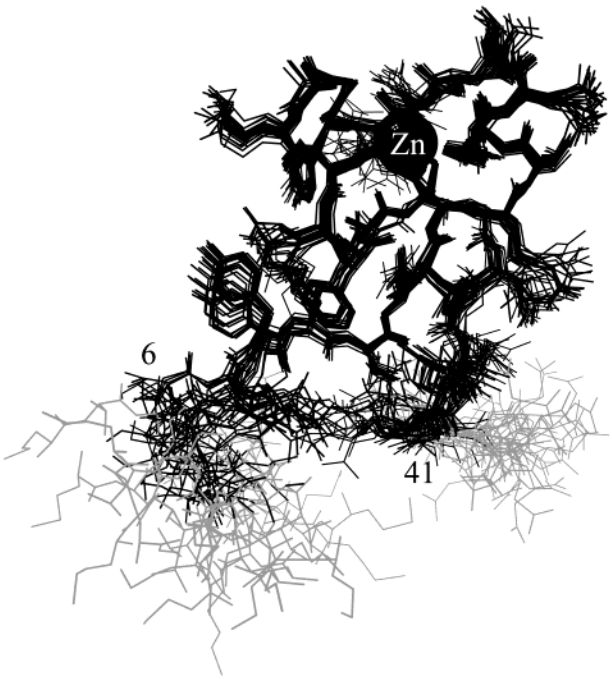

FIGURE 1: Region of the structure of the first CCCH domain of Nup475 used for the synthesis of the peptide Nup475−1D. The less ordered regions that are not included in Nup475-1D are shown in gray.

each CCCH domain might bind to a single UUAUU sequence. To test this hypothesis, we prepared a peptide that corresponded to the folded region of the first CCCH domain observed during the structure determination studies. The ability of this peptide, hereafter termed Nup475-D1, to bind to a series of RNA oligonucleotides was examined using a fluorescence anisotropy-based assay. We found that Nup475-D1 bound the oligoribonucleotide UUUAUUU with a dissociation constant in the low micromolar range and was selective for the sequence of the oligonucleotide and slightly preferred RNA over the same sequence of DNA.

EXPERIMENTAL PROCEDURES

Preparation of Nup475-D1. A peptide, Nup475-D1, with the sequence TSSRYKTEL**C**RTYSESGR**C**RYGAK**C**Q-FA**H**GLGELRQ was synthesized on a Milligen/Biosearch 9050 peptide synthesizer using 9-fluorenylmethoxycarbonyl chemistry. Upon completion of the synthesis, the peptide was cleaved from the resin and deprotected by treatment with a solution of trifluoroacetic acid (80%), thioanisole (5%), ethanedithiol (3%), and anisole (2%) for 2 h. The crude peptide was then filtered through glass wool and precipitated with ether followed by thorough washing with ether. Prior to purification, the peptide was resuspended in phosphate-buffered saline (PBS, pH 7.4) and treated with excess dithiothreitol (DTT) at 55 °C for 2 h to ensure that the cysteine thiols of the peptide were fully reduced. The peptide was then purified on a Vydac C18 reversed-phase HPLC column with an acetonitrile gradient containing 0.1% TFA. The peptide eluted from the column at 30% acetonitrile. Collected fractions were dried under a 95% nitrogen/5% hydrogen atmosphere in a Savant SpeedVac concentrator. All peptide manipulations were performed in this atmosphere to prevent cysteine oxidation, and all solvents were degassed with helium prior to use. The mass of the peptide was confirmed using MALDI mass spectrometry [calcd, 4144.66 Da ($M + H^+$); observed, 4144.60 Da].

Metal Binding Titrations. The affinity of Nup475-D1 for cobalt was measured by spectrophotometrically monitoring the titration of a solution of the peptide with Co(II). The relative affinity of the peptide for Zn(II) was determined by monitoring the displacement of Co(II) by Zn(II) (*13*). The experiments were performed in 200 mM HEPES and 50 mM NaCl at pH 7.0 and 25 °C under anaerobic conditions (5% hydrogen and 95% nitrogen) using a Perkin-Elmer Lambda 9 spectrophotometer.

Oligonucleotide Probes. 3′-Fluorescein (F)-labeled RNA oligonucleotides with the sequences UUUAUUU-F, UUUGUUU-F, UUUUUUU-F, and UUGAGUU-F were obtained from Dharmacon Research Inc., and a 3′-fluorescein-labeled DNA oligonucleotide with the sequence d(UUUAUUU-F) was obtained from the biopolymer facility at the Johns Hopkins University School of Medicine. All oligonucleotides were purchased in PAGE-purified, deprotected, and desalted form. Upon receipt, the oligonucleotides were resuspended in doubly distilled water and quantified.

Binding Studies. The binding of Nup475-D1 to the fluorescently labeled oligonucleotides was assessed using a fluorescence anisotropy assay. The measurements were taken on an ISA/Horida group Fluorolog-3 spectrofluorometer configured in the L format. Excitation and emission wavelength/band-pass were 490 nm/2 nm and 525 nm/6 nm, respectively. In a typical experiment, 500 μL of a 10 nM solution of the oligonucleotide in 50 mM Tris, 100 mM NaCl, and 0.05 mg/mL bovine serum albumin at pH 7.7 was added to an 0.875 mL Spectrosil far-UV quartz window fluorescence cuvette (Starna Cells, Inc.). The anisotropy of the free oligonucleotide was then recorded. Nup475-D1 was then titrated into the cuvette from a 500 μM stock solution (1.5 equiv of $ZnCl_2$, 50 mM Tris, and 100 mM NaCl at pH 7.7) in a stepwise fashion and the resultant change in anisotropy recorded. The peptide was added until the anisotropy values reached saturation. To fit the data, the anisotropy, r, was converted to fraction bound, F_{bound} (the fraction of peptide bound to DNA at a given DNA concentration), using the equation

$$F_{bound} = \frac{r - r_{free}}{(r_{bound} - r)Q + (r - r_{free})}$$

where r_{free} is the anisotropy of the fluorescein-labeled oligonucleotide and r_{bound} is the anisotropy of the oligonucleotide−peptide complex at saturation. Q refers to the quantum yield and is calculated from the fluorescence intensity changes that occur over the course of the experiment (I_{bound}/I_{free}). F_{bound} was plotted against the protein concentration and fitted using a simple one-site binding model:

$$P + D \rightleftharpoons PD \qquad \frac{[P][D]}{[PD]} = K_d$$

$$F_{bound} = \{P_{total} + D_{total} + K_d - [(P_{total} + D_{total} + K_d)^2 - 4P_{total}D_{total}]^{1/2}\}/2D_{total}$$

where P is the peptide (Nup475-D1) concentration and D is the DNA concentration. Although the volumes in the cuvettes did not change more than 10% over the course of the

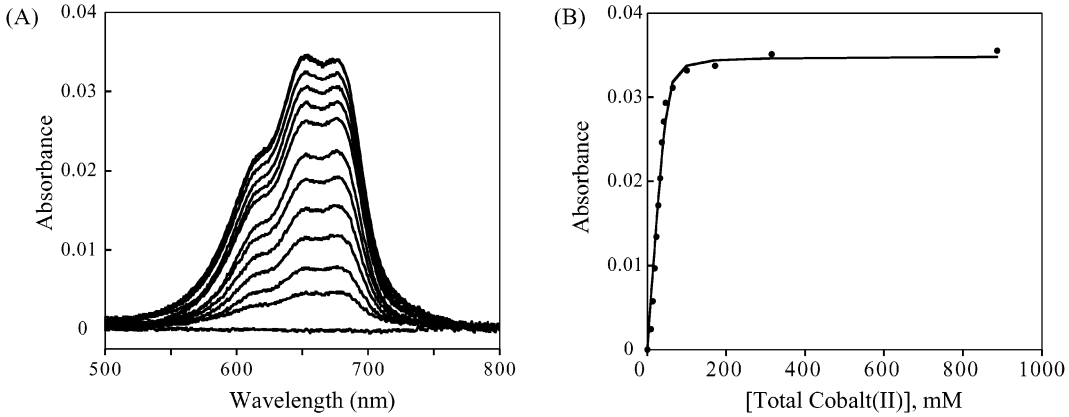

FIGURE 2: (A) Change in the absorption spectrum in the visible region when Nup475-D1 is treated with cobalt(II). (B) Plot of the level of saturation of Nup475 with cobalt(II) as a function of the concentration of cobalt(II) added.

experiments, all concentrations and fluorescence intensity changes were corrected before fitting the data.

RESULTS

Preparation of a Soluble, Single-Domain Peptide of Nup475. To prepare a soluble peptide that corresponded to a single CCCH domain of Nup475, the structured region observed in the NMR structure determination of a larger fragment was used as a guide (*12*). A synthetic peptide comprising the 36 amino acids that correspond to residues 6−41 in the larger fragment was prepared and purified. As anticipated, the peptide was highly soluble in water and could be concentrated to 2 mM without visible precipitation.

To determine if the peptide bound metal and folded, titrations with cobalt(II) and zinc(II) were performed. Cobalt(II) is a good spectroscopic probe of zinc-binding proteins because it will bind to mixed sulfur/nitrogen ligand sets in a tetrahedral coordination geometery like zinc(II), but displays well-defined optical transitions. These transitions include charge transfer bands between 200 and 300 nm and d−d transitions in the visible region (*14*). Figure 2 shows the results of a titration of Nup475 with Co(II) in the visible region (500−800 nm). The shape of the absorption curve is typical of a Cys_3His ligand set (*14*). A plot of the change in absorption at 655 nm versus cobalt(II) concentration is shown in Figure 2B. These data have been fit to a 1:1 peptide−metal binding equilibrium using nonlinear least-squares analysis. The results of this titration gave a dissociation constant K_d of 2×10^{-6} M (allowable range of 0.5×10^{-7} to 4×10^{-6} M) and an extinction coefficient of 670 M^{-1} cm^{-1} at 655 nm. To probe the affinity of Nup475 for zinc(II), a solution containing the Nup475-1D−Co(II) complex with a 1200-fold excess of a Co(II) solution was titrated with Zn(II) and the decreases in the Nup475-1D−Co(II) absorbances were monitored. These data could be fit with the use of nonlinear least-squares analysis to yield a K_d for the Nup475-1D−Zn(II) complex of 2×10^{-10} M.

Binding Interactions between Nup475-1D and Fluorescein-Labeled Oligonucleotides. A fluorescence anisotropy assay was developed to determine the binding affinity of Nup475-1D for oligonucleotides. By titrating a solution of the Nup475-1D−Zn(II) complex into a cuvette containing a fluorescently labeled RNA oligonucleotide and monitoring the change in anisotropy, *r*, we could determine the binding affinity of the peptide for specific oligonucleotides. The data were fit to 1:1 binding isotherms, as described in Experimental Procedures.

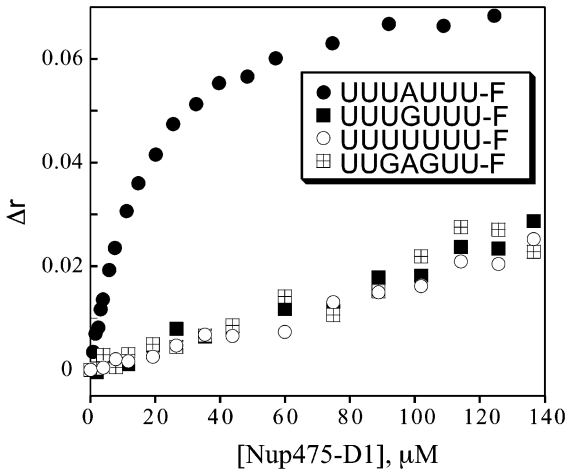

FIGURE 3: Comparison of the change in anisotropy, *r*, upon addition of the zinc(II) complex of Nup475-D1 to the oligonucleotides UUUAUUU-F, UUUGUUU-F, UUUUUUU-F, and UUGAGUU-F. The data for the curve with UUUAUUU-F could be fit to yield a dissociation constant of 4.7 ± 0.5 μM.

Analysis of natural binding sites and SELEX experiments have shown that the optimal binding partner for the full-length Nup475 is the RNA sequence UAUUUAUU (*6*, *10*). On the basis of the presence of two tandem CCCH domains in Nup475 and the repetitive nature of the binding site, we suspected that each of the two zinc fingers contacts half of this sequence, which is an adenosine residue and flanking uridine residues. We hypothesized that a single finger of Nup475 would bind to a single UUAUU sequence. Thus, we examined the binding of Nup475-1D to UUUAUUU-F and compared the results to binding of Nup475-1D to UUUGUUU-F, UUUUUUU-F, and UUGAGUU-F. These latter oligonucleotides lack either the central adenosine or the two flanking uridine residues. Figure 3 shows the results of these titrations. When Nup475-1D was titrated into UUUAUUU-F, significant changes in anisotropy occurred. In contrast, only small changes in anisotropy were observed using UUUGUUU-F, UUUUUUU-F, or UUGAGUU-F. The results using UUUAUUU-F could be fit to a 1:1 binding equilibrium from which a dissociation constant of 4.7 ± 0.5 μM was determined. The results for the other oligonucleotides could be fit to dissociation constants of >60 μM

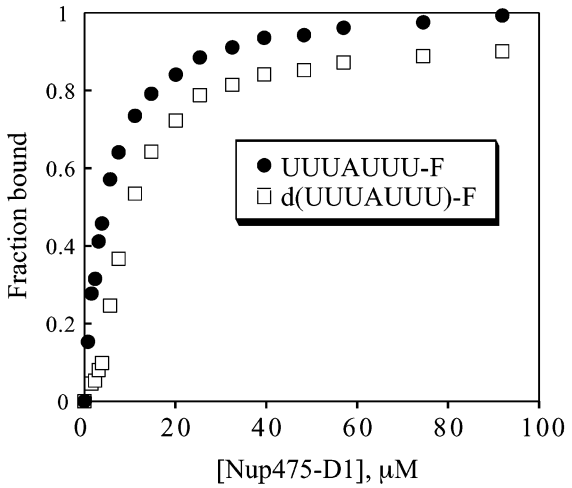

FIGURE 4: Comparison of the change in anisotropy (in terms of fraction bound) upon addition of the Nup475-D1−Zn(II) complex to single-stranded DNA with the sequence d(UUUAUUU)-F vs the corresponding sequence of RNA.

assuming that the values for the final anisotropy and *Q* are similar to those for UUUAUUU-F. These data indicate that a single CCCH domain peptide binds an appropriate RNA oligonucleotide with micromolar affinity and discriminates more than 10-fold against sequences with even single-base substitutions.

To compare the affinity of Nup475-1D for DNA with that for RNA, Nup475-1D was titrated into fluorescein-labeled DNA with the sequence d(UUUAUUU) and the change in anisotropy monitored. Figure 4 shows the results of the titration of Nup475-1D DNA compared with the same sequence of RNA. The data could be fit to yield a dissociation constant of 15 ± 2 μM for the DNA complex assuming 1:1 complex formation, approximately 3 times higher than that for the corresponding RNA complex. Some curvature in the binding curve at low protein concentrations suggests that more than one binding process may be occurring with the DNA oligonucleotide, but the RNA oligonucleotide is bound more tightly in any case.

DISCUSSION

We are interested in understanding the molecular basis of RNA recognition by Nup475. Most proteins that contain zinc-binding domains show modularity in metal binding (*15*, *16*), and some show modularity in nucleic acid interactions (*17*). This study has been directed toward two goals. First, we sought to find a single CCCH domain peptide that folded well and was sufficiently soluble for detailed biophysical studies. Previous attempts have yielded peptides that bound metal ions with appropriate affinities, but were poorly soluble and not well-folded based on NMR studies (*11*). Through the use of information obtained from structural studies of a larger Nup475 fragment (*12*), we have prepared a 36-amino acid peptide that binds cobalt(II) and zinc(II) with high affinity, is quite soluble, and is well-folded (based on NMR studies, data not shown). The dissociation constants for the cobalt(II) and zinc(II) complexes of this peptide, Nup475-D1, are comparable to those observed for previous Nup475-derived peptides and are similar to values obtained for other zinc-binding domain peptides such as those corresponding to TFIIIA-like CCHH zinc finger domains (*14*).

The biological function of Nup475 is based on its ability to bind to specific RNA sequences found in certain mRNAs, including that for the cytokine TNF (*5*). Previous studies had indicated that both CCCH domains from Nup475 were required for RNA binding monitored by gel mobility shift assays (*6*). We have utilized a solution-phase assay utilizing fluorescence anisotropy changes in oligonucleotides labeled with fluorescein to monitor nucleic acid binding. We found that the single CCCH domain peptide as its zinc(II) complex could bind RNA oligonucleotides in a sequence-selective manner. In particular, the oligonucleotide UUUAUUU-F was bound with a dissociation constant of approximately 5 μM. The presence of the central adenosine as well as the two flanking uridine residues was required for this relatively high affinity binding. NMR studies are underway to elucidate the structural basis for the sequence selectivity. Furthermore, replacement of the backbone with deoxyribose led to a 3-fold decrease in binding affinity. These observations are consistent with a model in which the two tandem CCCH domains in Nup475 bind similar UUAUU sequences and do so with positive cooperativity. This cooperativity leads to affinity for intact Nup475 or fragments containing both CCCH domains that is sufficiently high that binding can be detected by gel mobility shift assays. Our results with Nup475-D1 lay the groundwork for more detailed binding studies with larger Nup475 fragments. In addition, these studies may be important for other CCCH domain-containing proteins such as *Caenorhabditis elegans* PIE-1 that contain the CCCH domain that are more widely separated and appear to function independently of one another (*18*).

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