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Expression, purification, crystallization and preliminary X-ray analysis of full-length human RIG-I

The human innate immune system can detect invasion by microbial pathogens through pattern-recognition receptors that recognize structurally conserved pathogen-associated molecular patterns. Retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) are one of the two major families of pattern-recognition receptors that can detect viral RNA. RIG-I, belonging to the RLH family, is capable of recognizing intracellular viral RNA from RNA viruses, including influenza virus and Ebola virus. Here, full-length human RIG-I (hRIG-I) was cloned in *Escherichia coli* and expressed in a recombinant form with a His-SUMO tag. The protein was purified and crystallized at 291 K using the hanging-drop vapour-diffusion method. X-ray diffraction data were collected to 2.85 Å resolution; the crystal belonged to space group \( P2_1 \), with unit-cell parameters 

\[
\begin{align*}
\alpha &= \beta = \gamma = 90^\circ \\
a &= b = c &= 216.43 \text{ Å}
\end{align*}
\]

1. Introduction

The human innate immune system senses viral infection by various types of viral components. Pattern-recognition receptors play essential roles in recognizing different pathology-associated molecular patterns such as viral DNA, RNA and glycoproteins, leading to the activation of a signalling cascade for various immunological responses. Amongst the families of pattern-recognition receptors, the retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), RIG-I and melanoma differentiation-associated protein 5 (MDA-5), detect viral RNA (vRNA) in the cellular environment and activate host immunological responses by producing pro-inflammatory cytokines and type I interferons (IFNs; Yoneyama *et al.*, 2005).

Members of the RLH family share a similar architecture consisting of a conserved helicase domain and a C-terminal regulatory domain (CTD). In addition, RIG-I and MDA-5 contain two tandem caspase activation and recruitment domains (CARDs) at their N-terminus critical for the activation of the downstream immune response cascade (Loo *et al.*, 2008; Takahasi *et al.*, 2009). The helicase domain is responsible for vRNA interaction, ATP binding and hydrolysis. The CTD has been suggested to inhibit antiviral signalling in the absence of vRNA by unfolding partially and interfering in the formation of active CARDs (Luo *et al.*, 2011, 2012). Linking the helicase domain and the CTD is a V-shaped pincer, which plays roles in vRNA sensing and domain coordination (Cui *et al.*, 2008).

Despite being similar in domain organization, RIG-I and MDA-5 display distinctively different specificity in the recognition of the vRNA. RIG-I triggers innate immune responses during infection by paramyxoviruses, orthomyxoviruses, flaviviruses and rhabdoviruses, while MDA-5 initiates the response during picornavirus infection (Loo *et al.*, 2008). In addition, while MDA-5 requires double-stranded RNA (dsRNA) of at least 100 base pairs in length for IFN initiation (Wu *et al.*, 2013), RIG-I only requires dsRNA of a minimum length of ten base pairs (Kohlway *et al.*, 2013).

Upon vRNA recognition, the RIG-I CARDs are released and ubiquitinated by tripartite motif-containing protein 25 (TRIM25), a RING-finger E3 ubiquitin ligase (Gack *et al.*, 2007). The ubiquitinated RIG-I CARDs then recruit the adaptor protein interferon-β promoter stimulator 1 (IPS-1) via homotypic CARD–CARD inter-
actions (Kawai et al., 2005; Potter et al., 2008). The binding of IPS-1 to RIG-I triggers the activation of downstream signalling cascades for the elicitation of innate immune responses (Seth et al., 2005; Yoneyama et al., 2005).

X-ray crystallographic structures of truncated forms of human RIG-I (hRIG-I) containing the helicase and CTD domains in complex with dsRNA are available (Cui et al., 2008; Jiang et al., 2011; Luo et al., 2011, 2012). The NMR structure of the hRIG-I CARD domain alone has also recently been reported (Ferrage et al., 2012). The structure of the truncated form of duck RIG-I containing the CARD and helicase domains has also been reported by Kowalinski and coworkers (PDB entry 4a2w; Kowalinski et al., 2011) and shares only 53% sequence identity with the human protein. Solution of the X-ray crystallographic structure of full-length hRIG-I in its inactive state, together with the existing structures of truncated forms of hRIG-I and duck RIG-I, would provide insights into the molecular mechanism of the activation of this protein upon the detection of viral proteins in human host cells. In this report, we describe the cloning, overexpression, purification and X-ray crystallographic analysis of the inactive form of full-length hRIG-I crystallized in the absence of RNA.

2. Materials and methods

2.1. Protein expression and purification

The cDNA encoding the full-length hRIG-I was amplified using PCR and inserted into the bacterial expression vector Champion pET-SUMO (Invitrogen, USA) via the method of TA cloning. The resultant plasmid encodes full-length hRIG-I with an N-terminal His-SUMO protein tag. The pET-SUMO-hRIG-I plasmid was transformed into Escherichia coli BL21 Rosetta 2 (DE3) competent cells (Novagen, USA) for protein expression. The transformed cells were grown in Luria broth (LB) with 34 μg ml⁻¹ kanamycin and 34 μg ml⁻¹ chloramphenicol at 310 K, and protein expression was induced with a final concentration of 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) once the OD₆₀₀ had reached 0.6–0.8. The temperature was lowered to 289 K and the bacterial cell culture was incubated for a further 20 h for protein expression. The cell pellet was harvested by centrifugation at 4000g for 15 min at 277 K. The cells were resuspended and sonicated on ice with 60 ml resuspension buffer (1× PBS pH 7.4, 500 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol) containing Complete EDTA-free protease-inhibitor cocktail (Roche, Germany). The lysate was clarified by high-speed centrifugation at 35 000g at 277 K for 30 min. The supernatant was then passed through a HisTrap HP column (GE Healthcare, Sweden) equilibrated with resuspension buffer. Proteins were eluted in elution buffer [1× PBS pH 7.4, 500 mM NaCl, 5%(v/v) glycerol, 2 mM β-mercaptoethanol, 500 mM imidazole]. The fraction containing His-SUMO-tagged hRIG-I was dialysed overnight at 277 K with dialysis buffer [25 mM HEPES pH 7.4, 500 mM NaCl, 10%(v/v) glycerol, 2 mM DTT] and at the same time digested with SUMO protease (Invitrogen, USA) at a ratio of 1:100. The cleavage mixture was loaded onto a HisTrap HP column equilibrated with resuspension buffer. Cleaved hRIG-I was eluted in the flowthrough, while the His-SUMO tag and uncleaved hRIG-I were eluted in the elution buffer. Cleaved hRIG-I was further purified using a Superdex 200 HiLoad 16/60 size-exclusion column (GE Healthcare, Sweden) with gel-filtration buffer [25 mM HEPES pH 7.5, 500 mM NaCl, 5%(v/v) glycerol, 2 mM DTT]. Fractions containing hRIG-I were analysed by 10% SDS–PAGE (Fig. 1), pooled and concentrated using Amicon Ultra centrifugal concentrators (50 kDa cutoff, Millipore, USA). The concentration of hRIG-I was determined spectrophotometrically at 280 nm with a NanoVue Plus spectrophotometer (GE Healthcare, Sweden) and calculated using the mass extinction coefficient (E₁₃₆) of hRIG-I, which is 9.35.

2.2. Crystallization

A Mosquito liquid handler (TTP LabTech, UK) was used to set up high-throughput crystallization trials of 576 conditions using the sitting-drop vapour-diffusion method in MRC 2-drop 96-well crystallization plates. In each well, 100 nl 15 mg ml⁻¹ hRIG-I in gel-filtration buffer was mixed with an equal volume of crystallization solution at 291 K. Of the 576 conditions screened, initial hits were observed in Protein Complex Suite (Qiagen, USA) condition No. 88 (1.4 M sodium malonate pH 6.0) and Grid Screen Sodium Malonate (Hampton Research, USA) condition C3 (1.9 M sodium malonate pH 6.0). Optimization of crystals was set up manually in VDX 24-well crystallization plates (Hampton Research, USA) with a reservoir volume of 500 μl with a varying range of sodium malonate concentration and pH. Following optimization, diffraction-quality crystals of

![Figure 1](image1.png)

**Figure 1**
10% SDS–PAGE analysis of purified hRIG-I. Lane 1, molecular-mass markers (labelled in kDa). Lane 2, concentrated hRIG-I after gel filtration.

![Figure 2](image2.png)

**Figure 2**
Optimized crystal of hRIG-I. This crystal was formed using 1.0 M sodium malonate pH 5.5. The dimensions of the optimized crystals were approximately 0.05 × 0.05 × 0.05 mm.
hRIG-I were grown using 1.0–1.4 $M$ sodium malonate pH 5.5 at 291 K by hanging-drop vapour diffusion with 1 μl hRIG-I at a concentration of 15 mg ml$^{-1}$ in gel-filtration buffer mixed with 1 μl crystallization solution.

2.3. Data collection and processing

The crystal was soaked in cryoprotectant containing 3.4 $M$ sodium malonate pH 5.5 for 1 min prior to flash-cooling in liquid nitrogen. Data were collected on beamline 13B1 at the National Synchrotron Radiation Research Centre (NSRRC), Hsinchu, Taiwan. Data collection from the native hRIG-I single crystal was performed at 100 K with an exposure of 45 s per frame and 1° oscillation at a wavelength of 1 Å. A total of 45 frames were collected using an ADSC Quantum 315 CCD detector. Diffraction intensities were integrated and scaled using HKL-2000 (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1.

3. Results and discussion

Recombinant His-SUMO-tagged hRIG-I was expressed in $E$. coli BL21 Rosetta 2 cells (Novagen, USA) and was successfully purified with the His-SUMO tag cleaved (Fig. 1). The cleaved full-length hRIG-I (residues 1–925) exhibited a monomeric form in solution as it eluted at a size of 105 kDa from the size-exclusion chromatography column. The final yield of purified hRIG-I was ~0.5 mg of protein per litre of LB. Initial hits from crystallization screening appeared after 14 d. After optimization of the crystallization conditions, single bipyramidal-shaped crystals of hRIG-I were formed with dimensions of 0.05 x 0.05 x 0.05 mm in 5–7 d (Fig. 2).

A complete native data set was collected and processed to 2.85 Å resolution using a single native hRIG-I crystal on beamline 13B1 at the NSRRC, Taiwan (Fig. 3). The space group is $F\overline{2}3$, with unit-cell parameters $a = b = c = 216.43$, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit is estimated to contain one copy of RIG-I, with a calculated crystal volume per unit molecular weight $V_M$ of 2.01 Å$^3$ Da$^{-1}$, corresponding to a solvent content of 38.8% (Matthews, 1968).

| X-ray source | 13B1, NSRRC |
| Wavelength (Å) | 1.00 |
| Space group | $F\overline{2}3$ |
| Unit-cell parameters (Å, °) | $a = b = c = 216.43$, $\alpha = \beta = \gamma = 90^\circ$ |
| Resolution (Å) | 25.0–2.85 (2.95–2.85) |
| Observed reflections | 110526 |
| Unique reflections | 19544 |
| Multiplicity | 57 (5.6) |
| Completeness (%) | 99.7 (100) |
| $R_{merge}$ | 0.134 (0.710) |
| Average $I/\sigma(I)$ | 9.01 (1.62) |

$R_{merge} = \sum_{i} \sum_{hkl} |I_{i}(hkl)| - \langle I(hkl) \rangle|/\sum_{i} \sum_{hkl} I_{i}(hkl)$. 

Figure 3
Diffraction image of the hRIG-I crystal collected at the NSRRC. The resolution at the edge is 2.80 Å. The inset shows an enlarged view of the diffraction at the edge of the detector.
Solution of the structure of hRIG is currently being actively pursued by molecular replacement using hRIG-I ΔCARD (PDB entry 2ykg; Luo et al., 2011), RIG-I ΔCTD or the helicase domain from duck RIG-I (PDB entries 4a2w and 4a2p; Kowalinski et al., 2011) as search probes.

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References
