Acta Crystallographica Section D
**Biological Crystallography**
ISSN 0907-4449

# Expression, crystallization and preliminary X-ray analysis of the two amino-terminal Ig domains of the neural cell adhesion molecule (NCAM)

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Received 24 March 1999
Accepted 22 June 1999

The two amino-terminal Ig domains of the rat neural cell adhesion molecule, NCAM, have been expressed in the yeast strain *Pichia pastoris*. The double domain consisting of 191 amino acids was overexpressed, purified and crystallized. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a$ = 47.1, $b$ = 122.5, $c$ = 72.9 Å and $\beta$ = 98.3°. Assuming there are four double domains per asymmetric unit, $V_m$ is estimated to be 2.41 Å$^3$ Da$^{-1}$ and the solvent content to be 42.3%. A native data set has been collected from a single flash-frozen crystal to 1.85 Å resolution.

## 1. Introduction

The neural cell adhesion molecule NCAM is a cell-surface glycoprotein belonging to the immunoglobulin (Ig) superfamily. Cell adhesion molecules (CAMs) are involved in intercellular recognition and adhesion; NCAM is one of the most well characterized CAMs. NCAM is expressed on the surface of most cells in the peripheral and central nervous systems (Linnemann & Bock, 1989; Linnemann *et al.*, 1993), in muscle (Andersson *et al.*, 1993) and in the heart (Reyes *et al.*, 1991).

In the brain, three major isoforms of NCAM are expressed, with molecular weights of 190, 135 and 115 kDa. Two isoforms are transmembrane, while the third is coupled to the membrane *via* a glycosylphosphatidylinositol (GPI) anchor. The extracellular part is the same overall in all isoforms (however, alternative splicing events can occur) and consists of five N-terminal Ig domains and two membrane proximal fibronectin type III (F3) domains (Goridis & Brunet, 1992; Chothia & Jones, 1997).

The functions of the protein are many and diverse. NCAM is a morphoregulatory molecule, involved in the generation and maintenance of correct tissue morphology (Edelman, 1988). In the developing nervous system, NCAM plays a role during formation of the neural tube (Bronner-Fraser *et al.*, 1992) and, later, NCAM is a regulator of neurite outgrowth (Walsh & Doherty, 1997). The signal transduction cascade underlying NCAM-mediated neurite outgrowth might take place through an interaction with the FGF receptor (Hall *et al.*, 1996). Other investigations point towards a role of NCAM in the process of learning and memory (Lüthi *et al.*, 1994; Rønn *et al.*, 1995).

In many NCAM-mediated events, a homophilic binding interaction, *i.e.* binding of NCAM on one cell to NCAM on another cell, seems pivotal. Even though it has been a well established fact for many years that NCAM binds to NCAM (Rutishauser *et al.*, 1982), the mechanism of this interaction remains controversial. The proposed mechanisms of homophilic binding range from adhesion between only one Ig domain on each molecule to the interaction of all five Ig domains (Frei *et al.*, 1992; Rao *et al.*, 1992, 1993, 1994; Zhou *et al.*, 1993; Thomsen *et al.*, 1996; Ranheim *et al.*, 1996). Frei *et al.* (1992) and Thomsen *et al.* (1996) suggest that IgI and IgII are involved in NCAM–NCAM binding. Recently, more evidence for an interaction between IgI and IgII, based on direct affinity studies, has been put forward (Kiselyov *et al.*, 1997).

Three-dimensional structure determination of all seven extracellular domains, separately and in various combinations, is needed to fully clarify the NCAM structure and the mechanism of NCAM homophilic binding. The solution NMR structures of NCAM IgI and IgII have been reported separately (Thomsen *et al.*, 1996; Jensen *et al.*, 1999).

This is a report on the expression, purification, crystallization and preliminary X-ray analysis of the combined two N-terminal Ig domains of recombinant rat NCAM, IgI-II. Determination of this double-domain structure will give valuable information on the putative interactions between the domains and NCAM-mediated homophilic binding.

## 2. Expression and purification

The IgI-II domains of rat NCAM were produced as a recombinant protein in *Pichia pastoris*. Cloning and transfection procedures were performed as described previously (Jensen *et al.*, 1999).

A *P. pastoris* clone with a reasonable expression of NCAM IgI-II was selected and a culture of this clone was grown in YNBG medium [13.4 g yeast nitrogen base without amino acids with ammonium sulfate (Difco, Detroit), 2 ml 0.5 mg ml$^{-1}$ biotin, 10 g glycerol per litre of 100 m*M* potassium phosphate pH 6.0] at 303 K until saturation. pH was adjusted to 5.0 with 0.1 *M* KOH using a peristaltic pump connected to a pH meter.

The cells were centrifuged at 1500 rev min$^{-1}$ for 10 min at 298 K and resuspended in YNBM medium [13.4 g yeast nitrogen base without amino acids with ammonium sulfate (Difco, Detroit), 2 ml 0.5 mg ml$^{-1}$ biotin, 100 ml methanol per litre of 100 m*M* potassium phosphate pH 6.0] to start induction of protein expression. Methanol (50%) was added in a continuous flow of ∼10 ml h$^{-1}$ and cells were grown overnight. Owing to the presence of a signal peptide, the protein is secreted into the media. The culture was centrifuged at 2500 rev min$^{-1}$ for 15 min at 298 K, followed by 13000 rev min$^{-1}$ for 20 min at 298 K. The medium was concentrated at 277 K using Dow nitrocellulose filters with a cutoff value of 6 kDa. On a Sephadex G25 column (Pharmacia), the buffer was changed to 20 m*M* potassium phosphate pH 5.0, 10 m*M* NaCl, and small and large components of the medium were removed simultaneously. NCAM IgI-II was finally purified by ion exchange using a 5 ml Hi-Trap SP column (Pharmacia) and an NaCl gradient. All fractions were kept separate and analyzed by gel electrophoresis (SDS–PAGE) and silver staining. Only fractions giving rise to one band on the silver-stained gel were pooled and used for crystallization experiments. The yield was approximately 3–4 mg per litre of culture.

The authenticity of NCAM IgI-II was secured by amino-acid sequencing and mass spectroscopy. In non-reducing gel electrophoresis, the double domain migrates with an apparent molecular weight of 22 kDa, while a reducing gel shows migration corresponding to a molecular weight of ∼30 kDa.

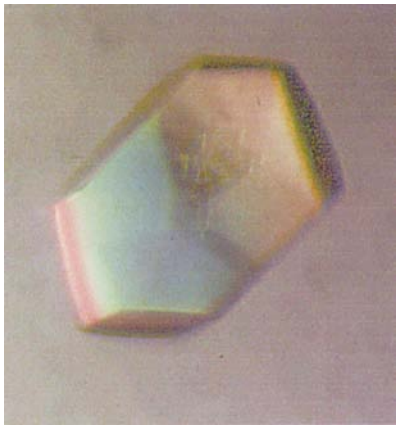

**Figure 1**
Crystal of rat NCAM IgI-II.

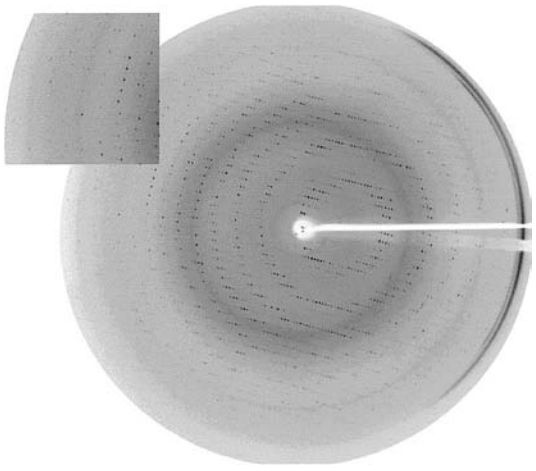

**Figure 2**
Diffraction pattern from an oscillation image of rat NCAM IgI-II. The inset shows diffraction at the edge of the image (1.7 Å resolution).

## 3. Crystallization

The initial screening for crystallization conditions was carried out in 1 + 1 µl hanging-drop experiments with 3.5 mg ml$^{-1}$ protein in 10 m*M* HEPES pH 7.5, 15 m*M* NaCl, using Crystal Screens I and II (Hampton Research). Very long crystal rods were obtained in 10% PEG 8000, 8% ethylene glycol, 0.1 *M* HEPES pH 7.5. Conditions were modified and beautiful hexagonal crystals were obtained within 4–5 d by addition of 1,2,6-trihydroxyhexane (THH) and $ZnCl_2$. However, these crystals did not show Bragg diffraction. Exchanging calcium for zinc and further optimization led to the final conditions of crystallization.

Initially, a shower of small crystals (dimensions 30 × 30 × 15 µm) were obtained from drops containing 1 µl protein solution (3.5 mg ml$^{-1}$ NCAM IgI-II in 10 m*M* HEPES pH 7.5, 15 m*M* NaCl) and 1 µl reservoir solution (14% PEG 4000, 10 m*M* $CaCl_2$). The drops were equilibrated against 0.5 ml reservoir solution at 293 K. Crystals appeared after 1–2 d and were subsequently macroseeded using 50 µm CryoLoops (Hampton Research) into a drop containing 1.2 µl protein solution and 1 µl reservoir solution (12–13% PEG 4000, 15 m*M* $CaCl_2$). The crystals grew to a maximum size of 200 × 200 × 500 µm within 2–3 d (Fig. 1). However, they were rather unstable and began to dissolve 3–4 d after seeding. The best data were obtained using the crystals while they were still growing or shortly after they reached their maximum size.

**Table 1**
Crystallographic parameters and data-collection statistics.

Numbers in parentheses refer to data in the outermost resolution bin.

| | |
|---|---|
| Space group | $P2_1$ |
| Unit-cell parameters (Å, °) | $a$ = 47.08, $b$ = 122.51, $c$ = 72.86, $\beta$ = 98.34 |
| Packing density (Å$^3$ Da$^{-1}$) | $V_m$ = 2.41 (4 mols/a.u.)† |
| Solvent content (%) | 42.3 |
| Resolution (Å) | 30–1.85 (1.92–1.85) |
| Total observations | 186351 |
| Unique reflections | 64828 |
| Redundancy | 2.9 |
| Average $I/\sigma(I)$ | 21.9 (3.4) |
| $R_{sym}$ ($I$) (%) | 4.7 (30.8) |
| Completeness (%) | 92.6 (90.0) |

† Estimated according to Matthews (1968).

## 4. Preliminary results

Initially, data were collected to 3.1 Å resolution at room temperature on an R-AXIS II image-plate system with a Rigaku rotating-anode Cu $K\alpha$ X-ray source. As the crystals suffered from radiation damage during data collection, conditions for cryo-protection were developed. Using a CryoLoop (Hampton Research), the crystal was quickly transferred through solutions of 20, 26 and 33% PEG 4000, 15 m*M* $CaCl_2$ before transfer to a stream of liquid nitrogen. Smaller crystals, around 60 × 60 × 200 µm, had to be employed for toleration of cryo-protection.

A complete native data set to 1.85 Å resolution has been collected at BM14, ESRF, Grenoble (Fig. 2). The data were collected using a 300 mm MAR Research image-plate detector (crystal-to-detector distance 290 mm). A total of 134 frames were collected, with oscillation steps of 1°. Processing and scaling of the data was performed using the programs *DENZO* version 1.9.1 and *SCALEPACK* version 1.9.0 (Otwinowski, 1993; Otwinowski & Minor, 1997). Crystallographic parameters and data-collection statistics are listed in Table 1.

The molecular-replacement method has been used in an attempt to solve the structure, using the programs *AMoRe* (Navaza, 1994) and *X-PLOR* (Brünger *et al.*, 1987). The search models were the NMR structure of NCAM IgI (Thomsen *et al.*, 1996) and the X-ray structures of ICAM-2 IgI-II (Casasnovas *et al.*, 1997) and VCAM-1 IgI-II (Jones *et al.*, 1995). However, this approach has not proven successful, and there may be several reasons for this. Firstly, there may be actual differences in the structures of these Ig domains. Secondly, there may be different angles and twists between the two domains. Thirdly, the search models comprise only one-eighth to one-quarter of the actual structure (four NCAM IgI-II molecules per asymmetric unit).

Isomorphous heavy-atom derivative data or MAD data are needed for solution of the structure. This work is in progress.

This work was supported by the Lundbeck Foundation, NeuroScience, Dansync and The Danish Biotechnology Programme. We thank Gordon Leonard and Andy Thompson at BM14, ESRF, Grenoble for expert technical assistance during data collection; Vladislav Soroka at the Protein Laboratory, University of Copenhagen for the cDNA construct encoding NCAM IgI-II; Flemming Poulsen at University of Copenhagen for providing equipment for protein expression and for amino-acid sequencing; Lars Fogh Iversen at Novo Nordisk A/S for mass-spectrum analysis of the protein sample.

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