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Purification, crystallization and preliminary X-ray diffraction analysis of the Kelch-like motif region of mouse Keap1

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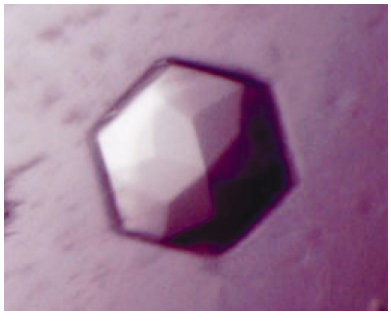

Keap1 (Kelch-like ECH-associating protein 1) is a negative regulator of the Nrf2 transcription factor in the cytoplasm. The Kelch/DGR (double-glycine repeat) domain of Keap1 associates with Nrf2 as well as with actin filaments. A recombinant protein containing both the Kelch/DGR domain and the C-terminal region of mouse Keap1 was expressed in *Escherichia coli*, purified to near-homogeneity and crystallized by the sitting-drop vapour-diffusion method. The crystal belongs to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 102.95$, $c = 55.21$ Å, and contains one molecule in the asymmetric unit. A complete diffraction data was collected to 2.25 Å resolution using an R-AXIS IV^{++} imaging plate mounted on an RA-Micro7 Cu $K\alpha$ rotating-anode X-ray generator.

1. Introduction

Keap1 (Kelch-like ECH-associating protein 1) is a member of a large family of proteins that contain an N-terminal broad complex, tram-track and bric-a-brac (BTB) domain (Bardwell & Treisman, 1994; Zollman *et al.*, 1994; Chen *et al.*, 1995) and a C-terminal Kelch/DGR (double-glycine repeat) domain (Xue & Cooley, 1993; Adams *et al.*, 2000). Keap1 negatively regulates the Nrf2 transcription factor, a critical regulator of the antioxidant-response element (ARE) dependent expression of cellular defence enzymes against oxidative and xenobiotic stresses (Itoh, Ishii *et al.*, 1999; Hayes *et al.*, 2000; Nguyen *et al.*, 2003). The induction of an array of ARE-dependent genes enhances cellular defences by restoring the cellular redox homeostasis, by minimizing oxidative DNA damage and by detoxifying reactive chemicals as well as carcinogens. Current models of the repressive regulation of the Nrf2 cellular defence system include nuclear deprivation of Nrf2 by physical sequestration through the Keap1–Nrf2 association in the cytoplasm (Itoh, Wakabayashi *et al.*, 1999) as well as the Keap1-dependent polyubiquitination and proteasome-mediated degradation of Nrf2 (McMahon *et al.*, 2003; Zhang & Hannink, 2003; Itoh *et al.*, 2003).

Recent studies have shown that Keap1 acts as an Nrf2-specific adaptor for Cul3-based E3 ligases (Kobayashi *et al.*, 2004; Cullinan *et al.*, 2004). Keap1 has been suggested to bridge Nrf2 physically to a Cul3-based E3 ligase for ubiquitin transfer. By functioning in cytosolic Nrf2 sequestration or by working as an adaptor for transferring ubiquitin to the Nrf2 molecule, Keap1 essentially requires a physical interaction with the N-terminal Neh2 domain of Nrf2 through its Kelch/DGR (Gln315–Thr598) domain (Itoh, Wakabayashi *et al.*, 1999). On the other hand, the Kelch/DGR domain is also important for co-localization with the actin cytoskeleton in the perinuclear region of the cytoplasm (Itoh, Wakabayashi *et al.*, 1999; Kang *et al.*, 2004). The Keap1–actin co-localization provides a scaffold for sequestering Nrf2 in the cytosol as well as for targeting Nrf2 to proteasome-mediated degradation, as the proteasome is also known to be co-localized with actin filaments and intermediate filaments. In addition, the extreme end of the C-terminal region (CTR; Ser599–Cys624) of mouse Keap1 has also been shown to be important for Keap1 repression of Nrf2 activity (Kang *et al.*, 2004). Structural analyses of these two C-terminal domains, encompassing residues 309–624 (DGR and CTR domains; referred to hereafter as mouse Keap1-DC), will provide insights into the basis of the Keap1 inter-

action with either Nrf2 or actin filaments, which both have a great impact on the regulation of cellular responses against toxic environmental stresses. Here, we report the purification, crystallization and preliminary crystallographic analysis of Keap1-DC from mouse.

2. Materials and methods

2.1. Chemicals

The pTXB3 expression vector and the chitin-binding beads were purchased from New England Biolabs. Buffer reagents used for purification were purchased from Sigma unless stated otherwise.

2.2. Cloning

The DNA fragment encoding mouse Keap1-DC (Thr309–Cys624) as well as the *Mxe* intein/chitin-binding domain fusion was constructed by the PCR overlap extension method using mouse Keap1 cDNA and pTXB3 as templates. *Nco*I and *Bam*HI restriction sites were added to the 5′ and 3′ ends, respectively, using the PCR primers. The resulting fragment was inserted into pET15b (Novagen) using the *Nco*I and *Bam*HI sites.

2.3. Expression and purification

The expression vector containing mouse Keap1-DC with a C-terminally fused *Mxe* intein/chitin-binding domain was expressed in *Escherichia coli* BL21 CodonPlus (DE3)-RIL (Stratagene) cultured in Luria Broth (LB) medium at 310 K. Overexpression of the recombinant fusion protein was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 m*M* and by shifting the culture temperature to 298 K. After 18 h further incubation, the cells were harvested and resuspended in 20 m*M* Tris–HCl pH 8.3, 1%(*v*/*v*) Triton X-100, 1 m*M* EDTA, 10 µg ml^{-1} DNase I, 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol (DTT) and Complete protease inhibitor (Roche). Cells were mechanically lysed by sonication (Branson Sonifier 450) on ice. The soluble protein fraction was then recovered by centrifugation at 27 000*g* for 30 min at 277 K. The recombinant mouse Keap1-DC fusion protein was captured by chitin-binding beads. The protein-bound affinity beads were washed with 40 column volumes of buffer *A* (20 m*M* Tris–HCl pH 8.3, 1 m*M* EDTA and 1 m*M* benzamidine–HCl) containing 0.3 *M* NaCl and 1% Triton X-100, followed by 40 column volumes of buffer *A*. On-column protein cleavage for the retrieval of native mouse Keap1-DC was initiated by incubating the beads for 24 h with one column volume of buffer *A* in the presence of 50 m*M* DTT and 50 m*M* mercaptoethane sulfonate (Mesna) at 277 K. The recovered mouse Keap1-DC was further purified on anion-exchange (Q2, BioRad) and Superdex S75 26/60PG columns (Pharmacia). The purified mouse Keap1-DC protein has an extra methionine at the N-terminus for translation initiation and an extra tyrosine at the C-terminus of Cys624, which was left over from the fusion protein after cleavage. The protein solution was exchanged into a buffer containing 20 m*M* Tris–HCl pH 8.3, 20 m*M* DTT and 10 m*M* benzamidine–HCl and then concentrated to 4 mg ml^{-1}. Finally, the protein was flash-frozen and stored in aliquots at 193 K.

2.4. Crystallization

Initial crystallization screening was performed with the sparse-matrix crystallization screening kits (Jancarik & Kim, 1991) Crystal Screens I and II from Hampton Research by the hanging-drop vapour-diffusion method in a 96-well plate (Corning, NY, USA). In each drop, 1.0 µl protein solution (4 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.3, 20 m*M* DTT and 10 m*M* benzamidine–HCl) and 1.0 µl reservoir solution were mixed and equilibrated against 100 µl reservoir solution. Small hexagonal rounded crystals of approximately 0.05 mm in diameter were grown within a week in the drop corresponding to condition No. 15 of Crystal Screen II (1.0 *M* lithium sulfate, 0.5 *M* ammonium sulfate and 0.1 *M* sodium citrate pH 5.6). Further optimization was carried out by varying the precipitant concentration against pH using 24-well plates (Q Plate II, Hampton Research) with 1.5 µl drops of protein solution mixed with 1.5 µl reservoir solution and equilibrated against 500 µl reservoir solution.

2.5. Data collection

Diffraction data were collected under cryogenic conditions using a Rigaku RA-Micro7 Cu $K\alpha$ rotating-anode X-ray generator operated at 40 kV and 20 mA and equipped with a Rigaku R-AXIS IV^{++} imaging-plate area detector and an X-stream low-temperature system. Prior to data collection, the crystals were transferred into a cryoprotectant containing 1.5 *M* lithium sulfate, 0.5 *M* ammonium sulfate and 0.1 *M* sodium citrate pH 5.2 for a few seconds and were then flash-cooled directly in a stream of cold gaseous N_2 at 90 K. The crystal-to-detector distance was set to 180 mm and the oscillation range was 1° with an exposure time of 5 min. A complete diffraction

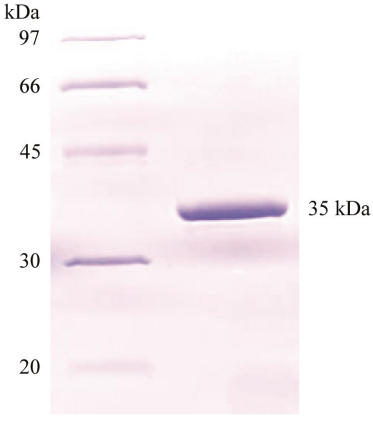

Figure 1
Coomassie-stained (12.5%) polyacrylamide gel of mouse Keap1-DC electrophoresed under denaturing conditions. Lane 1, low-molecular-weight range markers; lane 2, mouse Keap1-DC.

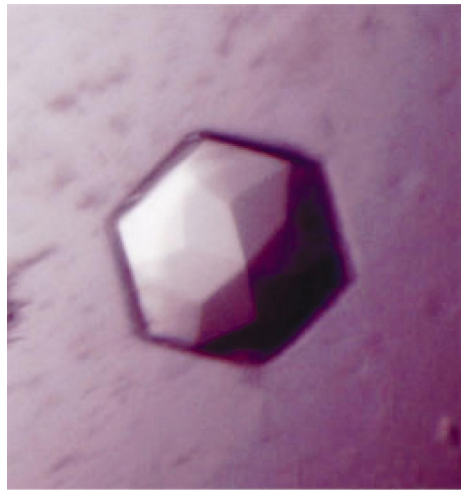

Figure 2
A single crystal of mouse Keap1-DC of approximately 0.2 mm in diameter.

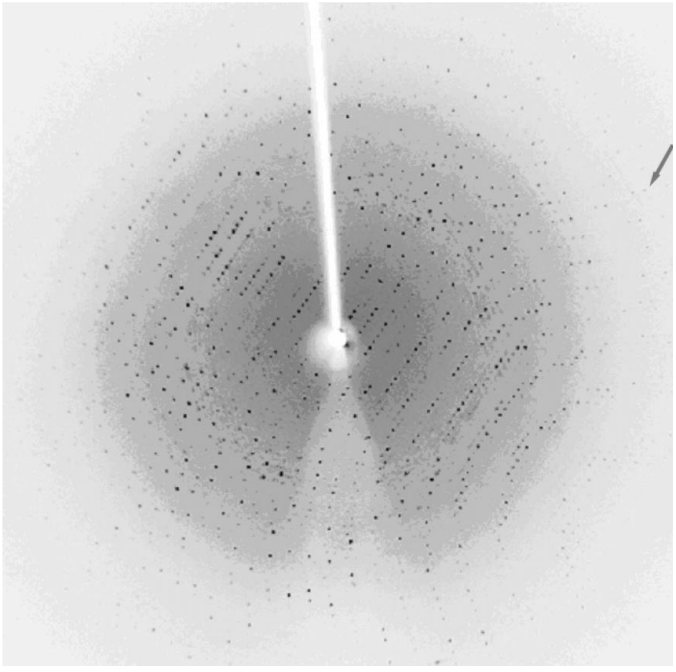

Figure 3
An X-ray diffraction image from a mouse Keap1-DC crystal. The diffraction spot shown by the arrow corresponds to 2.19 Å resolution. The crystal-to-detector distance was 180 mm, the oscillation angle was 1.0° and the exposure time was 300 s.

data set was obtained to 2.25 Å resolution. The data were processed and scaled using the *HKL*2000 suite (Otwinowski & Minor, 1997).

3. Results

The mouse Keap1-DC protein was expressed and purified to near-homogeneity as confirmed by SDS–PAGE (Fig. 1). After optimizing the crystallization conditions, good hexagonal rounded crystals of approximately 0.2 mm in diameter (Fig. 2) were obtained in the presence of 0.8 *M* lithium sulfate, 0.5 *M* ammonium sulfate and 0.1 *M* sodium citrate pH 5.2. The crystal diffracted beyond 2.2 Å resolution using our in-house facility (Fig. 3) and belongs to the hexagonal space group $P6_1$ (or its enantiomorph $P6_5$). The statistics of the processed data are shown in Table 1. A total of 117 495 measured reflections were merged into 15 961 unique reflections with an $R_{\rm merge}$ of 5.6% and 99.7% completeness to 2.25 Å resolution. Assuming that the crystal contains one molecule in the asymmetric unit, the molecular weight of the protein (34 674 Da) yields a solvent content of 49% and a Matthews coefficient of 2.4 Å^3 Da^{-1} (Matthews, 1968).

The crystal structure of RCC1 (regulator of chromosome condensation), which belongs to the Kelch-repeat superfamily, has recently been determined (Renault *et al.*, 1998). The RCC1 crystal structure consists of a seven-bladed β-propeller formed from internal repeats of 51–68 residues per blade. However, it showed poor sequence homology with the mouse Keap1-DC protein and also

Table 1
Data-collection and processing statistics.

Values in parentheses refer to the last shell (2.33–2.25 Å).

Space group	$P6_1$ or $P6_5$
Unit-cell parameters (Å)	$a = b = 102.95$, $c = 55.21$
Resolution range (Å)	50.0–2.25
Wavelength (Å)	1.5418
No. measured reflections	117495
No. unique reflections	15961
$R_{\rm merge}$† (%)	5.6 (15.4)
Completeness (%)	99.7 (97.5)
Redundancy	7.4 (7.1)

† $R_{\rm merge} = \sum |I(h) - \langle I(h)\rangle| / \sum I(h)$, where $I(h)$ is the observed intensity and $\langle I(h)\rangle$ is the mean intensity of reflection h over all measurements of $I(h)$.

failed to produce the correct structure solution by molecular replacement using the RCC1 structure as a model. Hence, a search for suitable heavy-atom derivatives is in progress to solve the structure by the MIR or MAD method.

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