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HIGH AFFINITY, DSRNA BINDING BY DISCONNECTED INTERACTING PROTEIN 1[†]

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Abstract

Disconnected Interacting Protein 1 (DIP1) appears from sequence analysis and preliminary binding studies to be a member of the dsRNA-binding protein family. Of interest, DIP1 was shown previously to interact with and influence multiple proteins involved in transcription regulation in *Drosophila melanogaster*. We show here that the longest isoform of this protein, DIP1-c, exhibits a 500-fold preference for dsRNA over dsDNA of similar nucleotide sequence. Further, DIP1-c demonstrated very high affinity for a subset of dsRNA ligands, with binding in the picomolar range for VA1 RNA and miR-iab-4 precursor stem-loop, a potential physiological RNA target involved in regulating expression of its protein partner, Ultrabithorax.

Keywords

dsRNA-binding protein; DIP1; gel retardation; high affinity; adenovirus VA1 RNA; iab-4 miRNA

A subset of RNA binding proteins can bind double-stranded RNA (dsRNA)¹ and function in splicing, RNA localization, translational control, RNA editing, post-transcriptional gene silencing, and cellular defense (reviewed in [1,2]). These proteins recognize and bind dsRNA through one or more dsRNA-binding domains (dsRBDs), which are 65–68 amino acids in length [3]. Proteins with dsRBDs bind similar dsRNA ligands with comparable affinity *in vitro*, generally in the nanomolar range [4–6]. Since most proteins in this family contain multiple dsRBDs, specificity for a particular dsRNA molecule is thought to arise from the spatial arrangement of multiple domains rather than activity of a single domain [7].

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¹ABBREVIATIONS:

DIP1, Disconnected Interacting Protein 1

DIP1-c, longest DIP1 isoform

dsRBD, double-stranded RNA-binding domain

DTT, dithiothreitol

HIV, human immunodeficiency virus

K_d , equilibrium dissociation constant

miRNA, microRNA

NATA, N-acetyl-tryptophanamide

RNAP, RNA Polymerase

Ubx, Ultrabithorax

The sequences of dsRBDs are loosely conserved and can be divided into two types: Type A domains bind dsRNA with higher affinity (nanomolar range), and Type B domains generally bind dsRNA weakly [3,7].

Disconnected Interacting Protein 1 (DIP1) contains two dsRBDs based on sequence similarity [8,9]. Interestingly, the closest sequence identity for DIP1 is found within the dsRBD sequences of RNA editase enzymes [8]. The first dsRBD is predicted to be a Type A domain, whereas the second dsRBD is a Type B [8,9]; however, isolated dsRBD1 does not bind dsRNA [9]. These results are inconsistent with the prevailing view that Type A domains bind dsRNA tightly, whereas Type B domains are for cooperative binding and/or to promote protein dimerization [10,11].

DIP1 was previously identified as a protein partner of Ultrabithorax (Ubx) [8], Disconnected [9], and Suppressor of variegation 3–9 [12], suggesting involvement in transcription regulation [8,9] and chromatin remodeling [12]. Indeed, we have shown that DIP1-c, the longest isoform, blocks transcription activation by Ubx using a yeast one-hybrid assay [8]. Although this function is not common for dsRNA-binding proteins, the dsRBDs in DIP1-c appear to be active, as shown in Northwestern blots of full-length protein [8] and the individual dsRBDs [9]. DIP1-c preferentially binds dsRNA over ssRNA and dsDNA [8], which is similar to other proteins with dsRBDs.

DIP1-c demonstrates unusual sequence selectivity based on its higher affinity for the adenovirus VA1 RNA *versus* the HIV TAR stem-loop RNA, both highly-structured RNA ligands, with remarkably high affinity for VA1 RNA, ~50 pM [8]. The highest affinity for dsRNA previously was demonstrated by XlrpA [13]. Although the basis of higher affinity association by DIP1-c is unknown, the observation suggests that dsRBDs in DIP1-c might discriminate among different ligands. In the present work, we examine further the dsRNA-binding behavior of DIP1-c and identify its high affinity for an interesting *Drosophila* microRNA precursor stem-loop sequence [14,15].

MATERIALS AND METHODS

Expression and Purification of DIP1-c

Expression and purification of DIP1-c has been described [8]. Given that the histidine tag was shown not to alter the affinity of DIP1-c for dsRNA [8], His₆-DIP1-c was used for these experiments. DIP1-c was dialyzed into 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 5% glucose, ~5 mM DTT following the Ni²⁺-nitrilotriacetic acid-agarose chromatography step [8].

Magnetic Circular Dichroism

Magnetic circular dichroism was used to determine the absorption coefficient of DIP1-c at 280 nm. N-acetyl-tryptophanamide (NATA, Aldrich) was used as a standard. Multiple DIP1-c samples were dialyzed in either a) 25 mM NaH₂PO₄, pH 7.5, 150 mM NaCl, 2% glucose or b) 50 mM NaH₂PO₄, pH 8.0, 150 mM NaCl, 5% glucose. All samples were analyzed from 280–320 nm on a J-500C Spectropolarimeter equipped with a 7020A power supply and UV-visible photomultiplier tube (Japan Spectroscopic Co.). Standard curves for NATA utilized the difference in values between the 292 nm peak and 320 nm plateau. Using the tryptophan concentration determined for DIP-c from this method allowed calculation of its molar absorption coefficient at 280 nm.

Nucleic acid preparation and handling

dsDNA40: Oligonucleotides bearing the sequence 5'-CCGGGCTGCACATGGTTAATGGCCAGTCCACGCGTAGATC-3' and its complement were annealed, radiolabeled, and utilized in gel retardation as described below for RNA. All RNA ligands were transcribed and dephosphorylated *in vitro* as described [8] with few modifications.

DNA Templates—All templates, except where noted, were synthesized oligonucleotides (Biosource, Inc.) with the appropriate sequence downstream of the T7 RNAP promoter annealed to the T7 RNAP promoter primer [8]. **Adenovirus VA1 RNA:** Full-length VA1 was synthesized as described [8]. VA1 20–108 template was produced by PCR amplification of bases 20–108 of full-length VA1. Other deletions of the VA1 20–108 sequence were synthesized oligonucleotides. **dsRNA40:** The templates were identical to dsDNA40 described above except the 40 bp sequences were preceded by 5'-GGGAGA-3', a sequence that promotes high yield using T7 RNAP [16]. **25dsRNA-4Loop:** An oligonucleotide bearing the sequence 5'-GGATCTCATCATGGCGGACGTACAG-3' and its complement were used to produce a 25 bp hairpin with the tetraloop 5'-ACUG-3'. **miR-iab-4 stem-loop:** The template bearing the sequence 5'-TCGTAAACGTATACTGAATGTATCCTGAGTGTATCCTATCCGGTATACCTTCAGTATACGTAACACGA-3' (miRBase, [17]), was synthesized as an oligonucleotide.

Folding RNA—RNA was diluted to 1 μ M or 10 nM in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, and was heated to $>95^{\circ}\text{C}$ and allowed to cool slowly. At 65°C , a final concentration of 10 mM MgCl_2 was added. RNA was either allowed to cool slowly to room temperature or immediately placed on ice.

Radiolabeling—RNA was radiolabeled as described [8], except the kinase reaction buffer was 40 mM Tris-HCl, pH 8.0, 10 mM MgCl_2 , 0.4 mM spermidine, 5% glycerol, 5 mM DTT, and the NICKTM gel filtration column (GE Healthcare) running buffer was 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl_2 .

Gel Retardation

Gel retardation assays were performed as described [8]. Stoichiometric measurements were performed with nucleic acid concentrations in the nanomolar range, whereas the nucleic acid concentration for binding affinity assays was in the sub-picomolar range. Binding was measured in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl_2 , 100 $\mu\text{g/mL}$ Bovine Serum Albumin, 5 mM DTT, 6.7% or 10% glycerol. All reactions involving VA1 RNAs, dsRNA40 and dsDNA40 were performed with buffer containing 10% glycerol; miR-iab-4 and 25dsRNA-4Loop reactions were performed in buffer containing 6.7% glycerol. DIP1-c was present in the appropriate concentration range. Data were acquired using a Fuji phosphorimager FLA-5000 and imaging software, ImageGuage 4.0 and MultiGuage 2.3 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Bound nucleic acid data were analyzed with Igor Pro version 4.02A (Wavemetrics, CA) and were fit to the equation:

$$Y=Y_{\max}\left(\frac{[DIP1]^n}{[DIP1]^n+K_d^n}\right)+b$$

Free nucleic acid data were fit to the equation:

$$Y = Y_{\max} - \left[Y_{\max} \left(\frac{[DIP1]^n}{[DIP1]^n + K_d^n} \right) \right] + b$$

where Y (fraction bound or free) varies, $Y_{\max}$ is the maximum bound dsRNA, K_d is the equilibrium dissociation constant, n is the Hill coefficient and was allowed to float, and b is the background radioactivity in the absence of protein. Where K_d values for bound and free data differed, free data were used to avoid distortions from artifacts of complex dissociation during gel electrophoresis. All K_d values were adjusted for activity from stoichiometric measurements at conditions of dsRNA ligand concentration $\gg K_d$ and were then averaged. The standard deviation was calculated for multiple experiments that encompassed at least two separate DIP1-c purifications.

RESULTS

Determining DIP1-c Protein Absorption Coefficient

Our laboratory has previously shown that DIP1-c, the longest isoform, preferentially binds dsRNA over ssRNA, and its affinity for structured RNA ligands, such as the adenovirus VA1 RNA, is >1000-fold higher than for dsDNA [8]. To perform a more detailed analysis of the RNA binding behavior of DIP1-c, the absorption coefficient for this protein was established to allow precise definition of protein concentration and hence, dissociation constants. Magnetic circular dichroism was used to measure the precise concentration of tryptophan [18] in a DIP1-c solution, which was used to calculate an extinction coefficient of $33,600 \pm 4000 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm.

DIP1-c High Affinity Binding to VA1 RNA

We have previously demonstrated DIP1-c binding to HIV TAR RNA and adenovirus VA1 RNA, with the latter having higher affinity ($K_d = 45 \pm 11 \text{ pM}$) (Figure 1D) [8]. To explore the key elements for binding, we dissected the VA1 RNA structure. The binding footprint for dsRBDs has been reported to be a minimum of 11 bp [20,21] or as large as 18–20 bp [13]. The secondary structure of full-length VA1 RNA, predicted using an online algorithm [19], identifies only one region in the structure that satisfies the 11 bp criterion (Figure 1A, arrows).

Encompassing bases 20–108 of full-length VA1 RNA, the “long arm” was tested for DIP1-c binding. DIP1 maintained high affinity for this truncated VA1 RNA ($K_d = 100 \pm 27 \text{ pM}$) (Figure 1D). Systematic removal of a third of the structure on either end of the long arm (Upper 2/3 or Lower 2/3) resulted in significant decreases in affinity (Figure 1D). To test the middle region of the VA1 long arm, we transcribed the middle 13 base pair duplex within a 25 base pair hairpin (25dsRNA-4Loop) (Figure 1B). DIP1-c bound 25dsRNA-4Loop with nanomolar affinity (Figure 1D). These binding parameters for DIP1-c are within the reported range of other proteins with dsRBDs [4–6]. DIP1-c associates nonspecifically with the Lower 1/3 of the long arm of VA1 RNA (Figure 1D). From these results, we conclude that elements beyond the extended dsRNA region of the VA1 long arm are important for higher affinity binding.

DIP1-c Binding to dsRNA and dsDNA

To compare dsRNA and DNA binding, an RNA duplex corresponding to a DNA duplex was transcribed *in vitro* with six base overhangs on each 5' end (dsRNA40) (Figure 2A). DIP1-c binds dsRNA40 with very high affinity ($K_d = 55 \pm 31 \text{ pM}$) (Figure 2C). As anticipated for this protein, a DNA duplex of the same sequence (dsDNA40) is bound ~500-fold more

weakly by DIP1-c ($K_d = 26 \pm 8$ nM) (Figure 2C). The higher affinity exhibited by DIP1-c for dsRNA40 contrasts the lower affinity measured for 25dsRNA-4Loop, an RNA hairpin of only 25 bp (Figure 1C). This observation suggests one factor that promotes higher affinity binding to VA1 RNA may be the length of the double-stranded segment of the long arm (bases 20–108).

DIP1-c Binds with High Affinity to a MicroRNA Precursor Stem-Loop

Based on the observation that DIP1 interacts with multiple proteins [8,9,12], including the Hox protein Ubx, multiple physiological RNA targets may exist. We therefore examined potential RNA targets associated with Ubx. There is growing evidence that miRNAs play important roles in the complex regulation of Hox genes [22,23]. Within this category of targets [24–26], one miRNA, miR-iab-4-5p, directly inhibits Ubx activity *in vivo* [22]. Further, *iab-4* is a genomic element that affects Hox gene expression, including *ubx*, as a long-range enhancer [14,15]. With the potential for DIP1 to interact with the products of long-range enhancers and simultaneously with transcription factors bound to relevant promoter regions, we examined DIP1-c binding to the miR-iab-4 precursor stem-loop and found very tight binding, $K_d = 45 \pm 11$ pM (Figure 3C).

DISCUSSION

dsRNA-binding domains are reported to recognize and bind through interactions with the sugar-phosphate backbone [3,27], although contacts between dsRBDs and ssRNA regions within a target have been observed by NMR [28,29]. The number of physiological dsRNA targets identified for proteins with dsRBDs is increasingly important [*e.g.*, 30–34]. Proteins with dsRBDs, which are presumed to bind nonspecifically, must possess mechanisms for recognizing targets among all RNA species *in vivo* [2], but the basis for this selectivity remains elusive. DIP1-c was isolated as a protein partner for an interesting set of proteins: a Hox transcription factor, (Ultrabithorax [8]), a zinc-finger transcription factor (Disconnected [9]), and a histone methyltransferase (Suppressor of variegation 3–9 [12]). DIP1-c, like other dsRNA-binding proteins, preferentially binds dsRNA tighter than ssRNA or dsDNA [8], and here, we have demonstrated a 500-fold preference for dsRNA over dsDNA of similar sequence.

Standard ligands to assess dsRNA binding include the adenovirus VA1, HIV TAR, and poly(I):poly(C) dsRNA [4–6], despite their apparent lack of physiological relevance. dsRNA-binding proteins generally bind these dsRNAs with affinities in the micromolar to nanomolar range [4–6]. Interestingly, DIP1-c binds to dsRNA in the picomolar range, with the highest affinity for dsRNA currently reported in the literature (although dsRNA-binding proteins may bind their physiological targets with higher affinity). The identification of physiological RNA ligands for many dsRNA-binding proteins remains largely elusive. The length of the duplex may play a role in higher affinity binding, although sequence specificity has not been ruled out [28]. A dependence on the RNA duplex length has been observed for XlrpA binding [13] and PKR binding and activity [35,36]; however, the ligands tested were perfect duplexes and of unknown physiological relevance.

DIP1-c demonstrates both high affinity and binding preferences on both non-physiologically- and potentially physiologically-relevant ligands. VA1 RNA is highly structured, but possesses only one region that satisfies the criterion of at least 11 contiguous base pairs for the dsRBD binding footprint [20,21]. Truncation of VA1 RNA weakens DIP1-c affinity to the nanomolar range, provided the perfect 13 bp region is present. Thus, DIP1-c recognizes the A-form helix of dsRNA, but other features of the VA1 RNA must contribute to higher affinity binding.

To examine binding to an interesting physiological dsRNA, we selected a target associated with the DIP1-c protein partner Ubx. Although six microRNAs are computationally predicted to target *ubx* mRNA [24–26], only one, miR-iab-4-5p, has been shown to directly inhibit Ubx activity *in vivo* [22]. This miRNA is produced by the cleavage of a stem-loop within the transcribed *iab-4* long-range enhancer [37]. DIP1-c binds with remarkably high affinity to the miR-iab-4 stem-loop ($K_d \sim 50$ pM), comparable to its affinity for full-length VA1 and dsRNA40. The similarity in miR-iab-4 and VA1 long arm RNA structures and the higher affinity for these RNAs suggests the potential for specific features (*e.g.*, loops, bulges, kinks, bubbles, and spatial arrangements of multiple duplex segments) to promote high-affinity binding.

DIP1 is a remarkably multi-functional protein with the potential for binding both dsRNA and multiple proteins, a duality that allows recruiting one of its partner proteins along with a specific RNA ligand. This multi-ligand binding suggests the potential for DIP1 to serve as a “bridge.” The studies presented here open new directions for investigations of dsRBD selection and impact on other functional aspects of DIP1. Identification of *in vivo* RNA targets will provide new avenues to explore the physiological consequences of DIP1 binding to these RNAs and its protein partners.

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REFERENCES

1. Tian B, Bevilacqua PC, Diegelman-Parente A, Mathews MB. The double-stranded-RNA-binding motif: interference and much more. *Mol. Cell. Biol.* 2004; 5:1013–1023.
2. Saunders LR, Barber GN. The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J.* 2003; 17:961–983. [PubMed: 12773480]
3. St. Johnston D, Brown NH, Gall JG, Jantsch M. A conserved double-stranded RNA-binding domain. *Proc. Natl. Acad. Sci. U.S.A.* 1992; 89:10979–10983. [PubMed: 1438302]
4. Green SR, Manche L, Mathews MB. Two functionally distinct RNA-binding motifs in the regulatory domain of the protein kinase DAI. *Mol. Cell. Biol.* 1995; 15:358–364. [PubMed: 7799944]
5. Tian B, Mathews MB. Functional characterization of and cooperation between the double-stranded RNA-binding motifs of the protein kinase PKR. *J. Biol. Chem.* 2001; 276:9936–9944. [PubMed: 11134010]
6. Wickham L, Duchaine T, Luo M, Nabi IR, DesGroseillers L. Mammalian Staufen is a double-stranded-RNA- and tubulin-binding protein which localizes to the rough endoplasmic reticulum. *Mol. Cell. Biol.* 1999; 19:2220–2230. [PubMed: 10022909]
7. Krovat BC, Jantsch MF. Comparative mutational analysis of the double-stranded RNA binding domains of *Xenopus laevis* RNA-binding protein A. *J. Biol. Chem.* 1996; 271:28112–28119. [PubMed: 8910425]
8. Bondos SE, Catanese DJ Jr, Tan XX, Bicknell A, Li L, Matthews KS. Hox transcription factor ultrabithorax Ib physically and genetically interacts with disconnected interacting protein 1, a double-stranded RNA-binding protein. *J. Biol. Chem.* 2004; 279:26433–26444. [PubMed: 15039447]
9. DeSousa D, Mukhopadhyay M, Pelka P, Zhao X, Dey BK, Robert V, Pelisson A, Bucheton A, Campos AR. A novel double-stranded RNA-binding protein, disco interacting protein 1 (DIP1), contributes to cell fate decisions during *Drosophila* development. *J. Biol. Chem.* 2003; 278:38040–38050. [PubMed: 12829713]

10. Hitti EG, Sallacz NB, Schoft VK, Jantsch MF. Oligomerization activity of a double-stranded RNA-binding domain. *FEBS Lett.* 2004; 574:25–30. [PubMed: 15358534]
11. Chang K-Y, Ramos A. The double-stranded RNA-binding motif, a versatile macromolecular docking platform. *FEBS J.* 2005; 272:2109–2117. [PubMed: 15853796]
12. Krauss, V.; O'Grady, M.; Hoffmann, J.; Schotta, G.; Fischer, A.; Kuhfittig, S.; Dorn, R.; Grigliatti, TA.; Reuter, G. KLETT, a Drosophila chromatin protein with dsRNA binding motifs interacts with the heterochromatin-associated SU(VAR)3–9 protein. 17th European Drosophila Research Conference in Edinburgh (UK); 2001. p. F8
13. Bass BL, Hurst SR, Singer JD. Binding properties of newly identified Xenopus proteins containing dsRNA-binding motifs. *Curr. Biol.* 1994; 4:301–314. [PubMed: 7922339]
14. Cumberledge S, Zaratzian A, S. Sakonju S. Characterization of two RNAs transcribed from the cis-regulatory region of the abd-A domain within the Drosophila bithorax complex. *Proc. Natl. Acad. Sci. U.S.A.* 1990; 87:3259–3263. [PubMed: 1692133]
15. Bae E, Calhoun VC, Levine M, Lewis EB, Drewell RA. Characterization of the intergenic RNA profile at abdominal-A and abdominal-B in the Drosophila bithorax complex. *Proc. Natl. Acad. Sci. U.S.A.* 2002; 99:16847–16852. [PubMed: 12481037]
16. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* 1987; 15:8783–8798. [PubMed: 3684574]
17. Griffiths-Jones S, Saini HK, van Dongen S, A.J. Enright AJ. miRBase: tools for microRNA genomics. *Nucleic Acids Res.* 2008; 36:D154–D158. [PubMed: 17991681]
18. Holmquist B, Vallee BL. Tryptophan quantitation by magnetic circular dichroism in native and modified proteins. *Biochemistry.* 1973; 12:4409–4417. [PubMed: 4750252]
19. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003; 31:3406–3415. [PubMed: 12824337]
20. Bevilacqua PC, Cech TR. Minor-groove recognition of double-stranded RNA by the double-stranded RNA-binding domain from the RNA-activated protein kinase PKR. *Biochemistry.* 1996; 35:9983–9994. [PubMed: 8756460]
21. Bycroft M, Grunert S, Murzin AG, Proctor M, Johnston DS. NMR solution structure of a dsRNA binding domain from Drosophila Stauf protein reveals homology to the N-terminal domain of the ribosomal protein S5. *EMBO J.* 1995; 14:3563–3571. [PubMed: 7628456]
22. Ronshaugen M, Biemar F, Piel J, Levine M, Lai EC. The Drosophila microRNA iab-4 causes a dominant homeotic transformation of halteres to wings. *Genes Dev.* 2005; 19:2947–2952. [PubMed: 16357215]
23. Brock HW, Hodgson JW, Petruk S, Mazo A. Regulatory noncoding RNAs at Hox loci. *Biochem. Cell Biol.* 2009; 87:27–34. [PubMed: 19234521]
24. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. *Genome Biol.* 2003; 5:R1. [PubMed: 14709173]
25. Stark A, Brennecke J, Russell RB, Cohen SM. Identification of Drosophila microRNA targets. *PLoS Biol.* 2003; 1:E60. [PubMed: 14691535]
26. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science.* 2004; 304:594–596. [PubMed: 15105502]
27. Ryter JM, Schultz SC. Molecular basis of double-stranded RNA-protein interactions: structure of a dsRNA-binding domain complexed with dsRNA. *EMBO J.* 1998; 17:7505–7513. [PubMed: 9857205]
28. Ramos A, Grunert S, Adams J, Micklem DR, Proctor MR, Freund S, Bycroft M, St Johnston D, Varani G. RNA recognition by a Stauf double-stranded RNA-binding domain. *EMBO J.* 2000; 19:997–1009. [PubMed: 10698941]
29. Stefl R, Xu M, Skrisovska L, Emeson RB, Allain FH. Structure and specific RNA binding of ADAR2 double-stranded RNA binding motifs. *Structure.* 2006; 14:345–355. [PubMed: 16472753]
30. Ferrandon D, Elphick L, Nusslein-Volhard C, Johnston DS. Stauf protein associates with the 3' UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. *Cell.* 1994; 79:1221–1232. [PubMed: 8001156]

31. Liu Y, Lei M, Samuel CE. Chimeric double-stranded RNA-specific adenosine deaminase ADAR1 proteins reveal functional selectivity of double-stranded RNA-binding domains from ADAR1 and protein kinase PKR. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:12541–12546. [PubMed: 11070079]
32. Chanfreau G, Buckle M, Jacquier A. Recognition of a conserved class of RNA tetraloops by *Saccharomyces cerevisiae* RNase III. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:3142–3147. [PubMed: 10716739]
33. Curtin SJ, Watson JM, Smith NA, Eamens AL, Blanchard CL, Waterhouse PM. The roles of plant dsRNA-binding proteins in RNAi-like pathways. *FEBS Lett.* 2008; 582:2753–2760. [PubMed: 18625233]
34. Heinicke LA, Wong CJ, Lary J, Nallagatla SR, Diegelman-Parenta A, Zheng X, Cole JL, Bevilacqua PC. RNA dimerization promotes PKR dimerization and activation. *J. Mol. Biol.* 2009; 390:319–338. [PubMed: 19445956]
35. Manche L, Green SR, Schmedt C, Mathews MB. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol. Cell. Biol.* 1992; 12:5238–5248. [PubMed: 1357546]
36. Lemaire PA, Anderson E, Lary J, Cole JL. Mechanism of PKR activation by dsRNA. *J. Mol. Biol.* 2008; 381:351–360. [PubMed: 18599071]
37. Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T. The small RNA profile during *Drosophila melanogaster* development. *Dev. Cell.* 2003; 5:337–350. [PubMed: 12919683]

Figure 1. DIP1-c binds the middle duplex region of VA1 RNA “long arm.”

A) Using an online algorithm [19], the VA1 sequence exhibits a three-armed structure with the “long arm” encompassing bases 20–108. The long arm, which has three main dsRNA segments separated by “bubbles,” contains a perfect 13 bp duplex in the middle region (marked by arrows). The other VA1 RNA deletion constructs are noted. B) 25dsRNA-4Loop RNA structure. The 25 bp hairpin contains the same perfect 13 bp duplex (marked by arrows) from VA1 RNA long arm. C) Gel retardation testing DIP1-c affinity for VA1 constructs. Depicted are representative gels for each construct; the first lane is dsRNA alone, and bound (B) and free (F) species are indicated. Radiolabeled RNA concentration in each reaction was set at ≤ 1 pM, and DIP1-c concentration increased left to right from 1.6 pM to 6.3 nM (full length, 20–108), 16 pM to 100 nM (Lower 2/3), 160 pM to 1 μ M (Upper 2/3, Lower 1/3), and 20 pM to 630 nM (25dsRNA-4Loop). D) Representative binding isotherms of DIP1-c for VA1 constructs. The data for Upper 2/3 and Lower 2/3 were derived from disappearance of the free species, and all others were from the appearance of bound species. Results from multiple determinations were utilized to provide averaged data in the text. Using only the VA1 long arm, bases 20–108, K_d decreases two-fold to ~ 100 pM, $n = 1.5$ (open circles). Further truncation (Upper 2/3 or Lower 2/3) further reduces the affinity significantly ($K_d \sim 6$ nM, $n = 1.0$ [open squares] and $K_d \sim 2$ nM, $n = 1.0$ [filled squares], respectively). The 13 bp region within the 25dsRNA-4Loop hairpin exhibits an affinity in the nanomolar range ($K_d \sim 5$ nM, $n = 1.1$, filled triangles). The Lower 1/3 portion of the long arm binds at non-specific levels ($K_d > 100$ nM, open triangles).

Figure 2. DIP1-c may bind preferentially to longer stretches of dsRNA

The ability of DIP1-c to bind larger stretches of dsRNA and dsDNA was investigated with gel retardation. Fractional saturation was determined using loss of free nucleic acid. A) Sequences of each ligand tested. B) Representative gels. The bound (B) and free (F) species are denoted. Nucleic acid concentrations were set at ≤ 1 pM in each reaction. The first lane on the left side contained no protein, and DIP1-c concentrations ranged left to right from 1.6 nM to 6.3 μ M (dsDNA40) and 1.6 pM to 6.3 nM (dsRNA40). C) Representative binding isotherms. DIP1-c bound dsRNA40 ($K_d = 55 \pm 31$ pM, $n = 1.7$, filled circles) nearly 500-fold tighter than dsDNA40 ($K_d = 26 \pm 8$ nM, $n = 1.2$, open circles).

Figure 3. DIP1-c binds miR-iab-4 precursor stem-loop with high affinity

A) Predicted secondary structure of the miR-iab-4 precursor stem-loop using an online algorithm [19]. This stem-loop gives rise to two miRNAs: miR-iab-4-5p (left side) and miR-iab-4-3p (right side), which are computationally predicted to bind Ubx mRNA [24–26] and impact Ubx function *in vivo* [22]. The mature miRNAs, predicted in miRBase [17], are highlighted by arrows. B) Representative gel with bound (B) and free (F) RNA denoted. RNA concentrations in each reaction were set at ≤ 1 pM. There was no protein in the first lane, and DIP1-c concentration ranged left to right from 1.6 pM to 3.2 nM. C) Representative binding isotherm. DIP1-c binds miR-iab-4 stem-loop with high affinity ($K_d = 45 \pm 11$ pM, $n = 1.6$).