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Crystallization and preliminary crystallographic characterization of the extracellular Ig-like domain of human natural killer cell activating receptor NKp44

Claudia Cantoni,[a,b,c]† Marco Ponassi,[d,e]† Roberto Biassoni,[f] Romana Conte,[f] Andrea Spallarossa,[b] Alessandro Moretta,[a,c] Lorenzo Moretta,[a,b,c] Martino Bolognesi[c,g] and Domenico Bordo[d]*

[a]Dipartimento di Medicina Sperimentale, Università di Genova, Italy, [b]Istituto Giannina Gaslini, Genova, Italy, [c]Centro di Eccellenza per Ricerche Biomediche, Università di Genova, Italy, [d]Laboratorio di Biologia Strutturale, Istituto Scientifico Tumori, Genova, Italy, [e]Laboratorio di Biologia Cellulare, Istituto Scientifico Tumori, Genova, Italy, [f]Laboratorio di Immunologia, Istituto Scientifico Tumori, Genova, Italy, and [g]Dipartimento di Fisica e INFM, Università di Genova, Italy

† CC and MP contributed equally to this work.

Correspondence e-mail: domenico@alcor.ge.infm.it

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The cytolytic activity of human natural killer (NK) cells is regulated by fine balancing between activating and inhibitory signals related to distinct families of inhibitory and triggering receptor molecules. The recent identification of NKp44, a triggering receptor selectively expressed by activated NK cells and playing an important role in the natural cytotoxicity of these cells, opens the way to the characterization of the mechanisms involved in NKp44-mediated NK-cell activation on a structural basis. For this purpose, the NKp44 extracellular Ig-like domain was cloned, overexpressed, refolded and crystallized. Diffraction intensities were collected to a resolution limit of 2.2 Å on a synchrotron source. NKp44 extracellular Ig-like domain crystals belong to the hexagonal space group $P6_222$ (or $P6_422$), with unit-cell parameters $a = b = 60.4$, $c = 197.2$ Å, $\gamma = 120^\circ$. Evaluation of the crystal packing parameter suggests the presence of one molecule (13.8 kDa) per asymmetric unit, with a solvent volume of 67%.

1. Introduction

Natural killer (NK) cells are the key elements that activate the immune system against transformed and virally infected cells. They mediate the efficient lysis of abnormal cells that express inadequate amounts of the major histocompatibility complex (MHC) class I molecules (Moretta *et al.*, 1996, 2000, 2001; Biron, 1997). Thus, NK cells are able to discriminate between normal MHC class I cells and cells that display either loss of or reduced expression of MHC class I molecules as a consequence of viral infection or tumour transformation. NK cell function is regulated by a subtle balance between inhibitory signals delivered by the MHC class I-specific receptors and activating signals produced by NK triggering receptors (Moretta *et al.*, 2000, 2001). In this regard, human NK cells are known to express a number of inhibitory receptors that recognize different groups of human leukocyte antigen (HLA) class I alleles present on normal cells (Moretta *et al.*, 1994, 1996). These receptors consist of an extracellular domain, hosting either two or three immunoglobulin (Ig)-like domains or a C-type lectin domain (Lopez-Botet *et al.*, 1997), a transmembrane hydrophobic segment and a cytoplasmic domain containing a conserved immunoreceptor tyrosine-based inhibitory motif (ITIM). The latter recruits SH2 domain-containing protein tyrosine phosphatases (SHP-1, SHP-2), resulting in the inhibition of NK cell cytolytic activity (Bolland & Ravetch, 1999; Long, 1999). The nature of NK-specific activating receptors has long remained elusive.

Three NK activating receptors, NKp46, NKp44 and NKp30, have recently been identified and characterized (Moretta *et al.*, 2000, 2001; Bottino *et al.*, 2000; Biassoni *et al.*, 2001). They are novel NK-specific triggering molecules collectively termed natural cytotoxicity receptors (NCR). They are responsible for NK-mediated cytolytic activity and belong to the Ig superfamily (Pessino *et al.*, 1998; Cantoni, Bottino, Vitale *et al.*, 1999; Pende *et al.*, 1999). NKp44, in particular, consists of an extracellular region (168 amino acids) and a cytoplasmic domain (63 amino acids) connected by a membrane-spanning segment (25 amino acids). Importantly, the transmembrane segment contains a charged residue (Lys) which promotes the association with ITAM-bearing signal transducing DAP12 molecules (Olcese *et al.*, 1997; Lanier *et al.*, 1998), likely *via* formation of a membrane-embedded salt bridge with an Asp residue present in the transmembrane portion of DAP12. The latter protein appears to be required for NKp44 surface expression and is involved in the transduction of the activating signal (Cantoni, Bottino, Vitale *et al.*, 1999).

The amino-acid sequence of NKp44 extracellular region harbours the signature of a novel Ig-like type V domain, characterized by the presence of a Trp residue (Trp36; see Fig. 1), two Cys residues (Cys22 and Cys91, forming a disulfide bond in Ig-like domains) and of two residues, Arg62 and Asp85, which define the

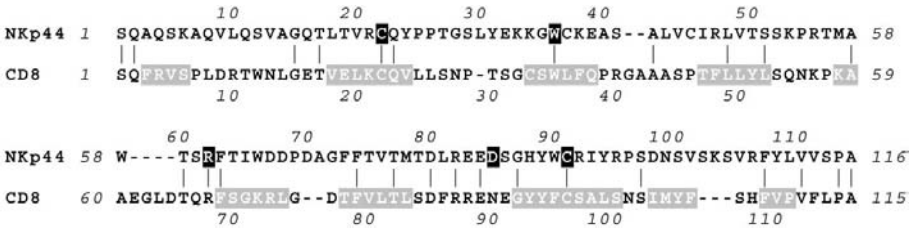

Figure 1
Sequence alignment of the NKp44 extracellular Ig-like domain and CD8 Ig-like domain. β-strand segments, represented as grey boxes, were identified in CD8 with the program *DSSP* (Kabsch & Sander, 1983). Residues defining the Ig-V signature are shown in black boxes.

typical Ig V domain signature (Williams & Barclay, 1988). The Ig-like domain is connected to the transmembrane segment by a 55 amino-acid linker peptide, displaying 14 predicted O-glycosylation sites. The amino-acid sequence homology of NKp44 with known human proteins is confined to the Ig-like V domain and limited to the poly-Ig receptor (25% sequence identity; Krajci *et al.*, 1991), the IRp60 receptor (27% sequence identity; Cantoni, Bottino, Augugliaro *et al.*, 1999) and the CMRF35 protein (29% sequence identity; Jackson *et al.*, 1992). A search for homologous proteins of known three-dimensional structure showed that NKp44 has weak but significant sequence homology (22% identical residues) with the CD8 surface molecule (Leahy *et al.*, 1992). The sequence-based alignment between the NKp44 Ig-like domain and CD8 is shown in Fig. 1.

To elucidate the mechanisms responsible for NKp44-mediated NK cell activation at the molecular level and to gain insight into the structural determinants involved in recognition of the yet unknown NKp44 physiological protein ligand, a crystallographic investigation on the Ig-like domain of NKp44 was undertaken.

2. Materials and methods

2.1. Expression, refolding and purification

NKp44 extracellular portion (nucleotides 393–728/accession No. AJ225109) was amplified starting from pCDNA3.1-NKp44 plasmid (Cantoni, Bottino, Vitale *et al.*, 1999) utilizing the following primers: 5′ GCGTACGGATCCCAGGCACAGTCCAAGGCTCAG (44 *Bam*HI, forward) and 5′ AACCCAAGCTTTTAAGATACCACCAGATAGAAT (44 *Hin*dIII, reverse). Amplification was performed with Pfu Turbo (Stratagene, CA) 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 347 bp PCR product was subcloned in pQE30 vector for prokaryotic expression (Qiagen GmbH, Germany), allowing the production of a recombinant protein with a 6×His tag at the N-terminus. The NKp44 nucleotide sequence was checked using a d-Rodhamine Terminator Cycle Sequencing Kit and a 377 Applied Biosystems Automatic Sequencer (Perkin Elmer Applied Biosystems, NJ, USA). The pQE30-NKp44 construct was transformed in M15(pREP4) *Escherichia coli* bacterial strain (Qiagen). Induction of recombinant NKp44 protein expression was obtained by growing a single colony overnight at 310 K, diluting this culture 1:100, growing it until an OD_{600} of 0.7 was achieved and adding 1 m*M* IPTG for 4 h at 310 K.

Bacterial cell pellets were recovered, resuspended 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 overnight. Cells were sonicated and washed with the following buffers: 50 m*M* Tris–HCl pH 8.0, 0.5% Triton-X 100, 100 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT; 2 *M* urea, 2 *M* NaCl, 50 m*M* Tris–HCl pH 8.0 and 10 m*M* DTT; 100 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA and 10 m*M* DTT. The inclusion bodies were dissolved under agitation in 6 *M* guanidinium–HCl, 100 m*M* Tris–HCl pH 8.0, 10 m*M* EDTA pH 8.0 and 0.1 m*M* DTT for 48 h at 277 K. Half of the supernatant was refolded in 20 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA pH 8.0, 25% glycerol, 3 m*M* reduced glutathione and 0.3 m*M* oxidized glutathione for 12 h. The remaining supernatant was then added and left to refold for a further 48 h. The solution containing the refolded protein was concentrated and then dialyzed overnight at 277 K against 100 m*M* NaH_2PO_4 and 10 m*M* Tris–HCl pH 8.0. The protein was purified with immobilized metal-affinity chromatography, using Ni–NTA agarose, as suggested by the manufacturers (Qiagen). Gel-filtration chromatography using a HiPrep Sephacryl S-100 column (Pharmacia Biotech, Sweden) was employed for the final purification step. The protein concentration was estimated by measuring the absorbance at 280 nm, employing the calculated extinction coefficient of 2.9 × 10^5 M^{-1} cm^{-1}.

Table 1
NKp44 Ig-like domain X-ray data-collection statistics.

Values in the last resolution shell (2.24–2.21 Å) are given in parentheses.

Source	ID14-2 beamline, ESRF
Wavelength (Å)	0.934
Temperature (K)	100
Resolution (Å)	2.21
Space group	$P6_222/P6_422$
Unit-cell parameters (Å, °)	$a = b = 60.4$, $c = 197.2$, $\alpha = \beta = 90$, $\gamma = 120$
Mosaicity (°)	0.39
No. of observations	353554
Unique reflections	11487
Data completeness (%)	99.6 (96.8)
$\langle I/\sigma(I)\rangle$	23.6 (5.7)
$R_{\rm merge}$† (%)	7.4 (52.3)

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

2.2. Crystal growth, data collection and processing

For crystallization purposes, the protein was brought to a concentration of 9.6 mg ml^{-1} in buffer containing 50 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl. Initial crystallization conditions were determined using the sparse-matrix method (Jancarik & Kim, 1991) as implemented in Hampton Crystal Screens I and II (Hampton Research, CA, USA). Refinement of preliminary crystallization conditions resulted in crystals of good quality which grew to their final size (hexagonal rods of about 0.1 × 0.05 × 0.05 mm) after a period of 7 d equilibration against a reservoir solution containing 0.1 *M* sodium acetate pH 4.8, 0.1 *M* ammonium acetate and 30%(*v*/*v*) PEG 400. Crystals were grown at 278 K by hanging-drop vapour diffusion by mixing an equal amount (1 µl) of protein and reservoir solutions. Prior to data collection, crystals were frozen at 100 K following immersion in a cryoprotectant solution identical to the crystallization mother liquor, but containing 35%(*v*/*v*) PEG 400.

Synchrotron X-ray data collection on a frozen native protein crystal was carried out at the ID14-2 beamline at ESRF Grenoble to a resolution limit of 2.21 Å (see Table 1). Data were processed using the programs *DENZO* and *SCALEPACK*, respectively (Otwinowski, 1993; Otwinowski & Minor, 1997).

3. Results and discussion

The NKp44 extracellular Ig-like domain was expressed in *E. coli*, refolded, purified and

crystallized. Analysis of the acquired diffraction pattern showed that NKp44 Ig-like domain crystals belong to the hexagonal space group $P6_222$ (or $P6_422$), with unit-cell parameters $a = b = 60.4$, $c = 197.2$ Å, $\gamma = 120^\circ$. Evaluation of the crystal packing parameter (Matthews, 1968) suggests that the lattice accommodates one molecule per asymmetric unit [V_M = 3.0 Å^3 Da^{-1}, 67%(*v*/*v*) solvent content]. The presence of two independent chains in the asymmetric unit [V_M = 1.5 Å^3 Da^{-1}, 35%(*v*/*v*) solvent content] cannot be ruled out on the basis of packing considerations, but is not supported by calculation of the protein self-rotation function. In this regard, inspection of the $\chi = 180^\circ$ section did not display any significant peak.

The structure solution of NKp44 was attempted *via* molecular-replacement methods using CD8, the closest structural relative of NKp44, as a search structure. For this purpose, the sequence alignment of NKp44 with CD8 (Fig. 1), identifying conserved regions and regions displaying insertions/deletions, was used to build suitably trimmed search models. Several molecular-replacement searches, as implemented in the programs *AMoRe* and *EMPR* (Navaza, 1994; Kissinger *et al.*, 2001), were run based on varying search models and experimental parameters. However, no solution could be recognized as allowing the correct orientation and translation of the template molecule(s) in the target crystal unit cell.

The elucidation of the molecular structure of the extracellular domain of the NKp44 triggering receptor will therefore be pursued either through multiple isomorphous replacement or *via* MAD techniques proceeding through expression of the SeMet-substituted protein.

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