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Expression, purification and preliminary crystallographic characterization of a novel segment from the neurofibromatosis type 1 protein

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Neurofibromin (MW 320 kDa) is the protein responsible for the pathogenesis of neurofibromatosis type 1 (NF1), one of the most common genetic diseases worldwide. The neurofibromin GAP-related domain (GRD, MW 38 kDa) possess a Ras-specific GTPase-activating protein property, which is at present its only clear biochemical function. This article describes the study of the bacterial production and preliminary X-ray crystallographic analysis of a neurofibromin fragment located at the C-terminal end of the GRD, which contains a region reported to be homologous to the yeast Sec14p lipid exchange protein. Of the three crystal variants obtained, a tetragonal form diffracted to a resolution of at least 2.3 Å.

1. Introduction

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen neurofibromatosis (Riccardi, 1992), is an autosomal dominant genetic disease with an incidence of 1 in 3500 births (http://www.nf.org). Affected patients display a complex clinical pattern predisposing them to the formation of benign to severe malignant tumours of the peripheral nerve system, learning disabilities and numerous poorly understood complications. The disease is caused by mutations in the NF1 gene (Upadhyaya *et al.*, 1994), which encodes a 320 kDa protein termed neurofibromin (DeClue *et al.*, 1991; Gutmann *et al.*, 1991). Neurofibromin functions as a Ras-specific GTPase-activating protein (GAP) with the ability to stimulate GTP hydrolysis on normal but not on oncogenic Ras. The segment responsible for this activity resides in a central portion of about 330 residues, called the GAP-related domain (GRD; Ballester *et al.*, 1990; Martin *et al.*, 1990; Xu *et al.*, 1990). Loss of neurofibromin contributes to the up-regulation of Ras in some tumours (Basu *et al.*, 1992; DeClue *et al.*, 1992). NF1 orthologues have been characterized in *Drosophila melanogaster* (The *et al.*, 1997) and *Saccharomyces cerevisiae* (Buchberg *et al.*, 1990); in the latter case, homology occurs in an extended portion of the GRD. While the RasGAP activity has been characterized on a structural (Scheffzek *et al.*, 1997, 1998) and on a biochemical level (Ahmadian *et al.*, 2003), very little is known about the potential functions of the remaining portion of the protein. This is particularly important as the RasGAP activity requires only ∼7% of the whole neurofibromin (Ahmadian *et al.*, 1996). In addition, a considerable number of missense mutations described in patients are found outside this domain (Fahsold *et al.*, 2000).

Employing a comparative structural approach, we are trying to identify functions other than RasGAP activity by analyzing the three-dimensional structure of neurofibromin and its fragments. Clearly, structural knowledge of any portion of neurofibromin should provide an important piece of information to analyze the potential effects of mutations identified in NF1 patients and to shed light on novel functions of neurofibromin. In this work, we focused on a segment covering approximately 280 residues in a region next to the C-terminus of the GRD.

Low but significant sequence homology with the yeast lipid-exchange protein Sec14p has been detected in this portion, which has been speculated to play a role in lipid-dependent modulation of the RasGAP activity (Aravind *et al.*, 1999). Inhibition of GAP activity by a number of lipids has previously been demonstrated (Bollag & McCormick, 1991; Golubic *et al.*, 1991), with conflicting results. We have overexpressed, purified and crystallized a fragment (MW 31.3 kDa, amino acids 1545–1816) containing the Sec14p-homology domain from human and *Drosophila* (MW 31.5 kDa, amino acids 1596–1868) neurofibromin. A number of crystal forms were obtained under different conditions.

2. Materials and methods

2.1. Cloning

cDNAs derived from human (residues 1545–1816) and *Drosophila* (residues 1596–1868) Sec14p-homologous coding regions were amplified by the polymerase chain reaction (PCR) using Pfu-Turbo polymerase (Strata-

gene) and cloned into a modified pET24d vector (Novagen) to provide an N-terminal 6×His tag followed by a tobacco etch virus (TEV) protease-cleavage site. *Escherichia coli* strain BL21(DE3)-Codon-plus-RIL (Novagen) was used for expression. Cells were grown in Terrific Broth (Sambrook & Russell, 2001) to an OD_{600} of 0.6, cooled to 288 K and induced with 0.2 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for 30 h at 288 K.

2.2. Protein expression and purification

Cell pellets were resuspended in 10 ml lysis buffer per gram of cells and passed through an EmulsiFlex C5 homogenizer (Avestin). The lysis buffer contained 20 m*M* Tris pH 7.5, 500 m*M* NaCl, 1% Triton X-100, 15 m*M* imidazole and 10 m*M* β-mercaptoethanol. The lysate was cleared by centrifugation (125 000*g* for 45 min at 277 K) in a type 45Ti rotor (Beckman) and applied onto Ni–NTA superflow resin (Qiagen) using a ratio of 1 ml of resin per 2.5 g cells. The column was initially washed with seven column volumes of lysis buffer and then with the same volume of lysis buffer without detergent (wash buffer). The protein was eluted in wash buffer containing 250 m*M* imidazole, mixed with TEV protease [at a ratio of 1:500(*w*:*w*)], dialyzed against wash buffer containing 1 m*M* EDTA for 20 h and concentrated to 2.5 ml in a Centriprep YM10 (Millipore). The buffer was exchanged to wash buffer using a PD10 column (AP biotech) before reloading onto the same Ni–NTA column. The protein collected in the flowthrough was concentrated to approximately 20 mg ml^{-1} with a Centricon YM10 (Millipore). The buffer was then exchanged to 20 m*M* Tris pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol before flash-freezing aliquots in liquid nitrogen.

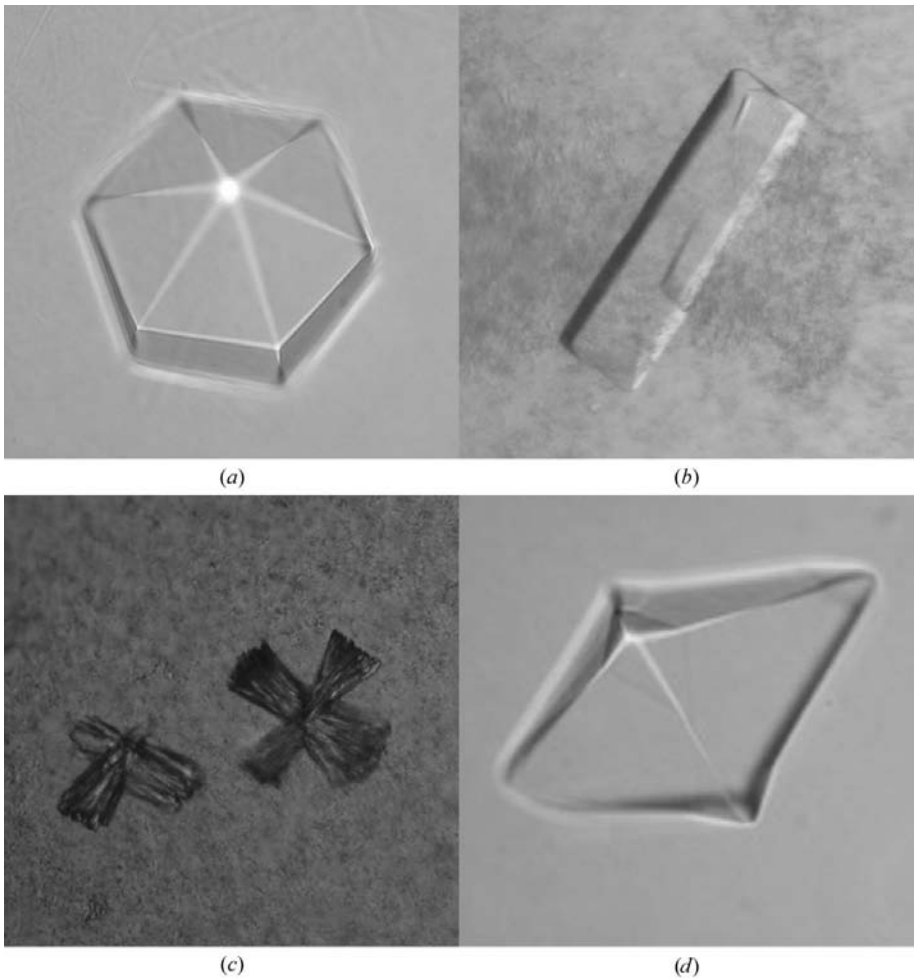

Figure 1
Various crystal forms of human and *Drosophila* neurofibromin segments containing the Sec14-homology domain. (*a*) Hexagonal crystals of the human protein. (*b*) Rod-like crystal of the *Drosophila* variant; the crystals often develop branches on one side of the rod. (*c*) Initial crystals of the human protein grown from 0.2 *M* MES pH 6, 0.2 *M* NaCl and 10%(*w*/*v*) PEG 4000. (*d*) Tetragonal crystal form of the human protein resulting from the refinement of conditions in (*c*) using a homemade salt/PEG screen (see text).

2.3. Crystallization and X-ray analysis

Crystallization of Sec14-homology domain constructs was performed with the hanging-drop method in VDX plates using Crystal Screens I and II (Hampton Research) or home-made crystallization screens (K. Scheffzek, unpublished work). For initial trials, equal volumes (1–2 µl) of protein solution (15 mg ml^{-1}) and crystallization screening buffer were gently mixed on a cover slip and suspended over a reservoir containing 400–500 µl crystallization screening solution. Experiments were carried out at room temperature (∼293 K) or at 277–280 K. For X-ray analysis, crystals were flash-cooled in liquid nitrogen after cryoprotection by a single transfer or by serial transfer into a solution containing mother liquor and 5–20% ethylene glycol. Diffraction data were measured at beamlines ID14-2, ID14-4 and ID29 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) or at beamline BW7A at EMBL c/o DESY (Hamburg, Germany). Data were acquired under cryogenic conditions (100 K, Oxford Cryosystems Cryostream) and recorded using ADSC Q210 CCD (beamline ID29), ADSC Q4R CCD (beamline ID14-2 and ID14-4) or MAR 165 mm CCD (beamline BW7A) detectors. Data were processed and scaled with the program package *XDS* (Kabsch, 1993) or *MOSFLM* v.6.1.1 (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

A deletion/extension method was used to identify soluble fragments of neurofibromin containing the Sec14p region. From a total of 24 constructs of various lengths covering the region between residues 1480 and 1944 of the human neurofibromin, only two yielded soluble (as assessed by a small-scale solubility assay; G. Stier, unpublished work) protein in large amounts. Both of these constructs ended at position 1816, with the N-terminus at position 1529 or 1545. Typical yields were around 5 mg protein per litre of culture (corresponding to 10 g of cells). The protein purification was according to standard protocols as described in §2.

Initial crystals of the human protein grew at room temperature (∼293 K) from hanging drops composed of equal amounts of protein (15 mg ml^{-1}) and reservoir solution (0.6 *M* NaH_2PO_4, 0.1 *M* sodium acetate pH 6). Hexagonal bipyramidal plates appeared after 14 d and grew to their full size within two weeks (Fig. 1*a*). They

Table 1
Summary of preliminary crystallographic analysis.

Value in parentheses are for the highest resolution shell.

Crystal form†	*A* (*Hs*NF1)	*B* (*Hs*NF1)	*C* (*Dm*NF1)
Crystal dimensions (µm)	100 × 100 × 50	300 × 300 × 200	150 × 40 × 40
Space group	$P6_{1(5)}22$	$P4_{1(3)}2_12$	$P2_12_12_1$
Unit-cell parameters (Å)	$a = b = 117.8$, $c = 97.8$	$a = b = 112.4$, $c = 124.2$	$a = 87.8$, $b = 119.9$, $c = 136.8$
Resolution (Å)	15–2.9 (2.9–3.14)	15–2.3 (2.3–2.41)	15–3.4 (3.4–3.51)
No. observations	68022 (9986)	337912 (35476)	120518 (17610)
No. unique reflections	8438 (1189)	38152 (4490)	19044 (2714)
Redundancy	8.1 (8.4)	8.8 (7.9)	6.3 (6.5)
Completeness (%)	96 (99.8)	98 (100)	97 (100)
R_{sym}‡ (%)	5.3 (29.9)	6.7 (30.9)	8.8 (29.9)
$I/\sigma(I)$	7.8 (2.2)	21 (5.8)	3 (1.8)
No. molecules in AU§	1	2	2
V_M (Å^3 Da^{-1})	3.1	3.2	5.6
Solvent content (%)	∼58	∼60	∼80

† *Hs*, *Homo sapiens*; *Dm*, *Drosophila melanogaster*. ‡ As defined in *XDS* (Kabsch, 1993) or *MOSFLM* (Collaborative Computational Project, Number 4, 1994). § As suggested from the Matthews parameter (Matthews, 1968) or self-rotation calculations using *POLARRFN* (Collaborative Computational Project, Number 4, 1994).

diffracted to no more than 3 Å at beamline ID29 of ESRF or comparable sources.

As we were not able to improve the quality of these crystals, we focused on a second crystal form that had previously been obtained from 0.2 *M* MES pH 6, 0.2 *M* NaCl and 10%(*w*/*v*) PEG 4000 but primarily grew in polycrystalline bundles (Fig. 1*c*). A self-made screen was performed employing different sodium salts and PEGs of various molecular weights at concentrations between 2.5 and 25%(*w*/*v*), which resulted in the formation of bipyramidal crystals in the presence of $Na_4P_2O_7$ (Fig. 1*d*). These crystals appeared within 12 h. Further optimization of $Na_4P_2O_7$ and PEG 4000 concentrations was performed in order to increase the crystal size. The optimized procedure was carried out for routine crystal production, as described in §2. The protein concentration was 15 mg ml^{-1} in a buffer consisting of 20 m*M* Tris, 50 m*M* NaCl, 1 m*M* β-mercaptoethanol, 1 m*M* EDTA and the reservoir solution (500 µl) was 0.25 *M* $Na_4P_2O_7$, 0.1 *M* MES pH 6, 12.5%(*w*/*v*) PEG 4000. Crystals appeared after 12 h at room temperature and reached final dimensions of 0.3 × 0.3 × 0.2 mm within 48 h. Two types of bipyramidal crystals have been observed: larger crystals with a larger central plane, which diffracted to 2.6–3 Å, and smaller ones with a higher diffraction potential.

The *Drosophila* protein crystallized at room temperature in hanging drops under various conditions. A multitude of small crystals was observed when the crystallization solution contained 1 *M* Na_2HPO_4/KH_2PO_4 pH 6.3, 0.1 *M* MES pH6, 5%(*w*/*v*) PEG 6000 or 30%(*v*/*v*) MPD, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate. One condition was further investigated using a homemade salt screen at various pH values, giving a condition (1.6 *M* ammonium sulfate, 0.1 *M* Bicine pH 9 or 0.1 *M* Tris–HCl pH 8) that resulted in crystal formation after 2 d at 291 K (Fig. 1*b*). The crystals appeared as rods with dimensions of approximately 150 × 40 × 40 µm. SDS–PAGE analysis confirmed the presence of the full protein segment in the crystals (not shown). Diffraction data were recorded to 3.4 Å resolution at ID14-4 at ESRF.

For X-ray data collection, crystals were transferred briefly to cryoprotecting solutions using rayon loops and either flash-cooled in liquid nitrogen or transferred quickly to the cryostream mounting. Serial transfers in cryoprotecting solutions with increasing concentrations of cryoprotectant or brief soaking of the crystals in medium-light paraffin oil or Paratone-N with subsequent elimination of residual external solvent did not improve diffraction. Cryogenic data collection was performed on beamlines ID14-2/29 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France or on beamline BW7B at EMBL Hamburg (c/o Deutsches Elektronen Synchrotron, DESY), Germany. Data processing was performed with the programs *XDS* (Kabsch, 1993) or *MOSFLM* (Collaborative Computational Project, Number 4, 1994). Data-collection statistics and crystal data are summarized in Table 1.

Our study describes the expression, purification and preliminary crystallographic analysis of a novel fragment from human and *Drosophila* neurofibromin. Considering the notoriously insoluble nature of fragments of this protein, these results represent important progress. A number of mutations located in the fragment studied here have been identified in NF1 patients. The three-dimensional structure of this fragment should shed light on new functions potentially not associated with its GAP activities.

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