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Expression, crystallization and preliminary X-ray analysis of extracellular modules of the neural cell-adhesion molecules NCAM and L1

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Recombinant proteins consisting of either the four or five amino-terminal immunoglobulin (Ig) modules of the rat neural cell-adhesion molecule NCAM or the whole extracellular part [six Ig and five fibronectin type III (F3) modules] of mouse L1 have been expressed in *Drosophila* S2 cells. The proteins have been purified and crystallized. The crystals of the recombinant protein containing the four amino-terminal Ig modules of NCAM diffract X-rays to ~4 Å resolution and belong to space group $P622$ or $P6_322$, with unit-cell parameters $a = b = 258.7$, $c = 182.4$ Å. No diffraction was observed for the other two protein constructs. This is a step towards determining the structure of multimodular constructs of cell-adhesion molecules that exhibit high structural flexibility.

1. Introduction

The neural cell-adhesion molecules NCAM and L1 are cell-surface glycoproteins belonging to the immunoglobulin (Ig) superfamily. NCAM and L1 are mainly expressed on the surface of cells in the central and peripheral nervous systems (Linnemann & Bock, 1989; Linnemann *et al.*, 1993). NCAM and L1 have diverse functions. Both proteins are known to mediate cell–cell interactions by a homophilic binding mechanism and to engage in heterophilic interactions with counter-receptors and various components of the extracellular matrix (Nybroe & Bock, 1990). NCAM plays a pivotal role during development of the nervous system, mediating adhesion between neural cells and modulating neuronal differentiation, axonal outgrowth and fasciculation. NCAM also regulates synaptic plasticity, underlying learning and memory consolidation (Rønn *et al.*, 2000). NCAM-mediated neurite outgrowth is modulated by the expression of ten amino acids encoded by the variable alternatively spliced exon (VASE or π-exon) in the fourth Ig module of NCAM (Lahrtz *et al.*, 1997). L1 is involved in myelination, neuronal cell migration, neurite outgrowth, axon fasciculation and pathfinding, and long-term potentiation in the hippocampus (Hortsch, 1996).

The extracellular part of NCAM consists of five Ig and two fibronectin type III (F3) modules and NCAM is expressed in three major isoforms: two transmembrane isoforms (180 and 140 kDa) and a glycosylphosphatidylinositol-anchored isoform (120 kDa; Goridis & Brunet, 1992; Chothia & Jones, 1997). L1 consists of six extracellular Ig modules and five F3 modules in the amino-terminal region followed by a transmembrane part and a highly conserved cytoplasmic tail (Gegelashvili *et al.*, 1993). The X-ray structure of the NCAM Ig1-2 modules (Kasper *et al.*, 2000) and the Ig1-3 modules (Soroka *et al.*, 2003) as well as NMR structures of the individual Ig1, Ig2 and Ig3 modules (Thomsen *et al.*, 1996; Jensen *et al.*, 1999; Atkins *et al.*, 2001) have been described previously. However, determination of the three-dimensional structures of larger fragments or of all seven extracellular modules of NCAM and all 11 extracellular modules of L1 are needed to fully clarify the molecular mechanisms underlying NCAM- and L1-mediated adhesion and signal transduction. Here, we present the expression, purification, crystallization and preliminary X-ray experiments of NCAM Ig1-4, NCAM Ig1-5 and all extracellular modules of L1.

2. Materials and methods

2.1. Expression and purification

The cDNA fragments encoding Ig modules 1–4 (residues 1–407) and Ig modules 1–5 (residues 1–502) of NCAM with a C-terminal His tag were synthesized by PCR using the rat NCAM-120 (without the alternatively spliced exon VASE) cDNA as a template. The cDNA fragment encoding the extracellular part of L1 (residues 33–1110) with a C-terminal His tag was synthesized by PCR using mouse L1 cDNA as a template. The amplified cDNA fragments were subcloned into the *Kpn*I/*Xho*I site of pMT/V5-His A plasmid (Invitrogen) (NCAM constructs) or into the *Sma*I/*Xho*I site of pMT/BiP/V5-His A plasmid (Invitrogen) (L1 construct). An *Escherichia coli* strain Top 10F′ (Invitrogen) was used for transformation and the recombinant clones were identified by restriction analysis of the plasmid DNA. The

recombinant plasmids encoding NCAM or L1 fragments were then cotransfected with the pCoBlast plasmid (Invitrogen) into *Drosophila* S2 cells (Invitrogen) according to the protocol supplied by the manufacturer. Stable clones were obtained using the antibiotic Blasticidin S. The recombinant proteins were expressed by induction of *Drosophila* S2 cells for 3 d after the cell density reached 4×10^6 cells ml^{-1}.

The proteins were isolated from components of the expression medium by gel-filtration on a Sephadex G-25 column (Pharmacia). The proteins were subsequently purified by affinity chromatography on an Ni–NTA column (Qiagen) and by ion-exchange chromatography on a 5 ml HiTrap SP or Q column (Pharmacia). The yields were 1–3 mg per litre of expression medium. The proteins were deglycosylated with PNGase F (New England Biolabs) at a concentration of 100 U per milligram of protein for 24 h at room temperature. The final step of purification was gel filtration on a Superdex 220 column (Pharmacia). The molecular weights of NCAM Ig1-4, NCAM Ig1-5 and L1 were ~40, 50 and 110 kDa, respectively, as estimated by SDS–PAGE. The authenticity of the proteins was confirmed by DNA sequencing, Western blotting and protein N-terminal sequencing. Dynamic light-scattering (DLS; DynaPro equipment, Protein Solutions) analysis was used to evaluate the molecular aggregation state of the molecules, which showed that the samples were monodisperse prior to crystallization.

2.2. Crystallization

The initial screening for crystallization conditions was carried out in 1 µl + 1 µl hanging-drop vapour-diffusion experiments with protein concentrations of 5.5 mg ml^{-1} (NCAM Ig1-4), 5 mg ml^{-1} (NCAM Ig1-5) and 3.5 mg ml^{-1} (L1) in 10 m*M* HEPES pH 7.5, 15 m*M* NaCl using Crystal Screens I and II (Hampton Research). Numerous small crystals of the L1 construct appeared in 1.5 *M* lithium sulfate, 0.1 *M* HEPES pH 7.5. The conditions were optimized and large crystals were obtained at room temperature within 1–2 d in 1.3 *M* lithium sulfate, 0.1 *M* HEPES pH 7.5. In contrast, no microcrystals of NCAM Ig1-4 or Ig1-5 were obtained from the Crystal Screens. However, the most promising conditions were optimized and large single crystals were obtained for NCAM Ig1-4 within 3 d in 1.4 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 5.5 and for NCAM Ig1-5 within 1–2 d in 1.2 *M* lithium sulfate, 0.1 *M* sodium acetate pH 5.0.

Owing to the weak diffraction of the crystals of the various constructs, several optimization procedures were applied. The effect of varying temperature, pH and precipitant and of using Additive Screens I and II (Hampton Research) and Detergent Screens I and II was investigated. The presence of even very small amounts of polyethylene glycols prevented crystallization by precipitating the protein. The presence of several other additives provided crystals, but without improvement of their diffraction properties. Successful post-crystallization soaking was reported for Mtcp1 crystals (Petock *et al.*, 2001), but in the case of Ig1-4 soaking the crystals with different concentrations of ammonium sulfate did not result in improved diffraction, even though improvement by dehydration at high concentrations of ammonium sulfate was expected (Heras *et al.*, 2003). A construct encoding the VASE exon in the fourth Ig module of NCAM was also prepared, but gave small and non-diffracting crystals of NCAM Ig1-5.

3. Data collection and processing

X-ray data were collected at room temperature and at 120 K using the crystallization solution containing 18% glycerol as a cryoprotectant on in-house equipment (MAR 345 image-plate system with a Rigaku rotating-anode Cu $K\alpha$ X-ray source) and at synchrotron sources (ID29, ESRF, Grenoble, France, X11, EMBL, Hamburg, Germany and I711, MAX-Lab, Lund, Sweden). The X11 beamline at EMBL, Hamburg was equipped with a MAR CCD detector. The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997).

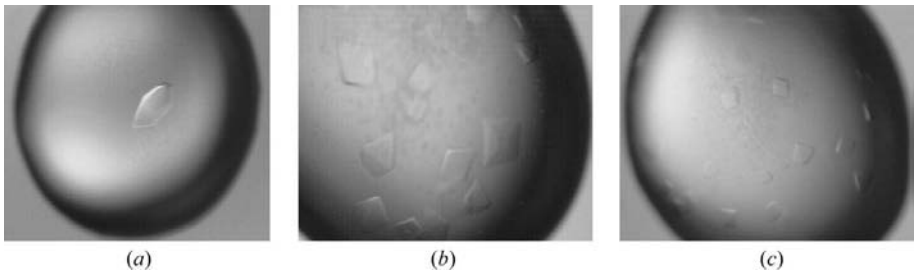

Figure 1
Crystals of (*a*) rat NCAM Ig1-4, (*b*) rat NCAM Ig1-5 and (*c*) extracellular part of mouse L1.

Table 1
Data-collection and processing statistics for NCAM Ig1-4 crystals.

Values in parentheses are for the highest resolution shell.

X-ray source	X11, EMBL, Hamburg
Wavelength (Å)	0.8111
Space group	*P*622 or $P6_322$
Unit-cell parameters	
a (Å)	258.7
b (Å)	258.7
c (Å)	182.4
Mosaicity (°)	0.48
Resolution (Å)	35–7.15 (7.47–7.15)
No. observations	31980
No. unique reflections	5417
Redundancy	5.9
Completeness (%)	96.7 (98.1)
$I/\sigma(I)$	13.2 (4.1)
R_{sym} (%)	11.5 (45.5)

4. Results and discussion

Crystals of NCAM Ig1-4 with dimensions of $0.4 \times 0.6 \times 0.6$ mm (Fig. 1*a*) diffracted at best to 4 Å resolution (ESRF, Grenoble; see Fig. 2*a*), but owing to time limitations a data set was not collected. However, a full data set was collected to 7.15 Å at EMBL, Hamburg (Table 1). The crystals of NCAM Ig1-4 exhibited a hexagonal lattice (*P*622 or $P6_322$). Assuming the presence of eight molecules of NCAM Ig1-4 per asymmetric unit, V_M was 2.7 Å^3 Da^{-1}, corresponding to a crystal solvent content of 54% using the Matthews formula (Matthews, 1968). Therefore, the asymmetric unit of the crystal seems to contain four dimers of NCAM Ig1-4, as NCAM Ig1-2 (Kasper *et al.*, 2000) and NCAM Ig1-3 (Soroka *et al.*, 2003) both crystallize forming the same dimer. Unfortunately, no diffraction was observed for crystals of NCAM Ig1-5 ($0.4 \times 0.5 \times 0.5$ mm; Fig. 1*b*) or L1 ($0.2 \times 0.3 \times 0.3$ mm; Fig. 1*c*).

It has previously been reported that multimodular proteins can be very flexible, resulting in crystals with poor-quality diffraction (Lombardo *et al.*, 1996). For example, crystals of the extracellular part of the multimodular protein neuroglian (the *Drosophila* homologue of L1) did not diffract X-rays (Huber *et al.*, 1994) and in crystals of the extracellular part of EGFR in complex with EGF it was not possible to locate the fourth module of the receptor (Ogiso *et al.*, 2002). This is probably also the case for the NCAM and L1 crystals. Electron-microscopy (EM) studies revealed

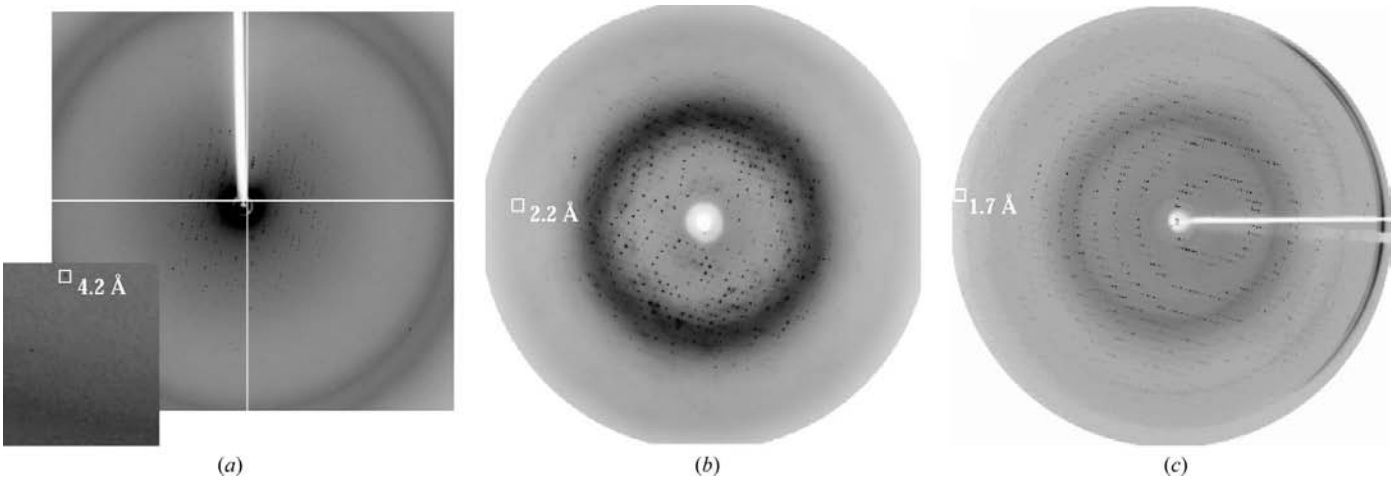

Figure 2
Diffraction images of (*a*) rat NCAM Ig1-4 (ID29, ESRF), (*b*) rat NCAM Ig1-3 (I711, MAX-Lab) and (*c*) rat NCAM Ig1-2 (BM14, ESRF).

that the extracellular parts of NCAM (Hall & Rutishauser, 1987; Becker *et al.*, 1989) and L1 (Drescher *et al.*, 1996; Hall *et al.*, 2000; Schürmann *et al.*, 2001) are capable of adopting different conformations. EM analyses of NCAM have revealed a bent rod-like structure. The angle of the bend was shown to vary considerably (50–140°), with an average value of 98° (Becker *et al.*, 1989), indicating internal flexibility of NCAM. L1 is observed to adopt both an extended rod-like and a more compact (possibly horse-shoe-like) structure (Schürmann *et al.*, 2001). On the basis of the crystal structures of the L1-homologous proteins hemolin (Su *et al.*, 1998) and axonin-1/TAG-1 (Freigang *et al.*, 2000), it was suggested that the Ig1-4 modules of L1 fold in a horseshoe-like shape.

Comparing the diffraction quality of crystals of the different extracellular constructs of NCAM crystallized so far (Ig1-2, Ig1-3, Ig1-4 and Ig1-5), a clear correlation is observed between the number of modules and the crystal quality. The best diffraction resolution was obtained for Ig1-2 crystals and gradually decreased with the increasing number of modules (Fig. 2). The type of conserved linker residues (Pro-Lys-Leu-Gln-Gly-Pro) between Ig4 and Ig5 in various species of vertebrate NCAMs is likely to introduce a bend between Ig4 and Ig5 modules and provide further flexibility, which agrees with electron-microscopy studies (Hall & Rutishauser, 1987; Becker *et al.*, 1989). Even though the crystals of NCAM Ig1-4 diffract to a modest resolution, some structural information may be provided on the largest fragment of an NCAM molecule available. The encouraging results obtained at ESRF, Grenoble show that data of improved resolution can be obtained from the crystals in future experiments, contributing to a more detailed knowledge of NCAM function.

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