Poly(A)-specific ribonuclease (PARN) is an oligomeric, processive and cap-interacting 3′ exoribonuclease that efficiently degrades mRNA poly(A) tails. Here we show that the RNA recognition motif (RRM) of PARN harbors both poly(A) and cap binding properties, suggesting that the RRM plays an important role for the two critical and unique properties that are tightly associated with PARN activity, i.e. recognition and dependence on both the cap structure and poly(A) tail during poly(A) hydrolysis. We show that PARN and its RRM have micromolar affinity to the cap structure by using fluorescence spectroscopy and nanomolar affinity for poly(A) by using filter binding assay. We have identified one tryptophan residue within the RRM that is essential for cap binding but not required for poly(A) binding, suggesting that the cap- and poly(A)-binding sites associated with the RRM are both structurally and functionally separate from each other. RRM is one of the most commonly occurring RNA-binding domains identified so far, suggesting that other RRMs may have both cap and RNA binding properties just as the RRM of PARN.

A eukaryotic mRNA is characterized by two structural features, the 5′ end located cap structure and the 3′ end located poly(A) tail (1). Both structures are recognized by specific sets of proteins and participate in mechanisms controlling the fate of mRNA, including mRNA processing, transport, translation, and stability (reviewed in Refs. 1–5). In the nucleus, the m7GpppG cap structure is recognized by cap-binding protein (2),3 a subunit of the nuclear cap-binding complex (CBC) (6), which in turn influences mRNA splicing (6), 3′ end formation (7), the nucleocytoplasmic transport (8), and poly(A) degradation (9). In the cytoplasm, CBC is replaced by the cytoplasmic cap-binding protein, also known as eukaryotic translation initiation factor 4E (eIF4E), which together with factors eIF4A and eIF4G, is responsible for initiation of cap-dependent mRNA translation (see Refs. 10 and 11) and references therein). Similarly, the poly(A) tail is recognized in the nucleus by the nuclear poly(A)-binding protein 1 (PABP1), which subsequently is replaced by the cytoplasmic poly(A)-binding protein (PABP) (see Refs. 12 and 13) and references therein). In particular, the cap and the poly(A) tail coordinate and influence protein synthesis and mRNA degradation (reviewed in Refs. 2, 3, 4, and 14). Both structures are recognized during two of the general pathways of eukaryotic mRNA degradation. In the deadenylation-dependent decapping pathway, the cap is recognized and removed after the initial deadenylation step, whereas hydrolysis of the cap is one of the final steps in the deadenylation followed by 3′-5′ degradation pathway.

Several poly(A) degrading activities have been characterized in eukaryotic cells (reviewed in Refs. 2, 3, and 14), e.g. the PAN2/PAN3 nuclease (15, 16), the CCR4-Caf-1 complex (17, 18), and poly(A)-specific ribonuclease (PARN) (19–24). Among these, PARN is unique because it interacts directly with both the cap structure and the poly(A) tail during deadenylation (22, 23, 25–27). The cap stimulates the rate of the PARN reaction and thereby amplifies PARN processivity (22, 25–27). It has been proposed that the cap binding property of PARN could play a critical role in regulating the competition between translation and mRNA degradation (Refs. 2, 5, 9, and 14 and references therein). For example, this property of PARN could abrogate ongoing protein synthesis and target the mRNA for degradation or vice versa could ensure that translation is not initiated on an mRNA already subjected to PARN-mediated degradation.

PARN is an oligomeric enzyme (22), and a recent crystal structure has revealed a dimeric composition (28). The active site of PARN has been characterized both biochemically (29, 30) and structurally (28) and is build up by four conserved acidic amino acid residues that coordinate catalytically essential divalent metal ions. In addition to the cap-
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binding and the active sites, PARN also contains two potential RNA-binding sites. One of these sites belongs to the recently identified R3H class of RNA-binding domains (Refs. 28 and 31 and Protein Data Bank entry 1U8G), whereas the second is a classical RNA recognition motif (RRM) (Protein Data Bank entry 1CVJ) (Fig. 1). However, no functional data has yet confirmed that any of these two domains bind RNA; neither has the cap-binding site been identified. It is of utmost importance to identify and characterize the cap- and poly(A)-binding sites of PARN to fully understand how PARN integrates its poly(A)-binding sites of PARN to fully understand how

FIGURE 1. mPARN(430–516) folds into a classical RRM. A, NMR structure of mPARN(430–516) (Protein Data Bank entry 1WHV) where tryptophan 449 (tryptophan 456 in human PARN) and tryptophan 468 (tryptophan 475 in human PARN) are highlighted in purple. B, crystal structure of RRM 1 of Poly(A) binding protein bound to A₁ (Protein Data Bank entry 1CIV). C, alignment of mPARN amino acid sequence 430–516 with the corresponding amino acid sequence from PARN in different species using the following PARN accession numbers from ENSEMBL. Homo sapiens, ENSMUSP00000055969; Rattus norvegicus, ENSRNOG0000003927; Monodelphis domestica, ENSMODP0000006050; Gallus gallus, ENSGALP000000004877; Xenopus tropicalis, ENSXETP00000024061; Danio rerio, ENSDARP00000028687; Takifugu rubripes, NEWSINFRUP00000154184; Ciona intestinalis, ENSCINP00000020262; A. gambiae, ENSAGAP00000018368. The NCBI accession number for PARN from A. thaliana was Q9LQ62. The location of the secondary structural elements in mPARN are indicated above the alignment. Sequences that are conserved more than 70% are colored in yellow, and the conserved tryptophans are highlighted in red. Structural drawings shown in A–C were made using the molecular graphics program Pymol (www.pymol.org).

EXPERIMENTAL PROCEDURES

Molecular Cloning—A DNA fragment corresponding to amino acids 443–560 in the human PARN sequence, PARN (443–560), was obtained by PCR amplification using plasmid pE₃PARN (30) as template and the primers (443–560)’ containing restriction site for Ndel and (443–560)’ containing restriction site for Bpu1102 I. The sequences of used primers are listed in Table S1. The obtained DNA fragment was cloned into pCR 2.1TOPO vector and subsequently subcloned into the pET19b vector between the Ndel and Bpu1102 I sites.

Site-directed Mutagenesis—All of the PARN mutants were generated from pE₃PARN using a QuikChange site-directed mutagenesis kit (Stratagen) following the manufacturer’s protocol. The mutations were introduced by using primers named as the corresponding mutation and with sequences as listed in Table S1. All of the mutations were confirmed by DNA sequencing.

Expression and Purification of Recombinant Polypeptides—His-tagged recombinant PARN, PARN(W219A), PARN
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(E455A), PARN(W456A), PARN(E455A,W456A), PARN(W475A), PARN(W526A), PARN(W531A), PARN(W639A), PARN(E455,W456,475A), PARN(443–560), and PARN(443–560,W456,475A) polypeptides were expressed from *Escherichia coli* strain BL21(DE3) as described (32). Soluble recombinant polypeptides were purified using Talon Metal Affinity Resin (Clontech) according to Nilsson and Virtanen (32). The amount of protein was measured using Bio-Rad protein assay kit, and purity was analyzed by SDS-PAGE followed by silver or Coomassie staining.

Fluorescence Spectroscopy—Recombinant PARN or PARN mutants expressed in *E. coli* and purified as previously described (32) were used. The protein was dialyzed into buffer D (20 mM Hepes-KOH, pH 7, 10% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol) containing 100 mM KCl. The protein samples were centrifuged for 5 min at 13,000 rpm in 4 °C and softly degassed prior to each measurement. Concentrations of the protein supernatants and ligands were determined spectrophotometrically on a Uvikon 933 UV-visible spectrophotometer (Kontron Instruments). For monomers of PARN, single Trp was applied (slit 2.5 nm, auto cut-off filter). Titrations based on all of the measurements, an excitation wavelength of 280 nm of 71,990, 66,490, 60,990, 33,920, and 22,920 M⁻¹ cm⁻¹ were used, respectively (33). Concentrations of m⁷GTP (Sigma, catalog number M-6133) and m⁷GpppG (GE Healthcare catalog number 27-4635) were determined according to Cai et al. (34).

Fluorescence measurements were performed on LS 50 B and LS 55 spectrophotometers (PerkinElmer Life Sciences) with an automatic correction for the photomultiplier sensitivity, in a thermostatted quartz micro or semi-micro cuvette (Hellma catalog number 104.002F QS and 119.004F QS), at 20.0 °C. For all of the measurements, an excitation wavelength of 280 nm was applied (slit 2.5 nm, auto cut-off filter). Titrations based on whole spectra recording (Fig. 2, A and B) were performed for PARN and single Trp → Ala PARN mutants, PARN(E455A,W456,475A), PARN(443–560), and PARN(443–560,W456,475A), extinction coefficients at 280 nm of 71,990, 66,490, 60,990, 33,920, and 22,920 M⁻¹ cm⁻¹ were used, respectively (33). Concentrations of m⁷GTP (Sigma, catalog number M-6133) and m⁷GpppG (GE Healthcare catalog number 27-4635) were determined according to Cai et al. (34).

The fluorescence intensities were corrected for the inner filter effect and the dilution of the sample. The values of the dissociation constants, *K_D*, were obtained by fitting the theoretical curve to the experimental data points, *F*, using the following full equilibrium binding equation,

\[
F = F(0) - [\text{cx}] \cdot (\Delta \phi + \phi_{\text{lig-free}}) + [\text{L}] \cdot \phi_{\text{lig-free}} \quad \text{(Eq. 1)}
\]

where the actual concentration of the protein-ligand complex, [cx], is as follows,

\[
[cx] = \frac{1}{2}([\text{L}] + [\text{P}] + K_D - \sqrt{([\text{L}] - [\text{P}] + K_D)^2 + 4K_D \cdot [\text{P}]})
\]

and *F*(0) is the initial fluorescence of the pure protein; *L* is the total ligand concentration; [P] is the protein concentration per monomer; Δφ is the difference between the fluorescence efficiencies of the apo-protein and the complex; \(\phi_{\text{lig-free}}\) is the fluorescence efficiency of the ligand. *K_D* and Δφ were free parameters of the fitting. The values of \(\phi_{\text{lig-free}}\) were fixed and taken from independent controlling titration experiments in pure buffer. Regressions were performed by means of a nonlinear, least squares method, using PRISM 3.02 (GraphPad Software) or Origin 7 software (OriginLab Corporation). The final *K_D* values were calculated as weighed averages from at least three independent titration series.

Circular Dichroism—CD spectra were measured on an Aviv spectropolarimeter (Lakewood, NJ) in 1.00-mm quartz cuvette (110-QS; Hellma) in 10 mM Hepes-KOH, pH 7.5, 5% glycerol, 0.75 mM MgCl₂, 0.1 mM EDTA, 0.25 mM dithiothreitol, 50 mM KCl, at 20.0 °C, with 4 s of integration time at each point in at least two scans. The buffers and the protein solutions were filtered through a 100-kDa membrane (Millipore) and degassed for 15 min prior to the experiments. The concentrations of PARN(443–560) and PARN(443–560,W456,475A) monomers were 20.9 and 11.5 μM, respectively. Molar ellipticity, [θ], was calculated for the full sequences of the tagged proteins.

Preparation of RNA Substrates—L3(A₃₀) RNA substrates with or without m⁷GpppG at their 5’ ends were synthesized by in vitro transcription as previously described (27). A₃₀–A₂₀ substrates were purchased from Dharnacon Research, Inc. Before usage the substrates were deprotected according to the instructions from the manufacturer. 10 pmol of A₃₀–A₂₀ were 5’-labeled with 20 pmol [γ⁻³²P]ATP (GE Healthcare; catalog number AA0068) using T4 polynucleotide kinase (USB; catalog number 70031Z), and the reactions were incubated in 37 °C for 45 min. The labeled oligonucleotides were resolved in 25% acrylamide gels, bands cut out and eluted in water. The final concentrations of labeled oligo(A) were 2.5–25 nM.

PARN Deadenylation Assay—The conditions for the deadenylation assays were 25 mM Hepes-KOH, pH 7.0, 100 mM NaCl, 0.1 μg/μl bovine serum albumin, and 15 mM MgCl₂. 10 mM PARN or PARN(E455,W456,475A) monomers were incubated with 50 mM capped or uncapped radioactively labeled L3A₃₀ RNA substrates. A 20-μl reaction was incubated at 30 °C, and 1-μl aliquots were taken out at indicated time points. The reactions were analyzed, and released AMP products were sep-
arated by one-dimensional TLC by spotting 1 μl of the reaction on a polyethyleneimine cellulose F plate (Merck; 5579) and using 0.75 M KH2PO4, pH 3.5 (H2PO4), as solvent. The plate was dried, exposed, and scanned by a 400S PhosphorImager (Molecular Dynamics).

**Electrophoretic Mobility Shift Assay**—10-μl reactions were performed in Buffer A (32 mM phosphate buffer, pH 7.0, 0.2 mM dithiothreitol, 100 mM KCl, 0.2 mM EDTA) using 5 μM of oligo(A) RNA and 0.2–8 μM of monomeric PARN or PARN mutant. The reactions were incubated for 15 min at room temperature. 5 μl of loading dye (8% glycerol, 0.15% bromphenol blue/xylene cyanole) was added to the reaction prior to loading the samples. The cap was binned using the samples of nonadenurinating gel (0.5× TBE, 6% 19:1 acrylamide/bisacrylamide v/v) prerun at 200 V, 5 W for 30 min in 4 °C. The gels were run for 3 h at 5 W in 4 °C or using the BioVectis DNA Pointer System for 33 min at constant 30 W at 10 °C, dried in a Bio-Rad gel dryer for 1 h, and finally exposed and scanned by a 400S PhosphorImager (Molecular Dynamics).

**Filter Binding Assay**—Reactions using 32P-labeled A5-A20 as the RNA were performed as described under “Electrophoretic Mobility Shift Assay.” After 15 min of incubation, the entire reaction mixture was bound to a Protran BA 85 Cellulose nitrate membrane (Schleicher & Schuell; catalog number 10 401191) preincubated in ice-cold Buffer A and mounted on a vacuum manifold with no vacuum applied. The membrane was washed with 0.75 ml of ice-cold Buffer A with vacuum applied and dried, and finally the amount of bound protein-RNA complex was quantified in a Beckman Coulter LC6500 scintillation counter. The equilibrium dissociation constant, Kd, was obtained by plotting experimental data and fitting curves with nonlinear regression (Origin 7 software, OriginLab Corporation) using the following binding equation,

$$[PL] = \frac{1}{2}([L_0] + [P_0] + K_d) - \sqrt{([L_0] - [P_0] + K_d)^2 - 4 \cdot [L_0][P_0]}$$

(Eq 3)

where [P0] and K_d were free parameters of the fitting, and [L0] is the dissociation rate constant, K_d, of the PARN-A20 complex was determined by setting up reactions as described above. At time 0 a 100-fold excess of cold A20 was added, and subsequently 10-μl aliquots were taken out at different time points and subjected to filter binding assay. The equation

$$[PL] = [PL]_0 \cdot e^{-k_d \cdot t}$$

(Eq 4)

was used to calculate the dissociation rate constant, k_d.

**Supplemental Data**—Supplemental information includes supplemental Fig. S1 and supplemental Table S1.

**RESULTS**

**The RRM of PARN Binds the Cap**—It has been shown that several cap-binding proteins, e.g., the translation initiation factor eIF4E (36–38), the CBP20 subunit of CBC (39–41), the DcpS scavenger enzyme (42), influenza A RNA polymerase (43) and, vaccinia virus cap modification enzyme VP39 (44), bind the cap by stacking of the 7-methylguanosine base of the cap structure between aromatic amino acids. PARN contains a large number of aromatic amino acid residues, making it likely that PARN-cap interaction can be investigated by fluorescence spectroscopy, just as it has been done for eIF4E and CBC (35, 45). To investigate this, we mixed PARN with m7GTP or m7GpppG cap analogs and monitored, using fluorescence spectroscopy, the fluorescence emission spectra of PARN after excitation at 280 nm (Fig. 2A). From the fluorescence spectra we determined, as described under “Experimental Procedures”, the equilibrium dissociation constant (K_d) of the PARN-cap interaction (Table 1). The calculated K_d value was in the low micromolar range and in the same range as previously suggested from kinetically determined inhibition constants (27) and at least 10-fold higher than for m7GTP or m7GpppG binding to CBC or eIF4E (35, 45).

A systematic mutational analysis was performed to investigate whether any of the six tryptophan residues in PARN could play a role in cap binding. The tryptophan residues were selected for mutagenesis because only six were present in the PARN sequence, whereas the other aromatic residues were significantly more abundant and because the fluorescence spectra indicated that tryptophan residues could be involved. Six separate PARN mutant polypeptides, wherein one at a time of the tryptophan residues was changed to alanine, were generated, and the cap binding properties of each mutant PARN polypeptide were investigated by affinity to 7- Me-GTP-Sepharose (supplemental Fig. S1) and by fluorescence titrations using m7GTP or m7GpppG as cap analogs (Fig. 2 and Table 1). One mutant polypeptide, PARN(W475A), was severely defective in cap binding, whereas a second mutant polypeptide, PARN (W456A), was slightly affected, as visualized by reduced binding to the 7-Me-GTP-Sepharose matrix (supplemental Fig. S1) and by increased calculated PARN-cap K_d values (Fig. 2 and Table 1). In eIF4E, a glutamate residue that immediately follows the cap-interacting tryptophans participates together with the tryptophan residue in cap recognition (35–37). A glutamate residue at position 455 of PARN could potentially play a similar role. Therefore, mutant PARN(E455A) was prepared, and its cap binding properties were investigated. PARN(E455A) showed no defect in its binding to the 7-Me-GTP-Sepharose matrix (data not shown) and a very small increase in the PARN-cap K_d value (Table 1). The triple mutant PARN(E455A,W456,475A) was as expected severely defective in cap binding (Fig. 2C and Table 1 and supplemental Fig. S1). Taken together these results suggest that Trp475 plays an essential role in PARN cap recognition, whereas Glu455 and especially Trp456 could play auxiliary roles.

The three amino acid residues Glu455, Trp456, and Trp475 are all located within a domain of PARN that folds into a classical RRM (Fig. 1) (reviewed in Ref. 46). It was therefore of interest to investigate whether this domain by itself could bind the cap structure. Toward this end we cloned a fragment of PARN (residues 443–560) comprising the RRM. The purified polypeptide PARN(443–560) was capable of binding the cap as revealed by fluorescence titrations and affinity to the 7-Me-GTP-Sepharose matrix with the binding constant, K_d, still in the micromolar range (Table 1, Fig. 2, B and C, and supplemental Fig. S1). As expected, the mutant PARN(443–560,W456,475A) polypep-
m7GpppG (in H9262 5 fragment containing the RRM domain of PARN by itself binds supplemental Fig. S1). Taken together our data reveal that a tide was severely defective in cap binding (Table 1, Fig. 2).

| TABLE 1 |

**Summary of PARN-cap equilibrium dissociation constants**

The values were determined by intrinsic protein fluorescence quenching in 20 mM Hepes-KOH, pH 7, 100 mM KCl, 10% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol at 20 °C.

| PARN polypeptide | $K_d$ (μM) | $\Delta K_d$ (μM) |
|------------------|------------|------------------|
| PARN | 1.59 ± 0.11 | 0.90 ± 0.02 |
| PARN(W456A) | 5.6 ± 1.4 | 6.4 ± 0.8 |
| PARN(W475A) | >1000 | >1000 |
| PARN(E455A,W456A,W475A) | >1000 | >1000 |
| PARN(E455A) | 3.3 ± 1.0 | 1.3 ± 0.5 |
| PARN(W219A) | 2.1 ± 0.5 | 0.8 ± 0.3 |
| PARN(W256A) | 2.0 ± 0.5 | 1.4 ± 0.4 |
| PARN(W531A) | 1.9 ± 0.5 | 1.4 ± 0.4 |
| PARN(W639A) | 1.6 ± 0.6 | 1.2 ± 0.6 |
| PARN(443–560) | 11.1 ± 0.2 | 6.96 ± 0.14 |
| PARN(443–560,W456A,W475A) | >1000 | >1000 |

*PARN mutants were generated by site-directed mutagenesis.

**FIGURE 2. Tryptophans 456 and 475 are important for cap binding of PARN.** A, fluorescence spectra of PARN at 5 μM in the presence of the following concentrations of m7GpppG (in μM, from top to bottom): 0, 0.6, 1.9, 4.3, 6.7, 9.2, 11.5, and 16.3. B, fluorescence spectra of 5 μM PARN(443–560) in the presence of the following concentrations of m7GpppG (in μM, from top to bottom): 0, 0.9, 2.2, 4.6, 7.0, 9.4, 11.8, and 16.5. C, binding curves rendered by fluorescence spectroscopy using m7GpppG as ligand and 0.2 μM PARN, PARN(E455A), PARN(W526A), PARN(443–560), PARN(W456A), PARN(W639A), PARN(W219A), PARN(443–560,W456A), PARN(443–560,W639A), and PARN(W219A) as the deadenylase. This suggests that bacterially expressed recombinant PARN to some extent can reconstitute the stimulatory effect the cap structure has on PARN activity (see also Ref. 9), although the effect is not as prominent as it is when using HeLa cell free S100 extracts (26) or PARN from calf thymus extracts and purified to apparent homogeneity (22, 25, 27). The reason for the inability to fully reconstitute the stimulatory effect using recombinant PARN purified from bacteria is not known but could be due to many reasons, e.g. lack of post-translational modifications.

**The RRM of PARN Binds Poly(A)—** The presence of a classical RRM within PARN suggests that the RRM could bind RNA besides binding the cap. To investigate this, we performed electrophoretic mobility shift assays (EMSA). Fig. 4 shows that both PARN and PARN(443–560) formed stable complexes with 32P-labeled A20 oligonucleotides, suggesting that the RRM of PARN binds RNA. The addition of increasing amounts of unlabeled A20, A10, or A5 oligonucleotides showed that both complexes were efficiently competed by A20, to some extent by A10, and not at all by A5 (Fig. 4). Further competition experiments...
showed that the complexes were efficiently completed by poly(A), to some extent by poly(G) and very inefficiently by poly(C) or heteropolymeric single-stranded RNA (Fig. 4). We have not been able to interpret competition experiments using poly(U), because of its poly(A) base pairing property. Finally, we investigated the oligonucleotide length requirement for protein-RNA complex formation using EMSA and filter binding assay (see “Experimental Procedures”) and found that more than 10 adenosine residues were required for efficient PARN-RNA or PARN(443–560)-RNA complex formation using EMSA (Fig. 5, A and C), whereas the filter binding assayed revealed a significant drop in the $K_D$ value for the PARN(443–560)-RNA interaction when the oligonucleotide was 10 nucleotides or longer (Fig. 6). To sum up, these data suggest that the RRM of PARN, besides binding the cap, binds RNA with a preference for poly(A) and that at least 10 adenosine residues are required for efficient poly(A) binding. Furthermore, the unaffected RNA binding properties of the PARN(443–560, W456, 475A) mutant polypeptide relative PARN(443–560) (Fig. 5, C and D) also suggest that the introduction of these two mutations does not severely affect the global fold of the RRM. Thus, we predict that the reduced affinity to the cap of the mutated RRM is related to a change of direct, local interactions with the mutated residues rather than being caused by a major conformational change of the mutated polypeptide. In support of this we did not observe any significant differences in the CD spectra of PARN(443–560) and PARN(443–560, W456, 475A) (Fig. 2F).

The Cap and RNA-binding Sites of the RRM Are Structurally and Functionally Separated from Each Other—The capacity of the PARN RRM to bind both cap and poly(A) implies that the two binding sites are located close to each other. However, it is not clear whether they are completely overlapping or whether they are distinct from each other. Furthermore, the two sites may very well be structurally separated but still functionally influence each other. For example, binding of the cap may pre-
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![Diagram](image)

**FIGURE 4.** The RNA binding of the RRM of PARN is poly(A)-specific. A, 1.5 μM PARN monomers were incubated with 5 nM labeled A20 RNA (lanes 1–18 and 20–40). In lane 19 PARN was omitted from the reaction. In lanes 1–6, 0.001, 0.01, 0.1, 1, 10, and 100 μM, respectively, of unlabeled A20 was included in the reactions. In lanes 7–12, 0.001, 0.01, 0.1, 1, 10, and 100 μM, respectively, of unlabeled A10 was included in the reactions. In lanes 13–18, 0.001, 0.01, 0.1, 1, 10, and 100 μM, respectively, of unlabeled A5 was included in the reactions. In lanes 21–25, 0.0001, 0.001, 0.01, 0.1, and 1 g/liter, respectively, of poly(A) was included in the reactions. In lanes 26–30, 0.0001, 0.001, 0.01, 0.1, and 1 g/liter, respectively, of poly(G) was included in the reactions. In lanes 31–35, 0.0001, 0.001, 0.01, 0.1, and 1 g/liter, respectively, of poly(C) was included in the reactions. In lanes 36–40, 0.0001, 0.001, 0.01, 0.1, and 1 g/liter, respectively, of 44-nucleotide-long RNA heteropolymer was included in the reactions. B, same as in A except that 8 μM of PARN(443–560) monomers were used instead of PARN. Formed complexes were analyzed by EMSA. O, C, and S denote the locations of origin of electrophoresis, RNA-protein complex, and free RNA, respectively.

unchanged. The active site and/or the R3H RNA-binding domain are two likely RNA interacting elements that very well could contribute to the enhanced kinetic rate constants of PARN relative to the RRM.

**DISCUSSION**

In this report we show that the RRM of PARN harbors both poly(A) and cap binding properties, suggesting that the PARN RRM can bind the two boundary marks, i.e. the cap structure and the poly(A)) tail, that define the extreme borders of a eukaryotic mRNA. Furthermore, our analysis strongly suggests that the two binding sites are separate from each other both functionally, because they cannot influence each other activities, and structurally, because they rely on different molecular determinants. Based on our studies, we therefore propose that the RRM of PARN plays a pivotal role in the two critical and unique properties that are tightly associated with PARN activity, i.e. recognition and dependence on both the cap structure and poly(A) tail during poly(A) hydrolysis. Interestingly, it has previously been shown that PARN activity can be recovered from mutant PARN polypeptides lacking the RRM (28), although such polypeptides are significantly less active than full-length PARN. Thus, the RRM of PARN is not the only domain responsible for adenosine specificity and efficient hydrolytic activity, even if our data strongly suggest that the RRM of PARN is a major structural and functional element important for proper PARN activity. In particular, the RRM is required both for cap and poly(A) binding and thereby contributes to the specificity of the enzyme. To fully understand the role of the RRM, it is crucial to solve a structure or structures consisting of PARN and the mRNA substrate with its cap structure in the PARN cap-binding site and a poly(A) tail with its 3’ end located adenosine residues in the active site of PARN.

The structure of the RRM domain of mouse PARN (mPARN) was recently determined by NMR analysis (Protein Data Bank entry 1WHV), and a schematic drawing of the structure is depicted in Fig. 1A. It is likely that the RRM of human PARN (hPARN) folds into a very similar structure because the corresponding region of hPARN only differs by one amino acid, i.e. glutamine residue 484 of mPARN is replaced by a lysine residue in hPARN (Fig. 1D). The amino acid numbering systems of mPARN and hPARN differ in this region by seven because of an insertion of seven amino acids in the human sequence N-terminally of the RRM domain. Thus, the two tryptophan residues Trp456 and Trp475 in hPARN correspond to residues Trp449 and Trp468 in mPARN, respectively. Interestingly, these two tryptophans are highly conserved throughout evolution (Fig. 1D). They are even conserved in the insect Anopheles gambiae, which has been shown to harbor cap-dependent PARN activity (23). However, they are not
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Conserved in Arabidopsis thaliana, which may indicate either that A. thaliana PARN (47) lacks cap binding capacity or that the polypeptide assigned as PARN in A. thaliana is only related to PARN in its nuclease domain. Experimental evidence for cap binding by the A. thaliana PARN remains to be obtained.

In the structure of the mPARN RRM, the residues Trp469 and Trp468 are located outside the β-sheet platform of the RRM (Fig. 1A), suggesting that the cap-binding site of PARN is not fully defined by the β-sheet platform. This is surprising because the corresponding β-sheet platform in the RRM of CBP20 harbors the cap-binding site of CBP20 (39–41). In CBP20 the inverted guanosine residue of the cap is stacked between amino acids Tyr20 and Tyr43 (Fig. 1B). A corresponding aromatic slot consisting of Phe475 and His442 can be found in the mPARN RRM. However, in the case of the mPARN RRM, the side chain of tyrosine residue 505 is stacked between the two side chains of Phe475 and His442, suggesting that this aromatic slot is unavailable for cap binding, given the assumption that the determined structure of the mPARN RRM is correct in this particular aspect.

Sequence-specific recognition of poly(A) by RRM motifs has previously been well described both biochemically and structurally for PABP-poly(A) interaction (Fig. 1C) (Ref. 48; reviewed in Ref. 46). In this case the β-sheet platforms of two RRMs forms a continuous binding surface that can encompass the docking of 11 adenosine residues, required for efficient RRM-poly(A) complex formation. Interestingly, several lines of evidence suggest that the PARN RRM may also function as an oligomer when interacting with poly(A). Early biochemical studies suggested an oligomeric composition of PARN (22), and the recent crystal structure revealed a dimeric complex consisting of two PARN subunits (28). Unfortunately, the RRM domain was not included in the polypeptide used for crystallization. Nevertheless, the dimeric structure of PARN provides a structural reason for the possibility that the RRM may function as an oligomer. Our current study is also in keeping with the possibility that the PARN RRM may function as an oligomer when binding poly(A). Significantly, Figs. 4–6 suggest that at least 10 adenosine residues are required for efficient PARN RRM-poly(A) complex formation.

One critical question is whether one subunit of the PARN RRM by itself can bind both poly(A) and cap simultaneously. This could very well be the case and would be in agreement with our biochemical and mutagenic data (Figs. 5 and 7 and Table 2). However, it cannot be excluded that the cap and the poly(A) tail bind to different subunits of a multisubunit RRM oligomer. Thus, even if the two binding sites are clearly separate from each other, both structurally and functionally, each of them may still only be functional on one subunit at the time. The location of the two tryptophan residues outside the β-sheet platform may provide enough binding surface to encompass binding of both the cap and the poly(A) tail simultaneously. Even if this very well could be the case, this scenario is highly speculative at the moment because it relies on the two critical assumptions that the cap interacts directly with at least one of the tryptophan residues (Trp469 or Trp468) and the poly(A) binds to the β-sheet platform. Although poly(A) binding to the β-sheet platform appears likely, because RNA binding to this platform is a general feature observed in determined RRM-RNA structures (46), our data do not unambiguously show that the cap interacts directly with any of the two tryptophans. It is,

![Figure 5](image1.png)

**Figure 5.** Amino acids important for cap binding are not involved in RNA binding of PARN. A, 0.2 (lanes 1, 6, and 11), 0.4 (lanes 2, 7, and 12), 0.8 (lanes 3, 8, and 13), 1.5 (lanes 4, 9, and 14), or 0 (lanes 5, 10, and 15) μM of PARN monomers were incubated with 5 nM labeled A20, A10, or A5, as indicated, in buffer A. B, the same as in A except that 0.5, 1.0, 2.0, and 4.0 μM, respectively, of PARN(E455A,W456,475A) monomers were used. C, the same as in A except that 1, 2, 4, and 8 μM, respectively, of PARN(443–560) monomers were used. D, the same as in A except that 1, 2, 4, and 8 μM, respectively, of PARN(443–560,W456,475A) monomers were used. Formed complexes were analyzed by EMSA. O, C, and S denote the locations of origin of electrophoresis, RNA-protein complex, and free RNA, respectively.

![Figure 6](image2.png)

**Figure 6.** RNA length requirement for binding RNA to the RRM of PARN. RNA binding properties of PARN(443–560) was investigated using filter binding assay. K_D values for poly(A) RNA of different length (as indicated) were determined in the presence of 15 nM PARN(443–560), as detailed under “Experimental Procedures.”

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TABLE 2
Summary of kinetic parameters of A20 RNA-protein interactions

| PARN polypeptide<sup>a</sup> | KD<sup>b</sup> | kd<sup>b</sup> | KD<sup>b</sup> | kd<sup>b</sup> |
|-----------------------------|-------------|-------------|-------------|-------------|
| PARN                        | 5 ± 1       | 14 ± 3 × 10⁻¹ | 5 ± 1       | 16 ± 2 × 10⁻³ |
| PARN(E455A,W456,475A)       | 4 ± 2       | 3.1 ± 0.4 × 10⁻³ | 7 ± 3       | 2.9 ± 0.4 × 10⁻³ |
| PARN(443–560)               | 8 ± 2       | 2.9 ± 0.4 × 10⁻³ | 11 ± 4      | 3.2 ± 0.4 × 10⁻³ |
| PARN(443–560,W456,475A)    | 4 ± 2       | 2.9 ± 0.4 × 10⁻³ | 11 ± 4      | 3.2 ± 0.4 × 10⁻³ |

<sup>a</sup> PARN or mutants thereof were expressed and purified as described under “Experimental Procedures.” The indicated polypeptides at [P<sub>r</sub>], being 1.5 nM were incubated with A<sub>20</sub> RNA substrate, and the amount of formed complex was monitored.

<sup>b</sup> Filter binding assays were performed, and equilibrium dissociation constants, K<sub>D</sub>, and dissociation rate constants, k<sub>d</sub>, were calculated as described under “Experimental Procedures” in the absence or presence of 50 μM cap as indicated. The listed values are the mean values ± experimental error of at least three independent experiments, each based on at least seven independent data points.

for example, possible that the tryptophan residues play a structural role that indirectly affects cap binding. In this case the quenching effect we observe by fluorescence spectroscopy upon cap binding could be the result of essential conformational changes that effect the microenvironment of the tryptophan indol rings. The heterodimerization of the U2AF<sub>65</sub> complex constitutes one such example where the microenvironment of one tryptophan residue within the RRM of U2AF<sub>65</sub> is drastically changed because of docking of the two subunits during heterodimerization (49).

It has been shown that PABP also interacts with both poly(A) and cap and that simultaneous interactions are dependent on one of the RRMs in PABP (50). However, in the case of PABP it has not been shown unambiguously that the RRM by itself can interact with the cap, as is the case for the PARN RRM. Moreover, PABP-cap interaction requires that the cap is linked to a short RNA moiety (50). Nevertheless, the PABP and the PARN cases indicate that many different RRMs may encompass both cap and RNA binding properties. This could be of biological significance because the RRM domain is a very widespread structural element and one of the most commonly occurring RNA-binding domains identified so far (51).

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