Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of the GST-fused human Bri3 N-terminal domain

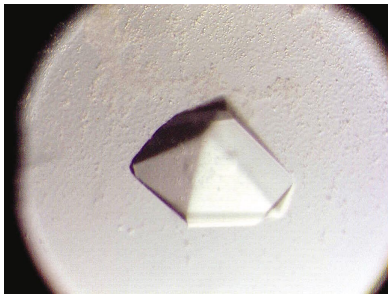
Qilu Ye,‡ Vinay Kumar Singh,‡ James Daniel Blonde and Zongchao Jia*

Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

‡ These authors made equal contributions.

Correspondence e-mail: jia@post.queensu.ca

Received 1 September 2004
Accepted 21 October 2004
Online 30 October 2004



Bri3 is a recently identified proline-rich transmembrane polypeptide up-regulated during TNF-mediated inflammation and immunity. The polyproline-rich N-terminal (residues 1–60) domain of Bri3 was affinity-purified to homogeneity as a glutathione-*S*-transferase (GST) fusion protein. Crystals were obtained in ∼3 d by the equilibrium vapour-diffusion method from a solution containing 1.5–2.2 *M* ammonium sulfate and 0.1 *M* bis-tris pH 6.0. The crystals belong to space group $P4_32_12$, with unit-cell parameters $a = b = 91.66$, $c = 57.53$ Å. An X-ray data set was collected to 1.6 Å resolution using synchrotron radiation, with an R_{sym} of 0.058 and a completeness of 95.3%. There is one molecule of the fusion protein in the asymmetric unit, which corresponds to ∼35% solvent content.

1. Introduction

Tumour necrosis factor-α (TNF) has been implicated in many diseases, such as rheumatoid arthritis, inflammatory bowel disease, cerebral malaria, diabetes, tumours, cardiovascular disease *etc.* (Aggarwal, 2003). Although the molecular mechanisms of TNF-induced cell death are gradually being unravelled, many genes involved in TNF-induced cell death (Mak & Yeh, 2002) remain unknown. Using suppressed subtractive hybridization from TNF-treated L929 cells, a 125-amino-acid transmembrane protein known as Bri3 has recently been identified (Wu *et al.*, 2003). Bri3 has a proline-rich N-terminal (amino acids 1–65) domain that is predicted not to be associated with the membrane. It has previously been shown that proline-rich regions of polypeptides commonly adopt a left-handed polyproline II (PPII) helical conformation that is very flexible (Creamer & Campbell, 2002). This flexibility poses a great difficulty for the protein to crystallize on its own. However, various studies have shown that the utilization of GST tags (Zhan *et al.*, 2001) and other large affinity tags (Kuge *et al.*, 1997) enhances the solubility of proteins and facilitates crystallization.

As a novel protein overexpressed during TNF-induced cell death, Bri3 is a promising lead for drug discovery. The proline-rich N-terminal domain of Bri3 containing the P*xx*P motif can be further explored in order to observe its ability to interact with the SH3 domains of various proteins, as previously shown for other proline-rich sequences (Kaneko *et al.*, 2003; Lewitzky *et al.*, 2004). SH3 domains bind to sequences that adopt a left-handed PPII helical structure in which the two invariant proline residues are found on the same face of the peptide and participate in hydrophobic interactions (Ravi *et al.*, 2004).

The determination of the three-dimensional structure of the proline-rich flexible N-terminal domain region of Bri3 will help to understand its interaction with its various binding partners. It may also shed new light on TNF-induced signalling pathways, which may eventually suggest new strategies for development of new drugs against TNF-related diseases. Here, we describe the crystallization and preliminary X-ray data of the GST-fused Bri3 N-terminal domain (amino acids 1–60), which we will call GST-Bri3N.

2. Cloning protein expression and purification

A construct for GST-Bri3N (amino acids 1–60) was generated from human cDNA using the primers 5′-GCGGATCCGCAATGGACCACAAGCCGCTGCTG-3′ (forward) and 5′-GCGTCGACGTGGATGTTGTAGACCCTGGG-3′ (reverse). The amplified PCR product was ligated into pCR 2.1-TOPO vector (Invitrogen) and subcloned as a *Bam*H1-*Sal*1 fragment into the *Bam*H1–*Sal*1 site of pGEX-4T-3. Recombinant GST-Bri3N was expressed in *Escherichia coli* BL21(DE3) strain. Cells were grown in 1 l Terrific Broth (Bioshop Inc., Canada) medium containing 50 µg ml^{-1} ampicillin at 310 K to an OD_{600} of 0.8, induced with 0.1 m*M* isopropyl-β-D-thiogalactopyranoside and grown for 4 h at room temperature. The cells were harvested and stored at 253 K until further use. Lysis was performed in PBS buffer (140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4) pH 7.3 containing 1 m*M* phenylmethanesulfonyl fluoride, 10 m*M* dithiotheritol and 0.1 mg ml^{-1} DNAse I using three cycles of brief sonication for 20 s pulsed with a 5 min gap between each cycle. The lysate was cleared by centrifugation and loaded onto a GSTrap column (Pharmacia) for affinity purification using the glutathione-*S*-transferase (GST) tag attached to the protein. After multiple washes with PBS, GST-Bri3N was eluted using 50 m*M* Tris–HCl containing 10 m*M* reduced glutathione pH 8.0 and was then purified to homogeneity by AKTA FPLC using Sepharose G-75 (16/60) size-exclusion column chromatography. The single peak was pooled and concentrated to 36 mg ml^{-1}. The presence of Bri3 in the GST-fused protein was confirmed by cleavage of the GST tag using one unit of thrombin (Sigma) per milligram of fusion protein. The cleavage product was run on a 20% Tris–tricine gel as described previously (Schagger & von Jagow, 1987). Fig. 1 shows a single band corresponding to Bri3N cleaved from GST-tagged fusion protein.

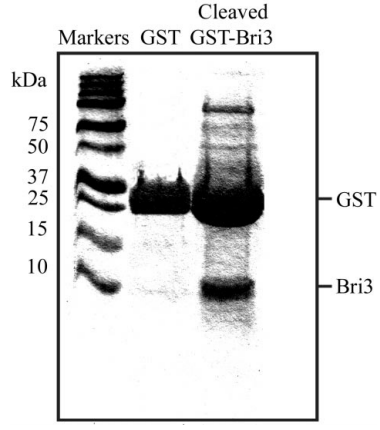


Figure 1
Tris–tricine polyacrylamide gel showing the cleavage of Bri3N from GST-fusion protein.

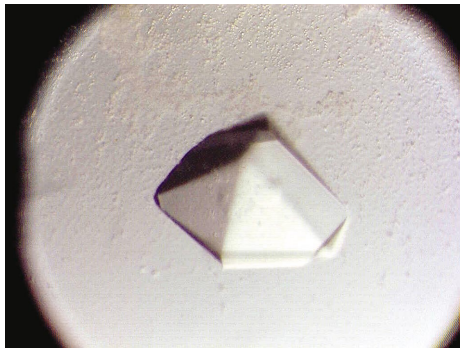


Figure 2
Typical GST-Bri3N crystal of approximate dimensions 0.3 × 0.3 × 0.2 mm.

Table 1
Statistics of X-ray diffraction data.

Values in parentheses are for the highest resolution shell.

Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 91.66$, $c = 57.53$
No. measured reflections	251419
No. independent reflections	30981
Resolution range (Å)	30.0–1.6 (1.66–1.60)
R_{sym}† (%)	5.8 (29.7)
Completeness (%)	95.3 (73.2)
Average $I/\sigma(I)$	38.1 (2.7)

† $R_{sym} = \sum|I(k) - \langle I\rangle|/\sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements.

3. Crystallization

GST-Bri3N was dialyzed into 10 m*M* phosphate buffer pH 7.0 for crystallization and concentrated to 10 mg ml^{-1}. The flowthrough from the concentrator was used as a negative control for crystallization. Initial crystallization trials for GST-Bri3N were performed using the equilibrium vapour-diffusion (hanging-drop) method at room temperature (293 K). Drops containing 2 µl protein sample (10 mg ml^{-1}) and 2 µl mother liquor were equilibrated against 1 ml reservoir solution. Sparse-matrix screens (Hampton Research) were performed as initial trials and the lead conditions were refined. The final condition contained 1.5–2.2 *M* ammonium sulfate and 0.1 *M* bis-tris pH 6.0. The crystals reached maximum dimensions of ∼0.3 × 0.3 × 0.2 mm (Fig. 2) within 3 d.

4. X-ray data collection

Data collection was carried out at the X6A station of Brookhaven National Laboratory using an ADSC Quantum 210 CCD detector at a wavelength of 0.9795 Å. Prior to data collection at 100 K, crystals were soaked in crystallization buffer containing 20%(*v*/*v*) PEG 400 for 2–3 min, followed by flash-cooling in a gas stream of liquid nitrogen. All data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to space group $P4_32_12$, with unit-cell parameters $a = b = 91.66$, $c = 57.53$ Å. Crystals diffracted well and a data set was collected to 1.6 Å resolution, with an R_{sym} of 0.058 and a completeness of 95.3% (Table 1). There is one GST-Bri3N molecule in the asymmetric unit, which corresponds to ∼35% solvent content.

Molecular replacement using a GST probe structure (PDB code 1bg5) was successful in space group $P4_32_12$. We are in the process of locating BRI3 density in order to achieve structure determination of the entire fusion protein.

This work was supported by a grant from the Canadian Institutes of Health Research to ZJ. We also acknowledge the Canadian Foundation for Innovation for support of the Protein Function Discovery Group. We thank BNL for synchrotron access and Melanie Adams for data collection. ZJ is a Canada Research Chair in Structural Biology and a recipient of the NSERC Steacie Fellowship.

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