Preparation of crystals for characterizing the Grb7 SH2 domain before and after complex formation with a bicyclic peptide antagonist

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Human growth factor receptor-bound protein 7 (Grb7) is an adapter protein involved in cell growth, migration and proliferation. It is now recognized that Grb7 is an emerging therapeutic target in specific cancer subtypes. Recently, the discovery of a bicyclic peptide inhibitor that targets the Grb7 SH2 domain, named G7-B1, was reported. In an attempt to probe the foundation of its interaction with Grb7, the crystallization and preliminary data collection of both the apo and G7-B1-bound forms of the Grb7 SH2 domain are reported here. Diffraction-quality crystals were obtained using the hanging-drop vapour-diffusion method. After several rounds of microseeding, crystals of the apo Grb7 SH2 domain were obtained that diffracted to 1.8 Å resolution, while those of the G7-B1–Grb7 SH2 domain complex diffracted to 2.2 Å resolution. The apo Grb7 SH2 domain crystallized in the trigonal space group $P6_3$, whereas the G7-B1–Grb7 SH2 domain complex crystallized in the monoclinic space group $P2_1$. The experimental aspects of crystallization, crystal optimization and data collection and the preliminary data are reported.

1. Introduction

Human growth factor receptor-bound protein 7 (Grb7) is an adapter protein that links membrane-bound and cytoplasmic tyrosine kinase receptors to downstream signalling pathways (Han *et al.*, 2001; Margolis *et al.*, 1992). These include the ErbB2 receptor, which signals cell growth, as well as focal adhesion kinase (FAK), which signals cell migration (Shen & Guan, 2004; Pradip *et al.*, 2013). Grb7 originally evoked interest in the context of cancer because it is co-over-expressed and co-amplified with ErbB2, the well established propagator of cell growth and clinical target for ErbB2-positive cancers (Stein *et al.*, 1994; Tanaka *et al.*, 1997; Kishi *et al.*, 1997; Walch *et al.*, 2004). Since then, however, it has been shown that Grb7 is independently connected with the development of aggressive, recalcitrant and advanced tumours (Ramsey *et al.*, 2010; Nadler *et al.*, 2010). It has therefore been proposed that targeting Grb7 provides an attractive strategy for the inhibition of multiple oncogenic pathways (Pero *et al.*, 2003; Giricz *et al.*, 2012).

Grb7 is composed of 532 residues that are arranged in well conserved protein domains including a Ras-associating (RA) domain, a pleckstrin homology (PH) domain and a C-terminal Src homology 2 (SH2) domain. In addition, Grb7 has a proline-rich N-terminal domain and another domain termed the BPS domain (between the PH and SH2 domains; Han *et al.*, 2001). Each domain is known to play a complementary role in the overall signalling function of Grb7. The SH2 domain has been the focus of antagonist design because it is the domain responsible for binding to the upstream partners of Grb7. The SH2 domain commences the first and hence the fate-determining step in the process of Grb7-dependent signalling (Han *et al.*, 2001; Daly, 1998). Moreover, it possesses a well defined and characterized binding pocket amenable to ligand-design efforts (Machida & Mayer, 2005). These factors mean the SH2 domain is an attractive module in the development of Grb7-based therapeutic agents. Indeed, this has been demonstrated by the discovery and characterization of the G7-18NATE peptide antagonist specifically designed to bind to the Grb7 SH2 domain (Pero *et al.*, 2002; Ambaye, Gunzburg *et al.*, 2011; Ambaye, Pero *et al.*, 2011; Gunzburg *et al.*, 2012).

Currently there exist three experimental structures corresponding to the SH2 domain of Grb7: a crystal structure of the apo form of the Grb7 SH2 domain (PDB entry 2qms; Porter *et al.*, 2007), a solution structure of the Grb7 SH2 domain bound to an ErbB2 peptide (PDB entry 1mw4; Ivancic *et al.*, 2003) and a crystal structure of the Grb7 SH2 domain bound to the cyclic peptide antagonist G7-18NATE (PDB entry 3pqz; Ambaye, Pero *et al.*, 2011). The X-ray structure of the apo Grb7 SH2 domain was reported previously by our group to 2.1 Å resolution. It concurs with the solution structure in its overall fold, having a pair of α -helices flanking a four-stranded β -sheet. This has contributed to the better understanding of the binding phenomenon underlying target specificity. However, the reported structure was found to contain a bound sulfate ion in the active site that constrained the site to mimic the holo form of the molecule. Thus, in order to obtain a truly apo form of the protein, and to potentially obtain structural information at higher resolution, we embarked on crystallization efforts under different conditions. In addition, we designed a rigid bicyclic analogue of the G7-18NATE peptide based on our biophysical and structural studies (Gunzburg *et al.*, 2013). This bicyclic peptide, named G7-B1 (Grb7 bicyclic peptide 1), was shown to have enhanced affinity for the Grb7 SH2 domain over G7-18NATE. The present work reports the crystallization and preliminary characterization of high-resolution data of the Grb7 SH2 domain both in the apo form and in complex with G7-B1.

2. Methods

2.1. Expression and purification of the Grb7 SH2 domain

The pGex2T plasmid containing the Grb7 SH2 domain insert encoding residues 415–532 of human Grb7 protein was obtained from collaborators (Janes *et al.*, 1997) and expressed in an *Escherichia coli* expression system as a GST-fusion protein in the BL21 (DE3) pLysS host strain (Porter *et al.*, 2005). Cell induction was achieved with 400 μ M isopropyl β -D-1-thiogalactopyranoside. Cell resuspension and lysis were carried out in ice-cold phosphate-buffered saline with 2 mM EDTA, 0.5% Triton X-100 by sonication. The fusion protein was purified by glutathione-affinity chromatography (GE Healthcare). The GST tag was removed with thrombin. Purification was continued by cation-exchange chromatography using a HiTrap HP column (GE Healthcare) after an overnight dialysis into 20 mM HEPES pH 7.4, 20% glycerol, 1 mM DTT at 4°C. This was followed by dialysis into 50 mM MES pH 6.6, 100 mM NaCl, 1 mM DTT and final purification by size-exclusion chromatography using a Sephadex 75 XK 16/60 column (GE Healthcare). The Grb7 SH2 domain was concentrated and stored at 4°C. The final purity and the progress of the purification steps were monitored by SDS-PAGE. The final concentration was determined with UV-visible spectroscopy using an extinction coefficient of 8480 cm⁻¹ M⁻¹ (Pace *et al.*, 1995).

2.2. Bicyclic peptide synthesis

The G7-B1 bicyclic peptide used for co-crystallization (sequence XFEGYDNXFPC, where X is *O*-allyl-serine) was prepared by solid-phase synthesis as a peptide amide using Fmoc-based tactics on a rink amide resin (1.0 mmol g⁻¹). The synthesis was carried out as reported previously (Gunzburg *et al.*, 2013). In brief, ring-closure metathesis (RCM) was employed to form the first ring between the *O*-allyl-serine groups on the resin. After successful RCM, the N-terminus was chloroacetylated and the peptide was cleaved from the resin and deprotected using trifluoroacetic acid (TFA). Thioether formation to form the second ring was achieved by treatment of the crude peptide in an aqueous base. The final bicyclic peptide structure is shown in

Fig. 1. The cyclized peptides were freeze-dried overnight and purified using preparative reverse-phase (RP) HPLC. Final peptide homogeneity and identity as well as the success of capping, RCM and thioether-formation reactions were monitored by analytical reverse-phase HPLC and electrospray ionization mass spectroscopy.

2.3. Crystallization

The purified protein sample was concentrated using an Eppendorf concentrator of molecular-weight cutoff 3500 at 4°C to 7 mg ml⁻¹, corresponding to about 511 μ M. The hanging-drop vapour-diffusion method was used to set up crystallization trials in Linbro 24-well tissue-culture plates. For the apo form of Grb7 SH2, 1 μ l protein solution was mixed with 1 μ l reservoir solution to make a final drop of size 2 μ l. For the G7-B1–Grb7 SH2 domain complex trials, a 1.5:1 molar ratio of the bicyclic peptide with Grb7 SH2 domain was prepared by adding the protein solution to the peptide dissolved in 0.1 M sodium phosphate pH 6, 1 mM DTT. The complex solution was mixed with the reservoir in a 1:1 ratio to form a 2 μ l drop on the coverslip, which was then sealed over the well with Vaseline. All of the protein and protein–peptide complex solutions were filtered prior to making the drops. A variety of commercially available screens including Index, PEG/Ion, SaltRx (Hampton Research; <http://hamptonresearch.com>) and JBScreens (Jena Biosciences; <http://jenabioscience.com>) were employed. All of the crystallization

Figure 1
Schematic and full chemical structure of the G7-B1 peptide antagonist. Single-letter amino-acid abbreviations are indicated in blue.

experiments were carried out at room temperature and were kept and monitored in a temperature-controlled room at 20°C.

2.4. Crystal optimization

Once initial crystals were obtained and confirmed to be protein by diffraction measurements, crystal optimization was effected by microseeding experiments (MacSweeney & D'Arcy, 2009). Specifically, the crystallization condition that produced the first crystal was prepared by hand and filled into four wells under the same conditions as the initial trial. The apo Grb7 SH2 domain and G7-B1–Grb7 SH2

Figure 2
The initial crystals of the apo Grb7 SH2 domain obtained from random screening. The crystal was found to diffract to 2.0 Å resolution.

domain solutions and drops were made as described above. A microseeding experiment was conducted by using a cat whisker to streak-seed the microcrystal into the newly set up drop over the four wells. The whisker was first wiped across 10 µl reservoir solution to reduce the number of seeds and was subsequently streaked over the four wells sequentially.

2.5. Data collection

Crystals were soaked in a cryoprotectant composed of 20%(v/v) glycerol in mother liquor prior to data collection. The crystals were then flash-cooled in liquid nitrogen and transferred to a stream of nitrogen gas at −173°C. X-ray data were collected at a wavelength of 0.954 Å on an ADSC Quantum 315r detector using the high-throughput protein crystallography beamline (MX2) at the Australian Synchrotron. Images were recorded with an oscillation angle of 1° and an exposure time of 3 s for the apo Grb7 SH2 domain crystal and with an oscillation angle of 0.5° and an exposure time of 2 s for the G7-B1–Grb7-SH2 domain complex crystal. Diffraction data were integrated with *XDS* (Kabsch, 2010) and intensities were scaled using *SCALA* (Evans, 2006; Winn *et al.*, 2011). Data-collection statistics are summarized in Table 1.

3. Results and discussion

Initial crystallization trials for both apo Grb7 SH2 domain and the G7-B1–Grb7 SH2 domain complex utilized Index, SaltRx, PEG/Ion (Hampton Research) and JBScreens (Jena Biosciences), each containing 96 conditions. Needles and spherulites were obtained in

Figure 3
The process of apo Grb7 SH2 crystal optimization by microseeding.

Table 1

Crystallographic data-collection statistics for apo Grb7 SH2 domain and the G7-B1–Grb7 SH2 domain complex.

Values in parentheses are for the outermost resolution shell.

	Apo Grb7 SH2	G7-B1–Grb7 SH2
Temperature (°C)	−173	−173
Wavelength (Å)	0.954	0.954
Space group	$P6_3$	$P2_1$
Unit-cell parameters (Å, °)	$a = b = 83.99$, $c = 75.95$, $\alpha = \beta = 90.0$, $\gamma = 120.0$	$a = 37.10$, $b = 63.87$, $c = 52.07$, $\alpha = \gamma = 90.0$, $\beta = 92.41$
Resolution (Å)	28.2–1.8 (1.9–1.8)	25.0–2.2 (2.3–2.2)
$R_{\text{merge}}^{\dagger}$ (%)	9.8 (74.8)	10.5 (50.1)
$R_{\text{p.i.m.}}^{\ddagger}$ (%)	3.0 (23.6)	6.5 (30.8)
$\langle I/\sigma(I) \rangle$	19.5 (3.7)	5.1 (2.4)
No. of unique reflections	27580 (3921)	12270 (1806)
Completeness (%)	99.7 (99.7)	99.6 (99.6)
Multiplicity	11.3 (10.8)	3.6 (3.6)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of individual reflections. $\ddagger R_{\text{p.i.m.}}$ is the multiplicity-weighted R_{merge} .

several conditions; however, the best initial crystal forms were observed in the Index screen: crystals of the apo Grb7 SH2 domain form appeared in 14 d in condition No. 33 of the Index screen [1.1 *M* sodium malonate, 0.1 *M* HEPES pH 7.0, 0.5% (v/v) Jeffamine ED-2001], while the G7-B1–Grb7 SH2 domain complex produced crystals that appeared in 10 d in condition No. 42 of the Index screen [0.1 *M* bis-tris pH 5.5, 25% (w/v) PEG 3350].

The initial apo Grb7 SH2 domain crystals from the trials diffracted to 2.0 Å resolution. However, we sought to optimize the crystallization process to determine whether crystals diffracting to higher resolution could be obtained. To this effect, a microseeding strategy using seeds from the condition that produced the initial crystals was utilized. This was carried out using a cat whisker to pick and spread the microcrystals into the freshly prepared crystallization condition [1.1 *M* sodium malonate, 0.1 *M* HEPES pH 7.0, 0.5% (v/v) Jeffamine ED-2001] in a new well. The initial apo Grb7 SH2 domain crystals from the microseeding experiment were so numerous that they never grew to a size sufficient for X-ray diffraction experiments, indicating that too much nucleation was occurring. Thus, a second round of microseeding experiments was carried out using the tiny crystals from the first round of seeding. This time the crystals grew to a relatively large size, but were still not of sufficient size for a diffraction experiment. Finally, a third round using the crystal seeds from the second seeding was conducted to successfully grow single crystals that diffracted to 1.8 Å resolution. Fig. 2 shows the initial few crystals

Figure 4

Monoclinic crystals of the G7-B1–Grb7 SH2 domain complex. Diffraction-quality crystals were obtained after microseeding.

of the apo Grb7 SH2 domain protein obtained from the random screening, while Fig. 3 shows the optimization process pursued. As can be seen, the crystals become fewer and larger during the crystal growth by seeding.

In contrast, the initial crystals obtained for the G7-B1–Grb7 SH2 domain complex from the random screen were very small. However, high-quality crystals were obtained after just the first round of microseeding of a freshly prepared crystallization condition [0.1 *M* bis-tris pH 5.5, 25% (w/v) PEG 3350] in a new well, which needed no further rounds of optimization. The final crystals of the Grb7 SH2

(a) **(b)****Figure 5**

Diffraction images of Grb7 SH2 domain crystals. (a) Diffraction image of the trigonal crystals of the apo Grb7 SH2 domain; (b) diffraction image of the G7-B1–Grb7 SH2 domain co-crystal.

domain in complex with the bicyclic peptide were found to diffract to 2.2 Å resolution. Fig. 4 shows the co-crystals obtained after the optimization.

The crystallization conditions were different to each other and also differed from the previous conditions in which the Grb7 SH2 domain has been crystallized [apo Grb7 SH2, PDB entry 2qms, 100 mM sodium citrate pH 6.1, 22.5% PEG 4000, 0.2 M ammonium sulfate, 5% glycerol; G7-18NATE–Grb7 SH2 domain complex, PDB entry 3pqz, 17.5% (w/v) PEG 3350, 0.2 M MgCl₂, 0.1 M glycine, 10% (w/v) glycerol, 0.1 M bis-tris pH 6.0]. However, both of these crystallization conditions, as well as the previous conditions, all contained small oxygen-containing polyatomic ions that are potential binders at the SH2 domain cationic phosphate recognition pocket.

A total of 180 images were collected with an oscillation angle of 1.0° for the apo Grb7 SH2 domain, while 720 images with a 0.5° oscillation angle were collected for the G7-B1–Grb7 SH2 complex, all at –173°C maintained by a stream of nitrogen gas. See Table 1 for details and Fig. 5 for representative diffraction images. The apo Grb7 SH2 domain was found to crystallize in a trigonal point group with space group *P*6₃ and the G7-B1–Grb7 SH2 domain complex formed a monoclinic crystal with space group *P*2₁. Cell-content analysis is consistent with both crystals containing two chains of the protein molecule per asymmetric unit. For the apo Grb7 SH2 domain, the Matthews coefficient obtained was 2.81 Å³ Da^{–1}, corresponding to 56% solvent content (Matthews, 1968). Similarly for the G7-B1–Grb7 SH2 domain crystal the Matthews coefficient was 2.25 Å³ Da^{–1}, with a solvent content of 45%.

Initial phases for these data sets have been found with molecular replacement using the previous 2.1 Å resolution apo Grb7 SH2 domain as a molecular-replacement model (PDB entry 2qms). The electron density for the G7-B1–Grb7 SH2 domain complex clearly revealed the presence of the peptide bound to the Grb7 SH2 domain protein. We anticipate that these high-resolution structures will provide greater detail than has previously been available for the analysis of the Grb7 SH2 domain and its ligand interactions.

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