Specific Recognition of HIV TAR RNA by the dsRNA Binding Domains (dsRBD1–dsRBD2) of PKR

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PKR (double-stranded RNA-dependent protein kinase) is an important component of host defense to virus infection. Binding of dsRNA to two dsRBDs (double-stranded RNA binding domains) of PKR modulates its own kinase activation. How structural features of natural target RNAs, such as bulges and loops, have an effect on the binding to two dsRBDs of PKR still remains unclear. By using ITC and NMR, we show here that both the bulge and loop of TAR RNA are necessary for the high affinity binding to dsRBD1–dsRBD2 of PKR with 1:1 stoichiometry. The binding site for the dsRBD1–dsRBD2 spans from upper bulge to lower stem of the TAR RNA, based on chemical shift mapping. The backbone resonances in the 40 kDa TAR–dsRBD1–dsRBD2 were assigned. NMR chemical shift perturbation data suggest that the β1–β2 loop of the dsRBD1 interacts with the TAR RNA, whereas that of the dsRBD2 is less involved in the TAR RNA recognition. In addition, the residues of the interdomain linker between the dsRBD1 and the dsRBD2 also show large chemical perturbations indicating that the linker is involved in the recognition of TAR RNA. The results presented here provide the biophysical and spectroscopic basis for high-resolution structural studies, and show how local RNA structural features modulate recognition by dsRBDs.

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Introduction

PKR (double-stranded RNA-dependent protein kinase), an interferon-induced protein kinase, is an important component of host defense, which is responsible for initiating innate immune responses to virus infection.¹,² PKR is produced in a latent form. Upon viral infection, the PKR activates itself by binding to double-stranded RNA (dsRNA), which many viruses produce in the processes of their replication in the host cell. Activated PKR phosphorylates eukaryotic translational initiation factor (eIF2) and blocks protein synthesis at the level of initiation.¹ PKR is also involved in apoptosis, signal transduction, cell growth, and differentiation.³–⁶

PKR contains two tandem copies of double-stranded RNA binding domain (dsRBD) and a Ser/Thr protein kinase domain.⁷ The N-terminal dsRBD1–dsRBD2 of the PKR binds to dsRNA and acts as a modulator of the C-terminal kinase domain activation. dsRBD motifs have been found in many proteins from all kingdoms, as well as animal viruses.⁸ Proteins containing dsRBDS, such as Xenopus rbpa, Drosophila Staufen, Dicer, RNase III, and ADAR family of adenosine deaminase, have functionally diverse biochemical roles in transcription, RNA processing, mRNA localization and translation.⁹ According to several structures of dsRBDS, dsRBD forms a compact protein domain with a α-β-β-α topology in which two α-helices packed against the same ace of a three-stranded antiparallel β-sheet.⁷,¹⁰ The structure of the N-terminal dsRBD1–dsRBD2 of PKR has been solved by NMR.¹¹ Each dsRBD adopts the canonical α-β-β-α topology and the two dsRBD domains are

Abbreviations used: PKR, double-stranded RNA-dependent protein kinase; dsRNA, double-stranded RNA; TAR, tat activation response region; RBD, RNA binding domain; ITC, isothermal titration calorimetry; TROSY, transverse relaxation optimized spectroscopy.

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connected by an interdomain linker, which is unstructured and very flexible.

The RNA binding characteristics of tandem PKR dsRBD1–dsRBD2 have been generally determined. dsRBD1–dsRBD2 binds to dsRNA but not RNA–DNA hybrids or dsDNA and the minimum RNA length for high-affinity binding of the dsRBD1–dsRBD2 is 16 base-pairs of canonical A-form dsRNA. In addition to canonical A-form dsRNA, many RNAs including 3’-UTR of z-tropomyosin, interferon-γ mRNA, human hepatitis δ agent RNA, adenovirus VA 1 RNA, Epstein-Barr Virus EBER-1 and -2 RNAs and HIV TAR, regulate PKR activation. These RNAs contain various structural features including unpaired or non-Watson–Crick base-pairs, bulges, internal loops, hairpin loops, junctions, and pseudoknots. How structural features of an RNA modulate PKR recognition and activity must be defined.

The molecular basis for dsRBD recognition of dsRNA has been revealed in several structures of single dsRBD–dsRNA complexes solved by NMR and X-ray crystallography. A single dsRBD recognizes two successive minor grooves and the intervening major groove on one face of a primarily A-form RNA helix. The first z-helix (z1) of the dsRBD interacts with a minor groove of the A-form RNA helix or UUCG or AGNN tetraloop. The loop between β1 and β2 of the dsRBD interacts with a successive minor groove. The second z-helix (z2) interacts with intervening major groove. These contacts are mainly involved with 2’-hydroxyl groups of the ribose sugar. These structures explain how dsRBDs specifically recognize dsRNAs over ssRNA or dsDNA.

Despite being armed with these structures, PKR dsRBD1–dsRBD2 recognition of cognate dsRNA still remains unclear. First, PKR dsRBD1–dsRBD2 has two tandem repeated dsRBDs, whereas all of the solved structures are single dsRBD–dsRNA complexes. Second, even though the target RNAs of PKR dsRBD1–dsRBD2 contain various structural features including unpaired or non-Watson–Crick base-pairs, bulges, internal loops, hairpin loops, junctions, and pseudoknots, the contribution of these structural features of dsRNAs to protein recognition are still unclear. Moreover, all of the solved structures are dsRBD complexed with a canonical double-stranded RNA or a dsRNA with hairpin loop.

Here, we use HIV-1 TAR RNA as a model ligand for PKR dsRBD recognition. The long-term goal of this work is to correlate RNA recognition with PKR activation. Many prior studies have investigated PKR activation by using poly(I–C) nucleotides. In contrast, TAR RNA is an activator of PKR with a defined size, and includes structural features beyond simple duplex, such as bulges and hairpin loops. We have investigated the biophysical features of the interaction between PKR dsRBD1–dsRBD2 and HIV TAR RNA. We have defined the affinity and specificity of the RNA–protein interactions, and have mapped the interaction surface between PKR dsRBD1–dsRBD2 and TAR RNA using NMR spectroscopy.

Results

Characterization and thermodynamic profiles of TAR RNA binding of dsRBD1, dsRBD2, and dsRBD1–dsRBD2 by ITC

We first investigated the interaction of TAR RNA with PKR dsRBDs using isothermal titration calorimetry (ITC). We characterized the binding of PKR dsRBD1, dsRBD2, and dsRBD1–dsRBD2 to HIV TAR RNA. Figure 2 shows representative ITC profiles resulting from the injection of dsRBD1–dsRBD2, dsRBD1, or dsRBD2 into a solution of TAR RNA at pH 6.5 with 100 mM NaCl.

The ITC data for the interaction of TAR RNA with dsRBD1–dsRBD2 were fit with a model for two binding sites; one is a single high affinity site and the second is a low affinity site, which is in good agreement with previous gel shift data. The dissociation constant for the first binding (Kd1) is 70 nM with a 1:1 stoichiometry (Figure 2). Compared to the first tight binding site, the dissociation constant for the second binding (Kd2) is 23 μM with more than 1:2 stoichiometry, which is 300-fold higher than the Kd1 of the first tight binding. Thus, this result suggests that the first dsRBD1–dsRBD2 binds to a unique high-affinity site on the TAR RNA in 1:1 stoichiometry, whereas the second binding site is far weaker and may involve binding of multiple dsRBD1–dsRBD2 to a single TAR RNA.

In contrast, titration of the TAR RNA with single dsRBD1 demonstrates 15-fold lower affinity for dsRBD1 and 1175-fold lower affinity for dsRBD2 compared to binding of the dsRBD1–dsRBD2 di-domain. dsRBD1 binds to the TAR RNA with a 1:1 stoichiometry, whereas the dsRBD2 binds to the TAR RNA with more than a 1:3 stoichiometry. Therefore, the dsRBD2 shows very weak and non-specific binding to the TAR RNA. This is consistent with previously reported mutation data showing that dsRBD1 has a higher affinity for the poly(I–C) nucleotide or long dsRNA than dsRBD2.

Overall binding free energy (∆G) calculated using experimentally determined binding association constants revealed that dsRBD1–dsRBD2 di-domain produced favorable ∆G enhancements of 1.6 kcal/mol and 4.2 kcal/mol, relative to dsRBD1 and dsRBD2, respectively. Binding of dsRBDs to the TAR RNA resulted in favorable ∆H values, −6.0, −7.6, and −11.1 kcal/mol, and unfavorable ∆S values, −2.6, −5.7, and −4.3 cal/mol, for dsRBD1, dsRBD2, and dsRBD1–dsRBD2, respectively. Binding of dsRBD1–dsRBD2 to TAR RNA resulted in a more favorable ∆H value than those of dsRBD1 or dsRBD2. These thermodynamic data clearly demonstrate that both dsRBDs in the PKR are required for high affinity, single-site binding to TAR.
RNA with a significant favorable ΔΔG relative to those of single dsRBDS.

**Thermodynamic profiles of TAR RNA structure and length on PKR dsRBD1–dsRBD2 binding by ITC**

TAR RNA is composed of secondary structure features beyond Watson–Crick base pairs, such as bulges, a hairpin loop, and a G–U base-pair (Figure 1). In order to investigate the effect of TAR RNA structure on PKR dsRBD1–dsRBD2 binding, we used ITC to compare the binding affinities of the dsRBD1–dsRBD2 to TAR RNAs of different lengths and with changes to the secondary structure (Figure 1 and Table 1).

![Figure 1](image-url)

**Figure 1.** Sequences and secondary structures of RNAAs and PKR dsRBD1–dsRBD2 used in this study. (a) Alignment of PKR dsRBD1 and dsRBD2 is shown. The consensus sequence (>50%) of dsRBDS and a schematic representation of the secondary structure are indicated. (b) Secondary structure of HIV TAR RNA, variants, and model double-stranded RNA used in this ITC study. (From the left, TAR RNA, bulge deletion variant, uucg tetraloop variant, top_40 variant, and model double-stranded RNA.)

Affinities of dsRBD1–dsRBD2 for TAR RNA were compared to that for a perfectly paired dsRNA helix of the same length, capped by a GUGA tetraloop (Figure 1). dsRBD1–dsRBD2 has a higher affinity for TAR RNA than the canonical dsRNA (Table 1). The dissociation constant for complex formation with canonical dsRNA is 3.7 μM, which is 48-fold higher than that of TAR RNA, and dsRBD1–dsRBD2 binds to the canonical dsRNA with >2:1 stoichiometry. These results are supported by native gel electrophoresis on TAR dsRBD1–dsRBD2 complexes. Binding of dsRBD1–dsRBD2 to TAR RNA gave rise to one shifted band, which corresponds to 1:1 complex (Figure 3). This result implies that secondary features of the TAR RNA contribute to high
affinity, 1:1 complex formation with dsRBD1–dsRBD2.

To investigate the structural features of TAR RNA that contribute to the high affinity dsRBD1–dsRBD2 interaction, we made a number of RNA variants, including deletion of the bulge (UCU), modification of the hexaloop to –UUCG– tetraloop, and truncation of dsRNA length. The interaction of dsRBD1–dsRBD2 with these variants was examined by ITC (Figure 1 and Table 1).

All three RNA structural variants had lower affinities for dsRBD1–dsRBD2 relative to the wild-type TAR. The $K_D$ of the bulge-deletion variant and the UUCG tetraloop variant complexes are 2.1 μM and 4.1 μM, respectively, which are 48-fold and 53-fold higher than that of TAR RNA. Binding of dsRBD1–dsRBD2 to the bulge-deletion mutant showed 2:1 protein-RNA stoichiometry by both ITC data (Table 1) and native gel shift (Figure 3). Our data thus show that the hexaloop and the bulge of the TAR RNA are important for high affinity binding to the dsRBD1–dsRBD2 with 1:1 stoichiometry.

**dsRBD1–dsRBD2 binding site mapping on TAR RNA by imino proton perturbation**

The interaction between the TAR RNA and the dsRBD1–dsRBD2 was further analyzed using NMR spectroscopy. First, the resonances of the free TAR RNA were assigned using a series of NMR experiments with unlabeled and $^{13}$C, $^{15}$N-labeled RNAs using standard experimental protocols. These data confirmed the formation of the apical bulge and stem, which have been previously characterized extensively by NMR.\(^{24–26}\) In addition, we observed formation of the highly paired lower stem.

NMR titration experiments were used to monitor formation of the RNA–protein complex. dsRBD1–dsRBD2 was added to TAR RNA and a series of 1D $^1$H NMR spectra were collected at protein:RNA ratios of 0:1, 0.5:1, and 1:1. As the molar ratio of dsRBD1–dsRBD2 relative to TAR RNA was increased, the intensity of some resonances decreased proportionally while the same number of new peaks appeared, indicating that the interaction between the dsRBD1–dsRBD2 and the TAR RNA was in slow exchange on the NMR time scale. These chemical shift changes were completed when the molar ratio of dsRBD1–dsRBD2 to TAR RNA reached $\approx 1.0$, which supports a 1:1 binding stoichiometry of the protein–RNA complex, in agreement with the ITC data (Figure 2 and Table 1) and the gel-mobility shift assay (Figure 3). In contrast, titration of the dsRBD1–dsRBD2 into a shorter TAR variant, the top_40 RNA (Figure 1),

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**Table 1. Thermodynamics parameters for the binding of PKR dsRBD2 with HHIV TAR RNA and variants**

<table>
<thead>
<tr>
<th>RNA</th>
<th>$K_D$ (μM)</th>
<th>$n$</th>
<th>$ΔH^o$ (kcal mol$^{-1}$)</th>
<th>$ΔG^o$ (kcal mol$^{-1}$)</th>
<th>$ΔS^o$ (kcal mol$^{-1}$ K$^{-1}$)</th>
<th>$ΔG^o$ (kcal mol$^{-1}$)</th>
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<tr>
<td>TAR</td>
<td>0.07</td>
<td>1.09</td>
<td>±0.01</td>
<td>−11.17±0.01</td>
<td>−9.88±0.30</td>
<td>−4.26±0.96</td>
</tr>
<tr>
<td>TAR_UUCG</td>
<td>4.13</td>
<td>2.48</td>
<td>±0.03</td>
<td>−12.93±0.28</td>
<td>−7.46±0.01</td>
<td>−18.074±0.95</td>
</tr>
<tr>
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<td>1.95</td>
<td>±0.28</td>
<td>−10.58±0.71</td>
<td>−8.14±0.01</td>
<td>−8.06±2.29</td>
</tr>
<tr>
<td>Top_40</td>
<td>2.83</td>
<td>2.07</td>
<td>±0.08</td>
<td>−14.13±0.71</td>
<td>−7.66±0.03</td>
<td>−21.35±2.24</td>
</tr>
<tr>
<td>TAR_noGU</td>
<td>0.34</td>
<td>2.11</td>
<td>±26.07</td>
<td>−21.19±0.40</td>
<td>−8.95±0.01</td>
<td>−37.05±3.34</td>
</tr>
<tr>
<td>LS8_GUGA</td>
<td>3.70</td>
<td>2.73</td>
<td>±0.13</td>
<td>−17.10±1.09</td>
<td>−7.52±0.03</td>
<td>−31.62±3.69</td>
</tr>
</tbody>
</table>

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**Figure 2.** ITC of the interaction of the TAR RNA with dsRBD1–dsRBD2 (a), dsRBD1 (b), and dsRBD2 (c). Each protein solution was titrated into a TAR RNA solution, and the heat absorbed was monitored by ITC. The RNA solution conditions were 10 mM sodium phosphate (pH 6.5) with 100 mM NaCl.
showed progressive line-broadening of the imino proton resonances, indicating formation of a low affinity complex in intermediate exchange. These NMR data (not shown) are consistent with the ITC data presented above.

Since free TAR RNA and the complex with dsRBD1–dsRBD2 was in slow exchange, free, bound, and chemical exchange cross-peaks were observed in the 2D $^1$H–$^1$H nuclear Overhauser effect spectroscopy (NOESY) spectrum of the 0.5:1 complex. Based on the assignments of the free TAR RNA, the imino proton resonances of TAR RNA complexed with the dsRBD1–dsRBD2 were assigned from the exchange cross-peaks; these

**Figure 3.** Native gel mobility shift for dsRBD1–dsRBD2 binding to TAR RNA and variants. (a) Secondary structure of HIV TAR RNA, bulge deletion variants, and dsTAR with UUCG tetraloop. (b) Native gel mobility shift experiment for dsRBD1–dsRBD2 binding to TAR RNA and variants. Concentration of each RNA is 2 μM. dsRBD1–dsRBD2 was added 2 μM and 4 μM for making 1:1 and 1:2 complexes in 10 mM sodium phosphate (pH 6.5) with 100 mM NaCl and 5 mM β-mercaptoethanol.
assignments were confirmed by NOESY experiments on the 1:1 complex. The imino proton regions of 1D $^1$H NMR spectra of free TAR and TAR–dsRBD1–dsRBD2 complex are shown in Figure 4. Imino proton resonances of U10, G11, G12, U13, U14, G21, U38, and G43 of TAR RNA shifted upon PKR dsRBD1–dsRBD2 binding. The imino proton resonance of U40 is only observed in the complex. The perturbed imino proton resonances upon dsRBD1–dsRBD2 binding were mapped on the secondary structure of the TAR RNA (Figure 4). These results show that the binding site for the dsRBD1–dsRBD2 spans the upper bulge to lower stem of the TAR RNA.

**Binding site mapping on the dsRBD1–dsRBD2 by chemical shift perturbation**

To probe the RNA interaction interface on dsRBD1–dsRBD2, we compared the $^1$H–$^{15}$N transverse relaxation optimized spectroscopy (TROSY) spectrum of free $^{15}$N-labeled PKR dsRBD1–dsRBD2 to that of the complex with the TAR RNA. The prior backbone assignments of free PKR dsRBD1–dsRBD2 were confirmed here using standard methods. The chemical shifts of both $^1$H and $^{15}$N nuclei are sensitive to their local chemical environment and therefore can be used as probes for interactions between the labeled protein and

![Figure 4](image-url)

**Figure 4.** Imino proton regions of 1D $^1$H NMR spectra of free TAR RNA (a) and complex with dsRBD1–dsRBD2 (b). Assignments are indicated by numbers above the peaks. The perturbed imino proton resonances upon dsRBD1–dsRBD2 binding were mapped on the secondary structure of the TAR RNA (c).
unlabeled RNA. Upon formation of a 1:1 complex, some cross-peaks did not change at all or only slightly shifted (Figure 5). However, more than 80% of cross-peaks shifted significantly, so that the proximity-based assignments by comparison of the two spectra would have been erroneous. In addition, as shown in the imino proton titration, because of the slow exchange between the free and RNA-bound states, the resonance assignment of dsRBD1–dsRBD2 complexed with the TAR RNA could not be followed by the addition of the TAR dsRBD1–dsRBD2 complexed with the TAR RNA alone and dsRBD1–dsRBD2

Therefore, de novo backbone assignments of the dsRBD1–dsRBD2 in the complex with the TAR RNA (40 kDa) were required.

NMR study of large RNA–protein complex (>40 kDa) is challenging. To date, two large RNA–protein complexes (~40 kDa) have been determined by NMR,

which are similar in molecular mass to the PKR dsRBD1–dsRBD2–TAR RNA complex. In the U1A–PIE RNA complex, the U1A protein (~10 kDa) forms a homodimer. In the NC-MoMuLV yRNA complex, NC protein is about 7 kDa. In contrast, PKR dsRBD1–dsRBD2 is about 20 kDa. The complexity of the protein spectrum is severe in PKR dsRBD1–dsRBD2–TAR RNA complex. Moreover, the interaction of dsRBDs is mediated by a-helices, which have resonances in the most overlapped portion of the NMR spectrum (Figure 5).

To acquire protein backbone assignments, TROSY-based triple resonance experiments of [70% ²H, ¹³C, ¹⁵N]dsRBD1–dsRBD2 in 1:1 complex with unlabeled TAR RNA were acquired. The sequential assignments between Q49 and D53 in HNCA spectrum are shown in Figure 6. The amino acid residues between Q49 and D53 are located in b2 and loop 3 of dsRBD1, which are opposite sites of the interface with RNA in the complex based on previous dsRBD–dsRNA structures. By contrast, some resonances of amino acid residues at the interface could not be observed in the triple resonance experiments. Presumably, these signals at the interface are broadened by intermediate time scale dynamics between non-magnetically equivalent conformers of the complex. To get more information for assignments, we prepared [¹⁵N]Lys selectively labeled protein and acquired two ²H–¹⁵N TROSY spectra of dsRBD1–dsRBD2 alone and dsRBD1–dsRBD2–TAR RNA complex (Figure 5). Using several different amino acid selectively labeled protein samples, the assignments of the backbone ²H–¹⁵N TROSY cross-peaks were completed.

Based on the resonance assignments of the free dsRBD1–dsRBD2 and the dsRBD1–dsRBD2–TAR complex, changes in ²H and ¹⁵N chemical shifts upon TAR RNA binding are mapped onto the three-dimensional structure of the free dsRBD1 and dsRBD2 in Figure 7. In both dsRBDs, the residues with large chemical shift perturbations are located on helix a1 and helix a2. This is consistent with the previous structures of single dsRBD–dsRNA complexes.

By contrast, the third interacting regions, b1–b2 loop, of dsRBD1 and dsRBD2 show different chemical shift perturbation upon TAR RNA binding. Residues in the b1–b2 loop of the dsRBD1 show large chemical shift perturbations whereas the b1–b2 loop of the dsRBD2 shows small chemical shift perturbations. Therefore, our perturbation data suggest that the b1–b2 loop of the dsRBD1 interacts with the TAR RNA, whereas that of the dsRBD2 is less involved in the TAR RNA recognition. Supporting this suggestion, our ITC data show that the TAR RNA has much higher affinity to isolated dsRBD1 compared to the dsRBD2. The residues of the interdomain linker between the dsRBD1 and the dsRBD2 also show large chemical

Figure 5. 2D ²H–¹⁵N TROSY spectra of the uniformly ¹⁵N-labeled dsRBD1–dsRBD2–TAR RNA complex (a) and free [¹⁵N]Lys-selectively labeled dsRBD1–dsRBD2 (blue) and complexed with TAR RNA (red) (b).
perturbations (Figure 7), clearly contribute to binding of the dsRBD1–dsRBD2 domain.

Discussion

To understand the origins of specific activation of PKR by RNA ligands, we have investigated the structural and thermodynamics aspects of dsRBD1–dsRBD2 binding to TAR RNA. Our data confirmed prior observations that both dsRBD domains are required for high-affinity binding to TAR RNA. The dsRBD1–dsRBD2 formed a 1:1 complex with a $K_D$ of 70 nM, whereas the individual dsRBDs formed weaker, higher stoichiometry complexes.

We have investigated the biophysical features of the interaction between PKR dsRBD1–dsRBD2 and HIV TAR RNA by using ITC and NMR. Prior biochemical experiments clearly demonstrated that dsRBD1–dsRBD2 binds to TAR RNA, and that binding leads to activation of PKR by autophosphorylation. Since many studies have reported PKR activation assay by using poly(I·C) nucleotides, we chose TAR RNA as an example of defined ligand that could be a better target for mechanistic and structural studies. Prior elegant biophysical and biochemical studies have defined that TAR RNA binds with high affinity to PKR dsRBD1–dsRBD2, and have broadly mapped the interaction.

As previously observed, the length of an RNA duplex region is critical for high-affinity binding of
dsRBD1–dsRBD2 to TAR RNA. Bevilacqua & Cech clearly showed that at least 16 bp of helical RNA are needed for dsRBD1–dsRBD2 binding to dsRNA.\(^{12}\) Truncation of the lower stem region of TAR (Top_40 RNA), which removes nine base-pairs and a bulge, decreases protein–RNA affinity by 14-fold as shown by ITC. The resulting TAR mutant has 16 potential base-pairs interrupted by bulges, but this context is insufficient for specific complex formation. NMR data on formation of a complex between this lower stem truncation mutant and dsRBD1–dsRBD2 show exchange broadening, consistent with formation of an unstable, non-specific complex.

Helical distortions modulate the affinity of PKR dsRBDs–TAR RNA complexes. Full-length, wild-type TAR forms a tight, 1:1 stoichiometry complex, as shown by NMR, native gels and ITC; the \(K_D\) of this complex is 70 nM, while a weaker 2:1 protein–RNA complex forms with a \(K_D\) of 2–5 \(\mu\)M. dsRBD1–dsRBD2 has a 48-fold higher affinity to the TAR RNA than a canonical \(A\)-form dsRNA with GUGA tetraloop (Table 1). The disruption of high-affinity binding in the dsRNA favors formation of >2:1
protein–RNA complex stoichiometry. The presence of the TAR apical loop sequence and three nucleotide –UCU– bulge are also required for high-affinity. The $K_D$ values of the bulge-deletion variant and the UUCG tetraloop variant are 2.1 μM and 4.1 μM, respectively, which is 48-fold and 53-fold lower than that of TAR RNA.

NMR titration experiments mapped the regions of TAR that interact with dsRBD1–dsRBD2 (Figure 4). The binding region for the dsRBD1–dsRBD2 spans from the upper bulge to the lower stem of the TAR RNA. Because this titration experiment focused on the NMR spectral region of the imino protons, which are normally observed in base-paired regions, we could not get information on the bulge and the hexaloop. However, we can map the two binding sites: one is around the bulge and the loop and the other is the lower stem around the G·U base-pair (Figure 4). Thus, the PKR dsRBDs interact with regions of structural distortion in TAR, consistent with the thermodynamic data discussed above and previous hydroxyl radical probing experiments. Specific recognition of a hairpin loop by a dsRBD is also found in the structure of the Rnt1p dsRBD–dsRNA containing an AGNN tetraloop. It was shown that the binding affinity to the Rnt1p dsRBD is lowered about fourfold by the substitution of the AGGA tetraloop to a GUGA tetraloop. As with major groove recognition of TAR by the HIV-1 Tat element, the shape of the minor groove in TAR may be altered by the presence of the three nucleotide bulge. In addition to the hairpin loop and bulge of the TAR RNA, the G·U base-pair in the lower stem also contributes to the high affinity to the dsRBD1–dsRBD2 (Table 1). The G·U pair has two distinguishing chemical features compared to the Watson–Crick base-pair. One is the exocyclic amino group of the G·U pair located in the minor groove and the other is a deep electronegative environment in the major groove because of the presence of co-planar guanosine N7, guanosine O6, and uridine O4. Any of these features might contribute to the interaction with the PKR dsRBD1–dsRBD2.

In both dsRBDs, the residues with large chemical shift perturbations were mapped on the helix α1, the β3–α2 loop, and the N-terminal end of helix α2. This is consistent with the previous structures of single dsRBD–dsRNA complexes (Figure 8). According to these structures, three regions of the dsRBD make contact with the dsRNA. The first region, helix α1, interacts with the minor groove of the dsRNA and the successive major groove of the dsRNA is recognized by the second region, which is between the β3–α2 loop and the N-terminal end of helix α2. Therefore, our perturbation data show that both the dsRBD1 and the dsRBD2 of the PKR interact with the TAR RNA using the helix α1 and the β3–α2 loop and the N-terminal end of helix α2 such as other single dsRBDs (Figure 8).

By contrast, the third interacting region, β1–β2 loop, of the dsRBD1 and dsRBD2 shows different chemical shift perturbation upon TAR RNA binding. In other structures, the β1–β2 loop interacts with the second minor groove, which is 10–13 bp away from the first recognized minor groove and as a result, a dsRBD binds to one face of the dsRNA helix without wrapping around it. Interestingly, the residues in the β1–β2 loop of the dsRBD1 show large chemical shift perturbations, whereas the

![Figure 8. Comparison of the dsRNA binding sites of dsRBDs. PKR dsRBD1 (a) and dsRBD2 (b) of the PKR dsRBD1–dsRBD2 complexed with TAR RNA, Xlrbpa (c) complexed with the model dsRNA, Staufen dsRBD3 (d) complexed with dsRNA containing UUCG tetraloop, Rnt1p dsRBD (e) complexed with snR47h RNA (from the top left clockwise). The interaction residues are shown in red.](image-url)
residues in the β1–β2 loop of the dsRBD2 show small chemical shift perturbations. Most dsRBDs have one or two positively charged residues in the β1–β2 loop that interact with the minor groove (Figure 1). The β1–β2 loop of dsRBD1 has R39 and R40, which show large chemical shift perturbation upon TAR binding, whereas the dsRBD2 has no charged residue in the β1–β2 loop and shows little chemical shift perturbations. This is also consistent with the previous mutagenesis data. Changing Gln to Ala at positions 19 and 110 in dsRBD1 and dsRBD2 reduced dsRNA binding to 20 and 45% of control levels, respectively. On the other hand changing Phe to Ala at residue 41 in dsRBD1 was a severe mutation (10% of the control level), but the equivalent change at residue 131 in the dsRBD2 was only slightly deleterious (90% of the control level). Therefore, our perturbation data suggest that the β1–β2 loop of the dsRBD1 interacts with the TAR RNA, whereas that of the dsRBD2 is less involved in the TAR RNA recognition. dsRBD2 recognizes the TAR RNA using two regions instead of three regions as the dsRBD1 does. Our ITC data support this suggestion, as TAR RNA has higher affinity for dsRBD1 compared to the dsRBD2.

In addition to these three regions, the residues of the interdomain linker between the dsRBD1 and the dsRBD2 also show large chemical perturbations (Figure 7). Previous NMR studies showed that the 15 residue linker that connects the two dsRBDs of PKR is very flexible and therefore does not fix the relative position of the two dsRBDs to each other in the RNA-free state. Even though there is no solved structure of the multi-dsRBDs complexed with dsRNA, we can get some information from the structures of the complexes of multiple single-stranded RNA binding domains and the cognate single-stranded RNAs. The interdomain linker of ssRBDs has 10 to 12 residues, which are very flexible in the free form. Upon RNA binding, the linker forms a 3–10 helix and interacts with the target RNA. Therefore, large chemical shift perturbations of the linker residues of the PKR dsRBD1–dsRBD2 might be due to direct interaction with the TAR RNA or conformational change of the linker or both. In either way, our chemical shift perturbation data show that the linker is involved in TAR RNA recognition.

To specify the TAR RNA recognition interface of dsRBD1–dsRBD2, and to reveal the TAR RNA recognition mechanism, the high-resolution structure of the dsRBD1–dsRBD2 TAR RNA complex is needed. The results presented here provide the biophysical and spectroscopic underpinning for these high-resolution structural studies.

Materials and Methods

Sample preparation

Human PKR dsRBD1 (1-85), dsRBD2 (99-169), and dsRBD1–dsRBD2 (1-169) were expressed with His-tag in *Escherichia coli* BL21(DE3)RIG cells. The proteins were purified by Ni-NTA column (Qiagen), thrombin cleavage, and gel filtration. The single dsRBDs were purified by Superdex 75 (26/60) gel filtration whereas Superdex 200 (26/60) was used for the purification of the dsRBD1–dsRBD2 didomain. The fractions were concentrated by Vivaspin (cutoff 5000 Da). All final protein samples were stored in 10 mM phosphate buffer (pH 6.5), 100 mM NaCl, 5 mM β-mercaptoethanol. Protein concentrations were determined by the absorbance at 280 nm (A280) using a molar extinction coefficient.

RNA samples were prepared by *in vitro* transcription using T7 polymerase as described. Instead of using a BioRad column, we use Superdex 200 (26/60) gel filtration for better resolution. RNA samples are concentrated by Vivaspin (cutoff 5000 Da). All RNA samples were kept in 10 mM phosphate buffer (pH 6.5), 100 mM NaCl. Molar extinction coefficients for the double-stranded RNA were calculated from the A260 values at 20 °C by extrapolation from the values at 90 °C.

Isothermal titration calorimetry

Measurements were performed on a VP-Microcalorimeter (MicroCal, Northampton, MA, USA) at 30 °C. The protein solution was prepared in 10 mM phosphate buffer (pH 6.5), 100 mM NaCl, 5 mM β-mercaptoethanol.

All RNA solutions were prepared in 10 mM phosphate buffer (pH 6.5), 100 mM NaCl.

Each titration involved a single 2 μl and a series of 10 μl injections of protein solution into the RNA solution. Titration curves were fitted by a non-linear least squares method with ORIGIN software (MicroCal). The parameters $K_a$ (binding affinity, $K_a=1/K_d$), $ΔH$ (binding enthalpy), and $n$ (stoichiometry) were estimated from the fitted curve. From the values of $K_a$ and $ΔH$, the change in free energy ($ΔG$) and in entropy ($ΔS$) were calculated with the equation: $ΔG=-RT ln K_a$, $ΔG=ΔH−TΔS$, where $R$ is the universal molar gas constant, and $T$ is the absolute temperature.

Native-gel mobility-shift assay

Samples were prepared in phosphate buffer (pH 6.5) containing 100 mM NaCl, 5 mM β-mercaptoethanol. After 5 min of incubation, binding reactions were loaded onto a running 10% (w/v) native gel (BioRad). The running buffer contained 0.5× TBE (50 mM Tris base, 41.5 mM boric acid, 1 mM EDTA, final pH 8.3). Electrophoresis was performed for 3 h at 70 V, at 4 °C. The gel was stained with 0.1% (w/v) toluidine blue solution.

NMR experiments

All NMR spectra were collected on Varian INOVA 500, 600, and 800 NMR spectrometers at Stanford Magnetic Resonance Laboratory (SMRL).

Several RNA and protein samples were prepared: unlabeled RNA, unlabeled dsRBD1–dsRBD2, 2H, 2H-labeled RNA, and 70% 2H, 2H, 2H-labeled dsRBD1–dsRBD2. Samples were 0.3–0.8 mM in each component.

To assign exchangeable resonances of RNA and DNA–protein complex, 2D NOESY (150 ms and 250 ms mixing times), 1H–2H TROSY, 3D 1H–15N NOESY–HSQC, and 2D HNNO-COSY were collected in 90% H2O/10% D2O at 298 K or 303 K. Non-exchangeable resonances of RNA were assigned by 2D NOESY (50 ms, 150 ms, and 250 ms mixing times), 2D TOCSY, 2D TROSY, 3D HCH-
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References


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