Expression and crystallographic characterization of the extracellular domain of human natural killer cell triggering receptor NKp46

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Human natural killer (NK) cells are regulated in their cytolytic activity by a delicate interplay between activating and inhibitory signals related to distinct families of triggering and inhibitory receptor proteins. NKp46 is a major NK cell-specific triggering receptor involved in the recognition and lysis of human and murine tumour and virally infected cells. It consists of an extracellular portion, composed of two Ig-like domains, a transmembrane segment and a small cytoplasmic domain. To shed light on the molecular-recognition events involved in NK cytotoxicity triggering mechanisms, the NKp46 extracellular region was cloned, overexpressed, refolded and crystallized. X-ray diffraction data could be collected to a resolution limit of 1.93 Å. Crystals of the NKp46 extracellular region belong to the hexagonal space group $P6_1$ (or $P6_5$), with unit-cell parameters $a = b = 85.48$, $c = 59.91$ Å, $\gamma = 120°$; the asymmetric unit contains one protein chain (197 amino acids).

1. Introduction

Natural killer (NK) cells are key elements in the immune system that direct the selective lysis of cells undergoing tumour transformation or viral infection (Biron, 1997). Their activation leads to the disruption of abnormal cells expressing inadequate amounts of major histocompatibility complex class I molecules (Moretta et al., 2000, 2001; Biron, 1997). In humans, NK-cell activity is determined by a fine balance between inhibitory signals, mediated by cell-surface inhibitory receptors able to recognize human leukocyte antigen (HLA) class I molecules, and activating signals delivered by NK cells triggering receptors involved in HLA-independent natural cytotoxicity (Moretta et al., 2001).

The inhibitory receptors include killer immunoglobulin receptors (KIR), which are type I transmembrane proteins hosting two or three C2-type immunoglobulin-like (Ig-like) domains (Biassoni et al., 2001; Lanier, 1998; Long, 1999; Pende et al., 1996). A second group of inhibitory receptors is represented by CD94/NKG2A, which are type II transmembrane proteins belonging to the C-type lectin superfamily (Braud et al., 1998; Carretero et al., 1997; Lee et al., 1998; McFarland et al., 2003).

The triggering receptors involved in NK-cell-mediated natural cytotoxicity (natural cytotoxicity receptor, NCR) have been characterized more recently (Biassoni et al., 2001; Moretta et al., 2000, 2001). Three major NCRs (NKp44, NKp46, NKp30) are deemed to recognize still undisclosed cellular ligands on the surface of tumour cells, thus promoting their killing. NCR expression is strictly confined to NK cells; however, NKp46 and NKp30 are expressed on both resting and activated NK cells, whereas NKp44 is selectively expressed on activated NK cells (Pessino et al., 1998; Pende et al., 1999; Cantoni et al., 1999).

NKp46 has been identified as a major activating receptor in the recognition and lysis of both human and murine tumour cells (Pessino et al., 1998). Human NKp46 is a type I transmembrane protein composed of an extracellular portion of about 230 amino acids, consisting of two extracellular C2-type Ig-like domains, a transmembrane region containing the basic amino acid Arg (about 20 amino acids) and a short cytoplasmic domain (about 30 amino acids). Signal transduction of NKp46 receptor is dependent on its association with CD3ζ and FceRIγ ITAM-bearing molecules.

In the context of our ongoing studies on the structure of NCRs aiming to shed light on their molecular mechanisms of target recognition and NK-cell activation, we have overexpressed and purified the extracellular region of human NKp46 (NKp46_EC, 197 amino acids). Crystals of NKp46_EC suitable for a high-resolution crystallographic investigation have been grown and characterized.

2. Materials and methods

2.1. Expression, refolding and purification

NKp46_EC (nucleotides 112–675/accession No. AJ001383) was amplified starting from the VR1012-NKp46 plasmid (Pessino et al., 1998)

using the following primers: 5′-GCGTA-AGGATCCACTCTCCCAAAACCGTTC (46 *Bam*HI, forward) and 5′-AACCCAAG-CTTTTAGCCAGTGACCAGGAGC (46 *Hin*dIII, reverse). Amplification was performed with Pfu Turbo (Stratagene, USA) for 20 cycles (45 s at 368 K, 45 s at 331 K and 2 min at 345 K), followed by a 7 min elongation step at 345 K. The 569 bp PCR product was subcloned in the pQE30 vector for prokaryotic expression (Qiagen GmbH, Germany), allowing the production of a recombinant protein with a $6\times$His tag at the N-terminus. The nucleotide sequence for the NKp46_EC was checked using a d-Rhodamine Terminator Cycle Sequencing Kit and a 377 Applied Biosystems Automatic Sequencer (Perkin Elmer Applied Biosystems, NJ, USA).

The M15(pREP4) *Escherichia coli* strain (Qiagen) was transformed with the pQE30/NKp46_EC construct. NKp46_EC expression was achieved by growing a single colony overnight at 310 K, diluting this starter culture 1:200, growing it to an OD_{600} of 0.8 and then adding IPTG to a final concentration of 2 m*M* before incubating for a further 3.5 h at 310 K. The bacterial cell pellet was recovered and resuspended at 277 K in 100 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA pH 8.0, 10 m*M* DTT, 0.5 mg ml^{-1} lysozyme and left at 277 K for 12 h under agitation. Sonication was used to disrupt the bacterial cells. The recovered pellet containing inclusion bodies was suspended and washed on ice in 50 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA pH 8.0, 100 m*M* NaCl, 0.5% Triton-X 100, 1 m*M* DTT. The inclusion bodies were first washed in 50 m*M* Tris–HCl pH 8.0, 2 m*M* NaCl, 2 m*M* urea, 10 m*M* DTT and then in 100 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA pH 8.0, 10 m*M* DTT and dissolved under agitation in 6 *M* guanidinium–HCl, 100 m*M* Tris–HCl pH 8.0, 10 m*M* EDTA pH 8.0, 0.1 m*M* DTT for 48 h at 277 K. After centrifugation (229 000*g*) for 30 min at 277 K, half of the supernatant was allowed to refold in 100 m*M* Tris–HCl pH 8.5, 2 m*M* EDTA pH 8.0, 0.9 *M* arginine–HCl, 0.1 *M* L-arginine, 6.3 m*M* cysteamine, 3.7 m*M* cystamine at 277 K. After 12 h, the remaining supernatant was added and left to refold for further 48 h. The solution containing the refolded NKp46 was then concentrated to about 40 ml using Centricon Plus 80 membranes (Amicon, USA) following the manufacturer's instructions and dialyzed overnight at 277 K against 100 m*M* NaH$_2$PO$_4$, 10 m*M* Tris–HCl pH 8.0. Finally, the dialyzed protein was purified by immobilized metal affinity chromatography (Ni–NTA agarose, Qiagen) followed by gel-

Table 1
X-ray data-collection statistics for NKp46_EC.

Values in parentheses correspond to the highest resolution shell (1.96–1.93 Å).

Source	BW7B beamline, DESY
Wavelength (Å)	0.8431
Temperature (K)	100
Resolution (Å)	40.0–1.93
Space group	$P6_1/P6_5$
Unit-cell parameters (Å, °)	$a = b = 85.48$, $c = 59.91$, $\alpha = \beta = 90$, $\gamma = 120$
Mosaicity (°)	0.19
No. of observations	160306
Unique reflections	18944
Data completeness (%)	100 (100)
$\langle I/\sigma(I) \rangle$	22.9 (2.4)
R_{merge}† (%)	7.5 (76.5)

† $R_{merge} = \sum_{i,h} |(I_{h,i} - \langle I_h \rangle)| / \sum (I_i)$, where I is the observed intensity of the reflection h.

filtration chromatography on a HiPrep Sephacryl S-200 column (Pharmacia Biotech, Sweden) using 30 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl as elution buffer. The protein concentration was evaluated by its absorbance at 280 nm using the calculated extinction coefficient of 1.4 M^{-1} cm^{-1}.

2.2. Crystal growth, data collection and processing

To explore crystal-growth conditions NKp46_EC was brought to a concentration of 9.0 mg ml^{-1}. Initial crystallization conditions were screened using the sparse-matrix method (Jancarik & Kim, 1991) as implemented in Hampton Crystal Screens I and II (Hampton Research, USA). After refinement of the preliminary growth conditions yielding crystalline precipitates, good-quality crystals were produced by equilibration against a reservoir solution containing 100 m*M* HEPES buffer pH 7.8, 15% PEG 8000 and 20%(*v/v*) ethanediol at 277 K. The hanging-drop vapour-diffusion setups were prepared by mixing equal amounts (0.9 µl) of the protein and reservoir solutions. Well shaped hexagonal bipyramidal crystals grew to maximum

dimensions of about $0.3 \times 0.2 \times 0.2$ mm in a period of about three months.

Synchrotron X-ray data collection was carried out on a frozen native protein crystal at the BW7B beamline (EMBL/DESY, Hamburg, Germany) to a resolution limit of 1.93 Å (see Table 1). The diffraction data were processed and scaled using the programs *DENZO* and *SCALEPACK*, respectively (Otwinowski, 1993; Otwinowski & Minor, 1997).

3. Results and discussion

The receptor extracellular portion NKp46, consisting of two C2-type Ig-like domains with a total MW of 22.7 kDa, was over-expressed in *E. coli* as His-tagged protein and obtained as inclusion bodies. The expressed protein was denatured and subsequently refolded following an appropriate redox-equilibrium procedure (based on the cystamine/cysteamine pair). After the purification phase, based on nickel-affinity and gel-filtration chromatography, the protein was crystallized and diffraction data were collected to 1.93 Å resolution from a frozen crystal. Analysis of the diffraction patterns yielded the crystal data shown in Table 1. Crystal-packing considerations indicate the presence of a single NKp46_EC chain in the crystallographic asymmetric unit (V_M = 2.78 $\text{Å}^3\,\text{Da}^{-1}$, 58% solvent content; Matthews, 1968).

The three-dimensional structure of NKp46_EC is presently under investigation using molecular-replacement methods. In this regard, a BLAST search against the current release of the PDB (Berman *et al.*, 2000) identified several proteins containing C2-type Ig-like domains, found not only in NK cells but also in other leukocyte cell types. Such structural homologues include the ligand-binding domain of the leukocyte immunoglobulin-like receptor (LIR-1; PDB code 1g0x), the human NK-cell inhibitory

Figure 1
Amino-acid sequence alignment of the expressed NKp46_EC segment and the leukocyte immunoglobulin-like receptor LIR-1 (PDB code 1g0x). LIR-1 β-strand segments are represented as grey boxes; Cys residues involved in disulfide bridges are shown in black boxes. The alignment was generated using *CLUSTALX* (Thompson *et al.*, 1997).

receptors (KIR2DL3 and KIR3DL1; PDB codes 1b6u and 1im9, respectively) and the extracellular domain of the NK-cell-activating receptor KIR2DS2 (PDB code 1m4k). In particular, the sequence similarity between NKp46_EC and LIR-1 (38% identical residues; Fig. 1) and the conservation in NKp46_EC of several residues involved in contacts between the two Ig-like domains of LIR-1 indicate the three-dimensional structure of the latter protein as the most suitable search model for a molecular-replacement approach to the NKp46_EC structure. However, such a hypothesis is based on the assumption that the mutual orientation of the two C2-type Ig domains is conserved in LIR-1 and NKp46_EC.

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