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Crystal and solution structure of the human RIG-I SF2 domain

RIG-I is a pathogen-recognition receptor that recognizes viral 5'-triphosphates carrying double-stranded RNA. Upon binding to these microbe-associated molecular patterns (MAMPs), RIG-I forms oligomers and promotes downstream processes that result in type I interferon production and induction of an antiviral state. Here, the crystal structure of the human RIG-I superfamily 2 ATPase domain crystallized in an unusually elongated and open conformation is reported. The elongated structure is probably induced in part by crystal packing, but nevertheless indicates that the domain is intrinsically very flexible. This flexibility might allow substantial structural changes upon substrate binding and oligomerization.

1. Introduction

The innate immune system detects invading pathogens *via* microbe-associated molecular patterns (MAMPs). Proteins belonging to the group of pattern-recognition receptors (PRR) have evolved to specifically sense MAMPs such as viral RNA or bacterial cell-wall components. The formation of PRR–MAMP complexes triggers signalling cascades that lead to host defence mechanisms by activation of nuclear factor- κ B and interferon regulatory factors. The resulting production of inflammatory cytokines and type I interferons induces an antiviral state and activates the adaptive immune response.

PRRs can be membrane-bound, such as the Toll-like receptors (TLRs), or cytoplasmic, with the RIG-I-like receptor family being one prominent example. The latter comprises the name-giving RIG-I (retinoic acid inducible gene-I), MDA-5 (melanoma differentiation-associated gene 5) and LGP-2 (Laboratory of Genetics and Physiology 2). These three proteins are involved in the sensing of a variety of viruses. RIG-I, for example, senses *Rabies virus*, *Hepatitis C virus*, influenza viruses and others (Goubau *et al.*, 2013; Hornung *et al.*, 2006; Kato *et al.*, 2006; Loo *et al.*, 2008; Saito *et al.*, 2008), while MDA-5 detects, for example, picornaviruses (Goubau *et al.*, 2013; Gitlin *et al.*, 2006). Structurally, RIG-I, MDA-5 and LGP-2 are closely related to DEAD-box helicases and form a family within the superfamily 2 RNA helicases/ATPases (Fairman-Williams *et al.*, 2010). Apart from the helicase (SF2) domain, RIG-I comprises two N-terminal CARDs (caspase activation and recruitment domains), which are essential for downstream signalling (Gack *et al.*, 2007; Meylan *et al.*, 2005; Saito *et al.*, 2007; Zeng *et al.*, 2010), and a C-terminal regulatory domain (RD) involved in substrate binding and specificity (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006; Schlee *et al.*, 2009; Schmidt *et al.*, 2009).

Recently, several structures have been reported that shed light on the working mechanism of RIG-I. The first structural information was obtained for the RIG-I RD alone (Cui *et al.*, 2008; Takahashi *et al.*, 2008) and in complex with 5'-triphosphate and blunt-end dsRNA (Kowalinski *et al.*, 2011; Lu *et al.*, 2010; Wang *et al.*, 2010), supporting the model of the RD being responsible for the specificity of RIG-I by binding to 5'-triphosphate RNA (Vela *et al.*, 2012). Further studies reported the structures of the RIG-I SF2 domain alone, RNA complexes of RIG-I SF2 and RIG-I SF2–RD, and the structure of RIG-I CARD–SF2 (Civril *et al.*, 2011; Ferrage *et al.*, 2012; Jiang *et al.*, 2011; Kohlway *et al.*, 2013; Kowalinski *et al.*, 2011; Luo *et al.*, 2011, 2012).

Table 1

Crystal parameters and data-collection and refinement statistics for RIG-I SF2.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	$P2_12_12_1$
Molecules per asymmetric unit	2
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 98.9$, $b = 113.0$, $c = 124.3$, $\alpha = \beta = \gamma = 90$
Solvent content (%)	53.2
Matthews coefficient ($\text{\AA}^3 \text{Da}^{-1}$)	2.63
Data-collection statistics	
Diffraction source	SLS beamline X06SA
Wavelength ($\text{\AA}$)	0.99987
Detector	PSI Pilatus 6M pixel
Diffraction protocol	Single wavelength
Data-processing statistics	
Resolution range ($\text{\AA}$)	50–2.70
No. of observed reflections	140661
No. of unique reflections	38287 (6016)
Completeness (%)	99.0 (98.4)
Multiplicity	3.67 (3.59)
$\langle I/\sigma(I) \rangle$	20.1 (2.2)
R_{meas} (%)	5.2 (77.4)
Refinement	
Resolution ($\text{\AA}$)	49.06–2.71
No. of reflections used	38264
R_{work} (%)	21.9 (31.5)
R_{free} (%)	25.8 (36.8)
No. of atoms	
Total	8183
Protein	8126
Ligand	21
Water	36
Wilson B factor	68.53
Average B factors ($\text{\AA}^2$)	
Protein	71.03
Ligand	67.69
Water	68.52
R.m.s. deviations	
Bond lengths ($\text{\AA}$)	0.006
Bond angles ($^\circ$)	0.907
Ramachandran plot analysis	
Favoured (%)	96.0
Allowed (%)	4.0
Disallowed (%)	0
PDB code	4on9

The available structures allow the formulation of a mechanism for RIG-I activation. In the inactive state the second CARD domain contacts the insertion domain of the RIG-I SF2 (Hel2i), leading to an auto-inhibited state. RNA ligands bind to the RIG-I RD and SF2 domain and compete with CARDS at the helical insertion domain (Hel2i). In the resulting clamp-like structure of RIG-I SF2 around the dsRNA substrate, ATP binding leads to further compaction. This clamp formation results in the release of the two CARD domains from the auto-inhibited state (Ferrage *et al.*, 2012; Jiang *et al.*, 2011; Kohlway *et al.*, 2013; Kowalinski *et al.*, 2011; Leung & Amarasinghe, 2012; Luo *et al.*, 2011, 2012). The exposed CARDS interact with ubiquitin and CARDS of a downstream adaptor (Peisley *et al.*, 2014).

Here, we report the crystal structure of the human RIG-I SF2 in an unusual elongated conformation. Additional SAXS (small-angle X-ray scattering) studies support the conformational flexibility of this domain, which could play an important role in the formation of higher order structures or in initial substrate recognition.

2. Materials and methods

2.1. Cloning, expression and purification of Hs-RIG-I HD

Human RIG-I SF2 (hsRIG-I SF2; comprising residues 230–793 of the full-length protein) was cloned into pETDuet vector (Novagen) via *EcoRI* and *NotI* restriction sites. Transformed *Escherichia coli*

Rosetta (DE3) cells were grown to an OD_{600} of 0.8 before induction with IPTG (0.2 mM final concentration). Expression was carried out overnight at 18°C. The harvested cells were resuspended in lysis buffer (50 mM Tris pH 7.3, 500 mM NaCl, 10 mM imidazole, 4 mM DTT) and lysed by sonication. The N-terminally His₆-tagged hsRIG-I SF2 was first purified by Ni-affinity chromatography and the hsRIG-I SF2-containing elution fractions were dialyzed against 20 mM Tris pH 7.3, 100 mM NaCl, 4 mM DTT. In a second step this protein solution was applied onto a combination of anion- and cation-exchange resins (HiTrap Q/SP FF, GE Healthcare) and the flow-through was collected as the construct does not bind to these columns under the buffer conditions used. As a final purification step, size-exclusion chromatography was performed (Superdex S200 prep grade, GE Healthcare, running buffer 20 mM Tris pH 7.3, 100 mM NaCl, 4 mM DTT). Purified hsRIG-I SF2 could be concentrated to 60 mg ml^{−1} and aliquots were flash-frozen in liquid nitrogen.

2.2. Crystallization and data collection

HsRIG-I SF2 crystals were grown in hanging-drop vapour-diffusion geometry at 15°C. Crystals suitable for data collection were obtained by mixing 1 μl hsRIG-I SF2 solution with 1 μl reservoir solution [100 mM HEPES pH 6.8, 100 mM ammonium sulfate, 6.7% (v/v) 2-propanol, 9% (v/v) PEG 4000] with a total reservoir volume of 250 μl in the well. Crystals appeared after 4 d. For cryo-protection, the crystals were soaked in reservoir solution containing 25% ethylene glycol and flash-cooled in liquid nitrogen. Diffraction data were collected to 2.7 $\text{\AA}$ resolution on the X06SA beamline of the Swiss Light Source, Villigen, Switzerland. Diffraction data were indexed and integrated using *XDS* and scaled with *XSCALE* (Kabsch, 2010a,b). Statistics of the collected data set are shown in Table 1.

2.3. Structure solution and refinement

The structure of hsRIG-I SF2 was solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007) within the *CCP4* program suite (Winn *et al.*, 2011). Calculation of the Matthews coefficient with the space-group parameters (Table 1) and the molecular weight of hsRIG-I SF2 suggested two molecules per asymmetric unit, with a solvent content of 53.2% (Matthews, 1968). Molecular replacement was performed using an approach with the three isolated domains of the mouse RIG-I SF2 (PDB entry 3tbk; Civril *et al.*, 2011) as search models. For refinement of the initial maps a randomly chosen 5% of the reflections were used as a test set for cross-validation (Brünger, 1992). Refinement was performed in cyclic rounds of manual model building using *Coot* (Emsley *et al.*, 2010) and refinement using *PHENIX* (Afonine *et al.*, 2012). As a last step, water molecules were added automatically using *PHENIX* and subsequently manually checked and refined again. The statistics of the refined model are shown in Table 1.

2.4. Validation and deposition

The stereochemistry of the hsRIG-I SF2 domain was analyzed by *PROCHECK* (Laskowski *et al.*, 1996). All residues lie within the favoured and allowed regions of the Ramachandran plot (see Table 1). The atomic coordinates and structure factors of hsRIG-I SF2 have been deposited in the Protein Data Bank as entry 4on9.

2.5. Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering experiments were conducted at the X33 beamline at the European Molecular Biology Laboratory/

Deutsches Elektronen-Synchrotron, Hamburg, Germany. Samples were measured using a wavelength of $1.5 \text{ \AA}$ and with a q range from 0.06 to 6 nm^{-1} (detector distance $d = 2.7 \text{ m}$). Scattering data were recorded with a Pilatus 1M detector. Prior to measurement, samples were purified by size-exclusion chromatography and additionally centrifuged. All samples were monodisperse as could be judged from size-exclusion chromatography and dynamic light-scattering

(Malvern-Viscotek 802 DLS) measurements. Scattering of the running buffer of this size-exclusion column (20 mM Tris pH 7.3, 100 mM NaCl, 4 mM DTT) was used for buffer correction of the protein sample measurements. Scattering data were recorded for hsRIG-I SF2 samples with concentrations of 2.5, 5 and 10 mg ml^{-1} (determined spectrophotometrically using $A_{280 \text{ nm}}$ and the molar extinction coefficients). The samples did not show signs of radiation

Figure 1

(a) Crystal structure of human RIG-I SF2. Subdomains are represented in different colours: Hel1, red; Hel2, brown; Hel2i, blue; bridging domain, orange. (b) Superposition of the ATP-binding site of the RIG-SF2 ADP-AlF₃-bound structure (PDB entry 4a36, shown as lines; Kowalinski *et al.*, 2011) and the corresponding region of the structure reported in this study (shown as sticks). The well coordinated sulfate ion occupies the space of the β -phosphate of the nucleotide-bound state. (c) Overlay of human (blue, this study), mouse (red, PDB entry 3tbk; Civril *et al.*, 2011) and duck (orange, PDB entry 4a2p; Kowalinski *et al.*, 2011) RIG-I SF2 crystal structures. (d) Cartoon representation of the crystallographic dimer with one monomer shown as in (a) and the second one in light blue. (e) Guinier plot [$\ln(I)$ versus s^2] of hsRIG-I SF2 SAXS data with linear regression (for points shown in red) used for determination of radius of gyration ($R_g = 3.37 \pm 0.01 \text{ nm}$). The lower plot shows the residuals of the linear regression, indicating that the sample does not show aggregation or repulsive effects. (f) Comparison of measured hsRIG-I SF2 SAXS data (5 mg ml^{-1}) with theoretical scattering curves of RIG-I SF2 crystal structures calculated using CRY SOL (Svergun *et al.*, 1995). HsRIG-I SF2 (this study, PDB entry 4on9, blue curve), $\chi^2 = 15.0$; mouse (PDB entry 3tbk, red curve), $\chi^2 = 2.6$; duck (PDB entry 4a2p, orange curve), $\chi^2 = 2.9$.

damage, which was assessed by automatic and manual comparison of eight consecutive 15 s exposure frames. Data were processed using *PRIMUS* from the *ATSAS* package (Konarev *et al.*, 2006). The protein molecular weight in solution was determined from the zero-angle scattering intensity $I(0)$ obtained from extrapolation of the Guinier plot [$\ln I(s)$ versus s^2] region obeying the Guinier approximation for globular proteins ($s \times R_g < 1.3$) and from the Porod volume. BSA (66 kDa) was used as an $I(0)$ reference. Theoretical scattering curves were calculated using *CRY SOL* (Svergun *et al.*, 1995).

2.6. Size-exclusion chromatography coupled right-angle laser light scattering (SEC–RALS)

SEC–RALS data were measured using a Malvern/Viscotek TD270 right-angle laser light scattering detector and a VE3580 refractive-index detector connected to an analytical ÄKTAmicro chromatography system equipped with a 24 ml 10/300 GL Superdex S200 column (GE Healthcare). A BSA standard (66 kDa) was used to calibrate the system. For data evaluation the program package equipped with the instrument was used (*OmniSEC*, Malvern/Viscotek). The RIG-SF2 construct ($c = 3 \text{ mg ml}^{-1}$) eluted as expected as a sharp single peak without any signs of aggregation in the SEC run.

3. Results and discussion

Human RIG-I SF2 crystallizes in the primitive orthorhombic space group $P2_12_12_1$ with two molecules per asymmetric unit. Traceable electron density could be found for the core hsRIG-I SF2 residues Phe241–Asp792 in chain *A* and Lys242–Ser793 in chain *B* and 36 solvent molecules interpreted as water, four sulfate ions and one chloride ion. The resolution of the structure is 2.7 Å and the model could be refined to an R_{work} and R_{free} of 0.219 and 0.258, respectively. As described previously (Civril *et al.*, 2011; Jiang *et al.*, 2011; Kowalinski *et al.*, 2011; Luo *et al.*, 2011), the structure comprises the two RecA-like domains Hel1 (242–444) and Hel2 (455–466 and 609–742) as well as the insertion domain Hel2i (271–603) and the bridging domain (745–793) (Fig. 1*a*). One sulfate ion coordinated by the P-loop (266–271) of the Hel1 domain occupies the space of the β -phosphate of ADP (Fig. 1*b* and Supplementary Fig. S1¹).

In contrast to the C-shaped conformation of the SF2 domain observed previously, hsRIG-I SF2 crystallizes in an unusual elongated conformation in which Hel1 and the bridging domain are bent away from the rigid Hel2i–Hel2 module with an angular span of about 45° (Fig. 1*c*). The α -helical linker (445–454) and the short N-terminal stretch at the beginning of the bridging domain (743–745) serve as hinges. Conformational flexibility of RIG-I SF2 crystal structures has been observed before (Kowalinski *et al.*, 2011), but the structure described here is substantially more elongated than the previously described structures.

Two RIG-I SF2 domains build up a crystallographic dimer (Fig. 1*d*), with the Hel2i domain contacting the Hel1 domain of the neighbouring molecule and *vice versa*. The molecular weight determined by SEC–RALS ($M_w^{\text{SEC–RALS}} = 64 \text{ kDa}$; Supplementary Fig. S2) and SAXS data indicate that hsRIG-I SF2 is monomeric in solution ($M_w^{\text{theoretical}} = 66.0 \text{ kDa}$), but it is possible that in RNA-activated RIG-I oligomers some contacts are mediated by the SF2 domains in addition to the CARD–CARD interactions.

To address solution conformations of the hsRIG-I SF2, we measured SAXS of the hsRIG-I SF2 construct. We could not observe signs of aggregation as judged from the linear Guinier plot (Fig. 1*e*). The molecular weight derived from SAXS data in the concentration range $c = 2.5$ – 10 mg ml^{-1} could only be explained by a monomeric hsRIG-I SF2 species in solution; even the highest concentration did not show signs of higher oligomers [$M_w^{I(0); c=10 \text{ mg ml}^{-1}} = 60.1 \text{ kDa}$; $M_w^{\text{Porod}; c=10 \text{ mg ml}^{-1}} = 55.5 \text{ kDa}$]. We then compared the experimental scattering data with the theoretical scattering curve of our crystallographic model. The obvious differences between the theoretical scattering curve of our model and the solution scattering curve (Fig. 1*f*) suggest that the crystallographically observed conformation is influenced by crystal packing (Supplementary Fig. S3) and does not represent a major conformation in solution. Notably, the solution scattering of the human RIG-I SF2 construct is very similar to the theoretical scattering curves of mouse and duck RIG-I SF2 crystal structures (Civril *et al.*, 2011).

In summary, the data support the current model that RIG-I SF2 possesses a high degree of conformational flexibility necessary for the formation of the different observed clamp conformations upon RNA and ATP binding and ATP-hydrolysis state, respectively (Kohlway *et al.*, 2013; Luo *et al.*, 2011, 2012). Therefore, the structure described here, even though not populated to a major degree in solution, expands the conformational spectrum of RIG-I SF2 that is likely to be needed to recognize specific MAMPs and form oligomeric structures for type I interferon activation.

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¹ Supporting information has been deposited in the IUCr electronic archive (Reference: TB5069).

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