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Preliminary crystallographic studies of BRCA1 BRCT–ABRAXAS complex

The BRCA1 holoenzyme complex plays an important role in DNA damage repair. ABRAXAS is a newly discovered component of this complex and its C-terminal region directly binds to the BRCA1 BRCT domain. Single crystals of the BRCA1 BRCT–ABRAXAS complex grown by co-crystallization belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 187.18$, $c = 85.31$ Å. Diffraction data were collected on the BM-14 beamline at the ESRF. Molecular-replacement calculations using *Phaser* led to three molecules in the asymmetric unit and a high solvent content of 76%.

1. Introduction

BRCA1 and BRCA2 are multifunctional proteins that are crucial for ensuring genome stability of human cells. Different domains of BRCA1 bind different interacting partners to perform a variety of functions including double-stranded DNA damage repair. Mutations in the BRCA1 and BRCA2 genes account for only 20% of familial breast and ovarian cancers, suggesting that the remainder arise for reasons that have still to be identified. Recent genetic analysis of cancer-patient families has identified *abraxas* as a new breast-cancer susceptibility gene and also as a novel target for therapeutic intervention (Solyom *et al.*, 2012). The protein ABRAXAS, recently discovered to be part of the BRCA1 DNA damage-repair complex, is one of the key adaptor proteins, which not only directly binds BRCA1 but also bridges interactions of BRCA1 with other interacting partners such as RAP80 and MERIT40. Through its interactions with RAP80, ABRAXAS is responsible for recruiting the BRCA1 complex to the DNA damage site (Shao *et al.*, 2009).

Cell lines containing the R361Q mutant of ABRAXAS were found to be hypersensitive to ionizing radiation. ABRAXAS is thus an essential factor in DNA damage resistance, repair and cell-cycle checkpoints (Eroles, 2013). ABRAXAS binds directly to the BRCA1 BRCT domain, which has been established to be a phosphopeptide-recognition module (Yu *et al.*, 2003; Wang *et al.*, 2007). The phosphopeptide-recognition sequence is pS- X_1 - X_2 -F (where pS is phosphorylated serine, X_1 is an aromatic amino acid, X_2 is a hydrophobic amino acid and F is phenylalanine; Yu *et al.*, 2003; Rodriguez *et al.*, 2003; Manke *et al.*, 2003). Two other proteins involved in DNA damage response and containing this signature motif for binding to BRCA1 BRCT are BACH1 and CtIP. However, ABRAXAS is unlike BACH1 and CtIP both in the location of the signature sequence and in the mechanism of localization at the DNA damage site. The signature sequence is located in the internal part of the sequences in BACH1 and CtIP, whereas it is at the carboxyl-terminus of ABRAXAS. Campbell and co-workers have studied the binding to BRCA1 and MDC1 of model tetrapeptides with and without free carboxyl ends. They observed that BRCA1 and MDC1 prefer oligopeptides with free carboxyl ends, with the preference being much greater in the case of MDC1. Through crystal structures of complexes, these authors have identified the environment around the conserved Arg1699 of BRCA1 as the factor determining the degree of specificity (Campbell *et al.*, 2010). In this context, the present study involving a longer oligopeptide with a free carboxy-terminus will provide information about the influence of additional amino acids on the interaction specificity.

Structural integrity of the BRCA1 BRCT domain is essential in performing the tumour suppressor function. The majority of breast and ovarian cancer-causing germline mutations in BRCA1 are located in the BRCA1 BRCT domain (Williams *et al.*, 2003). These mutations invariably disrupt phosphorylation-dependent interactions with cellular binding partners such as ABRAXAS, BACH1 and CtIP (Yu *et al.*, 2003; Manke *et al.*, 2003). ABRAXAS actually contains two potential ATM-phosphorylation serines, Ser404 and Ser406, at the C-terminus (Wang *et al.*, 2007). Through site-directed mutagenesis, phosphorylation at Ser406 alone was shown to be essential for binding to BRCA1 BRCT. Phosphorylation of Ser406 appears to be physiologically relevant, as the presence of phosphorylated Ser406 in cells exposed to ionizing radiation was confirmed using a phospho-specific antibody (Wang *et al.*, 2007). However, ABRAXAS phosphorylated at both the Ser404 and Ser406 residues was found to be selectively enriched on the incidence of ionizing radiation. To gain

insight into BRCT–phosphopeptide binding, we have undertaken biophysical and crystallographic studies of the interaction between the BRCA1 BRCT domain and differently (mono- and di-) phosphorylated C-terminal oligopeptides from ABRAXAS. Here, we report the crystallization of BRCA1 BRCT complexed with the ABRAXAS peptide phosphorylated at Ser406.

2. Materials and methods

2.1. Gene cloning of the BRCA1 BRCT domain

The BRCT domain (1646–1859) of BRCA1 was PCR amplified using the forward primer 5'-GTCGGATCCGAGAACCTGTACTTTCAGGGTGTGAACAAACGTATGTCCATG-3' and the reverse primer 5'-GTCGAATCCCTATTAGGGGATCTGGGGTATCATGTATGG-3'. The primers are designed in such a way as to accommodate a *Bam*HI restriction site and a TEV protease site (ENLYFQG) in the forward primer and an *Eco*RI site in the reverse primer. The PCR product of 687 base pairs and the empty vector pGEX-KT (a kind gift from Dr John Ladias) were digested by *Bam*HI and *Eco*RI and then ligated using the Quick Ligation Kit (New England Biolabs, USA). The potential clones were selected after screening for the presence of the insert and were finally confirmed by DNA sequencing.

2.2. Expression and purification of the BRCA1 BRCT domain protein

The BRCA1 BRCT protein was expressed in *Escherichia coli* bacterial strain BL21(DE3) using standard protocols. The BRCT domain fusion protein was purified from soluble cell-free lysate by affinity chromatography using glutathione Sepharose 4B resin (GE Healthcare). All purification steps were carried out at room temperature (~295 K). The GST fusion tag was removed by TEV protease and the reaction mixture was passed through a gel-filtration column (HiLoad Superdex 200) to obtain a monomeric population of BRCT. The TEV protease with a 6×His tag was removed by passing through a Ni²⁺–nitrotri-acetic acid (Ni–NTA) column. The purity of the BRCT domain was checked by resolving on 12% SDS–PAGE and the identity of the protein was confirmed by MALDI–TOF/TOF. The BRCT protein was concentrated to 25 mg ml^{−1} using a Centricon-10 (a centrifugal concentrator with 10 kDa molecular-mass cutoff) prior to crystallization.

2.3. Interaction analysis of BRCA1 BRCT with ABRAXAS

The oligopeptide of sequence (NH₂-G₃₉₉-F-G-E-Y-S-R-pS-P-T-F₄₀₉-COOH) from the C-terminus of ABRAXAS was obtained commercially (USV Biotech). The interaction between BRCA1 BRCT and ABRAXAS at 298 K was studied using an isothermal titration calorimeter (ITC-200; GE Healthcare). The protein and the peptide concentrations used were 0.020 and 0.200 mM, respectively. The ITC experiment was designed as a series of 18 injections, each of volume 2 µl, at time intervals of 210 s under constant stirring at 1000 rev min^{−1}. The heat of dilution was measured for subtraction during analysis of the exothermic data from the sample. The analysis software (*Origin 7.0*) provides values of the stoichiometry *N*, the binding constant *K_a* and the enthalpy change ΔH . All ITC experiments were repeated thrice and average values are reported.

2.4. Crystallization of the BRCA1 BRCT–ABRAXAS peptide complex

The ÄKTA FPLC-purified BRCA1 BRCT domain protein (25 mg ml^{−1}) was mixed with the ABRAXAS peptide (10 mg ml^{−1}) in a 1:1.5 molar ratio and incubated at 277 K overnight. Screening for

Figure 1
(a) The gel-filtration chromatogram of the BRCA1 BRCT protein eluted at the expected elution volume ~80 ml from a Superdex 200 column. (b) The gel-filtration fractions of purified BRCA1 BRCT protein (~25 kDa) resolved on a 12% SDS–PAGE gel.

Table 1

Crystal and diffraction data statistics.

Values in parentheses are for the outermost resolution shell.

No. of crystals	1
Beamline	BM-14, ESRF
Wavelength (Å)	0.9800
Temperature (K)	100
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	$a = b = 187.18$, $c = 85.31$
Mosaicity (°)	1.23
Resolution limit (Å)	76.47–3.8 (4.01–3.80)
Total No. of reflections	106886
Unique reflections	15491
Multiplicity	6.9
$\langle I/\sigma(I) \rangle$	9.5 (2.0)
Completeness (%)	99.9 (100)
$R_{\text{merge}}^{\dagger}$ (%)	9.1 (87.2)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean weighted intensity after rejection of outliers.

crystallization conditions was performed at 295 K by the hanging-drop vapour-diffusion method in 24-well crystallization plates using Crystal Screen and Crystal Screen 2 (Hampton Research). Drops prepared by mixing 2 μl protein solution with an equal volume of reservoir solution were equilibrated against 500 μl reservoir solution.

2.5. X-ray diffraction data collection

The X-ray diffraction data were collected at 100 K on the BM-14 beamline at the ESRF, France. A total of 150 frames, with a 1.0° oscillation of the crystal per frame, were collected at a crystal-to-detector distance of 350 mm and with an exposure time of 30 s.

3. Results and discussion

3.1. Protein preparation

Most of the BRCA1 BRCT domain expressed in *E. coli* accumulated in inclusion bodies. The typical yield from the soluble fraction was about 2.0 mg per litre of bacterial culture. The elution profile from the gel-filtration column (Fig. 1*a*) and the SDS-PAGE (Fig. 1*b*) established the monomeric status, purity and molecular mass of the purified BRCT. The molecular mass of 24 633.21 Da obtained from mass spectrometry was in agreement with expectations.

3.2. Interaction analysis with the ABRAXAS peptide

Fig. 2 shows the results of the calorimetric studies. From these observations, the binding affinity, ΔH and stoichiometry of the complex are estimated to be 1.2 μM , $-1.019 \times 10^4 \text{ cal mol}^{-1}$ and 1, respectively. The binding affinity of the ABRAXAS peptide to BRCT is lower than that of the BACH1 peptide (0.9 μM ; Shiozaki *et al.*, 2004), but higher than that of CtIP peptide (3.7 μM ; Varma *et al.*, 2005).

3.3. Crystallization

The BRCA1 BRCT–ABRAXAS peptide complex in 50 mM Tris pH 7.5, 300 mM NaCl crystallized in several conditions from Crystal Screen and Crystal Screen 2. The present diffraction analysis was carried out with crystals grown using 0.1 M MES monohydrate pH 6.5, 0.2 M ammonium sulfate, 30% polyethylene glycol monomethyl ether 5K. The crystal used for diffraction data collection was approximately $0.1 \times 0.08 \times 0.06 \text{ mm}$ in size (Fig. 3).

3.4. Data collection and processing

The crystals were soaked in a cryoprotectant solution obtained by augmenting the well solution with 30% glycerol. The crystals diffracted to 3.8 Å resolution at 100 K. The diffraction data were indexed and integrated using *iMosflm* (Battye *et al.*, 2011). Scaling

Figure 2

Representation of the ITC result obtained from the interaction of BRCA1 BRCT domain with the ABRAXAS peptide.

Figure 3

Single crystal of the BRCA1 BRCT–ABRAXAS complex.

and merging of the diffraction data were performed using the *CCP4* suite (Winn *et al.*, 2011). A summary of the data-collection and processing statistics is shown in Table 1. The crystal belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 187.2$, $c = 85.3$ Å. Crystals of BRCT complexed with a variety of oligopeptides reported in the literature belonged to the hexagonal space group $P6_122$. This is the first complex of BRCA1 BRCT to be crystallized in a tetragonal space group. A V_M (Matthews, 1968) value of $2.53 \text{ Å}^3 \text{ Da}^{-1}$ would correspond to the presence of six BRCA1 BRCT molecules in the asymmetric unit with 51% solvent content. Structure solution using the molecular-replacement method is being attempted with PDB entry 1t15 (Clapperton *et al.*, 2004) as the search model. Initial molecular-replacement calculations using *Phaser* (McCoy *et al.*, 2007) suggest space group $P4_12_12$ and the presence of only three molecules in the asymmetric unit, with a solvent content of 76%. Crystals containing a large amount of solvent are generally not well ordered, and the low diffraction resolution observed here might indeed be a consequence of the large solvent content.

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