BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2010,** *49,* 8237–8239 8237
DOI: 10.1021/bi100672x

Structure and RNA Interactions of the Plant MicroRNA Processing-Associated Protein HYL1†

Rodolfo M. Rasia,*[,‡,§] Julieta Mateos,[‡] Nicolás G. Bologna,[‡] Paula Burdisso,[‡] Lionel Imbert,[§] Javier F. Palatnik,[‡] and Jerome Boisbouvier*[,§,∥,⊥]

[‡]*Instituto de Biología Molecular y Celular de Rosario, División Biología Molecular, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, S2002LRK Rosario, Argentina,* [§]*CNRS, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France,* [∥]*CEA, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France, and* [⊥]*Université Joseph Fourier, Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France*

Received April 30, 2010; Revised Manuscript Received August 24, 2010

ABSTRACT: HYL1 is a double-stranded RNA binding protein involved in microRNA processing in plants. HYL1 enhances the efficiency and precision of the RNase III protein DCL1 and participates in microRNA strand selection. In this work, we dissect the contributions of the domains of HYL1 to the binding of RNA targets. We found that the first domain is the main contributor to RNA binding. Mapping of the interaction regions by nuclear magnetic resonance on the structure of HYL1 RNA-binding domains showed that the difference in binding capabilities can be traced to sequence divergence in $\beta2-\beta3$ loop. The possible role of each domain is discussed in light of previous experimental data.

Short noncoding RNA molecules have emerged as a major mechanism of gene regulation in higher eukaryotes. MicroRNAs (miRNAs), key members of the family of small noncoding RNA regulators, consist of 21 nucleotide (nt) molecules processed from endogenous transcripts. The mature miRNA functions within an effector RNA-induced silencing complex (RISC), in which its role is to recognize target mRNAs through base pair complementarity. The degree of complementarity determines whether translation is inhibited or the target mRNA is broken down (*1*).

The biogenesis of miRNAs is a complex process with several apparent differences between the mechanisms in plants and animals (*2*, *3*). miRNA precursors (pri-miRNAs) are transcribed in the nucleus by RNA polymerase II. The actual miRNAs are located within stem−loop structures in the pri-miRNA and are released through the action of RNase III-type enzymes. In plants, DICER-LIKE1 (DCL1) excises the mature miRNA in a stepwise manner aided by the dsRNA-binding protein HYL1 and the zinc-finger protein SERRATE (*4*−*6*). Finally, HEN1, a methyl transferase, methylates the 2′-hydroxyl group of the 3′ end of the miRNA released, which is then incorporated into AGO complexes (*7*, *8*).

A number of studies suggest that HYL1 is a main player in miRNA biogenesis in plants. Although not lethal, *hyl1* null mutations severely impair miRNA maturation and lead to an accumulation of pri-miRNA (*9*). HYL1 was shown to interact directly with DCL1 in vitro, and both proteins colocalize to small nuclear bodies in live cells (*4*, *5*). Even though DCL1 is itself capable of processing pri-miRNA to give ≈21 nt products, the cleavage is not specific. Precise excision of the miRNA from the precursor is achieved only in the presence of both HYL1 and SERRATE (*6*, *10*). More recently, HYL1 was shown to participate in miRNA strand selection (*11*). Sequence analysis of HYL1 shows that the protein contains two double-stranded RNA binding domains (dsRBDs) separated by a short linker. Several other accessory proteins involved in small RNA metabolism also feature dsRBDs in the absence of a catalytic motif [e.g., DGCR8 (*12*), PACT, TRPB (*13*), and RDE-4 (*14*)]. dsRBDs selectively bind dsRNA in a sequence-independent manner that is believed to be driven by recognition of the three-dimensional (3D) structure of their cognate RNAs (*15*, *16*). This behavior fits nicely with the involvement of dsRBDs in the recognition of miRNA precursors: pre-miRNAs do not share sequence similarity but do display a common stem−loop structure. To date, the molecular mechanism of HYL1 function has remained elusive. This work attempts to unveil the structural features of the dsRNA-binding domains of HYL1 and their interactions with miRNA precursors and processing intermediates.

We first produced three constructs of HYL1, one for each dsRBD (residues 1−100 and 97−167, termed HYL1-1 and HYL1-2, respectively) and one spanning both dsRBD domains (residues 1−167, termed HYL1-12). The HYL1-12 construct has previously been shown to be sufficient to rescue *hyl1* mutants in vivo (*17*). Size exclusion chromatography and solution nuclear magnetic resonance (NMR) studies indicated that the individual dsRBDs and the double-domain construct are monomeric. Both isolated dsRBDs yielded well-dispersed, ^{15}N HSQC spectra. The spectra of the individual dsRBDs combine to almost exactly reproduce the spectrum of the double-domain construct (Figure 1 of the Supporting Information), indicating that the dsRBDs in HYL1 behave as independently folding units and that no interdomain interactions exist in the full-length protein. Assignment of backbone resonances for each of the single-domain constructs was determined from a series of triple-resonance NMR experiments (*18*). The backbone structure of each dsRBD was determined with CS-Rosetta using a total of four residual dipolar coupling constraints per residue and the chemical shifts of backbone atoms (*19*, *20*). The overall fold of both domains (Figure 1) showed the canonical $\alpha\beta\beta\beta\alpha$ topology characteristic of dsRBDs.

To investigate the RNA binding capabilities of HYL1, we performed gel retardation experiments using the three HYL1 constructs described and the minimal functional regions of

†This work was funded by grants from HFSP (RG-0057/2006) to J.B. and J.F.P., ANR (06-JCJC-0034) to J.B., and ECOS-Sud (A06B04) to J. B. and J.F.P. and Grant PICT-2007-00720 to R.M.R.

*To whom correspondence should be addressed. R.M.R.: e-mail, rasia@ibr.gov.ar; phone, +54-341-4351235; fax, +54-341-4390465. J. B.: e-mail, jerome.boisbouvier@ibs.fr; phone, +33 4 38 78 95 60; fax, +33 4 38 78 54 94.

Published on Web 08/25/2010

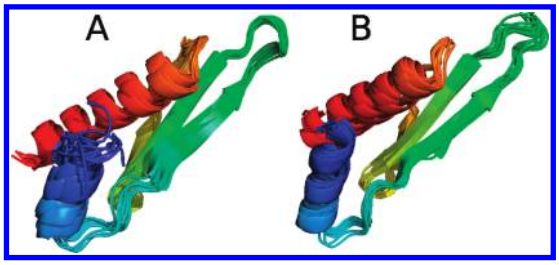

FIGURE 1: Solution structure ensembles of the dsRBDs of HYL1. The 10 lowest-energy conformers of HYL1-1 (A, residues 19−89) and HYL1-2 (B, residues 104−173) domains are shown (BioMagResBank entries 17143 and 17141 and Protein Data Bank entries 2l2n and 2l2m, respectively). They superimpose with an average backbone pairwise root-mean-square deviation of 1.51 ± 0.36 Å for HYL1-1 and 1.27 ± 0.25 Å for HYL1-2. The structures are displayed with a color gradient from the N-terminus (blue) to the C-terminus (red).

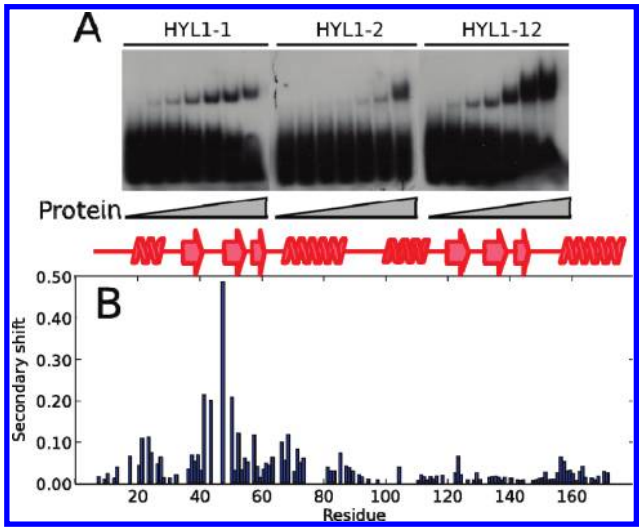

FIGURE 2: (A) Interaction of pre-miR319 with the three HYL1 constructs measured by electrophoretic mobility shifts. Protein concentrations from left to right were 0, 0.088, 0.28, 0.88, 2.8, 8.8, and 28 μM, respectively. (B) NMR mapping of the interaction of the miR319−miR319* construct with HYL1-12. The secondary shifts were calculated as $[(\Delta\delta^{15}N/6)^2 + (\Delta\delta^{1}H)^2]^{1/2}$. The secondary structure elements in the domains are shown schematically above panel B.

miR319 and miR172 precursors (Figure 2 and Figures 2−4 of the Supporting Information) (*21*, *22*). We observed that each isolated domain can interact with precursor miRNAs, though HYL1-1 interacts more strongly (K_D = 740 nM) than HYL1-2 (K_D > 10 μM). The double-domain construct shows an affinity similar to that of the first domain. These results suggest that HYL1-1 is the dominant RNA binding domain in HYL1 and that HYL1-2 may contribute to a lesser extent.

To identify the regions involved in RNA binding, we characterized the interaction between HYL1 and RNA by solution NMR spectroscopy. We recently demonstrated that *Arabidopsis thaliana* miR319a is processed from loop to base, in contrast to miRNA processing in animals. DCL1 performs four sequential dicing events to release the miRNA−miRNA* duplex. The last dicing step involves a processing intermediate that contains the miRNA−miRNA* section and the base of the precursor (Figure 2 of the Supporting Information) (*21*). Processing of this intermediate requires HYL1. It has also been shown that HYL1 participates in miRNA strand selection (*12*). These data suggest the miRNA−miRNA* duplex is the smallest physiologically relevant binding partner for HYL1. We therefore analyzed the

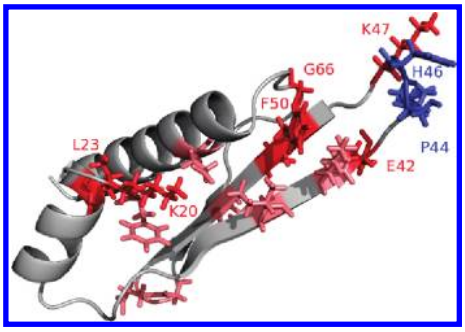

FIGURE 3: NMR mapping data represented on the structure of HYL1-1. Residues with secondary shifts of >0.15 are colored red and those with secondary shifts of >0.1 pink. Residues colored blue show no signal in the HSQC spectrum.

interaction of HYL1 with an RNA duplex encompassing the miRNA−miRNA* region from *A. thalinana* miR319 (Figure 2 of the Supporting Information). Both domains display changes in the $^{1}H-^{15}N$ HSQC spectrum upon addition of the miRNA−miRNA* duplex. In HYL1-1, a subset of signals of the free protein disappear, with the concomitant detection of a new set of signals. This observation indicates the formation of a protein−RNA complex that exists in slow exchange on the NMR time scale. In contrast, in HYL1-2 the intensity of several signals decreases without any major change in chemical shift, suggesting the formation of a weaker complex in the intermediate exchange regime. These data imply that HYL1-1 binds target RNA with a higher affinity than HYL1-2 and are in agreement with the gel retardation experiments. The perturbations induced upon RNA binding in each dsRBD are mirrored in the double-domain construct, indicating that both domains are functionally independent in the context of the full-length HYL1 protein in both free and RNA-bound forms (Figure 2B).

Residues of the first dsRBD of HYL1 that interact with RNA were identified by observing which NMR signals were perturbed upon addition of RNA (Figures 2B and 3 and Figures 5 and 6 of the Supporting Information). The regions identified in HYL1-1 are coincident with the RNA binding motifs conserved in canonical dsRBDs: residues in the N-terminal part of helix 1, the $\beta1-\beta2$ loop, the $\beta3-\alpha2$ loop, and the $\beta1-\beta2$ loop are the most affected.

For the second dsRBD of HYL1, NMR spectra do not show significant changes following the addition of RNA (Figure 2B). Nevertheless, resonances from nuclei in the N-termini of helices 1 and 2 are substantially broadened, suggesting weak binding of RNA in the intermediate exchange regime (Figures 5 and 6 of the Supporting Information). It is noteworthy that in this domain the $\beta1-\beta2$ loop, which is one of the main determinants of RNA binding in dsRBDs, does not seem to be affected by the binding of the RNA.

A. thaliana has four other proteins, termed DRB2−DRB5, that share the same domain architecture as HYL1. The amino acid sequence of the $\beta1-\beta2$ loop is conserved between dsRBD1 of HYL1 and all the dsRBDs of the DRB2−DRB5 proteins (Figure 7 of the Supporting Information). However, there is little conservation with the corresponding $\beta1-\beta2$ loop sequence in dsRBD2 of HYL1. In contrast, this region of HYL1-2 is well-conserved among HYL1 homologues of different plants (Figure 7 of the Supporting Information).

There are several potential reasons that could explain the disparity between the binding affinities determined for the two dsRBDs of HYL1. The presence of two or more dsRBDs is

commonplace in RNA binding proteins and has been shown to lead to an enhancement in the affinity for dsRNA through cooperative interactions. In PKR, an interferon-induced human protein kinase that contains two tandem dsRBDs, the affinity of the double domain is 1 order of magnitude higher than the affinity of the first dsRBD, which is in turn 1 order of magnitude higher than the affinity of the second dsRBD (*23*). Therefore, the function of HYL1-2 may be to increase the affinity of the whole protein for the target RNA. Domain 2 could also participate in protein−protein interactions necessary for the recruitment of miRNA processing enzymes. Certain dsRBDs have partially lost their dsRNA binding activity and function instead as sites for protein dimerization or kinase activation, e.g., the second motif of PKR or the third motif of PACT (*24*−*26*). In fact, HYL1-2 was recently shown to interact specifically with the DUF283 domain of its partner protein, DCL1 (*27*). In light of the interaction mapping and the sequence alignment data shown here, it is tempting to speculate that the sequence differences in the $\beta1-\beta2$ loop of dsRBD2 of HYL1 enable this domain to perform a more specialized role, potentially involving protein−protein rather than protein−RNA interactions.

While this work was under review, a paper describing a structural and functional analysis of the dsRBDs of HYL1 was published (*28*). The crystal structures of the free dsRBDs reported by Yang et al. superimpose very well with the NMR structures we report here (heavy backbone atom root-mean-square deviations of 1.18 and 1.21 Å for HYL1-1 and HYL1-2, respectively). Furthermore, the first dsRBD was also reported to be the major RNA binding site in HYL1, and the RNA binding surface identified in the reported crystal structure corresponds well to what we observed in solution. Interestingly, Yang et al. report that dimerization of the second dsRBD could be important for the function of HYL1. In our hands, this noncanonical dsRBD behaves exclusively as a monomer in solution, which suggests that HYL1-2 is more likely to be involved in the recognition of other proteins involved in miRNA biogenesis (*27*).

In summary, we have shown that the two dsRBDs of HYL1 contribute differently to RNA binding. We have mapped the regions of HYL1 that interact with RNA and determined the backbone structure of the two dsRBDs of the protein. We have ascertained that sequence divergence in the $\beta1-\beta2$ loop of dsRBD2 has a role in the differential contribution of each domain to RNA binding. These findings will guide further research that aims to establish the molecular function of HYL1 in plant miRNA biogenesis.

ACKNOWLEDGMENT

We thank Dr. Silvana Spinelli for help in the setup of RNA band shift assays, Dr. B. Brutscher for the BEST-type NMR pulse programs, Dr. Michael Plevin for suggestions and careful reading of the manuscript, and the Grenoble Partnership for Structural Biology for access to integrated structural biology platforms.

SUPPORTING INFORMATION AVAILABLE

Detailed protocols, NMR spectra of the domains of HYL1 free and in the presence of RNA, and sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Filipowicz, W., Bhattacharyya, S. N., and Sonenberg, N. (2008) *Nat. Rev. Genet. 9*, 102–114.
2. Jones-Rhoades, M. W., Bartel, D. P., and Bartel, B. (2006) *Annu. Rev. Plant Biol. 57*, 19–53.
3. Rana, T. M. (2007) *Nat. Rev. Mol. Cell Biol. 8*, 23–36.
4. Song, L., Han, M., Lesicka, J., and Fedoroff, N. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 5437–5442.
5. Fujioka, Y., Utsumi, M., Ohba, Y., and Watanabe, Y. (2007) *Plant Cell Physiol. 48*, 1243–1253.
6. Dong, Z., Han, M., and Fedoroff, N. (2008) *Proc. Natl. Acad. Sci. U.S.A. 105*, 9970–9975.
7. Huang, Y., Ji, L., Huang, Q., Vassylyev, D. G., Chen, X., and Ma, J. (2009) *Nature 461*, 823–827.
8. Montgomery, T. A., Howell, M. D., Cuperus, J. T., Li, D., Hansen, J. E., Alexander, A. L., Chapman, E. J., Fahlgren, N., Allen, E., and Carrington, J. C. (2008) *Cell 133*, 128–141.
9. Han, M., Goud, S., Song, L., and Fedoroff, N. (2004) *Proc. Natl. Acad. Sci. U.S.A. 101*, 1093–1098.
10. Kurihara, Y., Takashi, Y., and Watanabe, Y. (2006) *RNA 12*, 206–212.
11. Eamens, A. L., Smith, N. A., Curtin, S. J., Wang, M., and Waterhouse, P. M. (2009) *RNA 15*, 2219–2235.
12. Sohn, S. Y., Bae, W. J., Kim, J. J., Yeom, K., Kim, V. N., and Cho, Y. (2007) *Nat. Struct. Mol. Biol. 14*, 847–853.
13. Kok, K. H., Ng, M. J., Ching, Y., and Jin, D. (2007) *J. Biol. Chem. 282*, 17649–17657.
14. Parker, G. S., Maity, T. S., and Bass, B. L. (2008) *J. Mol. Biol. 384*, 967–979.
15. Tian, B., Bevilacqua, P. C., Diegelman-Parente, A., and Mathews, M. B. (2004) *Nat. Rev. Mol. Cell Biol. 5*, 1013–1023.
16. Stefl, R., Xu, M., Skrisovska, L., Emeson, R. B., and Allain, F. H. (2006) *Structure 14*, 345–355.
17. Wu, F., Yu, L., Cao, W., Mao, Y., Liu, Z., and He, Y. (2007) *Plant Cell 19*, 914–925.
18. Lescop, E., Schanda, P., and Brutscher, B. (2007) *J. Magn. Reson. 187*, 163–169.
19. Rohl, C. A., and Baker, D. (2002) *J. Am. Chem. Soc. 124*, 2723–2729.
20. Shen, Y., Lange, O., Delaglio, F., Rossi, P., Aramini, J. M., Liu, G., Eletsky, A., Wu, Y., Singarapu, K. K., Lemak, A., Ignatchenko, A., Arrowsmith, C. H., Szyperski, T., Montelione, G. T., Baker, D., and Bax, A. (2008) *Proc. Natl. Acad. Sci. U.S.A. 105*, 4685–4690.
21. Bologna, N. G., Mateos, J. L., Bresso, E. G., and Palatnik, J. F. (2009) *EMBO J. 28*, 3646–3656.
22. Mateos, J. L., Bologna, N. G., Chorostecki, U., and Palatnik, J. F. (2010) *Curr. Biol. 20*, 49–54.
23. Tian, B., and Mathews, M. B. (2001) *J. Biol. Chem. 276*, 9936–9944.
24. Green, S. R., Manche, L., and Mathews, M. B. (1995) *Mol. Cell. Biol. 15*, 358–364.
25. Daher, A., Longuet, M., Dorin, D., Bois, F., Segeral, E., Bannwarth, S., Battisti, P. L., Purcell, D. F., Benarous, R., Vaquero, C., Meurs, E. F., and Gatignol, A. (2001) *J. Biol. Chem. 276*, 33899–33905.
26. Peters, G. A., Hartmann, R., Qin, J., and Sen, G. C. (2001) *Mol. Cell. Biol. 21*, 1908–1920.
27. Qin, H., Chen, F., Huan, X., Machida, S., Song, J., and Yuan, Y. A. (2010) *RNA 16*, 474–481.
28. Yang, S. W., Chen, H. Y., Yang, J., Machida, S., Chua, N. H., and Yuan, Y. A. (2010) *Structure 18*, 594–605.