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Expression, crystallization and preliminary X-ray analysis of the extracellular Ig modules I–IV and F3 modules I–III of the neural cell-adhesion molecule L1

Nikolaj Kulahin,[a,b]* Christina Kasper,[b] Ole Kristensen,[b] Jette Sandholm Kastrup,[b] Vladimir Berezin,[a] Elisabeth Bock[a] and Michael Gajhede[b]

[a]Protein Laboratory, Institute of Molecular Pathology, Panum Institute, Blegdamsvej 3C, DK-2200 Copenhagen, Denmark, and [b]Biostructural Research, Department of Medicinal Chemistry, Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark

Correspondence e-mail: kulahin@plab.ku.dk

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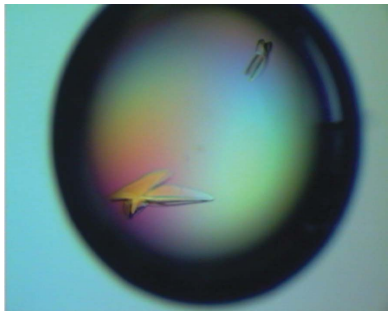


Four amino-terminal immunoglobulin (Ig) modules and three fibronectin type III (F3) modules of the mouse neural cell-adhesion molecule L1 have been expressed in *Drosophila* S2 cells. The Ig modules I–IV of L1 crystallized in a trigonal space group, with unit-cell parameters $a = b = 239.6$, $c = 99.3$ Å, and the crystals diffracted X-rays to a resolution of about 3.5 Å. The F3 modules I–III of L1 crystallized in a tetragonal space group, with unit-cell parameters $a = b = 80.1$, $c = 131$ Å, and the crystals diffracted X-rays to 2.8 Å resolution. This is a step towards the structure determination of the multimodular constructs of the neural cell-adhesion molecule L1 in order to understand the function of L1 on a structural basis.

1. Introduction

The neural cell-adhesion molecule L1 plays a pivotal role during development of the nervous system, mediating binding between neurons, neurite outgrowth and hippocampal long-term potentiation (Nybroe & Bock, 1990; Hortsch, 1996). The protein is known to mediate cell–cell interactions by homophilic binding, *i.e.* L1 on one cell binds to L1 on an adjacent cell (Grumet & Edelman, 1984; Lemmon *et al.*, 1989; Doherty *et al.*, 1995). It also mediates heterophilic binding to various cell-surface molecules and components of the extracellular matrix (Kuhn *et al.*, 1991; Horstkorte *et al.*, 1993; Felsenfeld *et al.*, 1994; Williams *et al.*, 1994; Margolis *et al.*, 1996; Sammar *et al.*, 1997).

L1 consists of six amino-terminal immunoglobulin-like (Ig) modules followed by five fibronectin type III (F3) modules, a transmembrane domain and a highly conserved cytoplasmic tail (Kiryushko *et al.*, 2004).

L1 homophilic binding activity was mapped to the first four Ig domains of human L1 (Kunz *et al.*, 1998; De Angelis *et al.*, 1999; Haspel *et al.*, 2001). This suggestion is based on the crystal structures of two L1 homologous proteins, haemolin (21% amino-acid identity; Su *et al.*, 1998) and axonin-1 (26% amino-acid identity; Freigang *et al.*, 2000). These structures reveal an unusual compact horseshoe-shaped form. This is in contrast to most tandem repeats of Ig modules, which are observed to be in a linear and extended conformation. In a study of L1 by electron microscopy, it was reported that Ig I–IV may also adopt a horseshoe-shaped conformation (Schürmann *et al.*, 2001). Such a structure might help the first four Ig domains to cooperate during their initial folding (Freigang *et al.*, 2000).

It has been demonstrated that the L1 F3 modules II and III promote signal transduction and neurite extension (Appel *et al.*, 1995). F3 module III potentiates L1 clustering and integrin ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) recruitment and this module is critical for L1-mediated neurite extension *in vitro*. Moreover, plasmin-mediated cleavage of L1 at the dibasic residues of F3 module III abolishes the multimerization and the ability of L1 to bind integrins. This indicates that the module is a key player in L1-mediated recognition processes and that proteolysis *in vivo* may provide regulation of L1-mediated cell adhesion (Silletti *et al.*, 2000).

Determination of the three-dimensional structures of extracellular modules of L1 is needed to clarify the molecular mechanisms underlying L1-mediated adhesion and signal transduction. Here, we

present data on the expression, purification, crystallization and preliminary X-ray analysis of the L1 modules Ig I–IV and F3 I–III.

2. Materials and methods

2.1. Expression and purification

cDNA fragments encoding Ig modules I–IV (Ig I–IV) and F3 modules I–III (F3 I–III) of L1 (residues 33–421 and 609–913, respectively) with a C-terminal His tag were synthesized by PCR using mouse L1 cDNA as a template. The amplified cDNA fragments were subcloned into the *Bgl*II/*Xho*I site of the pMT/BiP/V5-His A plasmid (Invitrogen). The *Escherichia coli* Top 10F′ strain (Invitrogen) was used for transformation and the recombinant clones were identified by restriction analysis. The recombinant plasmids encoding L1 fragments were then co-transfected with the pCoBlast plasmid (Invitrogen) into *Drosophila* S2 cells (Invitrogen) according to the protocol supplied by the manufacturer. Stable clones were obtained using blasticidin S selection. The recombinant proteins were expressed by induction of *Drosophila* S2 cells at a cell density of 4×10^6 cells ml^{-1} for 3 d. The proteins were isolated from components of the expression medium by gel filtration on a Sephadex G-25 column (Pharmacia), exchanging the buffer to phosphate-buffered saline (PBS; Sigma). This step was performed to remove the components of the expression medium (Drosophila-SFM; Invitrogen) since they strip the nickel ions from the resin used in the next purification step. The proteins were subsequently purified by affinity chromatography on a Ni–NTA column using Ni–NTA Superflow resin (Qiagen). Since *Drosophila* S2 cells secrete a lot of histidine-rich proteins, to avoid unspecific binding to the column imidazole was used at a concentration of 5 m*M* during loading of the sample onto the column and at a concentration of 10 m*M* during washing of the column. The affinity chromatography was followed by ion-exchange chromatography on a 5 ml HiTrap SP 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 500 U per milligram of protein for 24 h at room temperature. The final step of purification was gel filtration on a Superdex 200 column (Pharmacia). The molecular weights of L1 Ig I–IV and L1 F3 I–III were 40 and 30 kDa, respectively, as estimated by SDS–PAGE. The authenticity of the proteins was confirmed by DNA sequencing and N-terminal protein 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

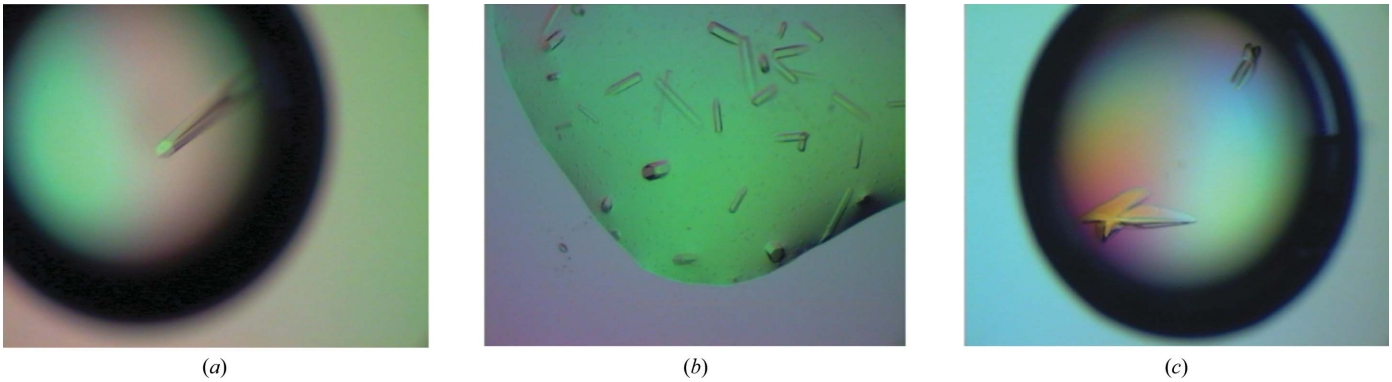
Initial screening for crystallization conditions was carried out in 1 + 1 µl hanging-drop vapour-diffusion experiments with protein concentrations of 4.5 mg ml^{-1} (L1 Ig I–IV) and 3.5 mg ml^{-1} (L1 F3 I–III) in 10 m*M* HEPES pH 7.5, 15 m*M* NaCl using Crystal Screens I and II (Hampton Research). Numerous small needle-like crystals of L1 IgI–IV appeared in 10% PEG 8000, 8% ethylene glycol, 0.1 *M* HEPES pH 7.5. The conditions were optimized and large crystals were obtained at 293 K within 3–5 d in 6% PEG 4000, 5% glucose, 0.1 *M* HEPES pH 7.0 (Fig. 1*a*). Unfortunately, the crystals (type I) only diffracted X-rays to about 10 Å resolution. Other promising conditions from the Crystal Screens were optimized and large single crystals were obtained at room temperature within 60–80 d in 1.6 *M* lithium sulfate, 0.1 *M* MES pH 6.0 (Fig. 1*b*). These crystals (type II) of L1 Ig I–IV diffracted X-rays to 3.5 Å resolution. Large crystals of L1 F3 I–III appeared at room temperature within 2–3 d in 12% PEG 20 000, 0.1 *M* MES pH 6.5. Optimization of the conditions resulted in crystallization at 279 K within 2–3 d in 15% PEG 6000, 0.1 *M* MES pH 6.5 (Fig. 1*c*). The crystals of L1 F3 I–III diffracted X-rays to 2.8 Å resolution.

3. Data collection and processing

X-ray data were collected at 120 K using 30% glucose (L1 Ig I–IV type I crystals), 12% glycerol (L1 Ig I–IV type II crystals) and 20% glycerol (L1 F3 I–III crystals) as cryoprotectants. Data were collected at beamlines BW7A and BW7B, EMBL, Hamburg, Germany equipped with a MAR CCD and a MAR Research image-plate detector, respectively. The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997).

4. Results and discussion

Crystals of L1 Ig I–IV with dimensions of $0.1 \times 0.1 \times 0.3$ mm (Fig. 1*b*) diffracted X-rays to 3.5 Å resolution at best and a data set was collected (Table 1). The L1 Ig I–IV data was indexed in a trigonal lattice ($P3_121$ or $P3_221$). Assuming the presence of six molecules per asymmetric unit, V_M was estimated to be 3.2 Å^3 Da^{-1}, corresponding to a solvent content of 61%, using the Matthews formula (Matthews, 1968). Inspection of the native Patterson function did not suggest that non-crystallographic translational symmetry was present. However, the self-rotation function indicated that a non-crystallographic threefold symmetry axis was located at $\theta = 70^\circ$ and $\varphi = 180^\circ$. Crystals of L1 F3 I–III had dimensions of $0.4 \times 1 \times 1$ mm (Fig. 1*c*) and



(*a*) (*b*) (*c*)

Figure 1
Crystals of mouse L1. (*a*) Ig I–IV type I crystals, (*b*) Ig I–IV type II crystals and (*c*) crystals of F3 I–III.

Table 1
Data-collection and processing statistics for mouse L1 Ig I–IV crystals (type II).

Values in parentheses are for the highest resolution shell.

X-ray source	BW7B, EMBL, Hamburg
Wavelength (Å)	0.84
Space group	$P3_121$ or $P3_221$
Unit-cell parameters	
a (Å)	239.6
b (Å)	239.6
c (Å)	99.3
Mosaicity (°)	0.94
Resolution (Å)	61–3.43 (3.62–3.43)
No. of observations	87741
No. of unique reflections	33271
Redundancy	2.6
Completeness (%)	75.5 (75.5)
$I/\sigma(I)$	7.4 (2.2)
R_{sym} (%)	8.8 (34.6)

Table 2
Data-collection and processing statistics for mouse L1 F3 I–III crystals.

Values in parentheses are for the highest resolution shell.

X-ray source	BW7A, EMBL, Hamburg
Wavelength (Å)	1.5
Space group	$P4_22_12$
Unit-cell parameters	
a (Å)	80.1
b (Å)	80.1
c (Å)	131.0
Mosaicity (°)	0.42
Resolution (Å)	52–2.83 (3.06–2.83)
No. of observations	242074
No. of unique reflections	17722
Redundancy	13.7
Completeness (%)	97.9 (100)
$I/\sigma(I)$	4.5 (1.9)
R_{sym} (%)	12.5 (37.1)

belonged to the tetragonal space group $P4_22_12$. The crystals diffracted X-rays to about 2.8 Å resolution and a data set was collected (Table 2). Assuming the presence of one molecule per asymmetric unit, V_M was estimated to be 3.1 Å^3 Da^{-1}, which corresponds to a crystal solvent content of 60%.

We attempted to solve the structures of both L1 Ig I–IV and F3 I–III by the molecular-replacement method. The structures of haemolin Ig I–IV (21% amino-acid identity) and axonin-1 Ig I–IV (26% amino-acid identity) were employed as models for crystals of L1 Ig I–IV and that of neuroglian F3 I–II (33% amino-acid identity) was used as a search model for crystals of L1 F3 I–III. The modules of the homologous proteins were used separately and simultaneously to find the best search models. Despite the use of various programs [*AMoRe* (Navaza, 1994), *Beast* (Read, 2001), *CNS* (Brünger *et al.*, 1998), *MOLREP* (Vagin & Teplyakov, 1997), *Phaser* (Storoni *et al.*, 2004)], no obvious solutions were identified. This indicates that it is not trivial to solve the structures of L1 Ig I–IV and L1 F3 I–III by molecular-replacement methods. We are therefore searching for heavy-atom derivatives to enable structure determination either by isomorphous replacement or by anomalous dispersion methods. The structures will illuminate the basis of L1 homophilic binding (Ig I–IV modules) and L1 clustering (F3 I–III modules), contributing to a more detailed knowledge of L1 function.

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