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Crystallization studies of the murine c-di-GMP sensor protein STING

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The innate immune response is the first defence system against pathogenic microorganisms, and cytosolic detection of pathogen-derived DNA is believed to be one of the major mechanisms of interferon production. Recently, the mammalian ER membrane protein STING (stimulator of IFN genes; also known as MITA, ERIS, MPYS and TMEM173) has been found to be the master regulator linking the detection of cytosolic DNA to TANK-binding kinase 1 (TBK1) and its downstream transcription factor IFN regulatory factor 3 (IRF3). In addition, STING itself was soon discovered to be a direct sensor of bacterial cyclic dinucleotides such as c-di-GMP or c-di-AMP. However, structural studies of apo STING and its complexes with these cyclic dinucleotides and with other cognate binding proteins are essential in order to fully understand the roles played by STING in these crucial signalling pathways. In this manuscript, the successful crystallization of the C-terminal domain of murine STING (STING-CTD; residues 138–344) is reported. Native and SeMet-labelled crystals were obtained and diffracted to moderate resolutions of 2.39 and 2.2 Å, respectively.

1. Introduction

Cyclic di-GMP (c-di-GMP) is a unique secondary messenger that controls a plethora of cellular activities such as biofilm formation, biogenesis of flagella and pili, secretion of pathogenic factors etc. in diverse bacteria (Romling et al., 2005; Jenal & Malone, 2006; Romling & Amikam, 2006; Hengge, 2009; Schirmer & Jenal, 2009). Its synthesis via GGDEF-domain-containing diguanylate cyclases (DGCs) and degradation via EAL-domain-containing (Tal et al., 1998; Simm et al., 2004; Tischler & Camilli, 2004; Romling et al., 2005) or HD-GYP-domain-containing (Slater et al., 2000; Ryan et al., 2006) phosphodiesterases (PDEs) has been well investigated in recent years. However, it remains unclear how many distinct c-di-GMP receptors are available and how these receptors execute their functions upon c-di-GMP binding in the cell, although a wide variety of different protein-based or RNA-based recognition motifs for c-di-GMP have been discovered, including those from the transcriptional factors Clp (Leduc & Roberts, 2009; Chin et al., 2010; Tao et al., 2010), FleQ (Hickman & Harwood, 2008) and VspT (Krasteva et al., 2010), from RNA-processing polynucleotide phosphorylase (PNPase; Tuckerman et al., 2011), from degenerate GGDEF or EAL domains (Navarro et al., 2009, 2011), from PilZ-domain proteins (Amikam & Galperin, 2006; Benach et al., 2007; Li et al., 2009; Ko et al., 2010; Habazettl et al., 2011; Li et al., 2011; Liao et al., 2012) and from riboswitches (Kulshina et al., 2009; Smith et al., 2009, 2011). The search for novel c-di-GMP receptors is still ongoing (Romling, 2011; Sondermann et al., 2011; Ryan et al., 2012).

Another unique cyclic dinucleotide, c-di-AMP, has recently been discovered and found to play roles in regulating cell-cycle progression (Romling, 2008; Witte et al., 2008; Corrigan et al., 2011; Oppenheimer-Shaanan et al., 2011) as well as controlling cell size and envelope stress (Corrigan et al., 2011). Interestingly, both c-di-AMP and c-di-GMP have been found to activate a host type I interferon response (Karaoilis et al., 2007; McWhirter et al., 2009; Woodward et al., 2010; Jin et al., 2011; Sauer et al., 2011) and the C-terminal domain of the STING protein (STING-CTD) has been identified as the direct innate immune sensor of c-di-GMP (Burdette et al., 2011), providing...
a scaffold to specify and promote phosphorylation of IFN regulatory factor 3 (IRF3) by TANK-binding kinase 1 (TBK1) (Tanaka & Chen, 2012). The phosphorylated IRF3 then dimerizes and translocates into the nucleus to bind at the IFNB promoter to induce interferon expression (Bowie, 2012). Structural studies are required to better characterize the interactions between STING and cyclic dinucleotides or other cognate binding proteins, which will allow a more detailed understanding of the roles played by STING in these crucial self-defence signalling pathways in eukaryotic cells.

To date, however, no such information about STING and/or its complexes is available. In this manuscript, we report the first successful crystallization of the murine STING138–344 domain. Native and SeMet-labelled crystals have been obtained and diffracted to resolutions of 2.39 and 2.2 Å, respectively.

2. Materials and methods

2.1. Reagents

c-di-GMP was produced by an enzymatic method using an altered thermophilic DGC enzyme as described previously (Rao et al., 2009).

2.2. Cloning and purification

The whole *Mus musculus* (murine) STING gene was synthesized using a cost-effective PCR-based two-step DNA-synthesis method (Xiong et al., 2008). The codons were optimized and designed using the DNAWorks software (Hoover & Lubkowski, 2002) to achieve a higher level of expression in *Escherichia coli*. The optimized oligomer and primer sequences used for STING gene assembly are listed in Table 1.

In order to obtain STING protein with improved solubility, we constructed a series of STING gene fragments of different lengths (Fig. 1a). The STING gene fragments STING138–333, STING138–344, STING138–378, STING179–344 and STING179–333 were chosen based on their hydropheric profiles. The STING138–333, STING138–344 and STING138–378 truncations were PCR-amplified directly from the whole synthesized gene template by using the same forward primer S5’-TACTTCCAATACCATGCAGTCTGACACCTGGAGAAGTAGC-3’ with different reverse primers S5’-TATCCACTTCCAATAGTCTGACACCTGGAGAAGTAGC-3’ for the STING138–333 domain.
5'-TATCCACTTCCAATGTCACGCATTCATG GTCACTTCCATTCGTAC-3' for the STING138–344 domain and 5'-TATCCACTTCCAATGTCACGCATTCATG GTCACTTCCATTCGTAC-3' for the STING138–333 domain, while the STING179–333 and STING179–344 truncations were synthesized using the same forward primer 5'-TATCCACTTCCAATGTCACGCATTCATG GTCACTTCCATTCGTAC-3' with different reverse primers 5'-TATCCACTTCCAATGTCACGCATTCATG GTCACTTCCATTCGTAC-3' for the STING179–333 domain and 5'-TATCCACTTCCAATGTCACGCATTCATG GTCACTTCCATTCGTAC-3' for the STING179–344 domain.

The obtained PCR fragment exhibited the correct size in an agarose-gel electrophoresis experiment and was confirmed by DNA sequencing. A ligation-independent cloning (LIC) approach (Aslanidis & de Jong, 1990; Stols et al., 2001; Wu et al., 2005) was then used to obtain the desired constructs. The final constructs code for an N-terminal His$_6$ tag, a 17-amino-acid linker and the STING138–333, to obtain the desired constructs. The final constructs code for an R138A mutation under the control of a T7 promoter. Overexpression of the His$_6$-tagged proteins was induced by the addition of 800 µM IPTG to 450 ml M9 medium (to give a final IPTG concentration of 0.5 µM) at 293 K for 18 h. The cells were harvested, resuspended in lysis buffer (20 mM Tris–HCl pH 8.0, 80 mM NaCl) and ruptured using a microfluidizer (Microfluidics). Most of the target protein was found to be present in the soluble fraction after centrifugation. Surprisingly, exclusion of the last putative transmembrane-containing region (residues 138–179; Burdette et al., 2011) only gave inclusion bodies; only truncations starting from residue 138 delivered soluble proteins. The three soluble truncated proteins were purified by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma) equilibrated with a buffer consisting of 20 mM Tris–HCl pH 8.0, 80 mM NaCl and eluted with a gradient of 50–300 mM imidazole in the same buffer. The fractions containing the STING138–333, STING138–344 and STING138–378 domains were monitored using 13% SDS–PAGE and recombined. The His$_6$ tag and linker were further cleaved from the STING138–333, STING138–344 and STING138–378 domains using tobacco etch virus (TEV) protease at 289 K for 16 h (Fig. 1b). The final product contained an extra tripeptide SNA at the N-terminal end after cleavage of the His$_6$-tag and linker sequence (MHHHHHHHSTSVDLGTVYLFQ) from the ligation vector. For crystallization, the STING138–333, STING138–344 and STING138–378 domains were further purified on a Sephadex gel-filtration column (AKTA; Pharmacia Inc.) using the lysis buffer. SeMet-labelled STING138–344 was further prepared in order to solve the phase problem. The labelled domain was generated in a similar way except that it was produced using the non-auxotrophic E. coli strain Rosetta (DE3) as the host in the absence of methionine but with ample amounts of SeMet (100 mg l$^{-1}$). The M9 medium consisted of 1 g NH$_4$Cl, 3 g KH$_2$PO$_4$ and 6 g Na$_2$HPO$_4$ supplemented with 20% (w/v) glucose, 0.3% (w/v) MgSO$_4$ and 10 mg FeSO$_4$ in 1 l double-distilled water. Induction was performed at 293 K for 18 h by the addition of IPTG to 450 ml M9 medium (to give a final IPTG concentration of 0.5 mM). Purification of the SeMet-labelled STING138–344 protein was performed using the same procedure as used for the native protein.

### 2.3. Crystallization

For crystallization, native STING138–333, STING138–344 and STING138–378 and SeMet-labelled STING138–344 domains were concentrated to approximately 6.5 mg ml$^{-1}$ in 20 mM Tris–HCl pH 8.0, 80 mM sodium chloride using an Amicon Ultra-10 (Millipore). Appropriate volumes of 0.5 mM c-di-GMP were also added to the solutions of the native and SeMet-labelled STING138–333, STING138–344 and STING138–378 domains to prepare samples for STING–c-di-GMP co-crystallization at a 2:1 ligand:protein ratio.

![Figure 2](image2.png)

**Figure 2**

Crystals of the STING138–344 and SeMet-labelled STING138–344 domains. (a) STING138–344 crystals grown in 2% PEG 400, 1.6 M ammonium sulfate, 0.1 M MES monohydrate using the hanging-drop vapour-diffusion method at 298 K. These crystals reached average dimensions of 0.95 × 0.95 × 0.1 mm after one week. (b) SeMet-labelled STING138–344 crystals grown in 1.6 M potassium/sodium phosphate, 0.1 M Na HEPES pH 7.5. These crystals reached average dimensions of 0.1 × 0.1 × 0.15 mm after one week.

![Figure 3](image3.png)

**Figure 3**

Diffraction pattern of SeMet-labelled STING138–344 protein collected using a MAR CCD detector on the BL13B1 beamline at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The exposure time was 8 s, the oscillation range was 1° per frame and the crystal-to-detector distance was 300 mm. The edge of the detector corresponds to a resolution of 2.2 Å.
crystallization communications

3. Results and discussion

In this manuscript, we report the successful cloning, protein expression and purification of the STING\textsuperscript{138–344} protein and the crystal screening and preliminary X-ray data analyses of native and SeMet-labelled STING\textsuperscript{138–344} proteins. Initially, we constructed a series of STING clones with different N-terminal and C-terminal sequences (Fig. 1a). Unexpectedly, constructs starting from residue 179 that lacked a putative transmembrane segment gave proteins in inclusion bodies, and only constructs starting from residue 138 (STING\textsuperscript{138–333}, STING\textsuperscript{138–344} and STING\textsuperscript{138–378}) that contained a putative transmembrane segment gave soluble protein. As shown in Fig. 1(b), the His\textsubscript{6} tag and linker of the STING\textsuperscript{138–333}, STING\textsuperscript{138–344} and STING\textsuperscript{138–378} domains could be successfully cleaved by TEV protease at 289 K for 16 h to obtain the domains with a purity of >95%. The domains contained an extra tripeptide SNA at the N-terminal end after cleavage of the His\textsubscript{6}-tag and linker sequence (MHIIIIIIIIIIIISTSVDLGTENLYFQ) from the ligation vector. These domains were further purified by gel-filtration chromatography. However, no crystal formation was observed for the STING\textsuperscript{138–333} and STING\textsuperscript{138–378} domains. Hence, only the STING\textsuperscript{138–344} domain was further studied.

Since STING does not seem to share homology with any known immunosensors and may represent a novel category of microbial detector (Burdette et al., 2011), solution of its structure using a molecular-replacement approach is unlikely. Therefore, in order to obtain the essential phase information, we further screened the SeMet-labelled STING\textsuperscript{138–344} domain for crystallization. Luckily, crystals of the SeMet-labelled STING\textsuperscript{138–344} domain were obtained after one week. Further refinements of the STING\textsuperscript{138–344} protein/complex are now in progress.

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