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Preliminary crystallographic characterization of the Grb2 SH2 domain in complex with a FAK-derived phosphotyrosyl peptide

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Growth factor receptor-bound protein 2 (Grb2) is an adaptor protein with a single SH2 domain that specifically binds to focal adhesion kinase (FAK) when residue Tyr925 of FAK is phosphorylated. The Grb2–FAK interaction is associated with cellular integrin-activated signal transduction events leading to the activation of the Ras-MAPK pathway. Crystals of the Grb2 SH2 domain in complex with a phosphopeptide corresponding to residues 921–930 of FAK have been obtained using the sitting-drop vapour-diffusion technique. The crystals belonged to space group $P3_121$, with unit-cell parameters $a = b = 102.7$, $c = 127.6$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0^\circ$. A diffraction data set was collected from a flash-cooled crystal at 100 K to 2.49 Å resolution using synchrotron radiation. Structure determination by molecular replacement and analysis of the detailed structure of the complex are currently in progress.

1. Introduction

Focal adhesions are integrin-mediated points of contact between the cell surface and the extracellular matrix. In response to cues in the extracellular milieu by the assembly and disassembly of focal adhesions (Bershadsky *et al.*, 2003; Ridley *et al.*, 2003), these sites modulate cell adhesion, migration, proliferation, differentiation and survival. FAK (focal adhesion kinase) plays a crucial role in the regulation of focal adhesions by its kinase activity and subsequent tyrosine phosphorylation of multiple structural and signalling molecules. The C-terminal domain of FAK (Fig. 1), *i.e.* the focal adhesion targeting region (FAT), is necessary and sufficient for localizing FAK to focal adhesions (Hildebrand *et al.*, 1995). The FAT domain (PDB code 1k40) is a four-helix bundle that can exist as a monomer and a dimer owing to domain swapping of helix 1 (Arold *et al.*, 2002; Hayashi *et al.*, 2002). When phosphorylated at residue Tyr925 on the first helix, FAT can be recognized by the SH2 domain of Grb2 (Schlaepfer *et al.*, 1994) and subsequently facilitates activation of the FAK-mediated Ras-MAPK pathway (Schlaepfer & Hunter, 1997). However, the typical conformation of the phosphotyrosyl peptide bound to the Grb2 SH2 domain is a β-turn structure (Rahuel *et al.*, 1996). There is controversy regarding the possible adaptation of the phosphorylated Tyr925-containing motif (pY925) on the Grb2 SH2 domain. Therefore, structural determination of the complex would be an important contribution to understanding the interaction. Here, we present the crystallization and preliminary X-ray diffraction analysis of the Grb2 SH2 domain in complex with a FAK pY925 phosphotyrosyl peptide.

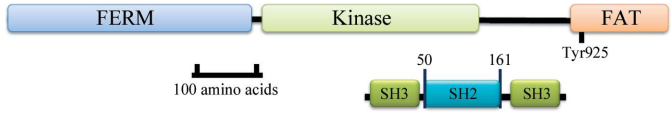

Figure 1
Schematic drawing showing the domain organization of the two proteins FAK (top) and Grb2 (bottom). The FERM, kinase and FAT domains of FAK are indicated, as is the Tyr925 phosphorylation site. The SH2 domain of Grb2 is shown from sequence number 50 to 161 and is flanked on each side by an SH3 domain.

2. Materials and methods

2.1. Protein expression and purification

The cDNA corresponding to residues 50–161 of human Grb2 protein (Fig. 1) was amplified by PCR and cloned into the pGEX-2T expression vector (GE Healthcare Sciences) to fuse it with an N-terminal GST tag for purification purposes. *Escherichia coli* BL21 cells transformed with the construct were grown in 6 l Luria broth to an OD_{600} of 0.6–0.8 and induced with 1.0 m*M* isopropyl β-D-1-thio-galactopyranoside for 3–4 h at 310 K. The cells were harvested at 6000*g* for 30 min, resuspended in buffer *A* (20 m*M* phosphate buffer pH 7.2, 100 m*M* NaCl) and lysed by sonication. The lysate was then centrifuged at 13 500*g* for 30 min and the supernatant was loaded onto a glutathione Sepharose column (GE Healthcare Sciences) equilibrated with buffer *A* and washed with the same buffer containing 1% Tween-20. The SH2 domain was released from the immobilized GST tag after incubation with thrombin and the reaction was blocked by the addition of phenylmethanesulfonyl fluoride to a final concentration of 1 m*M*. Fractions containing the desired protein were pooled and dialyzed against buffer *B* (20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl). Prior to setting up crystallization trials, the purity of the protein was checked using SDS–PAGE and verified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS). Approximately 10 mg purified protein was obtained from 1 l culture. The protein was concentrated to 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl by ultracentrifugation for crystallization trials.

2.2. Crystallization

The FAK pY925 phosphotyrosyl peptide (NDKVpYENVTG) composed of residues 921–930 of human FAK was commercially synthesized and verified by MALDI–TOF MS (GenScript Co., USA). A twofold molar excess of the pY925 phosphopeptide was incubated with Grb2 SH2 (10 mg ml^{-1}) before the crystallization trials. Crystals of Grb2 SH2 complexed with the FAK pY925 phosphotyrosyl peptide were grown by sitting-drop vapour diffusion at 298 K. A typical sitting drop was prepared by mixing 1.0 µl protein solution and an equal volume of reservoir solution. Initial crystallization conditions were obtained using kits from Emerald BioSystems (Bainbridge Island, Washington, USA). The final optimized condition (Wizard I condition 27) consisted of 2.0 *M* sodium/potassium phosphate, 100 m*M* CAPS pH 10.5 and 200 m*M* Li_2SO_4. Crystals with dimensions of about 0.3 × 0.1 × 0.1 mm grew in four weeks at 298 K during equilibration against 300 µl reservoir solution (Fig. 2).

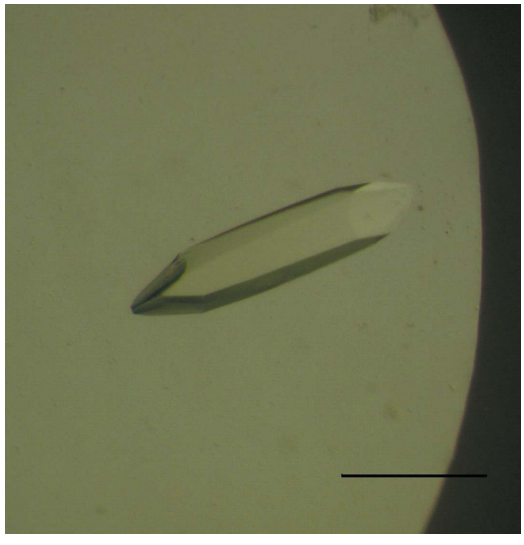

Figure 2
Crystal of the Grb2 SH2–FAK pY925 complex. The dimensions of the crystal are 0.3 × 0.1 × 0.1 mm. The scale bar is 0.2 mm in length.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P3_121$
Unit-cell parameters (Å, °)	$a = b = 102.7$, $c = 127.6$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	30–2.49 (2.56–2.49)
Total reflections	52263 (4987)
Unique reflections	27719 (2701)
Completeness (%)	99.8 (99.4)
R_{merge}† (%)	4.5 (49.7)
Average $I/\sigma(I)$	40.7 (3.6)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of the individual replicates of a given reflection *hkl* and $\langle I(hkl)\rangle$ is the average intensity over all replicates of that reflection.

2.3. Data collection and processing

X-ray diffraction data were collected on beamline BL13C1 equipped with a Q315 area detector at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. The crystal was transferred into a cryoprotectant solution containing 20% glycerol in mother liquor for 5 s and then flash-cooled in liquid nitrogen to 100 K. Diffraction images were indexed, integrated and scaled using *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The crystal belonged to the orthorhombic space group $P3_121$, with unit-cell parameters $a = b = 102.7$, $c = 127.6$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$. Assuming the presence of six molecules of 13 kDa protein in the asymmetric unit, the calculated Matthews coefficient (V_M) was 2.49 $Å^3$ Da^{-1} (Matthews, 1968). The solvent content of the crystal was calculated to be 50.6%. A complete data set has been obtained to 2.49 Å resolution, corresponding to an

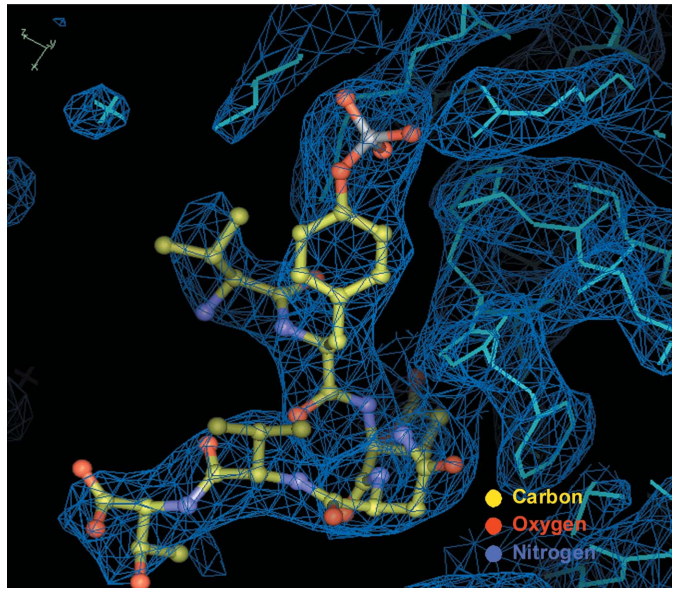

Figure 3
Electron-density map of the Grb2 SH2–FAK phosphotyrosyl peptide complex contoured at 1.5σ. The phosphopeptide is depicted with C atoms in yellow, O atoms in red and N atoms in purple.

R_{merge} of 4.5%. Details of the data-collection statistics are summarized in Table 1.

Molecular-replacement calculations were performed on the Grb2 SH2–FAK pY925 complex using the program *Phaser* (McCoy *et al.*, 2007). The monomeric Grb2 SH2 domain (PDB code 1jyr; Nioche *et al.*, 2002) was used as the search model for the Grb2 SH2–FAK pY925 complex. However, the first electron-density map obtained by rigid-body refinement showed discontinuity between residues 121 and 123 as well as some slight movement in one subdomain of the dimeric molecule. Model fitting using *Coot* (Emsley & Cowtan, 2004) then revealed that the dimeric SH2 domain contained two swapping subdomains. Subsequent iterative rounds of structural refinement using the rigid-body, simulated-annealing, conjugate-gradient energy-minimization and *B*-factor refinement options of *CNS* (Brünger *et al.*, 1998) were used to reduce the crystallographic *R* factor to below 25% in this space group. Interestingly, electron density corresponding to the pY925 motif could only be located in three of the six SH2 binding pockets (Fig. 3). Hence, free and pY925-bound forms of Grb2 SH2 could be obtained and compared in the same crystal. The complete structure determination of the complex will be discussed in a separate paper to address the issue of whether or not a conformational change of FAK–FAT is needed for binding to the Grb2 SH2 domain.

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