Biochemical and Biophysical Research Communications 414 (2011) 506–511

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Disconnected Interacting Protein 1 binds with high affinity to pre-tRNA and ADAT ☆

Daniel J. Catanese Jr. *, Kathleen S. Matthews

Department of Biochemistry and Cell Biology, Rice University, 6100 Main St., Houston, TX 77005, USA

ARTICLE INFO

Article history:
Received 9 September 2011
Available online 24 September 2011

Keywords:
dsRNA-binding protein
DIP1
ADAT
High affinity
tRNA
Gel retardation

ABSTRACT

Disconnected Interacting Protein 1 (DIP1), a member of the double-stranded RNA-binding protein family based on amino acid sequence, was shown previously to form complexes with multiple transcription factors in *Drosophila melanogaster*. To explore this protein further, we have undertaken sedimentation equilibrium experiments that demonstrate that DIP1-c (longest isoform of DIP1) is a dimer in solution, a characteristic common to other members of the dsRNA-binding protein family. The closest sequence identity for DIP1 is found within the dsRBD sequences of RNA editase enzymes. Consistent with this role, we demonstrate binding of DIP1-c to a potential physiological RNA target: pre-tRNA. In addition, DIP1-c was shown to interact with ADAT, a tRNA deaminase that presumably modifies pre-tRNAs. From these data, we hypothesize that DIP1 may serve an integrator role by binding its dsRNA ligand and recruiting protein partners for the appropriate metabolism of the bound RNA.

1. Introduction

Disconnected Interacting Protein 1 (DIP1) is a member of the double-stranded RNA (dsRNA) binding protein family and, based on sequence alignment, contains two dsRNA-binding domains (dsRBDs) [1,2]. DIP1-c, which is the longest isoform of DIP1, has been shown to bind a number of RNA targets *in vitro* [1,3]. Proteins with dsRBDs, motifs that are 65–68 amino acids in length [4], have been shown to function in a wide variety of RNA processes (reviewed in [5,6]).

Proteins with dsRBDs bind dsRNA with similar affinity (~nM range) (reviewed in [1,3]), and interaction is thought to occur mainly through the ribose-phosphate backbone and not the individual RNA bases [4,7]. How can these proteins discriminate among all RNA molecules in the cell? Currently, it is thought that specificity for dsRNA arises from the spatial arrangement of multiple dsRBDs [8], with the binding footprint for dsRBDs reported to be a minimum of 11 base pairs [9,10] or as large as 18–20 base pairs [11].

The function of DIP1 and the role of its dsRBDs are currently unknown, but its interactions with Ultrabithorax (Ubx) [1], Disconnected [2], and Su(var)3-9 [12], suggest this protein is involved in transcription regulation [1,2] and chromatin remodeling [12]. However, we suggest here that DIP1 may also contribute to tRNA maturation. Indeed, the closest sequence identity for DIP1 is found within the dsRBD sequences of RNA editase enzymes, such as RED1 [13]. Based on domain arrangements, DIP1-c appears to be comparable to the N-terminal half of human RED1 (Fig. 1). Interestingly, the C-terminal half of RED1 appears to mimic the domain structure of ADAT, a tRNA deaminase [14].

In the present work, we examine the assembly structure of DIP1-c and its association with ADAT to show that these two proteins interact *in vitro*. We further examine the dsRNA-binding behavior of DIP1-c and identify a potential physiological target of DIP1, pre-tRNAs [15], which are presumed to be modified by ADAT.

2. Materials and methods

2.1. Expression and purification of DIP1-c

Expression and purification of DIP1-c has been described [3]. Given that the histidine tag was shown not to alter DIP1-c affinity for dsRNA [1,3], His_6-DIP1-c was used for these experiments.

2.2. Analytical Ultracentrifugation (AU)

Micromolar amounts of DIP1-c were dialyzed against 1 L of AU Buffer (20 mM NaH_2PO_4, 200 mM NaCl, 2% glucose, 0.1 mM

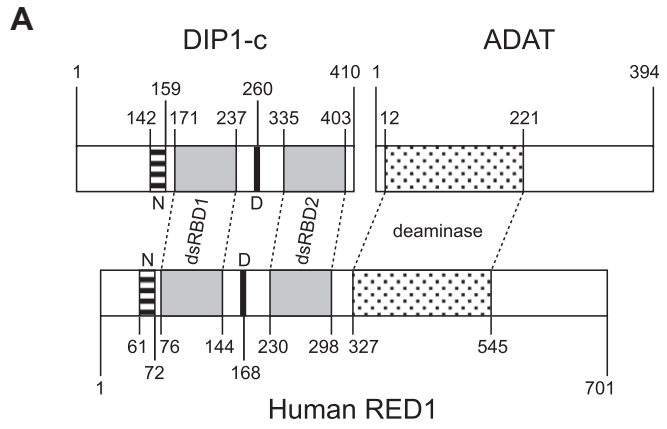
Abbreviations: ADAR, adenosine deaminase that acts on RNA; ADAT, adenosine deaminase that acts on tRNA; AU, analytical ultracentrifugation; DIP1, Disconnected Interacting Protein 1; DIP1-c, longest DIP1 isoform; dsRBD, double-stranded RNA-binding domain; dsRNA, double-stranded RNA; GST, glutathione S-transferase; K_d, equilibrium dissociation constant; miRNA, microRNA; RED1, human RNA editase 1; RNAP, RNA polymerase; ssRNA, single-stranded RNA; TNT, transcription and translation; $tRNA^{Ala}$, tRNA alanine; Ubx, Ultrabithorax.

☆ This work was supported by grants from the Robert A. Welch Foundation (C-576) and the National Institutes of Health (GM22441) to K.S.M. D.J.C. was trained on the National Institutes of Health Houston Area Molecular Biophysics Predoctoral Training Grant 5T32-GM08208.

* Corresponding author. Present address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, BCM-280, BCMC 220A, Houston, TX 77030, USA. Fax: +1 713 798 7375.

E-mail address: catanese@bcm.edu (D.J. Catanese Jr.).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.09.096



B

	Mouse ADAR2		Human RED1	
	% Id.	% Sim.	% Id.	% Sim.
DIP1-c dsRBD1	42	52	44	54
DIP1-c dsRBD2	34	51	35	53
ADAT deaminase domain	33	52	35	53

Fig. 1. Evolutionary correlation between DIP1-c, ADAT, and vertebrate ADAR2/RED1. (A) Alignment of domains within *Drosophila* DIP1-c and ADAT to human RED1. ADARs in all higher organisms contain two important motifs: dsRBDs and deaminase domains. Interestingly, vertebrate ADAR2 [22] (human RED1 shown) appears to be a fusion between two protein sequences that highly resemble DIP1-c and ADAT. DIP1-c resembles the N-terminal half of ADAR2/RED1, in which there are two dsRBDs (gray), a nuclear localization sequence (N, striped), and a DEAD box (D, black); ADAT contains a deaminase domain (polka dot) similar to the C-terminal half of ADAR2/RED1. (B) Sequence comparisons of DIP1-c and ADAT with mammalian ADARs. Shown are the percent identity (% Id.) and similarity (% Sim.) between each DIP1-c dsRBD and ADAT deaminase domain and the corresponding sequence within either mouse ADAR2 or human RED1.

dithiothreitol) at 4 °C with four exchanges with pH decreasing from pH 7.8, to pH 7.6, and two steps with pH 7.5. For each exchange, protein was dialyzed for at least 30 min. All sedimentation equilibrium experiments were performed at 20 °C according to the manufacturer's specifications on an Optima XL-A Analytical Ultracentrifuge (Beckman Coulter, Brea, CA) using an An-60 Ti Rotor, charcoal-filled epon 6-channel centerpieces, and Beckman XL-A version p4.5 software. Experiments were performed at four speeds (9000, 12,000, 15,000 and 18,000 RPM) with wavelengths scans of 235, 237, and 280 nm after 14 h at each speed. To ensure that sedimentation equilibrium had been achieved, a second scan was taken at least 1 h later at each speed.

Analysis of the AU data was performed using UltraScan 6.2 software [16]. Data were analyzed with a GlobalFit regime [17] using the "Ideal and Non-interacting species" model in the software. The parameters used were calculated based on DIP1-c sequence and buffer: Vbar (partial specific volume) was 0.72495 mL/g; density and viscosity of the buffer were 1.0169 g/mL and 1.0734 cp, respectively.

2.3. Cloning of GST–ADAT

The ADAT sequence (a gift from Mary O'Connell, Western General Hospital, Edinburgh, UK) was cloned between the EcoRI and XhoI sites of the pGEX-6P-2 vector (GE Healthcare, Piscataway, NJ). The resulting vector expresses ADAT fused with the glutathione S-transferase (GST) domain on its N-terminus.

2.4. Cloning of tRNA constructs

The sequence of *Bombyx mori* (silkworm moth) $tRNA^{Ala}$ (a gift from Walter Keller, University of Basel, Czech Republic) was placed under T7 RNA polymerase (RNAP) control in pUC118 (Takara Shuzo Company, Tokyo, Japan). Other constructs were cloned by synthesizing the entire sequence behind the T7 RNAP promoter (5′-TAATACGACTCACTATAG-3′) as two separate oligonucleotides (Biosources, Inc., Camarillo, CA). The sequences were flanked by BamHI and EcoRI sites for cloning into pUC18 (Novagen, Madison, WI). The clones designed were: *B. mori* pre-$tRNA^{Ala}$ [18], *Drosophila melanogaster* pre-$tRNA^{Ala}$ [19], and *D. melanogaster* $tRNA^{Ala}$ [19]. To allow for proper polymerase "runoff" during transcription, all of the above constructs include a restriction site 3′ of the template sequence of either SwaI (for 3′ poly-U constructs) or BstNI (for 3′ CCA constructs).

2.5. GST–ADAT pulldown assay

GST–ADAT was generated using transcription/translation (TNT) reactions supplemented with [^{35}S]-methionine in the T7 PCR Quick Master Mix (Promega, Madison, WI) (see Supplemental data in [1]). TNT reactions contained either template for DIP1-c, a plasmid carrying the sequence of *D. melanogaster* pre-$tRNA^{Ala}$ downstream of the T7 RNA polymerase promoter, or both plasmids. TNT reactions were mixed with lysates containing GST–ADAT or "empty" cell lysates. A similar experiment was performed with only the plasmid containing the DIP1-c sequence for the TNT reaction, with addition of exogenous *D. melanogaster* pre-$tRNA^{Ala}$ in amounts ranging from 50 pM to 50 nM. To ensure stability of the pre-$tRNA^{Ala}$ in the reaction, an RNase inhibitor (SUPERaseIN, Ambion, Austin, TX) was added. The pre-$tRNA^{Ala}$ was added to the wash buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$) to maintain the potential ternary complex of pre-$tRNA^{Ala}$, DIP1-c, and GST–ADAT throughout the experiment.

2.6. RNA preparation and handling

Pre-tRNA and tRNA templates were grown in 50 mL LB overnight cultures of either XL1-Blue (Stratagene, La Jolla, CA) or DH5α (Invitrogen Corp., Carlsbad, CA) *Escherichia coli* cells, and the plasmids were purified using a Qiagen miniprep kit (Valencia, CA). To allow for proper T7 RNAP polymerase "runoff," each construct was digested several hours by either SwaI (for 3′ poly-U constructs) or BstNI (for 3′ CCA constructs), based on manufacturer's instructions (New England Biolabs, Ipswich, MA). The digested plasmids were precipitated with ethanol and resuspended in water treated with diethylpyrocarbonate (Sigma, St. Louis, MO). RNA ligands were transcribed *in vitro*, gel-purified, dephosphorylated, folded, and radiolabeled as described [1,3].

2.7. RNA gel retardation

Gel retardation assays and analysis were performed as described [1,3].

3. Results

3.1. Establishing DIP1-c protein character for detailed RNA binding analysis

Precise binding parameters require determining the functional binding unit of DIP1-c. Other proteins with dsRBDs are reported to dimerize [6,20,21], and DIP1-c self-associates in a yeast two-hybrid assay (Peter Pelka, personal communication). To confirm this feature, analytical ultracentrifugation was used to determine the