Protein production, crystallization and preliminary crystallographic analysis of the four N-terminal immunoglobulin domains of Down syndrome cell adhesion molecule 1

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Down syndrome cell adhesion molecule 1 (Dscam1), a member of the immunoglobulin (Ig) superfamily, plays important roles in both the nervous and the immune systems. Via alternative RNA splicing, *Drosophila* Dscam1 encodes a vast family of Ig-containing proteins that exhibit isoform-specific homophilic binding. Whether different Dscam1 isoforms adopt the same dimerization mode is under debate, and the detailed mechanism of Dscam1 specificity remains unclear. In this study, eight different isoforms of Dscam1 Ig1–4 have been cloned, overexpressed, purified to homogeneity and crystallized. X-ray data were collected to 1.9–4.0 Å resolution. These structures will provide the opportunity to perform extensive structural comparisons of different Dscam1 isoforms and provide insight into its specificity.

1. Introduction

The DSCAM (Down syndrome cell adhesion molecule) gene was first isolated from the human chromosome band 21q22.2–22.3, which is a critical region for many neurological phenotypes of Down syndrome (Yamakawa *et al.*, 1998). *Drosophila* has four Dscam paralogues, and the founding member, Dscam1, functions in both the nervous and the immune systems (Pasquier, 2005; Zhan *et al.*, 2004; Watson *et al.*, 2005; Liu *et al.*, 2009).

Dscam1 is a type I transmembrane glycoprotein which consists of ten Ig domains, six fibronectin type III (Fn III) repeats, one transmembrane domain and one intracellular domain. Dscam1 can potentially generate more than 38 000 isoforms through alternative RNA splicing (Schmucker *et al.*, 2000). *In vitro* binding studies have shown that Dscam1 performs exclusive isoform-specific binding which is determined by the three variable Ig domains (Ig2, Ig3 and Ig7; Wojtowicz *et al.*, 2004). Dscam1 forms homodimers via matching interactions of the three variable Ig domains (Ig2/Ig2, Ig3/Ig3 and Ig7/Ig7) in a modular fashion, as proposed by Wojtowicz *et al.* (2007) and Sawaya *et al.* (2008).

The N-terminal four Ig domains (Ig1–4) of Dscam1 form a horseshoe configuration, which may be shared by many other neural receptors (Chen *et al.*, 2013; Liu *et al.*, 2011; Freigang *et al.*, 2000). Such a horseshoe conformation is conserved in the

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Dscam1 Ig1–8 structure (Sawaya *et al.*, 2008). However, there is a debate as to whether different Dscam1 Ig1–4 isoforms adopt the same dimerization mode (Meijers *et al.*, 2007, Sawaya *et al.*, 2008). The detailed mechanism of how Dscam1 acquires its exclusive isoform-specificity remains unclear.

Here, we report the cloning, expression, crystallization and X-ray crystallographic studies of eight different isoforms of Dscam1 Ig1–4. We combined a particular Ig2 isoform with various Ig3 isoforms or *vice versa*. These structures will provide us with the opportunity to perform extensive struc-

tural comparisons of different Dscam1 isoforms and to shed light on the specificity of Dscam1.

Table 1
Macromolecule-production information for Dscam1 Ig1–4.

Source organism	<i>D. melanogaster</i>
DNA source	cDNA
Expression vector	pMelBac-B
Transfection system	Bac-N-Blue
Baculovirus amplification host	Sf9
Expression host	High Five

Figure 1
Crystals of Dscam1 Ig1–4.

Table 2

Crystallization of Dscam1 Ig1–4 (the first number indicates the Ig2 isoform and the second number indicates the Ig3 isoform).

Isoform	1.9	6.9	6.44	4.44	7.44
Method	Hanging drop	Hanging drop	Hanging drop	Hanging drop	Hanging drop
Plate type	24-well	24-well	24-well	24-well	24-well
Temperature (K)	289	289	289	289	289
Protein concentration (mg ml ⁻¹)	15	15	13	12	10
Composition of reservoir solution	0.1 M bis-tris pH 5.5, 0.1 M ammonium acetate, 20%(w/v) PEG 10 000	0.1 M HEPES pH 7.5, 0.2 M ammonium sulfate, 25%(w/v) PEG 3350	0.1 M HEPES pH 7.5, 15%(w/v) PEG 3350	0.1 M HEPES pH 7.5, 0.1 M calcium chloride, 30%(w/v) PEG 400	0.2 M ammonium chloride, 17%(w/v) PEG 3350
Volume and ratio of drop	1:1	1:1	1:1	1:1	1:1
Volume of reservoir (μl)	500	500	500	500	500

Isoform	9.44, Zn	9.44, no Zn	4.4	8.4
Method	Hanging drop	Hanging drop	Hanging drop	Hanging drop
Plate type	24-well	24-well	24-well	24-well
Temperature (K)	289	289	289	289
Protein concentration (mg ml ⁻¹)	10	10	11	15
Composition of reservoir solution	0.1 M sodium cacodylate pH 6.4, 0.2 M zinc acetate, 20%(w/v) PEG 4000	0.1 M sodium acetate pH 4.6, 25%(w/v) PEG 1500	0.1 M HEPES pH 7.5, 0.5 M magnesium chloride, 20%(w/v) PEG 3350	0.1 M MES pH 6.5, 20%(w/v) PEG 3350
Volume and ratio of drop	1:1	1:1	1:1	1:1
Volume of reservoir (μl)	500	500	500	500

2. Materials and methods

2.1. Macromolecule production

The cDNA for *Drosophila* Dscam1 Ig1–4 was cloned into modified pMelBac-B vector (Invitrogen) using the NarI and HindIII restriction sites. The modified vector pMelBac-B has a honeybee melittin secretion signal for secreted expression, an N-terminal hexahistidine tag for purification and a TEV site for removing the tag. Details may be found in Table 1.

The protein was expressed in a baculovirus system. The recombinant plasmid containing the target gene was co-transfected with Bac-N-Blue DNA into *Spodoptera frugiperda* 9 (Sf9) cells (Invitrogen). The recombinant virus was amplified in Sf9 cells to prepare high-titre viral stocks. For expression, High Five cells with a density of $\sim 1.5 \times 10^6$ cells ml⁻¹ were infected with the recombinant baculovirus at a multiplicity of infection of between 5 and 10. 2–3 d post-infection, the cells were spun down and the medium was collected for purification.

The medium was filtered using a 0.45 μm cellulose acetate membrane (Corning) and concentrated to 100 ml using a Stirred Cell 8400 (Merck Millipore). The small volume of concentrated medium was dialyzed with binding buffer (20 mM Tris pH 7.4, 250 mM NaCl). The N-terminally hexahistidine-tagged Dscam1 Ig1–4 protein was then loaded onto a column of His-Tag purification resin (Roche) pre-equilibrated with binding buffer. The column was then washed extensively with ten column volumes of binding buffer plus 5 mM imidazole for the removal of nonspecifically bound contaminants. The target protein was finally eluted with binding buffer plus 200 mM imidazole.

The tag of the target protein was subsequent removed by overnight hydrolysis with TEV protease in the presence of 0.1% β-mercaptoethanol at 277 K and verified by 12% SDS-

PAGE. Hydrolysed protein was desalted with a HiTrap Desalting column (GE Healthcare) on an ÄKTApurifier FPLC system (GE Healthcare). The desalted protein was then applied onto a HiTrap Chelating HP column (GE Healthcare). The flowthrough was collected, concentrated to 0.5 ml with an Amicon Ultra centrifugal filter (Merck Millipore) and further purified on a Superdex 200 gel-filtration column (GE Healthcare) with 20 mM HEPES pH 7.5, 100 mM NaCl. All purification steps were carried out at 289 K and the purity was monitored by SDS-PAGE.

2.2. Crystallization

The purified protein was concentrated to 10–15 mg ml⁻¹ for crystallization. The concentrations of the protein samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Hanging-drop vapour-diffusion crystallization trials were performed at 289 K. Equal volumes of protein solution (10–15 mg ml⁻¹ in 20 mM HEPES pH 7.5, 100 mM sodium chloride) and reservoir solution were mixed and crystals appeared in 2–3 d (Fig. 1). The crystallization conditions for different isoforms of Dscam Ig1–4 are summarized in Table 2.

2.3. Data collection and processing

Before data collection, the crystals were soaked in reservoir solution supplemented with 20%(v/v) glycerol as a cryoprotectant for a few seconds and then mounted in nylon loops and flash-cooled in liquid nitrogen. The Dscam Ig1–4 data were collected on beamline 3W1A at Beijing Synchrotron Radiation Facility (BSRF) and beamline BL17U1 at Shanghai Synchrotron Radiation Facility (SSRF). The data were processed with XDS (Kabsch, 2010) or the HKL-2000

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Isoform	1.9	6.9	6.44	4.44	7.44	9.44, Zn	9.44, no Zn	4.4	8.4
Diffraction source	BSRF	SSRF	BSRF	SSRF	SSRF	BSRF	BSRF	SSRF	BSRF
Wavelength (Å)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Temperature (K)	100	100	100	100	100	100	100	100	100
Rotation range per image (°)	1	1	1	1	1	1	1	1	1
Exposure time per image (s)	20	1	30	1	1	30	30	1	20
Space group	$P2_1$	$P2_12_12_1$	$C2$	$P2_1$	$P1$	$P2_1$	$P2_1$	$P4_12_12$	$P2_1$
Unit-cell parameters									
a (Å)	92.9	63.7	193.6	88.9	61.7	66.7	66.7	100.2	92.5
b (Å)	59.8	90.5	63.4	59.8	88.2	57.3	56.7	100.2	61.3
c (Å)	93.6	169.6	90.1	89.6	94.3	129.9	128.7	351.8	95.2
α (°)	90	90	90	90	98.2	90	90	90	90
β (°)	92.8	90	99.9	109.1	98.8	93.7	92.1	90	92.7
γ (°)	90	90	90	90	90.1	90	90	90	90
Resolution range (Å)	50–2.50 (2.59–2.50)	50–2.35 (2.40–2.35)	50–3.40 (3.61–3.40)	50–2.20 (2.33–2.20)	50–1.90 (2.02–1.90)	50–3.20 (3.40–3.20)	50–2.90 (3.08–2.90)	50–4.00 (4.14–4.00)	50–2.95 (3.07–2.95)
No. of unique reflections	35836 (3379)	42006 (2696)	14910 (2314)	45397 (7212)	148198 (21493)	16383 (2581)	20863 (3321)	15793 (1552)	21045 (1407)
Completeness (%)	99.4 (94.4)	99.7 (98.2)	98.9 (96.8)	99.7 (99.1)	94.4 (81.9)	99.6 (98.3)	96.6 (97.0)	99.9 (99.4)	97.2 (98.0)
Multiplicity	4.5 (4.2)	7.9 (6.7)	3.8 (3.8)	4.8 (4.7)	2.2 (2.1)	4.9 (5.0)	3.0 (3.0)	9.6 (8.0)	2.7 (2.5)
$\langle I/\sigma(I) \rangle$	18.5 (2.3)	27.7 (4.6)	8.3 (2.1)	11.3 (2.6)	9.6 (2.5)	8.1 (2.0)	8.9 (2.1)	7.6 (2.0)	14.1 (2.2)
R_{meas} (%)	6.8 (52.7)	5.8 (38.0)	10.3 (69.5)	9.1 (63.9)	5.9 (27.2)	11.2 (65.8)	10.5 (62.4)	9.4 (66.7)	7.8 (46.1)
Solvent content (%)	54.9	52.1	57.0	50.0	58.8	52.7	51.9	72.5	56.6

program suite (Otwinowski & Minor, 1997). Crystallographic data statistics are summarized in Table 3.

3. Results and discussion

Various isoforms of the four N-terminal Ig-like domains of *Drosophila* Dscam1 were cloned into a modified pMelBac-B vector. To facilitate comparison, we produced Dscam1 Ig1–4 constructs using a particular Ig2 isoform combined with various Ig3 isoforms or *vice versa*. These constructs were successfully expressed in a baculovirus system and purified to homogeneity by affinity chromatography and size-exclusion chromatography. Crystals were produced using crystallization screening kits from Hampton Research, followed by refinement of the conditions by the variation of the precipitant, pH and protein concentration and the use of additives. The crystallization conditions for the different Dscam1 isoforms are shown in Table 1. Dscam1 Ig1–4 isoform 9.9 was crystallized under two conditions: with or without the bivalent cation zinc. The statistics of the diffraction data are presented in Table 2.

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