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Crystallization and preliminary X-ray diffraction studies on the catalytic domain of the chick retinal neurite-inhibitory factor CRYP-2

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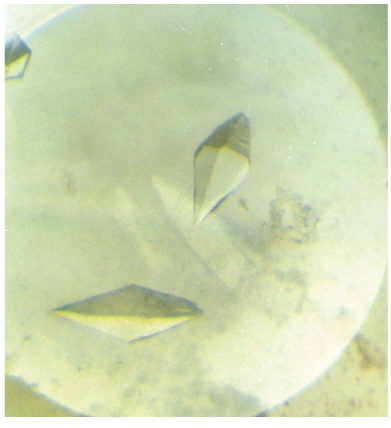
The receptor protein tyrosine phosphatase CRYP-2 has been shown to be an inhibitory factor for the growth of retinal axons in the chick. The extracellular receptor domain of CRYP-2 contains eight fibronectin repeats and studies using the extracellular domain alone demonstrated the chemorepulsive effect on retinal neurons. The precise role of the intracellular catalytic domain and the mechanism by which its activity is regulated is not known. Determination of the structure of the catalytic domain of CRYP-2 was proposed in an effort to understand the downstream signal transduction mechanism in this system. The cloning, expression, purification and crystallization of the catalytic domain of CRYP-2 are now reported. Preliminary crystallographic studies were performed on the diamond-shaped crystals, which grew under oil using the microbatch method at 298 K. Native X-ray diffraction data were collected to 2.9 Å resolution on a home source. The crystals belong to the trigonal space group $P3_121$, with unit-cell parameters $a = b = 68.26$, $c = 244.95$ Å. Assuming the presence of two molecules per asymmetric unit, the V_M value was 2.7 Å^3 Da^{-1} and the solvent content was 54.8%.

1. Introduction

Receptor protein tyrosine phosphatases (RPTPs) have been shown to play a major role in axon-guidance mechanisms in vertebrates. Genetic interactions (Sun *et al.*, 2001) link the RPTPs involved in axon guidance and, depending upon the spatial and temporal context, the relationships amongst these proteins vary from partial redundancy, collaboration to competition. These RPTPs have been extensively examined *in vivo* in the midline axon-guidance system of the fruit fly *Drosophila melanogaster* and the retinotectal system of the chick *Gallus gallus*. Five RPTP genes in the chick, CRYPα, CRYP-2, PTPμ, PTPγ and PTPα, have different but overlapping expression patterns throughout the retina and the tectum. PTPα is restricted to Mueller glia cells and the radial glia of the tectum, indicating a possible function in controlling neuronal migration (Ledig *et al.*, 1999). CRYPα, CRYP-2 and PTPμ are present in defined laminae of the tectum. The proteins CRYPα and CRYP-2 in particular are expressed throughout the retinal ganglion cell layer, from which axons grow out to their tectal targets. It is proposed that these two proteins respond directly to cell- or substrate-adhesion cues in the path of retinal growth cones but do not participate in topographic mapping itself as they do not occur in gradients in the retina (Ledig *et al.*, 1999). *In vitro* functional assays (Stepanek *et al.*, 2001) have shown that the extracellular domain of CRYP-2/cPTPRO is an anti-adhesive neurite-inhibitory molecule for retinal axons with potent growth-cone-collapsing activity.

CRYP-2 is a type-III receptor protein tyrosine phosphatase and has been classified into the PTPρ subfamily of RPTPs. Members of this class of RPTPs are of particular interest in the axon-guidance mechanism as they are exclusively expressed in neurons during axonogenesis. This family includes the mammalian RPTPs PTP-BK/GLEPP-1 as well as DPTP10D and DPTP99A from *Drosophila*. While the extracellular receptor domain of CRYP-2 alone has been demonstrated to possess chemorepulsive activity, the role of the cytosolic domain and the mechanism by which its activity is regulated



are as yet unclear. Control of the activity of the intracellular catalytic domains in this class of proteins can be orchestrated either by the extracellular receptor component or cytosolic interacting partners (Johnson &Van Vactor, 2003). In an effort to understand the role of this catalytic domain and the mechanism(s) by which its activity is regulated, we have cloned and overexpressed the catalytic domain of CRYP-2 in *Escherichia coli*. Here, we report the purification, crystallization and preliminary crystallographic studies performed on this catalytic domain.

2. Materials and methods

2.1. Protein purification and expression

The gene encoding CRYP-2 (residues 95–377; 0 in this scheme refers to the start of the intracellular domain) was cloned between the *Nhe*I and *Xho*I restriction sites of the bacterial expression vector pET-15b (incorporating an N-terminal polyhistidine tag) to simplify protein purification. After transforming the plasmid into BL21 (DE3) cells (Novagen Inc.), the cells were grown to an optical density at 600 nm of 0.5, whereupon the cells were induced with 1 m*M* IPTG (final concentration). Following this, the temperature for growth was lowered to 290 K and cells were grown for a further 6 h before they were spun down and stored at 193 K until use. The cells were resuspended in lysis buffer (50 m*M* sodium phosphate buffer, 250 m*M* NaCl pH 7.5). After sonication for 4 min on ice, the cell debris was separated from the crude cell lysate by centrifugation for 30 min at 10 000 rev min^{-1} in a Sorvall centrifuge. After equilibration with cobalt-containing Talon (Clontech Inc.) resin (approximately 4 ml of resin suspension was used for the cell-free lysate from 10 g cell paste) and a washing step with buffer *B* (50 m*M* sodium phosphate, 250 m*M* NaCl, 5 m*M* imidazole pH 7.5), the N-terminal six-His-tagged CRYP-2 was eluted from the column in the elution buffer (50 m*M* sodium phosphate, 250 m*M* NaCl, 200 m*M* imidazole pH 7.5). The partially purified protein (Fig. 1) was further subjected to size-exclusion chromatography on a Sephacryl Hiprep 16/60 S-200 HR column (Amersham Biosciences Inc.). Based on the elution volume of CRYP-2 in the size-exclusion chromatography experiment, we infer that this protein is a dimer in solution.

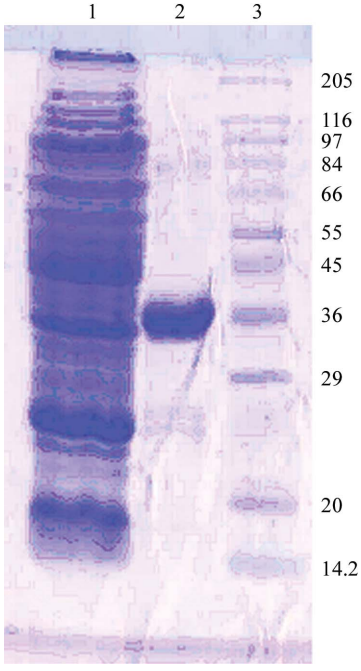


Figure 1
A 12% SDS–PAGE shows the overexpression and purification of the catalytic domain of CRYP-2. Lane 1, cell lysate prior to incubation with Talon resin. Lane 2, CRYP-2 after purification (post-gel filtration). Lane 3, molecular-weight markers in kDa (Sigma–Aldrich Inc., wide-range).

Table 1
Summary of data-collection, processing and refinement statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	1.54
Resolution (Å)	20–2.9
No. of reflections	25486
No. of unique reflections	11010
Redundancy	1.99 (1.97)
Completeness (%)	95.5 (80.1)
R_{sym}† (%)	6.03 (14.72)
$I/\sigma(I)$	7.7 (3.9)

† $R_{sym} = \sum_j |\langle I\rangle - I_j| / \sum \langle I\rangle$, where I_j is the intensity of the jth reflection and $\langle I\rangle$ is the average intensity.

2.2. Crystallization and data collection

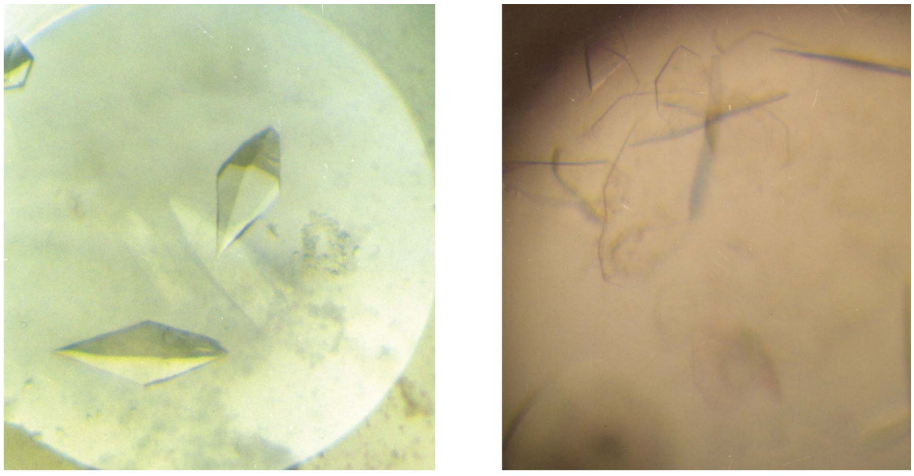
Initial screening for the crystallization conditions for this protein was performed using crystallization kits from Hampton Research (Crystal Screens 1 and 2 and PEG-Ion Screen). The conditions were examined using the hanging-drop method at 293 K, where the drop (4 µl) contained 2 µl protein solution and 2 µl well solution. Crystalline precipitates were observed in the PEG-Ion screen in conditions containing either magnesium nitrate or ammonium nitrate. A range of polyethylene glycols of different molecular weights were examined for their suitability as precipitants in an effort to obtain single crystals. Plate-like crystals were obtained in 4–5 d in a condition containing PEG 10K and magnesium nitrate, whereas diamond-shaped crystals were obtained using PEG 10K and ammonium nitrate (Fig. 2). The crystals were very susceptible to oxidation, resulting in a rapid deterioration of crystal quality as a function of time. The crystallization conditions were then optimized in the oil-drop method, which yielded crystals that were far superior to those obtained from the hanging-drop vapour-diffusion method. The crystals obtained from conditions containing magnesium nitrate diffracted much more poorly than those obtained using the ammonium nitrate condition. The crystal for which diffraction data is reported in this manuscript was obtained from a condition containing 15% PEG 10K with 0.6 *M* ammonium nitrate.

2.3. Data collection

A single crystal of CRYP-2 obtained using the ammonium nitrate condition was mounted on a cryoloop using 25% PEG 400 as the cryoprotectant. The cryoprotectant conditions were optimized to provide the lowest mosaicity values upon freezing. It was essential that the cryoprotectant buffer contained 1–5 m*M* DTT. The diffraction data were collected at 100 K on a MAR imaging-plate system mounted on a Rigaku RU-200 rotating-anode X-ray generator. The data were processed using *AUTOMAR* (http://www.marresearch.com/automar/automar/run.htm) and were scaled using the program *MARSCALE*.

3. Results and discussion

The crystals of the catalytic domain of CRYP-2 appeared after 3–4 d and grew to dimensions of 0.3 × 0.2 × 0.07 mm after about 7–9 d. The



(*a*) (*b*)

Figure 2
Crystals of CRYP-2 obtained in two different crystal forms. The diamond-shaped crystals seen in (*a*) (obtained in the presence of ammonium nitrate) diffract X-rays better than the plate-like crystal form in (*b*) (obtained using magnesium nitrate) and belong to the trigonal space group $P3_121$.

crystals diffracted to better than 2.9 Å resolution. A total of 25 486 measured reflections were merged into 11 010 unique reflections with an $R_{\rm merge}$ of 6.03%. The crystals belong to the primitive trigonal space group, with unit-cell parameters $a = b = 68.26$, $c = 244.95$ Å. Table 1 summarizes the data-collection statistics. Based on the molecular weight and the space group, the crystal was assumed to contain two protein molecules per asymmetric unit, giving a $V_{\rm M}$ value of 2.71 Å^3 Da^{-1}. The catalytic domain of RPTPμ (PDB code 1rpm; Hoffmann *et al.*, 1997) served as the starting model for solving the structure by molecular replacement using the program *MOLREP* (Vagin & Teplyakov, 1997). In a fully automated rotation and translation search, two copies of the domain could be located in the asymmetric unit and the ambiguity in the two enantiomeric space groups ($P3_121$ and $P3_221$) could be resolved in favour of $P3_121$. Model building and refinement of this structure are in progress.

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