If their surmise is correct, Dokland et al. (2004) will have used their structure of a tetramer to bring us closer to an image of the assembled flavivirus capsid.

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Structure, Vol. 12, July, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.str.2004.06.008

siRNAs at RISC

A recent report (Ma et al., 2004) describes the crystal structure of a "mini-siRNA" bound to a PAZ domain.

RNA interference (RNAi) has taken experimental biology by storm in recent years, as RNAi has quickly evolved from a biological curiosity in plants and nematodes into a powerful gene knock down technology in many eukaryotic systems, including worms, flies, and mammals. This evolutionary conserved pathway (reviewed in Hannon [2002]) is triggered in response to exogenous dsRNA introduced to cells, for example, by viral infection or transfection of in vitro synthesized dsRNA, as well as by the expression of endogenously encoded RNAi triggers, microRNAs (miRNA). Depending on the nature of the dsRNA trigger and the biological system in which it is expressed, RNAi results in the downregulation of a homologous target gene through the cleavage, translational repression, or transcriptional inhibition of its mRNA. The RNAi machinery has been linked to the establishment of heterochromatin and proper centromere function in fission yeast (Hall et al., 2002; Volpe et al., 2002) and appears to have prominent roles during the development of multicellular eukaryotic organisms through action of miRNAs (Carrington and Ambros, 2003).

Two core proteins are universally associated with RNAi-related silencing phenomena: Dicer and Argonaute. Dicer is an RNase III type nuclease that cleaves dsRNA to generate short interfering RNAs (siRNAs), which are dsRNA of 19–24 nucleotides with two-nucleotide 3′ overhangs and a 5′ phosphate at each end (Bernstein et al., 2001). Dicer also cleaves short hairpin miRNA precursors, which are in turn produced by a related nuclease called Drosha, to generate miRNAs (Lee et al., 2003). These structures, siRNAs or miRNAs, are loaded into an effector complex called RISC, the RNA-induced silencing complex, to select its target, a homologous mRNA for destruction in the case of siRNAs or, for miRNAs, blocking mRNA translation (Carrington and Ambros, 2003; Hammond et al., 2001; Martinez et al., 2002). RISC contains an Argonaute protein as its signature component, which has two characteristic domains, PAZ, also found in Dicer family proteins, and PIWI (Carmell et al., 2002; Cerutti et al., 2000). In *Drosophila*, loading appears to occur via an intermediate complex, the RISC loading complex that contains Dicer-2 and R2D2 (Liu et al., 2003).

Though there has been remarkable progress in defining the cast of characters involved in the RNAi pathway, we still do not understand how they work at the molecular level to orchestrate this process. This is where the structural biologists are starting to make an impact. Our first glimpse into the molecular machinery of RNAi came with three separate reports of the three-dimensional structures of the PAZ domains of Argonaute proteins (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). These implicated the PAZ domain as an RNA binding module for Argonautes. It was also suggested that the PAZ domain acts as a specificity determinant for siRNA or pre-miRNA structures since it appears to bind to the characteristic two-nucleotide 3′ overhang of siRNAs (Song et al., 2003). Now, Patel and his colleagues have determined the crystal structure of the human Argonaute1 (eIF2c1) PAZ with a 9-mer RNA that formed a "mini siRNA," an siRNA look-alike with a 7-base pair A-form duplex and a two-nucleotide 3′ overhang at each end (Ma et al., 2004). As for the two fly proteins, the human Ago1 PAZ domain adopts an OB-like β-barrel fold with an αβ module attachment and a cleft in between. The cleft was implicated by mutagenesis and NMR chemical shift perturbations in RNA binding (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). Now we can see how the siRNA actually binds. The RNA strand

Figure 1. The Structure of the PAZ-RNA Structure

Human Argonaute-1 PAZ is shown in gray and the RNA is shown in purple and blue. For clarity, only one of the PAZ domains is shown. The other is bound in the same manner to the blue strand. The helical portion of the mini-siRNA is contacted by the C-terminal tail and the top of the β-barrel of the OB-like fold. The overhang takes a sharp turn and inserts itself into the cleft.

in the duplex part of the mini-siRNA is contacted by the C-terminal tail of PAZ and the top of its β-barrel. The overhang then takes a sharp turn and inserts itself into the cleft (Figure 1). Since the cleft is filled with aromatic residues, one would expect to see stacking interactions between the bases in this single-stranded overhang end of the siRNA as is seen in several single-stranded nucleic acid binding proteins (Antson, 2000). Instead, PAZ had a surprise in its cleft. Two tyrosines and a histidine are holding onto the phosphate between the two nucleotides of the overhang. Another tyrosine contacts that phosphate through a water-mediated hydrogen bond, as well as the 2′OH of the penultimate base. The base at the 3′ end sits nicely atop a hydrophobic neighborhood, but the edge of the base, where its identity can be read, is exposed. There is also a conserved lysine that sits between the phosphate right before the sharp turn and the edge of penultimate base, which might assist in stabilizing the turn. PAZ doesn't appear to make many contacts to the 2′OHs. Instead, it is probably more sensitive to the characteristic distance between the phosphates of the A-form RNA helix, which would be very different for a B-type helix that is common for DNA. As for the other strand, the base at the 5′ end sits against the PAZ domain. Its 5′OH points away from the protein. However, the mini-siRNA that was used in the crystal structure lacks the 5′ phosphate, which is another characteristic feature of siRNAs, and that licenses the siRNA to go into the RNAi pathway. The addition of such a charged moiety may reorient that unconstrained end of the RNA toward the PAZ, or it is also possible that other portions of Argonaute hold on to that phosphate.

In the crystal structure, each strand is contacted by one PAZ domain resulting in a 2:1 PAZ-mini-siRNA complex. There are no contacts between the two PAZ domains, meaning that they can, in principle, be independently bound at two ends of a regular length siRNA, while the siRNA is still double stranded. However, the other regions of Argonaute or other components of RISC may obscure this type of dimerization. After all, PAZ is approximately 130 residues of 857 of the protein. In addition, the low RNA binding affinities of the PAZ domains of both *Drosophila* Ago1 and Ago2 imply that other portions of the protein may contribute to RNA binding (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003).

The 3′ end recognition feature of PAZ would be important for the only two protein families that contain these domains, the Dicers and the Argonautes, to distinguish between bona fide siRNAs from other RNA molecules. It might also be important as a docking module for Dicer to measure the appropriate distance from the end to the site of cleavage to generate 22-mers, especially for miRNA production (Carmell and Hannon, 2004; Song et al., 2003). As for RISC, we learned that the PAZ of Argonaute is holding onto the 3′ end of the siRNA. How is this then used as a guide to hook up with the mRNA, and who is slicing the mRNA? Perhaps the marriage of genetics, biochemistry, and now structural biology could help in answering these questions.

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Acknowledgments

I thank Ji-Joon Song and Patrick Paddison for their comments.

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Structure, Vol. 12, July, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.str.2004.06.007

Attack of the Killer Tomato Pathogens

The solution structure of the bacterial avirulence protein AvrPto sheds light on how this protein is translocated across the plant cell wall and how it binds to its host targets.

Pseudomonas syringae is a bacterial plant pathogen. Over 40 pathogenic variants, or pathovars, which infect different plants, have been identified. *P. syringae* does not reproduce inside plant cells, but instead colonizes the intercellular space outside the plant cell wall. These pathogens then inject effector proteins into host cells through a needle-like protuberance known as a type III secretion system (TTSS). *P. syringae* pv. *tomato* infects tomato and *Arabidopsis* and is an economically significant pathogen and also a well-studied paradigm for the γ proteobacteria. This subgroup includes *Pseudomonas* spp., *Yersinia* spp., and *Salmonella* spp., all of which use the TTSS to inject bacterial proteins into host cells.

Avirulence factors (avr) are a class of effector proteins injected via the TTSS. The *P. syringae* genome codes for some thirty effectors (Buell et al., 2003). Why there are so many effectors, and what their functions are, are areas of ongoing study. Injection of effectors into nonresistant hosts enhances bacterial virulence. AvrPto, for example, causes a decrease in expression of genes related to cell wall defense (Hauck et al., 2003). Resistance to a particular avr protein arises from host expression of a resistance (R) gene/protein. A host R protein recognizes a specific bacterial avr protein and induces the hypersensitive response (HR) in the host cell. The HR includes generation of reactive oxygen and nitrogen species, expression of defense-related genes, and localized cell death. This year, two structures of *P. syringae* type III effector proteins have been solved, a crystal structure of the AvrB protein from pv. *glycinea* (Lee et al., 2004) and a solution structure of the AvrPto protein from pv. *tomato* (Wulf et al., 2004 [this issue of *Structure*]).

AvrPto is an extended, three-helix bundle, 2.5 nm in diameter and 5 nm along the helical axis. Helices C and D are connected by a loop which adopts a conformation diagnostic of an Ω loop. Ω loops are large loops with a defined and stable conformation (Fetrow, 1995). They are generally found on the surfaces of proteins and are involved in a variety of functions, including mediating protein-ligand interactions. AvrPto exists in equilibrium among unfolded states and folded monomer and dimer states with a $\Delta G^0_{FU} \approx -0.4$ kcal/mol. The significance of the low intrinsic stability of the folded protein is unclear, although Wulf et al. hypothesize that it could allow chaperone-independent secretion of the protein through the ~2 nm diameter injection needle assembly. Effector proteins likely traverse the injection needle in an unfolded or semifolded state and then refold within the host cell. Intrinsic instability does not appear to be a requirement for secretion, however, as AvrB is relatively well behaved and does not have a known chaperone (C. Lee and F. Katagiri, personal communication). Wulf et al. also propose that the low stability could contribute to AvrPto-target binding free energy. One of AvrPto's host targets is the Ser/Thr kinase Pto. The association of AvrPto with Pto, in the presence of a third protein, Prf, elicits the HR in resistant plants (Pedley and Martin, 2003).

As a point of convenience, and often necessity, structural biologists will make mutations, truncations, or deletions to the protein of interest in order to increase solubility or the propensity to crystallize. For proteins or domains with no intrinsic catalytic activity, observation of a folded structure is frequently taken as sufficient evidence that the manipulations did not substantially affect function. Wulf et al. also found it necessary to truncate the N-terminal 28 and C-terminal 31 residues of AvrPto to obtain a protein amenable to high-resolution structural analysis. Previous work had shown that the AvrPto core (residues 31–124) retains binding to Pto (Chang et al., 2001), and Wulf et al. verified that their truncated AvrPto (TrAvrPto, residues 29–133) binds to its targets Api2 and Api3 in yeast two-hybrid screens. Further verification that truncated AvrPto retained function was obtained by coexpression of Pto and several truncation variants of AvrPto in *Nicotiana benthamiana* leaves. Here, a TrAvrPto fusion containing the N-terminal myristoylation motif (MTrAvrPto) was cotransfected with Pto into *Agrobacterium*, which was then used to infect *N. benthamiana*. Both wild-type and MTrAvrPto elicit an HR when coexpressed with Pto, clearly demonstrating that the truncated AvrPto maintained activity in planta.

The present structure of TrAvrPto clarifies previous mutation data (Chang et al., 2001; Shan et al., 2000). Random mutagenesis identified 12 point mutants which disrupted AvrPto:Pto interactions. Seven of these mu-