Biochimica et Biophysica Acta 1834 (2013) 1089–1098

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Distinct roles of the R3H and RRM domains in poly(A)-specific ribonuclease structural integrity and catalysis

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ARTICLE INFO

Article history:
Received 15 August 2012
Received in revised form 26 January 2013
Accepted 28 January 2013
Available online 4 February 2013

Keywords:
Poly(A)-specific ribonuclease (PARN)
The R3H domain
The RRM domain
RNA binding
Structural feature
Enzymatic property

ABSTRACT

Deadenylases specifically catalyze the degradation of eukaryotic mRNA poly(A) tail in the 3′- to 5′-end direction with the release of 5′-AMP as the product. Among the deadenylase family, poly(A)-specific ribonuclease (PARN) is unique in its domain composition, which contains three potential RNA-binding domains: the catalytic nuclease domain, the R3H domain and the RRM domain. In this research, we investigated the roles of these RNA-binding domains by comparing the structural features and enzymatic properties of mutants lacking either one or two of the three RNA-binding domains. The results showed that the R3H domain had the ability to bind various oligonucleotides at the micromolar level with no oligo(A) specificity. The removal of the R3H domain dissociated PARN into monomers, which still possessed the RNA-binding ability and catalytic functions. Unlike the critical role of the RRM domain in PARN processivity, the removal of the R3H domain did not affect the catalytic pattern of PARN. Our results suggested that both R3H and RRM domains were essential for the high affinity of long poly(A) substrate, but the R3H domain did not contribute to the substrate recognition of PARN. Compared to the RRM domain, the R3H domain played a more important role in the structural integrity of the dimeric PARN. The multiple RNA-binding domain architecture endows PARN the property of highly efficient catalysis in a highly processive mode.

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1. Introduction

In eukaryotic cells, the steady-state levels of transcripts are controlled by many factors including the rate of transcription, mRNA processing, mRNA transportation and mRNA decay. Among them, mRNA decay is increasingly recognized as an important control point in gene expression [1,2]. Eukaryotic mRNA stability is regulated by the untranslational regions and of particularly important, the 5′-cap structure and the 3′-poly(A) tail [2]. These two posttranscriptional modifications interact with eIF4E at the 5′-end and poly(A)-binding protein (PABP) at the 3′-end of the mRNA, which protect the mRNAs against the degradation by exoribonucleases [3]. The degradation of most eukaryotic mRNAs is initiated by the removal of the poly(A) tail from the 3′-end (deadenylation) by 3′-exoribonucleases with high preference of poly(A) as the substrate (deadenylases) [2,4,5]. Deadenylases have been found to participate in diverse intracellular physiological processes by modulating the length of the poly(A) tail [5,6]. According to the nature of the nuclease domain, deadenylases can be divided into two groups: the DEDD and EEP superfamilies [5]. The most well-studied members are CCR4, Caf1, Pan2–Pan3 and poly(A)-specific ribonuclease (PARN) [2,5].

The existence of diverse deadenylases implies that the eukaryotic cell may require distinct deadenylase in a specific physiological process. Actually, the deadenylase activity is regulated by both the intracellular localization and the recruitment by other proteins or complexes [5]. In both yeast and human cells, the deadenylation is mainly mediated by the CCR4-Not complex [4]. PARN is only conserved in vertebrates and may be important in regulated deadenylation such as early development and embryogenesis [7–10], stress response [11,12] and DNA damage response [13,14]. Due to its function in cell cycle regulation, PARN is proposed to play a role in cancer development and several inhibitors have been identified recently [15,16].

PARN belongs to the DEDD 3′-exonuclease superfamily, which contains four acidic residues in the active site to coordinate two divalent metal ions that are essential for catalysis and stability [17–21]. Besides the general deadenylase activity, PARN is unique in its catalytic properties and domain composition. PARN catalyzes the removal of the poly(A) tail in a highly processive mode [10,22], and the processivity can be amplified by the 5′-cap structure of the mRNA [23–25]. Structural

Abbreviations: ANS, 1-anilinonaphtalene-8-sulfonate; CD, circular dichroism; CTD, C-terminal domain; DTT, dithiothreitol; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PARN, poly(A)-specific ribonuclease; p74, the full length PARN; p62, the N-terminal fragment of PARN (residues 1–540); p60, the N-terminal fragment of PARN (residues 1–520); p54, the N-terminal fragment of PARN (residues 1–470); p46, the N-terminal fragment of PARN (residues 1–446); p60dH, p60 with the deletion of the R3H domain; p46dH, p60 with the deletion of the R3H domain; RRM, RNA-recognition motif; SEC, size-exclusion chromatography

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http://dx.doi.org/10.1016/j.bbapap.2013.01.038

studies indicate that the full-length PARN mainly exists as a dimer [19]. Sequence analysis reveals that each subunit of PARN contains three well-structured RNA-binding domains (the catalytic nuclease domain, the R3H domain and the RRM domain) and a C-terminal domain (CTD) (Fig. 1). CTD is predicted to be intrinsically unstructured [26,27] and a bipartite NLS motif is predicted to locate between the RRM domain and CTD [28]. The four domains of PARN assemble into a globular molecule with multiple interdomain and intersubunit binding interface [26,29], and the R3H domain stabilizes PARN via protecting the adjacent RRM domain against aggregation during unfolding [30]. Previous studies have clearly revealed that both the nuclease domain and the RRM domain have high preference in the binding of poly(A) [19,31,32], and the RRM domain contains the major cap binding site [29,31,33,34]. However, no direct evidence is available yet regarding the poly(A) binding property of the R3H domain. Notably, the other members in the deadenylase family generally contained only one poly(A) binding domain (DEDD domain or EEP domain) [2,5]. It is unclear why PARN requires three potential substrate-binding domains and whether all three domains are essential for the highly efficient and processive catalysis of the enzyme. To address these problems, we constructed a series of mutants lacking either one or two of the three RNA binding domains. The results showed that the R3H domain had the ability to bind various oligonucleotides with no preference, implying that the R3H domain did not contribute to the substrate specificity of PARN. The enzyme lacking the R3H domain existed as a monomer and possessed the deadenylase activity. A comparison of the biophysical and biochemical properties of the proteins indicated that the R3H domain was important to the structural integrity and high substrate affinity but not the processivity of PARN.

2. Materials and methods

2.1. Materials

Tris and kanamycin were purchased from AMRESCO. Imidazole, 1-anilinonaphtalene-8-sulfonate (ANS), sodium dodecyl sulfate (SDS), and polyadenylic acid potassium salts were obtained from Sigma-Aldrich, Inc. Cap analog m^7GpppG (catalog number: P171B), dithiothreitol (DTT) and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were purchased from Promega. The 6-mer oligo(A) (A6), oligo(U) (U6), oligo(G) (G6), oligo(C) (C6) and the random nucleotide 5′-GAUCAG-3′ (R6) without the 5′ phosphate group were synthesized by TaKaRa Biotechnology Co. Ltd (Dalian, China). All other reagents were local products of analytical grade.

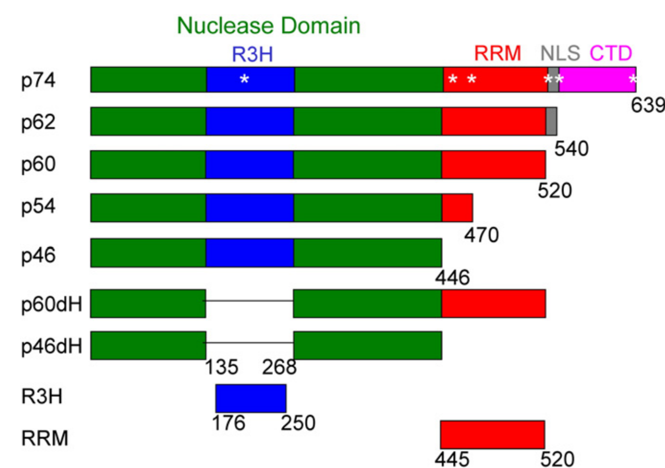

Fig. 1. Domain architecture of PARN and domain composition of PARN fragments constructed in this study. The locations of the Trp residues are marked with asterisks. The lengths of isolated R3H and RRM domains were optimized to get a better protein production yield.

2.2. Protein expression, purification and sample preparation

The plasmid containing the gene of wild type human PARN was kindly provided by Professor Anders Virtanen (Uppsala University, Sweden). The truncated proteins p62 (residues 1–540 AA), p60 (residues 1–520 AA), p54 (residues 1–470 AA) and p46 (residues 1–446 AA) were constructed using the following primers: p62-F, 5′-CGATGTCACATATGGAGATAATCAGGAGC-3′; p62-R 5′-GATCGCTCGAGCTAGTTTAACCGTTTGCTG-3′; p60-F, 5′-CGATGTCACATATGGAGATAATCAGGAGC-3′; p60-R 5′-GATCCTCGAGCTACTTCTCTTCCTGTTTTC-3′; p54-F, 5'-TCGCATATGGAGATAATCAGGAGCA-3'; p54-R, 5'-CGTGGATCCTCAGTTACCAAAGGCACTGAA-3'; p46-F, 5′-CGATGTCACATATGGAGATAATCAGGAGC-3′; p46-R, 5′-GATCCTCGAGTTAATGATCACGTTTAGGCTGC-3′. Two proteins (p60dH and p46dH) with the removal of the R3H domain and the helical linker ranging from residues 135 to 268 were constructed by two-step PCR method using the following primers: F, 5′-ATGGAATTCCATATTTAGGATTTTCTAGAGTC-3′ and R, 5′-GACTCTAGAAAATCCTAAATATGGAATTCCAT-3′. RRM (residues 445–520 AA) and R3H (residues 176–250 AA) were obtained using the following premiers: RRM-F, 5′-GCGCCATATGGATCATGTTCTCCATG-3′; RRM-R, 5′-GATCCTCGAGCTACTTCTCTTCCTGTTTTC-3′; R3H-F, 5′-GATCCATATGGATCAAAAGAAGTTTATTG-3′; R3H-R, 5′-TACTCTCGAGTCATTTGCGTTCTTCTTCATC-3′.

The recombinant proteins were overexpressed in *Escherichia coli* BL21 (DE3) and purified as described previously [18,35] with some modifications. In brief, the expression of p74, p62, p60 and p46 was induced by 0.1 mM IPTG at 16 °C for 24 h and the expression of p54, p60dH, p46dH, RRM and R3H was induced by 0.04 mM IPTG at 10 °C for 48 h. The proteins were purified by Ni^{2+}-affinity chromatography, and the final products were obtained from a Superdex 200 16/60 pre-grade column equipped on an ÄKTA purifier (GE Healthcare). The protein concentration was determined according to the Bradford method [36]. The protein samples were prepared in buffer A containing 20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA and 20% (v/v) glycerol, and were aliquoted and stored at −80 °C until used.

2.3. Spectroscopy

Far-UV circular dichroism (CD) spectra were recorded on a Jasco-715 spectrophotometer using a cell with a path length of 0.1 cm and a resolution of 0.2 nm. Intrinsic fluorescence was measured on a Hitachi F-2500 spectrophotometer using a 0.2 ml cuvette with a slit width of 5 nm for both excitation and emission and a spectral resolution of 0.5 nm. The fluorescence spectra excited at 295 nm are mainly contributed by the Trp fluorophores in proteins, while those excited at 280 nm are dominated by the Trp and Tyr fluorophores. ANS was used as an extrinsic probe to detect the hydrophobic exposure of the native proteins [37]. A 100-fold molar excess of ANS was added to the protein solutions, and ANS fluorescence was measured using an excitation wavelength of 380 nm after 45 min equilibration in the dark. All spectroscopic experiments were carried out using a protein concentration of 0.2 mg/ml. The spectroscopic samples are prepared in 20 mM Tris–HCl, pH 7.0, 100 mM KCl, 0.5 mM DTT, 2 mM EDTA and 10% (v/v) glycerol. The samples were degassed before use.

2.4. Enzyme assay

The enzymatic activity was measured according to the SEC method as described previously [38]. In brief, the standard reaction buffer for PARN contained 20 mM Tris–HCl, pH 7.0, 100 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA and 10% (v/v) glycerol. SEC experiments were performed on an ÄKTA purifier equipped with a Superdex 200 10/30 GL column (GE Healthcare). The concentration of the product AMP was evaluated by the peak area of AMP in the elution profile using the standard curve. The enzymatic kinetic constants K_m and V_{max} were

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