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Expression, crystallization and preliminary crystallographic analysis of the extracellular IgV-like domain of the human natural killer cell inhibitory receptor p75/AIRM1

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p75/AIRM1 (Siglec-7) is a sialic acid-binding Ig-like lectin recently identified as an inhibitory receptor on natural killer cells. The expression, *in vitro* folding, circular-dichroism spectroscopy, crystallization and preliminary X-ray characterization of the Ig-V like domain of p75/AIRM1 are reported. X-ray data were collected from a single crystal at 100 K, with a maximum useful diffraction pattern extending to 1.45 Å resolution on a synchrotron source. The crystal belongs to a primitive monoclinic space group, with unit-cell parameters $a = 32.65$, $b = 49.72$, $c = 39.79$ Å, $\alpha = \gamma = 90$, $\beta = 113^\circ$. The systematic absences indicate that the space group is $P2_1$. Assuming one molecule per asymmetric unit, V_M (the Matthews coefficient) was calculated to be 1.879 Å^3 Da^{-1} and the solvent content was estimated to be 32.01%.

1. Introduction

Mammalian lectins are classified according to their structural similarities and the types of carbohydrates they bind: C-type lectins, P-type lectins, galectins and immunoglobulin-type or I-type lectins (Drickamer, 1995), also termed sialoadhesins or Siglecs (sialic acid-binding Ig-like lectins). Siglecs are characterized by an N-terminal V-set Ig-like domain that binds to sialylated glycoconjugates and a variable number of C2-set Ig-like domains (Crocker & Varki, 2001). To date, 11 human Siglecs have been cloned (http://www.gene.ucl.ac.uk/nomenclature/genefamily/siglec.html) and each member is expressed in a specific cell type, normally on haemapoietic cells (Yu *et al.*, 2001; Angata *et al.*, 2002; Yousef *et al.*, 2002). The only member of the Siglec family not expressed on circulating haemapoietic cells is MAG/Siglec-4 (Kelm *et al.*, 1994), which is localized to the nervous system. Siglecs display high sequence similarity in their IgV-like ligand-binding domain. In contrast, their cytoplasmic regions show little sequence homology, indicative of different intracellular functions.

Some Siglecs are highly expressed on the surface of natural killer (NK) cells (Crocker & Varki, 2001). These cells are a subset of lymphocytes with the intrinsic ability to recognize and kill virally infected and tumour cells (Trincheri, 1989). p75/AIRM1, or Siglec-7, was identified as an inhibitory NK-cell receptor in a redirected killing assay in which an anti-p75/AIRM1 monoclonal antibody was used to cross-link p75/AIRM1 at the interface between the NK cell and the target cell (Falco *et al.*, 1999). It has been shown that p75/AIRM1 recognizes sialoconjugates (Ito *et al.*, 2001; Yamaji *et al.*, 2002; Crocker *et al.*, 1999; Nicoll *et al.*, 2003), but it is still not known whether the binding of p75/AIRM1 to sialoconjugates influences signalling. Moreover, the natural ligand has yet to be identified. Reported in this paper are the expression, refolding, circular-dichroism spectroscopy, crystallization and preliminary X-ray characterization of the IgV-like domain of p75/AIRM1 produced in a bacterial expression system.

2. Experimental

2.1. Cloning, expression, refolding and purification

The DNA sequence encoding the V-set domain of p75/AIRM1, corresponding to amino acids 19–150, was amplified by PCR from plasmid pCDNA-3.1 (Falco *et al.*, 1999) and cloned into the pT7-7 expression vector (Novagen) using the following oligonucleotide primers: 5′-TTT TTT TTC ATA TGC AGA AGA GTA ACC GGA AGG ATT ACT CGC-3′ and 5′-TTT TTT TTG GAT CCT TAC CTG TGG GTC AAG GCT GTC ACG TTC ACA GAG AGC-3′. Amplification was performed by denaturation at 367 K for 40 s, annealing at 331 K for 40 s and elongation at 345 K for 40 s for 30 cycles, followed by a final elongation step at 345 K for 5 min. The amplified fragment was digested with *Nde*I and *Bam*HI and cloned into the pT7-7 expression vector (Novagen), restricted with the same two enzymes, to construct pT75-1D. The nucleotide sequence was verified by DNA sequencing using the primer 5′-TAA TAC GAC TCA CTA TAG GG-3′. The protein was expressed in *Escherichia coli* strain BL21(DE3)pLysS (Novagen) transformed with the pT75-1D vector. Bacteria

were grown at 310 K in LB medium containing 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol to an absorbance of 0.6 at 600 nm; isopropyl-β-D-thiogalactoside was then added to a concentration of 1 m*M*. Culture growth was continued for 3 h. Cells were then harvested by centrifugation and resuspended in 100 m*M* Tris–HCl buffer pH 8.0 containing 2 m*M* EDTA and 10 m*M* DTT. The cells were disrupted by sonication using a Branson Sonifier for 4 min at 50% duty cycle. The pellet thus obtained was washed four times in 50 m*M* Tris–HCl buffer pH 8.0, 1 m*M* EDTA, 100 m*M* NaCl and 0.5% Triton-X 100 and once in the same buffer containing 2 *M* urea. Recombinant protein was obtained as inclusion bodies, which were dissolved in 25 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA, 0.1 m*M* DTT and 8 *M* urea. p75/AIRM1 was refolded *in vitro* by dilution into 25 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA, 0.4 *M* L-arginine, 8% glycerol, 3 m*M* reduced glutathione and 0.9 m*M* oxidized glutathione to a final concentration of 5 µg ml^{-1}. After 48 h at 277 K, the refolded mixture was concentrated by ultrafiltration using an Amicon YM 5K membrane. The retentate solution was dialyzed against 25 m*M* MES pH 5.5 containing 150 m*M* NaCl. The V-set domain of p75/AIRM1 eluted as a monomer of $M_r \simeq 15\,000$ from a Superdex-HR75 gel-filtration column (Amersham Pharmacia). Following dialysis against 25 m*M* MES pH 5.5, the protein (pI 8.5) was further purified using a MonoS cation-exchange column (Amersham Pharmacia), from which p75/AIRM1 was eluted with a linear NaCl gradient. The purified protein migrated as a single band on SDS–PAGE. N-terminal sequencing and monoclonal antibody-binding assays (data not shown) confirmed the identity and the immunoreactivity of the purified p75/AIRM1. Protein concentration was measured by absorbance at 280 nm using a calculated extinction coefficient of 30 560 M^{-1} cm^{-1}.

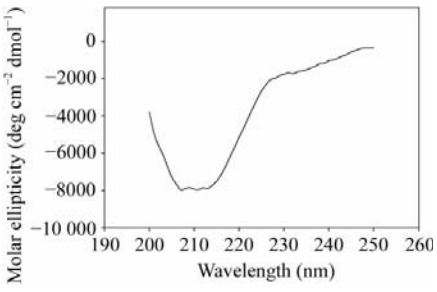

Figure 1
Circular-dichroism diagram of 0.100 m*M* p75/AIRM1 in 25 m*M* MES pH 5.5 at 277 K. The CD spectrum was recorded at 4 s intervals with a scan rate of 50 nm min^{-1} and a band with of 1 nm. Analysis of the CD spectrum indicates that p75/AIRM1 is folded and composed of β-strands as major secondary-structure elements (β = 40%).

2.2. Circular-dichroism spectroscopy

Measurement was conducted on a Jasco J-720 spectropolarimeter (Jasco, Inc.) at 277 K using a 0.005 cm cuvette. The spectra was recorded in the far-ultraviolet region between 250 and 200 nm at a scan rate of 50 nm min^{-1} with a 4 s interval and a bandwidth of 1 nm. The data was analyzed using the program *K2d* (Andrade *et al.*, 1993) and the figure was prepared using the program *Sigma Plot* 8.2 (SPSS Inc.).

2.3. Crystal growth, data collection and processing

The protein was concentrated to 3 mg ml^{-1} in 25 m*M* MES buffer pH 5.5. Crystallization trials were initiated using commercially available screens (Hampton Research Crystal Screen I/II; JBScreen Mixed, Jena Bioscience; Emerald Biostructures Wizard Screen I/II) at 277 K and room temperature. Although a broad crystallization screening was used, the protein only crystallized after five months at 277 K in hanging drops containing 1.5 µl protein solution and 1.5 µl of a reservoir solution composed of 20%(*w*/*v*) PEG 8000, 0.2 *M* NaCl, 0.1 *M* phosphate–citrate pH 4.2 (Emerald Biostructures Wizard Screen I solution 31). Any attempt to improve the crystal-growth rate by changing the experimental crystallization condition failed. For data collection, a crystal was harvested and briefly immersed in a cryoprotectant buffer containing 20%(*v*/*v*) ethylene glycol prior to flash-freezing in a nitrogen stream. Synchrotron data collection was carried out at the CHESS F1 beamline; the data were processed using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The crystal diffracted to 1.45 Å resolution and belongs to a primitive monoclinic space group, with unit-cell parameters a = 32.65, b = 49.72, c = 39.79 Å, $\alpha = \gamma = 90$, $\beta = 113°$. The systematic absences indicate that the space group is $P2_1$.

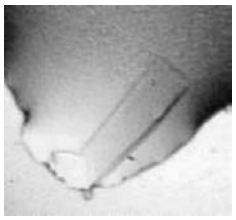

Figure 2
Crystal of the Ig-V like domain of p75/AIRM1 used for data collection. The crystal has approximate dimensions of 0.7 × 0.08 × 0.05 mm.

Table 1
p75/AIRM1 IgV-like domain X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell (1.51–1.45 Å).

Source	F1 beamline, CHESS
Wavelength (Å)	0.934
Temperature (K)	100
Resolution (Å)	1.45
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 32.65, b = 49.72, c = 39.79, $\alpha = \gamma = 90$, β = 113
Mosaicity (°)	0.59
No. of observations	238401
Unique reflections	20819
Data completeness (%)	89.3 (68.6)
$\langle I/\sigma(I)\rangle$	15.3 (2.5)
$R_{\rm merge}$† (%)	6.0 (38.8)

† $R_{\rm merge} = \sum_h \sum_i |I_{hi} - \langle I_h\rangle| / \sum_h \sum_i I_{hi}$.

3. Results

To study the structure and function of p75/AIRM1, we have expressed its extracellular Ig-V-like domain by *in vitro* folding from bacterial inclusion bodies. The protein was purified by gel-filtration chromatography and cation-exchange chromatography. The purified protein is monomeric, judging by gel-filtration chromatography, with a molecular weight of 15 650 Da, as measured by analytical mass spectrometry (data not shown). The structural integrity of p75/AIRM1 was assessed by circular-dichroism spectroscopy (Fig. 1), giving a predicted β-sheet content of 40%. This domain was crystallized at 277 K using the hanging-drop crystallization technique and a crystal suitable for X-ray data collection was obtained after five months (Fig. 2). Data from this single crystal were collected using a synchrotron source; the data-collection statistics are reported in Table 1. The crystal diffracts to 1.45 Å and belongs to the primitive monoclinic space group $P2_1$. We are in the process of solving the p75/AIRM1 structure by molecular replacement using as a search probe a recently reported Siglec-7 crystal structure (Alphey *et al.*, 2003) produced by a mammalian expression system. Knowledge of the p75/AIRM1 crystal structure at high resolution obtained using our bacterial expression system will be valuable in designing high-affinity oligosaccharide ligands to define the molecular mechanism by which this receptor inhibits NK-cell cytolytic activity. The structure can also serve as a template to model other Siglecs identified from searching the human genome database. Moreover, the ability to

produce recombinant p75/AIRM1 in milligram amounts should facilitate identification of its cellular or viral ligands.

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