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Crystallization of gankyrin, an oncoprotein that interacts with CDK4 and the S6b (rpt3) ATPase of the 19S regulator of the 26S proteasome

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Gankyrin is an oncoprotein overexpressed in hepatocarcinoma cells that binds to the cell-cycle regulator CDK4 and the S6b ATPase subunit of the regulatory component of the proteasome. It belongs to the family of ankyrin-repeat proteins that appear to mediate protein–protein interactions in diverse biochemical processes. Gankyrin has been crystallized from polyethylene glycol solutions and diffraction data have been obtained from these crystals that extend to 2.1 Å spacing.

1. Introduction

Gankyrin is overexpressed in human hepatocellular carcinomas, its name deriving from this observation (gann is cancer in Japanese) and the presence of six ankyrin repeats in its sequence (Higashitsuji *et al.*, 2000). Its overproduction leads to transformation in cultured NIH-3T3 cells and induces tumour formation in nude mice. Gankyrin overexpression correlates with hyperphosphorylation and degradation of the retinolastoma protein Rb1, a tumour suppressor factor. Gankyrin has been shown to bind to the cyclin D-dependent kinase CDK4, a cell-cycle regulator, one of whose substrates is Rb1 (Dawson *et al.*, 2002; Li & Tsai, 2002). Phosphorylation of Rb1 is an important step in enabling progression through the cell cycle and thus the activities of the relevant kinases are carefully controlled. Binding of gankyrin to CDK4 prevents the latter from binding to $p16^{INK4}$ (Li & Tsai, 2002). $p16^{INK4}$ is a member of the INK4 family of proteins which inhibit CDKs 4 and 6 and thus themselves have tumour suppressor functions. Although the INK4 proteins are also composed of ankyrin repeats, the mode of CDK4 binding by $p16^{INK4}$ is different to that of gankyrin, since gankyrin binding does not inhibit the kinase activity of CDK4. As a $p16^{INK4}$ antagonist, gankyrin functions as a promoter of cell-cycle progression.

Gankyrin has also been shown in yeast two-hybrid screens to form a complex with the S6b ATPase subunit of the regulatory complex of the proteasome. S6 is one of six ATPases, all of which belong to the AAA (ATPases associated with diverse cellular activities) superfamily (Ogura & Wilkinson, 2001). Among the ATP-dependent proteases, these components are associated with substrate unfolding and translocation prior to proteolysis (Schmidt *et al.*, 1999). In the case of the 26S proteasome, degradation takes place in the 20S core. Elsewhere, AAA subunits have been shown to function as molecular chaperones.

Gankyrin is a 25 kDa protein consisting of a polypeptide chain of 226 amino-acid residues containing five or possibly six ankyrin repeats together with an N-terminal extension of 38 residues. As a step towards a structural understanding of the interactions of gankyrin with its partner proteins and its role in cellular transformation, we have overexpressed, purified and crystallized gankyrin and collected diffraction data from the crystals that extend to 2.1 Å spacing.

2. Expression and purification

Gankyrin with an MGSSHHHHHHSSGLVPRGSH tag at its N-terminus was expressed from a pET28a plasmid derivative in *Escherichia coli* strain RosettaBlue (Novagen). Cells were grown in 1.5 l of LB kanamycin medium to A_{595} = 0.6. Expression of recombinant gankyrin was induced by the addition of IPTG to a final concentration of 1 m*M*. 3 h later, the cells were harvested by centrifugation and resuspended in 30 ml of 0.1 *M* sodium phosphate pH 7.6, 0.5 *M* NaCl, 1 m*M* β-mercaptoethanol (buffer *A*) and Complete EDTA-free protease inhibitors (Boehringer Mannheim). 30 mg of lysozyme was added and the cell suspension was twice frozen and thawed from 193 K. Cell lysis was completed by sonication (ten pulses of 60 s duration delivered over a period of 20 min) with an MSE SoniPrep 150 sonicator. The lysate was clarified by centrifugation at 34 500*g* for 30 min.

The soluble lysis fraction was supplemented with 10 m*M* imidazole pH 7.5 and loaded onto a nickel-chelating Sepharose (Amersham Biosciences) column equilibrated in buffer *A*. The column was washed with six column volumes of buffer *A* plus 10 m*M* imidazole, two

column volumes of buffer *A* plus 30 m*M* imidazole and developed with a 30–500 m*M* imidazole gradient in buffer *A*. Fractions containing gankyrin were identified by denaturing gel electrophoresis, pooled and dialysed against 20 m*M* Tris–HCl pH 8.4, 300 m*M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* dithiothreitol (DTT). 0.05 units of thrombin (Boehringer Mannheim) were added per milligram of protein and the N-terminal His tag was removed by overnight digestion at 277 K. The digestion mixture was dialysed against buffer *A* plus 10 m*M* imidazole pH 7.5 and polyhistidine-tagged material was removed by passage through a nickel-chelating Sepharose column. At this stage, gankyrin appeared to be pure as judged by Coomassie-stained denaturing polyacrylamide gel electrophoresis. Its appearance on similar gels run under native conditions was dependent, however, on the presence or absence of DTT (5 m*M*), when a single band or multiple bands were observed, respectively. We therefore treated the protein with 10 m*M* iodoacetic acid (IAA) to carboxymethylate exposed cysteine residues in a reaction carried out overnight in 50 m*M* Tris pH 8.0, 0.2 *M* NaCl (buffer *B*). After passage through a Superdex 75 16/60 column (Amersham Biosciences), native gels showed the IAA-modified gankyrin to be homogeneous, with a mobility slightly higher than that of the unmodified protein (Fig. 1). ESI mass spectrometry gave a MW value of 24 828 Da. This is 3 Da greater than that expected for gankyrin with the three additional residues (GSH) attached at the amino-terminus and in which two of the five cysteines have been acetylated.

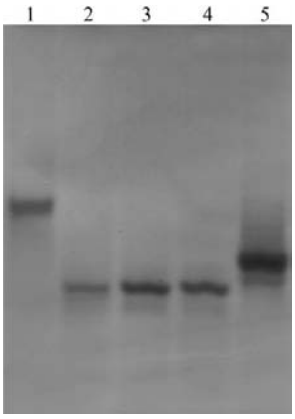

Figure 1
Coomassie-stained polyacrylamide gel electrophoresis of gankyrin under non-denaturing and non-reducing conditions. Lane 1, histidine-tagged fusion protein after nickel-chelation chromatography; lanes 2–4, gankyrin following modification with iodoacetic acid; lane 5, gankyrin following cleavage with thrombin.

3. Crystallization

Crystallization experiments utilized the hanging-drop vapour-diffusion method exploiting a variety of screening kits (Brzozowski & Walton, 2001; Jancarik & Kim, 1991). Protein was dissolved in 10 m*M* Tris–HCl buffer pH 7.5 at a concentration of 10 mg ml^{-1}. Needle-like crystals grew over a period of days in a number of drops containing polyethylene glycol (PEG), but these crystals were not suitable for structure determination. Crystals suitable for X-ray analysis grew from 24%(*w*/*v*) PEG 5000 monomethyl ether, 0.1 *M* MES [2-(*N*-morpholino)ethanesulfonic acid] pH 6.4 over a period of two months (Fig. 2).

4. Data collection

A single crystal cut from a cluster (Fig. 2) was scooped through a solution of 35% PEG 5000 monomethyl ether, 0.1 *M* MES pH 6.4 and placed in a stream of cold gaseous N_2 at 120 K. Diffraction data were collected on beamline ID29 (λ = 0.9756 Å) at the ESRF on a CCD ADSC Quantum 210 detector. The data were processed using *DENZO*, *SCALEPACK* and programs from the *CCP*4 suite (Otwinowski & Minor, 1997; Collaborative Computational Project, Number 4, 1994). The crystal belongs to space group $P2_12_12_1$, with unit-cell parameters a = 37.61, b = 64.64, c = 74.72 Å. If it is assumed that there is one monomer in the asymmetric unit, the calculated solvent content is 32.6% and the Matthews coefficient is 1.8 $Å^3$ Da^{-1}. 303 373 observations of 18 822 reflections in the resolution range 40–2.1 Å were recorded. The mean overall redundancy is 2.4 and is 2.1 in the highest resolution shell (2.18–2.10 Å). The data are 95.8% complete (89.4% in the highest resolution shell) with an overall $R_{\rm merge}$ on intensities of 0.067 (0.433); the mean $I/\sigma(I)$ value is 10.7 (2.5).

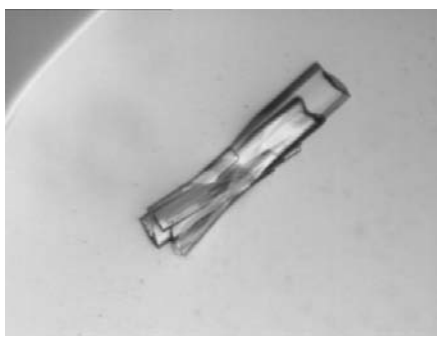

Figure 2
A cluster of gankyrin crystals with approximate dimensions 1 × 0.2 × 0.07 mm. A single crystal was cut from this cluster and used for data collection.

5. Discussion

The structures of several proteins containing ankyrin repeats are known; these feature 2.5–12 iterations of the ankyrin motif. The repeating ankyrin motif is usually made up of a core of 33 or so residues that form a $\beta_2\alpha_2$ structure containing a β-hairpin and helix–loop–helix (Sedgwick & Smerdon, 1999). Multiple ankyrin repeats stack together to form a structure resembling a cupped hand in which an extended α-helical bundle forms the palm and a perpendicularly oriented extended β-sheet forms the fingers. We are currently carrying out molecular-replacement calculations using coordinate sets from a number of these structures as search models. It will be particularly interesting to examine the structure of the 38-residue N-terminal extension and its organization with respect to the gankyrin domain. It will also be interesting to compare the structure of gankyrin with that of its yeast homologue Nas6p, the crystallization of which has recently been reported (Adachi *et al.*, 2002). These experiments represent the first step in understanding the interactions of gankyrin with its protein targets, which are at the heart of cellular biochemistry.

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