Recognition of viral RNA stem-loops by the tandem double-stranded RNA binding domains of PKR

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ABSTRACT

In humans, the double-stranded RNA (dsRNA)-activated protein kinase (PKR) is expressed in late stages of the innate immune response to viral infection by the interferon pathway. PKR consists of tandem dsRNA binding motifs (dsRBMs) connected via a flexible linker to a Ser/Thr kinase domain. Upon interaction with viral dsRNA, PKR is converted into a catalytically active enzyme capable of phosphorylating a number of target proteins that often results in host cell translational repression. A number of high-resolution structural studies involving individual dsRBMs from proteins other than PKR have highlighted the key features required for interaction with perfectly duplexed RNA substrates. However, viral dsRNA molecules are highly structured and often contain deviations from perfect A-form RNA helices. By use of small-angle X-ray scattering (SAXS), we present solution conformations of the tandem dsRBMs of PKR in complex with two imperfectly base-paired viral dsRNA stem-loops; HIV-1 TAR and adenovirus VA₁-AS. Both individual components and complexes were purified by size exclusion chromatography and characterized by dynamic light scattering at multiple concentrations to ensure monodispersity. SAXS *ab initio* solution conformations of the individual components and RNA-protein complexes were determined and highlight the potential of PKR to interact with both stem and loop regions of the RNA. Excellent agreement between experimental and model-based hydrodynamic parameter determination heightens our confidence in the obtained models. Taken together, these data support and provide a framework for the existing biochemical data regarding the tolerance of imperfectly base-paired viral dsRNA by PKR.

Keywords: HIV-1 TAR; VA₁; PKR; protein–RNA interactions; small-angle X-ray scattering

INTRODUCTION

The interferon response provides a crucial first line of defense against viral pathogens via specific protein production from the interferon-stimulated genes (ISGs) (Malmgaard 2004). These proteins mount a multipronged resistance to viral infection, at least in part by inhibition of host translation in order to slow viral progeny proliferation (Sadler and Williams 2008). Human double-stranded RNA (dsRNA)-activated protein kinase (PKR) is a key ISG that functions in the innate immune response to counteract viral infection via further amplification of the interferon response (Weber et al. 2006; Hovanessian 2007). Latent PKR interacts directly with dsRNA from replication by-products, mRNA transcripts, or the viral genome itself, which enables self-association and ultimately autophosphorylation of two threonine

residues that overhang the enzyme's active site (Thomis and Samuel 1993). Autophosphorylated PKR can then target its substrates for phosphorylation, including eukaryotic initiation factor 2 α (eIF2 α) at Ser51. Phosphorylation of eIF2 α inhibits the guanidine nucleotide exchange activity of the eIF2 heterotrimeric complex, thereby preventing translation initiation (Gale and Katze 1998; Sudhakar et al. 2000). Therefore, dsRNA recognition by PKR represents an effective means to attenuate both host and viral protein production (Sonenberg and Hinnebusch 2009).

Human PKR is a 551-amino-acid residue enzyme containing three distinct regions, each with specific functional roles in the response to dsRNA. The N-terminal region (residues 1–169) contains tandem dsRNA binding motifs (dsRBMs) that interact with RNA (Fig. 1A). NMR studies of PKR_{1–169} in isolation revealed that each dsRBM from PKR adopts a canonical fold containing a three-stranded anti-parallel β -sheet flanked by two α -helices with the tandem dsRBMs joined by a 23-amino-acid linker (Nanduri et al. 1998). The C-terminal region encompasses a Ser/Thr kinase domain responsible for target substrate recognition and phosphorylation. Structural

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FIGURE 1. Domain organization of PKR and secondary structures of viral dsRNAs. (A) Domain organization of PKR is shown, including the tandem dsRBMs (encompassing residues 1–169), the interdomain linker (residues 170–251), and the Ser/Thr kinase domain (residues 252–551). Key threonine residues in the activation loop that become autophosphorylated upon interaction with dsRNA are also indicated. Secondary structures of HIV-1 TAR (B) and the apical stem-loop of adenovirus VA₁ (VA₁-AS) (C) RNA are shown.

studies on the kinase domain in complex with eIF2 α detailed the overall Ser/Thr kinase fold as well as the features required for target substrate interaction (Dar et al. 2005). The dsRNA binding and kinase domains are joined by a third region, an 80-amino-acid flexible interdomain linker implicated in PKR self-association (Lemaire et al. 2005; VanOudenhoove et al. 2009). Previous studies have suggested significant flexibility in the two linkers (one between the dsRBMs, the other between the dsRBMs and kinase domains), allowing for two distinct conformations to be observed: an extended “open” conformation where dsRBMs and kinase domains are not in contact, and a collapsed “closed” conformation (Carpick et al. 1997; Gabel et al. 2006; Lemaire et al. 2006; McKenna et al. 2007; VanOudenhoove et al. 2009). Together, the data suggest a direct communication between domains that allows propagation of the activation signal from the dsRNA binding to the kinase domain via the interdomain linker.

Binding of dsRNA to dsRBMs significantly enhances the kinase activity of PKR (Thomis and Samuel 1993). The molecular basis for the interaction has been studied extensively with a variety of synthetic and biological dsRNA molecules, although no high-resolution structure of PKR in complex with RNA exists. Productive interaction requires residues from both dsRBM1 and -2 as well as the linker that joins them (Kim et al. 2006). One of the most unique features of PKR is its selectivity for dsRNA: Single-stranded polynucleotides, dsDNA, and DNA–RNA hybrids are not capable of high-affinity interaction (Gale and Katze 1998). Structures of a single dsRBM from proteins other than PKR in complex with perfectly duplexed dsRNA indicate that approximately 1.5 turns (~ 16 bp) of the A-form RNA helix comprising consecutive minor-major-minor grooves are recognized and that contacts are mediated primarily through the 2'-hydroxyl groups of the ribose sugar, explaining the observed selectivity (Bycroft et al. 1995; Kharrat et al. 1995; Ryter and Schultz

1998; Ramos et al. 2000; Gan et al. 2006; Stefl et al. 2010). In addition to a minimum length requirement (~ 16 bp), specific structural (bulges, loops, etc.) and nucleotide modifications (i.e., 5'-phosphorylation state) have been observed to be accommodated by PKR and, in some cases, significantly affect both the affinity for and activation of PKR (Bevilacqua and Cech 1996; Kim et al. 2006; McKenna et al. 2006; Puthenveetil et al. 2006; Nallagatla et al. 2007; Nallagatla and Bevilacqua 2008). As there is scant structural information about imperfectly duplexed RNA in complex with PKR, a detailed understanding of how PKR accommodates these deviations remains an unanswered question.

A well-characterized viral dsRNA regulator of PKR is the HIV-1 transactivation response (TAR) element, an RNA stem-loop structure spanning nucleotides +1 to +59 of the newly synthesized viral mRNA (Berkhout et al. 1989). TAR adopts an A-form RNA helix with distortions including a trinucleotide bulge and hexaloop (Fig. 1B; Puglisi et al. 1992; Aboul-ela et al. 1996). HIV-infected cells treated with interferon demonstrate a decreased production of HIV proteins, as well as HIV particles (Benkirane et al. 1997). The interferon-mediated response is ascribed to the activity of PKR in lymphocytes as transduction of PKR into T-cell progeny protects cells from HIV-1 infection and severely inhibits HIV-1 replication rates in infected cells (Dimitrova et al. 2005). An important link between host innate immune response and HIV-1 infection appears to be mediated by PKR through a specific interaction with HIV-1 TAR RNA. HIV-1 TAR RNA has been established as a regulator of PKR kinase activation, where high-affinity interaction requires the presence of 16 bp proximal to the loop as well as both the bulge and hexaloop (SenGupta and Silverman 1989; Gunnery et al. 1992; Maitra et al. 1994; Kim et al. 2006). Given that each individual dsRBM requires ~ 15 bp for interaction, it is unclear how the tandem dsRBM domains of PKR are interacting with TAR RNA. A recent study has suggested that defects (i.e., bulge or hexaloop) serve as anti-determinants to PKR binding and activation and that TAR unwinding and dimerization represents the activating species (Heinicke et al. 2009). However, the biological basis for TAR dimerization remains unresolved at present.

High-resolution information on the tandem dsRBMs of PKR in complex with dsRNA complex has been difficult to obtain, likely due to the established flexibility of both the linker between dsRBM1 and -2 and the inherent flexibility of stem-loop RNA structures. Here we present the solution conformation of tandem dsRBMs of PKR in complex with an imperfectly base-paired viral dsRNA using small-angle X-ray scattering (SAXS). *Ab initio* analysis of PKR_{1–169}, HIV-1 TAR, and the protein–RNA complex were performed to investigate how each dsRBM orients and interacts with TAR RNA. Dynamic light scattering (DLS) was employed to (1) investigate the homogeneity of macromolecules at various concentrations, (2) measure the hydrodynamic radius of individual macromolecules as well as their complexes, and

(3) compare the calculated values from SAXS models with experimentally determined values. This experimental approach was repeated using a second model RNA ligand, the apical stem of adenovirus VA_I RNA, in order to confirm the validity of the results with a second established binding partner of PKR. Together these results provide a plausible framework for the recognition of short, imperfectly base-paired RNA ligands by the tandem dsRBMs of PKR and highlight the effectiveness of SAXS approaches for systems with highly flexible components.

RESULTS

RNA-protein complex purification and characterization

In perfectly duplexed dsRNA, the tandem dsRBMs of PKR require a minimum of 15 bp (1.5 turns of the A-RNA helix) for interaction, and yet based on existing structures, it would be expected that each dsRBM would require the entire 15 bp. Additionally, PKR is capable of tolerating deviations from perfect duplexes, including bulges, internal loops, and loops for high-affinity binding. In order to study the structural basis for these interactions, we sought to determine the solution conformation of two dsRNA-PKR complexes by SAXS. Complexes were assembled with purified recombinant PKR₁₋₁₆₉, which contains both dsRBM1 and -2 and retains identical binding affinity to the full-length PKR (Fig. 2). DLS experiments were performed to study homogeneity of all macromolecules and to determine the hydrodynamic radius (r_H) of each species being studied at multiple concentrations. PKR₁₋₁₆₉ displays a narrow distribution of hydrodynamic radii at all concentrations examined, consistent with a monodisperse sample (2.80 ± 0.10 nm) (Fig. 3A). A linear distribution of r_H over multiple concentrations (2.50–6.50 mg/mL) was observed, suggesting minimal concentration dependence on the Stokes radius (Fig. 3B).

RNA model ligands were selected based on (1) their high affinity (~ 100 nM) for PKR₁₋₁₆₉ (Fig. 2A), (2) their ability to stimulate autophosphorylation and kinase activity of PKR, and (3) the fact that they are short, imperfectly base-paired dsRNA stem-loops. Based on these criteria, we selected HIV-1 TAR RNA (57 nucleotides [nt]) and the apical stem-loop of adenovirus VA_I RNA (52 nt) (Fig. 1B). Both RNAs were in vitro transcribed from linearized template, purified by size exclusion chromatography, and subjected to native gel electrophoresis. Both TAR (Fig. 2A, lane 1) and apical stem-loop of adenovirus VA_I (VA_I-AS) (data not shown) behave as a single species based on this analysis. DLS confirmed the r_H value consistent with monomeric TAR (2.7 ± 0.2 nm) over the concentration range examined, indicating minimal concentration dependence on Stokes radius, thus excluding the possibility of presence of aggregation (Fig. 3C). Identical observations were made for VA_I-AS ($r_H = 2.58 \pm 0.05$ nm) that has similar size to that of TAR, suggesting

FIGURE 2. RNA-protein complex purification by size exclusion chromatography. (A) EMSA of PKR₁₋₁₆₉ (0–1000 nM) binding to TAR RNA (100 nM) confirming the formation of a single complex. HiLoad 26/60 Superdex 75 size exclusion chromatography purification of the PKR₁₋₁₆₉-TAR complex (B) and the PKR₁₋₁₆₉-VA_I-AS complex (C). Concentration of elution fractions was monitored by in-line spectrophotometric detection at 260 and 280 nm simultaneously.

that both RNA have a similar shape in solution as r_H depends on size and shape of macromolecules (Fig. 3D). The determined hydrodynamic radii of the RNA species being studied are within error of each other, consistent with their size exclusion chromatography elution profiles.

Complexes containing PKR₁₋₁₆₉ and either TAR or VA_I-AS were assembled in the presence of excess RNA to minimize nonspecific protein binding and maximize 1:1 stoichiometry. At low concentrations, electrophoretic mobility shift assays (EMSAs) confirm the emergence of a single higher-molecular-weight species upon incubation of TAR with increasing concentrations of PKR₁₋₁₆₉ (Fig. 2A). Identical results were obtained with VA_I-AS-containing complex (data not shown). Complexes were then assembled at high concentration (μ M range) and purified away from free RNA by size exclusion chromatography (Fig. 2B,C). Given that solution

FIGURE 3. Monodispersity of individual components and RNA-protein complexes. (A) Representative DLS profile of PKR₁₋₁₆₉ at 3.50 mg/mL, before (solid line) and after (dotted line) SAXS data collection. The curves are superimposable, indicating no sample deterioration. The concentration dependence of the hydrodynamic radius (r_H) was determined by DLS for (B) PKR₁₋₁₆₉, (C) TAR, (D) VA₁-AS, (E) the PKR₁₋₁₆₉-TAR complex, and (F) the PKR₁₋₁₆₉-VA₁-AS complex. Each experiment was performed in quadruplicate; error, SD from the mean.

conformation data quality by SAXS is sensitive to sample heterogeneity, this step is essential to ensure the removal of uncomplexed dsRNA. The elution profile for both complexes shows two distinct peaks: a higher-molecular-weight peak corresponding to RNA-protein complex followed by a lower-molecular-weight peak corresponding to free RNA.

The DLS profiles of complexes are also linear with respect to increasing concentration, showing no signs of aggregation across the concentration range at which the experiments were performed (Fig. 3E,F). PKR₁₋₁₆₉ in complex with either TAR ($r_H = 3.06 \pm 0.04$ nm) or VA₁-AS TAR ($r_H = 3.11 \pm 0.03$ nm) is monodisperse and within error demonstrates similar r_H values, consistent with their elution profiles from size exclusion chromatography. As expected, an increase in r_H is

observed in the complex compared with the individual components, although relative to the largest component (PKR₁₋₁₆₉), these increases were relatively moderate for both TAR (9%) and VA₁-AS (11%). Taken together, these results confirm complex homogeneity and suggest a side-by-side orientation of both RNA-protein complexes.

The dsRNA binding domain of PKR adopts an extended conformation in solution

To understand the behavior of dsRNA-PKR₁₋₁₆₉ complexes in solution, we first studied the individual protein and RNA components in solution using SAXS. As our starting point, we first examined PKR₁₋₁₆₉ as the high-resolution

structure of this protein construct has already been determined in solution (Nanduri et al. 1998) and will serve to validate the quality of SAXS results. SAXS data collected at multiple concentrations were merged to generate a single scattering profile for PKR₁₋₁₆₉ for further data analysis (Fig. 4A, red circles). First, the scattering data were used to generate a distance distribution plot that presents a frequency histogram of all observed electron pairs in the molecule, enabling determination of key parameters, including the radius of gyration (r_G) and the maximum particle dimension ($D_{\max}$) using the program GNOM (Svergun 1992). A skewed bell-shaped distance distribution function was observed typical for elongated molecules that may contain flexible regions (Fig. 4B, red circles). The experimentally calculated r_G and $D_{\max}$ for PKR₁₋₁₆₉ are 2.53 ± 0.04 nm and 9.0 nm, respectively (Table 1). Based on the scattering data, multiple ab initio solution conformations for PKR₁₋₁₆₉ were generated using the program DAMMIF (Franke and Svergun 2009) and superimposed using DAMAVER (Volkov and Svergun 2003) to generate an average surface envelope of the protein (Fig. 5A). The molecule adopts an extended structure that appears to orient the individual dsRBMs at opposite ends joined by the linker. However, the relatively uniform broadness of the entire molecule (including both the dsRBMs and linker) is consistent with the flexible nature of the linker. The χ -value

obtained for individual ab initio models was approximately 0.9, illustrating an excellent agreement between experimental data and that obtained from the ab initio calculation. Furthermore, the normalized spatial discrepancy (NSD) parameter of 0.80 ± 0.04 among the ab initio models generated to create the average model indicates very little difference between individual PKR₁₋₁₆₉ models. In order to validate the ab initio calculations, the program HYDROPRO (de la Torre et al. 2000) was employed to calculate hydrodynamic parameters. The r_G of 2.62 ± 0.02 nm, $D_{\max}$ of 9.5 ± 0.05 nm, and r_H of 2.76 ± 0.06 nm are in good agreement with experimentally calculated hydrodynamic properties (Table 1). Identical DLS results were obtained after SAXS data collection, confirming absence of any radiation damage to the sample after exposure to X-rays (Fig. 3A).

To validate our ab initio modeling approach and to determine the orientation of individual dsRBMs within the solution conformation, we took advantage of the existing high-resolution solution structure of PKR₁₋₁₆₉ (Protein Data Bank [PDB] identification 1QU6) (Nanduri et al. 1998). This was accomplished by employing the program BUNCH (Petoukhov and Svergun 2005), which is able to utilize high-resolution information and identify optimal positions and orientations of individual domains (dsRBMs) for which the high-resolution structural information is available, as well as place dummy residues for regions where the high-resolution information is missing. In order to prevent model bias, the linker was severed in the center such that each dsRBM could rotate freely. All the structures calculated were nearly identical to each other as well as to the determined ab initio models that do not incorporate information from high-resolution data (Fig. 5B). Overall, the ab initio model comfortably accommodates each dsRBM and strongly suggests that the linker can adopt a number of different configurations. BUNCH data analysis also suggests that dsRBM1 is positioned into the shorter arm while dsRBM2 is positioned in the longer arm of the SAXS model. The hydrodynamic parameters calculated from ab initio models as well as for three-dimensional structures from BUNCH are not only nearly identical but also agree very well with those parameters determined experimentally, thereby validating our modeling approach (Table 1).

Solution conformation of HIV-1 TAR and adenovirus VA_I-AS RNA

SAXS data for TAR and VA_I-AS were collected at multiple concentrations and merged to generate their respective scattering profiles (Fig. 4A). The resultant distance distribution functions for both TAR and VA_I-AS demonstrate a bell-shaped curve with an extended tail that is consistent with an elongated stem-loop conformation (Fig. 4B). For TAR, the experimentally determined values of r_G and $D_{\max}$ are 2.60 ± 0.05 nm and 8.4 nm, respectively. The pair-distance distribution plot was utilized for ab initio analysis to obtain

FIGURE 4. Characterization of complexes by SAXS. (A) SAXS scattering profiles for PKR₁₋₁₆₉ (red circles), TAR (green diamonds), VA_I-AS (cyan squares), the PKR₁₋₁₆₉-TAR complex (purple plus signs), and the PKR₁₋₁₆₉-VA_I-AS complex (blue triangles). Each data point represents the merged raw data from multiple sample concentrations. (B) Dependence of the pair distribution function upon particle radius for each of the samples outlined in A.

TABLE 1. Comparison of experimental and predicted hydrodynamic parameters

Hydrodynamic parameter	PKR ₁₋₁₆₉			TAR			PKR ₁₋₁₆₉ -TAR			VA ₁ -AS			PKR ₁₋₁₆₉ -VA ₁ -AS		
	Experimental	DAMMIF	HYDROPRO BUNCH	Experimental	HYDROPRO DAMMIF	HYDROPRO MONSA	Experimental	HYDROPRO DAMMIF	HYDROPRO MONSA	Experimental	HYDROPRO DAMMIF	HYDROPRO MONSA	Experimental	HYDROPRO DAMMIF	HYDROPRO MONSA
r_H (nm) ^a	2.8 (±0.1)	2.76 (±0.06)	2.80 (±0.05)	2.7 (±0.2)	2.90 (±0.06)	3.00 (±0.06)	3.06 (±0.04)	2.90 (±0.06)	3.00 (±0.06)	2.58 (±0.05)	2.40 (±0.05)	3.1 (±0.1)	3.11 (±0.03)	2.40 (±0.05)	3.1 (±0.1)
r_G (nm) ^b	2.53 (±0.04)	2.62 (±0.02)	2.63 (±0.01)	2.60 (±0.05)	2.66 (±0.01)	2.53 (±0.02)	2.58 (±0.03)	2.66 (±0.01)	2.53 (±0.02)	2.45 (±0.02)	2.50 (±0.02)	2.60 (±0.01)	2.66 (±0.04)	2.50 (±0.02)	2.60 (±0.01)
D_{max} (nm) ^b	9.0	9.5 (±0.05)	9.8 (±0.05)	8.4	9.2 (±0.05)	9.00 (±0.04)	9.2	9.2 (±0.05)	9.00 (±0.04)	7.9	8.80 (±0.07)	9.1 (±0.04)	9.0	8.80 (±0.07)	9.1 (±0.04)
r_G/r_H	0.9 (±0.1)	0.95 (±0.08)	0.94 (±0.06)	1.0 (±0.2)	0.92 (±0.07)	0.84 (±0.08)	0.84 (±0.07)	0.92 (±0.07)	0.84 (±0.08)	0.95 (±0.07)	1.04 (±0.07)	0.8 (±0.1)	0.86 (±0.07)	1.04 (±0.07)	0.8 (±0.1)
χ	—	0.9	1.1	—	1.0	1.0/1.4/1.0 ^c	—	1.0	1.0/1.4/1.0 ^c	—	1.0	1.0/1.1/1.1 ^c	—	1.0	1.0/1.1/1.1 ^c
NSD	—	0.80 (±0.04)	0.9 (±0.1)	—	0.7 (±0.1)	0.58 (±0.03)	—	0.7 (±0.1)	0.58 (±0.03)	—	0.7 (±0.1)	0.76 (±0.02)	—	0.7 (±0.1)	0.76 (±0.02)

^aFrom DLS data.
^bFrom SAXS data.
^cValues for protein/RNA/complex, respectively.

FIGURE 5. Ab initio modeling of PKR₁₋₁₆₉, TAR, and VA_I-AS. (A) Averaged ab initio model of PKR₁₋₁₆₉ obtained from DAMMIF (top left) and 90° rotation (top right) around its major axis. (B) Superimposition of high-resolution NMR structures into the ab initio model, suggesting excellent agreement with the known NMR structure. (C) Averaged ab initio model of TAR obtained from DAMMIF, and its 90° rotation around its major axis. (D) Averaged ab initio model of VA_I-AS obtained by DAMMIF (bottom left) and its 90° rotation (bottom right) around its major axis.

the solution conformation of the RNA. The χ value of approximately 1.0 was obtained for each model, indicating good agreement between SAXS experimental data and the data calculated for each ab initio model. The NSD parameter of 0.7 ± 0.1 for ab initio models also suggests that individual ab initio models agree with each other almost perfectly. The averaged solution conformation presents an extended shape with a slight curvature. Interestingly, the model lacks significant definition in terms of distinct structural features such as the loop or internal bulge (Fig. 5C). The r_H (2.90 ± 0.06 nm), r_G (2.66 ± 0.01 nm), and $D_{\max}$ (9.2 ± 0.05 nm) values for the TAR models were calculated using HYDROPRO and are consistent with experimentally determined values (Table 1). As with the free protein (PKR₁₋₁₆₉), no significant sample deterioration due to radiation damage was observed by DLS after SAXS data collection (data not shown).

For the VA_I-AS RNA, an r_G of 2.45 ± 0.02 nm and $D_{\max}$ of 7.9 nm were obtained from the distance distribution function, which was further employed to generate several ab initio solution conformations. The resultant χ -values of those models of approximately 1.0 and the NSD parameter of 0.7 ± 0.1 provide a high level of confidence in our data analysis. VA_I-AS adopts an extended shape with a slight curvature but with a more pronounced definition in terms of three dis-

tinct bulges (Fig. 5D). VA_I-AS RNA is significantly more compact than TAR, which suggests that VA_I-AS adopts a more rigid conformation in solution than TAR. The slightly smaller r_G and $D_{\max}$ values may be due to the smaller RNA size (52 vs. 57 nt). The r_H (2.40 ± 0.05 nm), r_G (2.50 ± 0.07 nm), and $D_{\max}$ (8.80 ± 0.07 nm) values for the VA_I-AS models were calculated using HYDROPRO and are consistent with experimentally determined values (Table 1). DLS results before and after data acquisition were identical, confirming that no significant sample deterioration due to radiation damage occurred (data not shown).

Solution conformation of the PKR₁₋₁₆₉-TAR complex

To determine the molecular basis for the interaction between PKR and TAR RNA, we subjected purified complex to SAXS analysis. The merged scattering data from multiple concentrations of complex were used to generate the distance distribution function, which indicates an extended structure with corresponding r_G (2.58 ± 0.03 nm) and $D_{\max}$ (9.2 nm) values (Fig. 4A,B). The experimentally determined $D_{\max}$ for the complex is within error of each of the individual RNA and protein components, consistent with a side-by-side as opposed to end-to-end orientation of the complex. Individual ab initio solution conformations of the PKR₁₋₁₆₉-TAR complex were determined using the MONSA software suite, which exploits the differential scattering intensity of RNA and protein components to allow visualization of each molecule in the complex. The best-fit model based on comparison with experimental data is shown in Figure 6A. The RNA-protein interface curves along the length of the extended complex structure, with both dsRBMs and the linker region between them located in close proximity to the RNA. As opposed to the free TAR RNA model, the RNA stem-loop is clearly visualized upon complex formation with PKR₁₋₁₆₉, and both the loop and stem regions of the RNA are spatially close to PKR based on the MONSA analysis. The overall solution conformation of PKR₁₋₁₆₉ is similar to that of the free protein, and therefore, it appears as though dsRBM1 is oriented toward the loop of TAR, with the linker and dsRBM2 primarily mediating the interaction with the stem region of the RNA. Finally, the solution conformation is consistent with 1:1 stoichiometry, as other stoichiometries tested were unable to sustain reasonable fits to the raw data. For comparison, the average solution conformation from the individual MONSA fits is significantly less information-rich, yet defines a disc-shaped surface envelope (Fig. 7A). The determined χ -values of approximately 1.0 and a NSD value of 0.58 ± 0.03 for the complex indicates a quality fit between the calculated individual solution structures and the raw scattering data. Model-based parameter determinations are in good agreement with experimentally determined values, including the SAXS model-based and DLS-determined r_H values (Table 1).

FIGURE 6. Modeling of complexes with MONSA. MONSA uses scattering information for individual components and complex to resolve the structure of complex. (A) Best fit ab initio model of PKR₁₋₁₆₉-TAR complex where individual components can be visualized (PKR₁₋₁₆₉ in cyan, TAR in orange). (B) Best fit solution ab initio model of PKR₁₋₁₆₉-VA₁-AS (PKR₁₋₁₆₉ in cyan, VA₁-AS in green).

VA₁-AS and TAR adopt similar overall conformations in complex with PKR₁₋₁₆₉

Finally, we performed SAXS experiments on purified PKR₁₋₁₆₉-VA₁-AS to determine whether a similar solution conformation could be observed to the PKR₁₋₁₆₉-TAR complex. Experimental hydrodynamic parameters based on the distance distribution function calculated from the raw scattering data are within error of those determined for the TAR-containing complex (Fig. 4; Table 1). The overall topology of individual surface conformation models presents an extended structure in which, again, the dsRBMs of PKR track the length of the VA₁-AS RNA molecule (Fig. 6B). The entire length of the PKR₁₋₁₆₉ protein mediates the interaction, presumably via the dsRBMs and linker joining them. Unlike in the TAR-containing complex, the loop region of the VA₁-AS RNA was not pronounced, and no significant conformation change is observed in the RNA molecule upon protein binding. However, the overall disc-shaped topology is similar between the two complexes, as illustrated by the comparison of the solution conformations (Fig. 7B). As with all other molecules examined by SAXS, the model validation parameters (χ ; NSD) and hydrodynamic parameters (r_H ; r_G ; D_{max}) are also consistent with an accurate model determination (Table 1).

DISCUSSION

PKR belongs to a large family of dsRBM-containing proteins that vary in terms of the number of dsRBMs they contain

(typically one to five) and their cellular function (Saunders and Barber 2003). The structure of the individual dsRBMs from proteins other than PKR in complex with synthetic RNA duplexes suggest that the A-RNA helix is recognized in a sequence-independent manner via the 2'-hydroxyl groups of the ribose sugar in the minor grooves and nonbridging oxygen residues of the phosphodiester backbone in the intervening major groove (Bycroft et al. 1995; Kharrat et al. 1995; Ryter and Schultz 1998; Ramos et al. 2000; Gan et al. 2006). To date, no high-resolution structure of the dsRBMs of PKR in complex with either synthetic or natural ligands exists, nor does a clear understanding exist of how PKR is capable of interacting with imperfectly duplexed RNA ligands. Therefore we sought to structurally characterize the interaction between the tandem dsRBMs of PKR and two viral dsRNA ligands, HIV-1 TAR and a stem-loop form adenovirus VA₁ (VA₁-AS) (Fig. 1).

We chose to examine the conformation of individual components and complexes by SAXS, a solution-based structural technique that allows low-resolution determination of molecular shape and, importantly, can accommodate systems that are dynamic. While SAXS experiments directly determine r_G and D_{max} values, solution conformation determination requires additional assumptions that make model validation extremely important. All protein, RNA, and complexes were purified by size exclusion chromatography to remove heterogeneity and were confirmed monodisperse by DLS

FIGURE 7. Averaged MONSA models of complexes. (A) Averaged ab initio model of PKR₁₋₁₆₉-TAR (top left) and its 90° rotation (bottom left) around its major axis. (B) Averaged solution ab initio model of PKR₁₋₁₆₉-VA₁-AS (top right) and its 90° rotation (bottom right) around its major axis.

prior to SAXS experiments (Figs. 2, 3). All samples demonstrated a no dependence of r_H on sample concentration, confirming their suitability for SAXS experiments (Fig. 3). Confidence statistics ($\chi \sim 1$ in all cases) on individual models indicate an excellent fit with the raw scattering data. NSD values (less than 0.9 in all cases) confirm that the individual models share similar structural features. Model-based calculation of hydrodynamic parameters are within error of the experimentally determined values and, importantly, are cross-validated with the independently determined r_H values from DLS experiments. DLS experiments performed after SAXS data collection for each sample demonstrated absence of any sample degradation due to radiation damage. Finally, by using BUNCH modeling with the known NMR solution structure of PKR_{1–169}, it was possible to recapitulate the determined SAXS model and potentially orient the individual dsRBMs to specific regions of the model. Taken together, these results give us high confidence in the determined solution conformations.

Models of RNA–protein complexes were determined using MONSA, software that ultimately allows for the separate visualization of both protein and RNA components. The experimentally determined solution structure of TAR in complex with the tandem dsRBMs of PKR (PKR_{1–169}) highlights that nearly the entire length of the protein tracks a curved path to form contacts with the RNA stem–loop, involving both dsRBMs and the linker that joins them (Fig. 6A). This is consistent with the observation that high-affinity binding to TAR requires both dsRBMs of PKR, and NMR chemical shift perturbation experiments that have identified the binding interface to involve both dsRBMs and the linker (Kim et al. 2006). A striking feature of the model is the apparent decrease in flexibility of TAR RNA upon interaction with PKR_{1–169} and the ability to visualize both the stem and loop portions of TAR RNA (Fig. 6A; Supplemental Fig. S1). Based on our modeling results of the free protein, we propose that dsRBM1 is responsible primarily for interaction with the loop, whereas the linker and dsRBM2 primarily interact with the stem region of TAR RNA. These results are supported by preliminary NMR data that indicate that site-specific spin-labeling of dsRBM2 broadens imino proton resonances in the stem region far from the loop region (Supplemental Fig. S2). The model also suggests that the C-terminal end of the protein (dsRBM2) may be oriented such that the 80-residue interdomain linker and kinase domain are not directly affected by interaction with the RNA; similar observations have been made previously (McKenna et al. 2007). The RNA sequesters the tandem dsRBMs entirely, which would preclude the RNA from interacting with another molecule of PKR or an interaction between the dsRBMs and kinase domains from a single PKR molecule.

While the model resolution is not sufficient to confirm the nucleotides involved, the observed TAR loop structure suggests that a region larger than the expected hexaloop may

be involved in loop formation. The TAR RNA hexaloop is highly dynamic (Jaeger and Tinoco 1993) and has been shown to adopt significantly different conformations, depending on its association with ligands including small molecules, peptides, proteins, and other nucleic acid molecules (Bardaro et al. 2009; Fulle et al. 2010). Concerted motions in TAR RNA have been suggested as the basis for access to bound state conformations (Al-Hashimi et al. 2002). HIV-2 TAR undergoes similar conformational changes (Raghunathan et al. 2006). Other dsRBM-containing proteins such as *Saccharomyces cerevisiae* Rnt1p (Wu et al. 2004) and *Drosophila* Staufien (Ramos et al. 2000) have shown a preference for loop regions, and mutation of the TAR hexaloop, bulge, or stem results in a significant reduction in binding affinity to PKR (Kim et al. 2006). Therefore, there is significant experimental basis for recognition of the TAR loop via the dsRBMs of PKR.

Our SAXS, DLS, and size exclusion chromatography data are all consistent with a 1:1 complex stoichiometry under the conditions in which the experiments were performed. With the experimentally determined hydrodynamic parameters, we were unable to fit the acquired scattering data to any other complex stoichiometry. These results are consistent with previous NMR experiments in which the same complex stoichiometry is observed (Kim et al. 2006; McKenna et al. 2007). It is important to stress that this study has been focused on a bound conformation of the PKR–TAR complex, and no comment can be made on whether this complex represents an active conformation. TAR RNA dimerization has been suggested as a potential mechanism required for activation (Heinicke et al. 2009), and while our data cannot preclude this possibility, it does not directly support it either.

We additionally examined a second viral dsRNA stem–loop, VA₁-AS, in order to determine the generality of our results. Previously performed NMR experiments have indicated that identical amino acid residues in PKR are responsible for the interaction with both TAR and VA₁-AS and that similar regions of the stem–loop structure are responsible for interaction with PKR (McKenna et al. 2006). Free VA₁-AS is more conformationally constrained than TAR, and the same trend is observed once it is bound to PKR_{1–169}, as VA₁-AS undergoes only moderate conformation changes (Figs. 5, 6). However, the SAXS envelope of TAR and VA₁-AS-containing complexes have similar overall domain orientations and hydrodynamic parameters, consistent with a similar mode of interaction supporting previous studies (McKenna et al. 2006).

Overall, the work presented demonstrates how tandem dsRBMs are capable of tolerating a wide range of sequence and structural motifs to function in the innate immune response to viral infection. A rigorous characterization of additional dsRNA ligands, and the full length protein, will help to resolve the full spectrum of interactions possible for the dsRBMs of PKR.

MATERIALS AND METHODS

PKR₁₋₁₆₉ expression and purification

Recombinant human PKR₁₋₁₆₉ protein was expressed and purified according to the method previously described (McKenna et al. 2006). Affinity purified protein was subjected to size exclusion chromatography using a HiLoad 26/60 Superdex 75 gel filtration column (2.6 × 60 cm, GE Healthcare Life Sciences) in 50 mM Tris (pH 7.50), 100 mM NaCl, and 5 mM 2-mercaptoethanol (2-Me). The elution fractions were monitored by means of absorbance at 280 nm, and fractions containing purified protein were combined and concentrated using Millipore concentrator filters (M_w cut-off, 3000; Millipore). At all stages, protein purity is checked by SDS-PAGE, and the concentration was determined using the known extinction coefficient measured by spectrophotometry (NanoDrop 2000c, Thermo Scientific).

RNA preparation and purification

HIV-1 TAR and adenovirus VA₁-AS RNA were in vitro transcribed from linearized template under control of the T7 RNA polymerase promoter and purified by gel filtration chromatography according to the method previously (McKenna et al. 2006). Purified RNA were subsequently concentrated using Millipore concentrator filters (M_w cutoff, 3000; Millipore). The purity of the transcribed RNA was verified by native polyacrylamide gel electrophoresis (in 1× TBE buffer), and the concentration was determined by spectrophotometer.

RNA–protein complex formation

PKR₁₋₁₆₉–TAR and PKR₁₋₁₆₉–VA₁-AS complexes were prepared by incubating purified PKR₁₋₁₆₉ in the presence excess RNA in a buffer containing 50 mM Tris (pH 7.50) and 100 mM NaCl for 15 min at room temperature. After incubation, the mixture was applied on a HiLoad 26/60 Superdex 75 gel filtration column (2.6 × 60 cm, GE Healthcare Life Sciences). Elution fractions were assayed for the presence of RNA–protein complex via in-line spectrophotometer (260/280 nm simultaneously) and were confirmed by native PAGE. Fractions containing RNA–protein complex were pooled and concentrated in Millipore concentrators (M_w cut off, 3000; Millipore). The purity of both complexes was assessed by the native polyacrylamide gel electrophoresis, and the concentrations were determined spectrophotometrically using RNA extinction coefficients and assuming the complex had 1:1 stoichiometry. EMSAs were performed using native polyacrylamide gels as described previously (Booy et al. 2012), with the exception that samples were prepared in a different buffer (50 mM Tris at pH 7.50, 100 mM NaCl).

Dynamic light scattering

Prior to the examination by DLS, all samples were dialyzed for 2 h at 4°C against 50 mM Tris (pH 7.50), 100 mM NaCl. After dialysis, all the macromolecules were subjected to filtration through a 0.1-μm filter (Millipore), and samples were equilibrated at 20°C prior to data acquisition. The hydrodynamic radius and homogeneity of each sample were examined using the Zetasizer Nano S system (Malvern Instruments) equipped with a 4-mW laser ($\lambda = 633$ nm)

according to the method previously described (Meng et al. 2012). The DLS experiments for each sample were performed at multiple concentrations: 2.50–6.50 mg/mL for PKR₁₋₁₆₉, 0.70–1.30 mg/mL for TAR, 0.80–1.90 mg/mL for VA₁-AS, 0.30–1.10 mg/mL for the PKR₁₋₁₆₉–TAR complex, and 0.70–1.30 mg/mL for the PKR₁₋₁₆₉–VA₁-AS complex. DLS data were analyzed using DTS software (version 6.01, Malvern Instruments).

Small angle X-ray scattering

DLS data for PKR₁₋₁₆₉, RNA, and PKR₁₋₁₆₉–RNA complexes were collected prior to SAXS data collection to confirm that all the samples are highly pure and suitable for data collection. The SAXS data for TAR were collected at 0.7, 1.0, 1.3 mg/mL; for VA₁-AS at 1.0, 1.4, and 1.9 mg/mL; for PKR₁₋₁₆₉ at 3.5, 4.5, and 5.5 mg/mL; for PKR₁₋₁₆₉–TAR complex at 0.80, 1.20, and 2.10 mg/mL; and PKR₁₋₁₆₉–VA₁-AS complex at 0.60, 0.9, and 1.63 mg/mL (all in 50 mM Tris at pH 7.50, 100 mM NaCl buffer). The data collection was performed on a Rigaku S-MAX3000 equipped with a Rigaku MicroMax+002 microfocus sealed tube (Cu K α radiation at 1.54 Å) and a Confocal Max-Flux (CMF) optics system operating at 40 W (Rigaku). Scattering data were recorded using a 200-mm multiwire two-dimensional detector. The data for all three samples and buffer were collected for 3 h for each sample within the range of $0.008 \leq s \leq 0.26 \text{ \AA}^{-1}$ and processed according to the method previously described, where $s = 4\pi \sin \theta / \lambda$ (Patel et al. 2010, 2011, 2012). Briefly, the sample data were subtracted from the buffer data using the program PRIMUS (Konarev et al. 2003) followed by merging of buffer subtracted data for each sample and the complex. The data were further processed using the GNOM program (Svergun 1992) to obtain radius of gyration (r_G) and maximal particle dimension ($D_{\max}$). The ab initio modeling for protein and individual RNA molecules was performed using the program DAMMIF (Franke and Svergun 2009). The program MONSA (Svergun 1999) was employed to calculate ab initio models of protein–RNA complexes by fitting data for protein, RNA, and the complex simultaneously. The quality of the models was verified by the goodness of fit parameter (χ -value) after each model calculation. Ab initio models for each sample and complex were then rotated and averaged using the program DAMAVER (Volkov and Svergun 2003) to obtain a representative conformation where the NSD parameter was used to determine the quality of superimposition of ab initio models.

The three-dimensional structures of PKR₁₋₁₆₉ were calculated using the program BUNCH (Petoukhov and Svergun 2005), where high-resolution data from the PDB file 1QU6 (Nanduri et al. 1998) was used and fitted with the SAXS data. Both domains of PKR₁₋₁₆₉ were allowed to rotate freely and find their optimized positions and orientations according to the method described previously (Patel et al. 2011, 2012). The quality of the models was checked by means of the determined χ -value. To produce Figure 5B, individual bunch models were superimposed on average ab initio model using supcomb (version 2.0) (Kozin and Svergun 2001).

Comparison of hydrodynamic properties

The program HYDROPRO (de la Torre et al. 2000) was employed to calculate solution properties such as hydrodynamic radius, radius of gyration, and maximal particle dimension for each model calculated using SAXS data for PKR₁₋₁₆₉, free RNA, and the PKR₁₋₁₆₉–RNA

complexes. The input parameters for HYDROPRO calculations included molecular weight, partial specific volume ($\bar{v}$), as well as density and viscosity of the buffer. A buffer density of 1.0038 g/mL and a viscosity of 0.01026 Poise were calculated using the program SEDNTERP (Laue et al. 1992). The sequence molecular weight (18831.5) and partial specific volume (0.738 mL/g) of PKR₁₋₁₆₉ was calculated using the ProtParam utility available on the ExPaSy server and the SEDNTERP program, respectively. The sequence molecular weight for HIV-1 TAR (18354.4) and VA_I-AS (17165.6), as well as the partial specific volumes for HIV-1 TAR (0.5689 mL/g), and VA_I-AS (0.569 mL/g), were calculated using NucProt Calculator (Voss and Gerstein 2005). Additionally, the partial specific volume for the complex of PKR₁₋₁₆₉-HIV-1 TAR as well as PKR₁₋₁₆₉-VA_I-AS was calculated using Equation 1:

$$\bar{v}_{\text{complex}} = \frac{(M_{\text{PKR}}\bar{v}_{\text{PKR}} + M_{\text{RNA}}\bar{v}_{\text{RNA}})}{(M_{\text{PKR}} + M_{\text{RNA}})} \quad (1)$$

SUPPLEMENTAL MATERIAL

Two supplemental figures are included in order to improve the quality and clarity of the manuscript. A description of the data is enclosed within the appropriate figure legend.

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