Physiol. Res. 64 (Suppl. 1): S85-S93, 2015

Recombinant Expression, In Vitro Refolding and Characterizing Disulfide Bonds of a Mouse Inhibitory C-Type Lectin-Like Receptor Nkrp1b

L. HERNYCHOVÁ[1,2], H. MRÁZEK[1], L. IVANOVA[1,2], Z. KUKAČKA[1,3], J. CHMELÍK[1,3], P. NOVÁK[1,3]

[1]Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic, [2]Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic, [3]Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic

Received June 12, 2015
Accepted July 16, 2015

Summary

As a part of the innate immunity, NK (Natural Killer) cells provide an early immune response to different stimuli, e.g. viral infections and tumor growths. However, their functions are more complex; they play an important role in reproduction, alloimmunity, autoimmunity and allergic diseases. NK cell activities require an intricate system of regulation that is ensured by many different receptors on a cell surface which integrate signals from interacting cells and soluble factors. One way to understand NK cell biology is through the structure of NK receptors, which can reveal ligand binding conditions. We present a modified protocol for recombinant expression in *Escherichia coli* and *in vitro* refolding of the ligand-binding domain of the inhibitory Nkrp1b (SJL/J) protein. Nkrp1b identity and folding was confirmed using mass spectrometry (accurate mass of the intact protein and evaluation of disulfide bonds) and one-dimensional nuclear magnetic resonance spectroscopy. The intention is to provide the basis for conducting structural studies of the inhibitory Nkrp1b protein, since only the activating Nkrp1a receptor structure is known.

Key words

NK cell • C-type lectin-like receptor (CTLR) • Nkrp1 • Mass spectrometry • Disulfide bonds

Corresponding author

L. Hernychová or P. Novák, Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4. Czech Republic. E-mail: lucie.hernychova@biomed.cas.cz or pnovak@biomed.cas.cz

Introduction

Under physiological conditions, the immune system identifies molecules of a foreign origin, for example microbial and viral antigens or alloantigens. When self-molecules are recognized, autoimmune diseases may evolve. Apart from this mechanism of an organism's defense against infections, another essential ability of the immune system is to detect abnormal expression of self-molecules that occurs during cellular stress (Fine *et al.* 2010), viral infections (Williams *et al.* 2012) or tumorigenic processes (Carlyle *et al.* 2004), while avoiding autoimmunity. Such expression changes are identified by Natural Killer (NK) cells, therefore providing immune homeostasis.

NK cells were described by Kiessling *et al.* (1975). Although significant progress in this area has been accomplished over the last four decades, the precise mechanisms of their functions are not, however, fully understood yet. NK cells have a large amount of receptors with diverse functions on their surface that simultaneously send distinct signals into the cell. Consequently, NK cells are complex and important regulators of the immune response. They influence dendritic cells, macrophages and T and B cells (Vivier *et al.* 2008), so they are therefore involved in reproduction (Winger and Reed 2013), transplantation immunity (Villard 2011), allergic diseases (Mathias *et al.* 2014) and autoimmunity (Tian *et al.* 2012).

NK receptors are divided according to their

function into activating and inhibitory, among others (cytokine, chemokine and adhesion receptors). An NK cell is activated when signals from activating receptors reach the activation threshold and exceed inhibitory signals. Based on the ligands of NK receptors, hypotheses concerning NK cell recognition strategies of target cells have been described. The first of them is called *missing-self recognition* and suggests that NK cells detect down-regulation or the complete absence of self-molecules (Kärre *et al.* 1986), which normally impair their cytotoxic activity. These molecules mainly include MHC class I glycoproteins and also non-MHC ligands, e.g. Clr-b protein, which is recognized by the Nkrp1b receptor (Carlyle *et al.* 2004).

According to the second hypothesis – *stress induced-self recognition* – the expression of some self-molecules (usually MHC class I homologues) increases in response to different types of cellular stress. These molecules are ligands of activating NK receptors, therefore contributing to NK cell cytotoxic reaction, for example NKG2D receptor and ULBP-like transcript 1 (MULT1) binding pair (Carayannopoulos *et al.* 2002).

The last hypothesis is based on the recognition of *non-self* ligands. Activating NK receptors are capable of identifying foreign molecules, for instance a pair of murine Ly49H receptor and viral protein m157 (Smith *et al.* 2002).

Structurally, NK receptors are grouped into two classes – immunoglobulin and C-type lectin-like receptors (CTLR). The first receptor group is comprised of type I transmembrane proteins with two to four immunoglobulin domains, which are separated from the cell surface by a short stalk region (Lanier 2005). There are three protein families in this category: Killer Cell Immunoglobulin-Like Receptors (KIR), Leukocyte Immunoglobulin-Like receptors (LILR) and Natural Cytotoxicity Receptors (NCR). The most common ligands of these receptors are MHC class I molecules alone or in complex with presented peptides, host proteins, whose expression is influenced by the physiological condition of the cell, and viral molecules (Hecht *et al.* 2009, Campbell and Hasegawa 2013, Binici *et al.* 2013).

The second receptor group (CTLR) includes type II transmembrane proteins, which consist of a cytoplasmic domain, transmembrane region, stalk region and C-Type Lectin-Like Domain (CTLD). The CTLR group also includes three protein families: Ly49, CD94/NKG2 and Nkrp1. As well as immunoglobulin receptors, CTLRs interact with MHC class I, viral proteins and molecules whose expression is affected by cellular stress (Campbell and Hasegawa 2013).

Interactions are mediated through the CTLD. Its conserved structure is characterized by two α-helices, two antiparallel β-sheets and a long loop region, which constitutes the most variable and flexible part of the whole structure (Sovová *et al.* 2011). The domain is usually stabilized by two or three intramolecular disulfide bonds (Zelensky and Gready 2005). Some CTLRs form dimers, e.g. NKG2D or Ly49C (Li *et al.* 2001, Dam *et al.* 2003). In the case of Nkrp1 proteins, the existence of intermolecular disulfide bonds remains to be confirmed experimentally, since the number of cysteines in proteins differs among Nkrp1 family members and, in addition, is reliant on the respective mouse strain.

The murine Nkrp1 protein family contains five members with either an activating or an inhibitory function: Nkrp1a, b/d, c, f and g. *Nkrp1* genes are located in the Natural Killer Cell (NKC) gene complex (Yokoyama *et al.* 1991), their expression is variable and dependent on a mouse strain. For example, Nkrp1b was found to be expressed only in certain mouse strains, e.g. SJL/J, BALB/c or 129S1 (Kung *et al.* 1999, Carlyle *et al.* 2004, Chen *et al.* 2011) and only by approximately 60 % of all NK cells (Zhang *et al.* 2012). Apart from NK cells, Nkrp1 receptors can also be detected on the surface of NKT cells, T cells and dendritic cells (Appasamy *et al.* 1996, Aust *et al.* 2009).

Nkrp1 receptors were originally thought to bind carbohydrates in a calcium-dependent manner (Bezouska *et al.* 1994). However, later experiments showed that the interactions are of a protein nature (Iizuka *et al.* 2003, Carlyle *et al.* 2004, Kveberg *et al.* 2009, Kveberg *et al.* 2011, Aust *et al.* 2009) and calcium-independent due to a loss of amino acids essential for ion binding (Zelensky and Gready 2005). The ligands of Nkrp1 receptors are C-type Lectin-Related (Clr) proteins, which also belong among CTLRs (Yokoyama and Plougastel 2003). The expression of most Clr proteins is tissue-specific (e.g. Clr-d was detected only in an eye), with the exception of Clr-b, whose expression was found in all tested tissues except the brain. Such expression profile, in contrast with the unrestricted MHC class I expression, might be important for regulating immunosurveillance (Zhang *et al.* 2012).

Concerning the structure of Nkrp1 proteins, only Nkrp1a (B6) crystal and NMR structure are known (Kolenko *et al.* 2011, Rozbesky *et al.* 2014).

Additionally, a model of Nkrp1c (B6) using chemical cross-linking and molecular modeling was proposed (Rozbesky *et al.* 2013). The structures of other Nkrp1 receptors, however, remain to be solved. The genes responsible for encoding mouse Nkrp1 proteins, their functions, ligands and revealed structures are summarized in Table 1.

Since the X-ray and NMR structures of activating receptor Nkrp1a have been proposed and there is no structural model of the mouse inhibitory Nkrp1 receptor, we decided to establish a basis for structural characterization of Nkrp1b. Here we describe the recombinant expression in *Escherichia coli* and *in vitro* refolding of the ligand-binding domain (CTLD) of the inhibitory Nkrp1b (SJL/J) protein (GI: 13774943) using a fast protocol, yet achieving sufficient protein quantity and purity for conducting subsequent structural studies. Additionally, we used mass spectrometric techniques (accurate mass of the intact protein and characterizing disulfide bonds), which are also supported by one-dimensional NMR spectroscopy, to show evidence of protein folding.

Table 1. The mouse Nkrp1 protein family. Genes encoding proteins, their functions, known ligands and structures.

Gene	*Nkrp1a*	*Nkrp1b/d*	*Nkrp1c*	*Nkrp1e*	*Nkrp1f*	*Nkrp1g*
Protein function	activating	inhibitory	activating	*pseudogene*	activating	inhibitory
Ligand	?	Clr-b	?	-	Clr-c, d, g	Clr-d, g, f
Structure	X-ray NMR	?	MS	-	?	?

Materials and Methods

Materials

The pET-30a(+) expression vector was purchased from Novagen (USA) and the *Escherichia coli* BL-21 (DE3) strain from Stratagene (USA). The ENrich^{TM} SEC 70 10 × 300 column was received from Bio-Rad (USA). All the chemicals were obtained from Sigma-Aldrich unless otherwise stated and were of the highest available purity.

Protein expression

DNA construct pET-30a(+)-Nkrp1b (SJL/J) was transformed into competent *Escherichia coli* BL-21 (DE3) cells, which allow for high-level expression of recombinant proteins. Transformed cells producing Nkrp1b protein were then used to produce protein in 500 ml of LB medium with kanamycin (50 μg/ml) at 37 °C and 220 rpm in a 2 l Erlenmeyer flask. After reaching a density of OD = 0.6 (λ = 550 nm), the cells were induced by isopropyl-β-D-thiogalactopyranoside (0.1 mM) and grown for 4 h. Finally, the culture was centrifuged at 5000 g and 4 °C for 10 min.

Isolation and solubilization of inclusion bodies

Isolation and solubilization of inclusion bodies, as well as protein refolding, were carried out on the basis of Rozbesky *et al.* (2011) with several modifications that are stated below.

During the isolation of inclusion bodies, the proteinase inhibitors leupeptin and phenylmethyl-sulfonylfluoride (PMSF) with a final concentration of 1 μM and 1 mM were used. For the solubilization of inclusion bodies, we applied 8 ml of guanidine-HCl buffer (6 M guanidine-HCl, 50 mM Tris-HCl, 10 mM DTT, pH 8.0) for each 1 g of their wet weight.

Nkrp1b (SJL/J) protein refolding and purification

Protein refolding was performed by a rapid dilution method, which uses a hundredfold higher volume of refolding buffer containing 50 mM Tris-HCl; 1 M L-arginine hydrochloride; 100 mM $CaCl_2$; 9 mM cysteamin; 3 mM cystamin and 1 mM NaN_3 (pH 8.5). Before refolding, the buffer was chilled to 4 °C and supplemented by PMSF to a final concentration of 1 mM. The solution was stirred for 1 h at 4 °C, then transferred into dialysis tubes and dialyzed twice (for 4 h and 16 h)

against 8 l of buffer consisting of 15 mM Tris-HCl; 9 mM NaCl; 1 mM NaN_3 (pH 8.5).

In the next step, the protein solution was concentrated by ultrafiltration using a cellulose membrane and centrifugal filter units, both with a 10 kDa molecular weight cut-off (Millipore, USA). The protein sample was then purified by size-exclusion chromatography performed by an ENrich^{TM} SEC 70 10 × 300 column (flow rate 0.4 ml/min) using NGC Quest^{TM} (Bio-Rad, USA). The elution buffer was composed of 10 mM HEPES; 50 mM NaCl and 1 mM NaN_3 (pH 7.4). The quality of the acquired protein fractions was examined by SDS-PAGE under reducing and nonreducing conditions in a 15 % polyacrylamide gel. Finally, the protein concentration was estimated by Bradford assay (Bio-Rad, USA) with BSA standards in the range of 0-0.5 mg/ml with a step of 0.1 mg/ml.

Accurate mass of the intact protein

To determine the mass of the intact protein, 200 pmol of the purified sample was desalted using a Protein MacroTrap C4 column (Michrom, USA). The protein was eluted with 80 % acetonitrile with 0.5 % formic acid and analyzed using an Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI-FT-ICR MS) on 12T SolariX XR^{TM} (Bruker Daltonics, USA).

Disulfide bonds characterization

Disulfide bonds evaluation was performed as described (Pompach *et al*. 2009). Briefly, Nkrp1b protein was analyzed by SDS-PAGE in 15 % polyacrylamide gel under nonreducing conditions with addition of 200 μM cystamin as an oxidative agent. Protein was then digested in the gel by trypsin (Promega, USA) and Asp-N (Roche, Switzerland) proteinases at 37 °C overnight. The final concentration of both proteinases was 5 ng/μl. Obtained peptides were subjected to Liquid Chromatography (LC) ESI-FT-ICR MS and data were analyzed using Data Analysis 4.1 (Bruker Daltonics, USA).

One-dimensional ^1H NMR spectroscopy

The ^1H spectrum with presaturation was acquired on a 700 MHz Bruker Avance III spectrometer equipped with a TCI cryoprobe at 25 °C. Before the measurement was taken, 10 % D_2O was added to the protein sample dissolved in a buffer containing 10 mM HEPES; 50 mM NaCl and 1 mM NaN_3 (pH 7.4).

Results

Protein refolding and purification

Since the CTLD of mouse Nkrp1b (SJL/J) shares 94 % identity with mouse Nkrp1c (B6), a previously-published protocol was adopted (Rozbesky *et al*. 2011). Following a rapid dilution method in 800 ml of refolding buffer (50 mM Tris-HCl; 1 M L-arginine hydrochloride; 100 mM $CaCl_2$; 9 mM cysteamin; 3 mM cystamin and 1 mM NaN_3; pH 8.5), a subsequent dialysis and concentration of the protein sample were performed. Finally, Nkrp1b was purified using size-exclusion chromatography. Additionally, desired protein fractions were analyzed by SDS-PAGE (Fig. 1). As is shown in the figure, Nkrp1b folds as a monomer, which corresponds to the peak of retention volume of 11.5 ml (fractions 5-6). Although fraction 6 contains pure monomeric protein, interestingly, a significant amount of dimer is present in fraction 5. The fractions eluted earlier consist of protein aggregates. The yield of pure Nkrp1b protein obtained from 0.5 l of LB medium is usually over 5 mg.

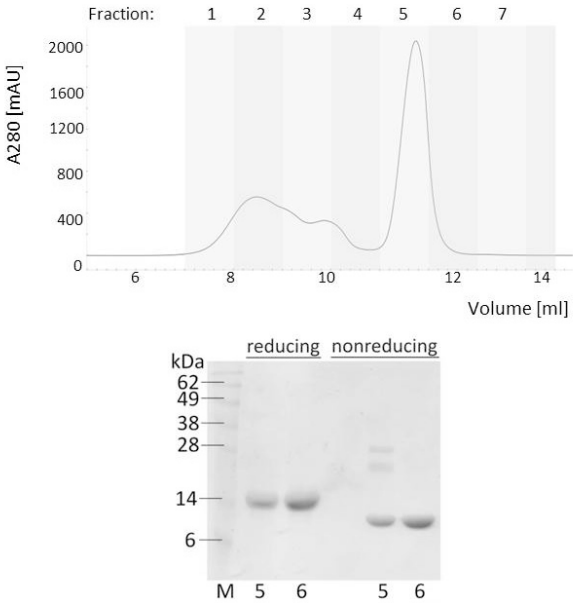

Fig. 1. Purification of the Nkrp1b protein using size-exclusion chromatography and consequent analysis of protein fractions by SDS-PAGE in a 15 % polyacrylamide gel. As both methods prove, protein forms monomer or monomer/dimer mixture, which is present in fractions 5 and 6.

Characterization of the Nkrp1b refolding

An accurate mass of pure desalted Nkrp1b protein was evaluated by ESI-FT-ICR MS to confirm its identity and folding (the sequence of the analyzed protein is shown in Fig. 2A). After mass spectrum (Fig. 2B)

deconvolution, the monoisotopic mass of the native protein was 15457.29 Da. Such experimental protein mass correlates with theoretical value (15457.30 Da) for the case where all cysteines in Nkrp1b are involved in disulfide bridges with a measurement error of 0.4 ppm. Also, the dispersion of amide (8-11 ppm) and methyl (–1.5-0.5 ppm) proton signals in the ^{1}H NMR spectrum (Fig. 2C) clearly shows that this protein is well folded.

Characterizing disulfide bonds

An LC-MS analysis of the Nkrp1b protein digested with trypsin and also Asp-N revealed three disulfide bonds between ^{94}C-^{105}C, ^{122}C-^{210}C and ^{189}C-^{202}C. Figure 3 demonstrates masses of the cross-linked peptides together with a schematic diagram of the disulfide bridges. Sequence numbering is in accordance with the entire protein sequence as it is presented in Figure 2A.

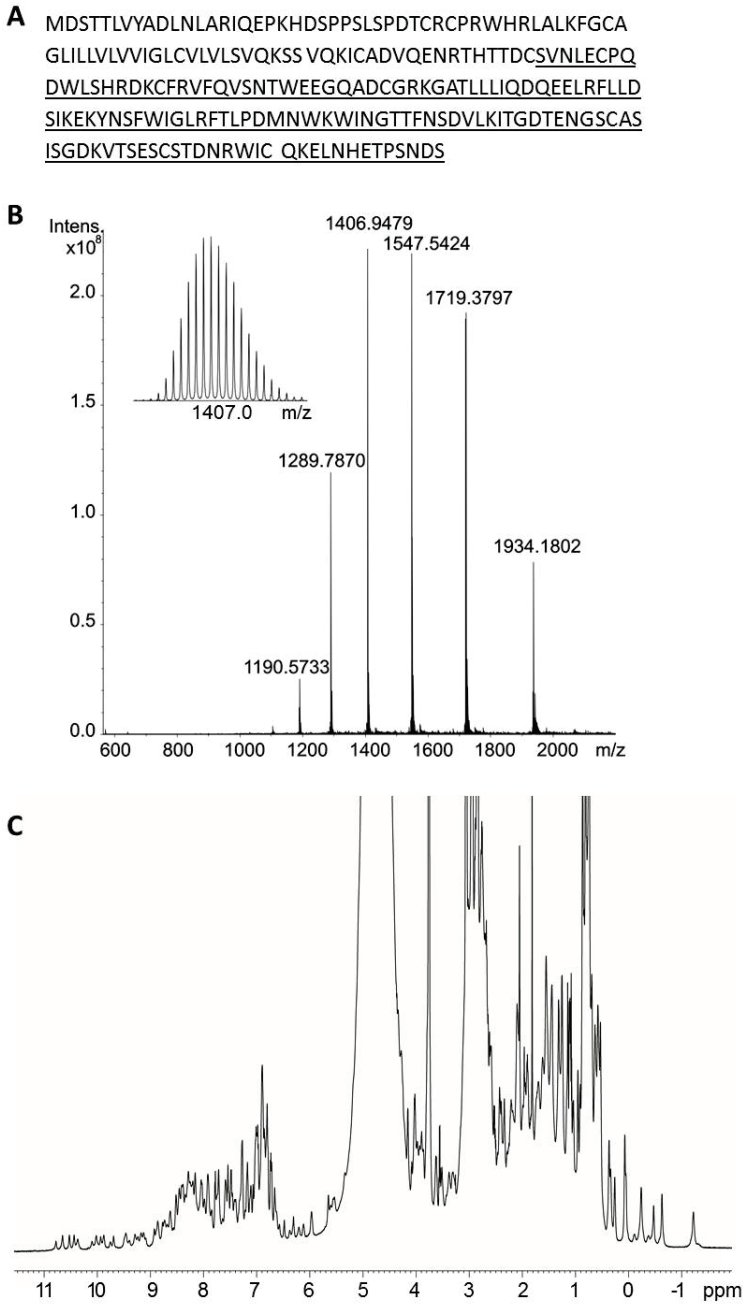

Fig. 2. Characterization of the Nkrp1b refolding. **(A)** Underlined amino acids in the whole Nkrp1b (SJL/J) protein sequence indicate a prepared protein part (CTLD). **(B)** The mass spectrum of the native Nkrp1b protein with a detail of the selected charge state (+11). Based on the calculated mass of Nkrp1b, all cysteines participate in the formation of disulfide bridges. Acquired experimental mass (15457.29 Da) corroborates protein identity, as the theoretical value (15457.30 Da) differs only by 0.4 ppm. **(C)** The ^{1}H NMR spectrum of Nkrp1b. Dispersion of amide (8-11 ppm) and methyl (–1.5-0.5 ppm) proton signals supports the results of ESI-FT-ICR MS that protein was folded successfully.

Proteinase	Disulfide bonded peptides	Linked cysteines	Theoretical mass of peptides	Experimental mass of peptides	Error
Trypsin	89 - 102/103 - 107	^{94}C - ^{105}C	2349.0867 Da	2349.0874 Da	0.3 ppm
Trypsin	108 - 125/208 - 223	^{122}C - ^{210}C	3951.7664 Da	3951.7639 Da	0.6 ppm
Trypsin	179 - 196/197 - 207	^{189}C - ^{202}C	2850.2004 Da	2850.1970 Da	1.2 ppm
Asp-N	89 - 97/98 - 120	^{94}C - ^{105}C	3806.7444 Da	3806.7432 Da	0.3 ppm
Asp-N	121 -133/205 - 221	^{122}C - ^{210}C	3468.7114 Da	3468.7097 Da	0.5 ppm
Asp-N	176 -194/195 - 204	^{189}C - ^{202}C	2920.3037 Da	2920.3027 Da	0.4 ppm

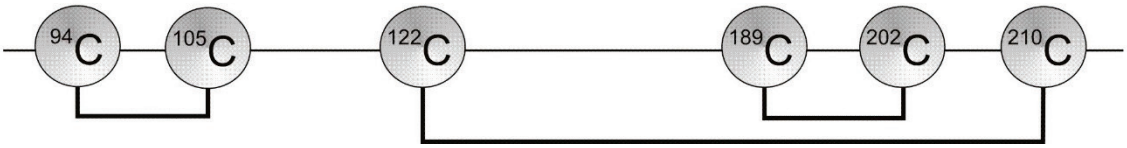

Fig. 3. Tryptic and Asp-N disulfide bonded peptides observed in an LC-ESI-FT-ICR MS analysis of the Nkrp1b CTLD and the scheme of disulfide linkages.

Discussion

The history of Nkrp1 receptors dates back to 1970s, when the first of them was described (Glimcher *et al.* 1977). It was named NK1.1 antigen, later known as Nkrp1c (Giorda and Trucco 1991, Ryan *et al.* 1992, Yokoyama and Seaman 1993). Research on Nkrp1 receptors covered their expression, which appeared to be stochastic (Zhang *et al.* 2012), occurring only in a limited number of cellular types (Appasamy *et al.* 1996, Aust *et al.* 2009) and additionally, dependent on the strain of mouse (Kung *et al.* 1999, Carlyle *et al.* 2004). Further investigation concerned Nkrp1 binding activity (Iizuka *et al.* 2003) and the signaling of several family members (Ljutic *et al.* 2005). However, little is known about their structure.

The effort to characterize the functions of Nkrp1 receptors is important for understanding NK cell regulation; and the receptor structure is the starting point for it. It can reveal conditions of ligand binding that triggers subsequent processes in a cell, or elucidate signaling.

In contrast with well characterized Ly49 family members (Dam *et al.* 2003), the only revealed structure of Nkrp1 receptors is activating Nkrp1a (B6) (Kolenko *et al.* 2011, Rozbesky *et al.* 2014). Furthermore, the protein sequence of inhibitory Nkrp1b (SJL/J) is more than 79 % identical to the activating Nkrp1c (B6) receptor. The essential differences are located within the cytoplasmic tail, which is involved in signaling processes (Ljutic *et al.* 2005). Interestingly, the ligand-binding CTLDs of both proteins are nearly indistinguishable in their amino acid sequence (Rozbesky *et al.* 2011). In addition, a similar activating-inhibitory pair of Nkrp1 molecules (Nkrp1f and Nkrp1g) is known for its high promiscuity with several Clr ligands (Kveberg *et al.* 2011). A modulation of Nkrp1b/Clr-b receptor/ligand pair during murine cytomegalovirus infection has recently been suggested (Aguilar *et al.* 2015).

We propose a modified protocol for preparing the mouse inhibitory C-type lectin-like receptor Nkrp1b (SJL/J) CTLD including recombinant protein expression in *Escherichia coli*, *in vitro* refolding using the chemical chaperone L-Arginine hydrochloride (based on Rozbesky *et al.* 2011) and one-step purification by size-exclusion chromatography. The quality of the *in vitro* refolded protein was validated using mass spectrometric techniques and one-dimensional NMR spectroscopy. Since the accurate mass of the intact protein showed all cysteines involved in disulfide bridge formation, disulfide bonds characterization was performed to support this result. The acquired connections between cysteines (^{94}C-^{105}C, ^{122}C-^{210}C and ^{189}C-^{202}C) are consistent with the common disulfide pattern of other CTLRs (Zelensky and Gready 2005, Rozbesky *et al.* 2013).

This protocol is suitable for conducting further

structural studies on Nkrp1b, including chemical cross-linking or crystallization, as the protein yield (>5 mg from 0.5 l of LB medium) and purity are adequate.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (CZ.1.07/2.3.00/30.0003); the European Regional Development Fund ''BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Vestec'' (CZ.1.05/1.1.00/02.0109), Charles University (project UNCE_204025/2012) and Institutional Research Concept (RVO 61388971). Access to instrumental and other facilities was supported by the EU (Operational Program Prague – Competitiveness project CZ.2.16/3.1.00/24023). The authors also gratefully acknowledge the support by project LO1509 of the Ministry of Education, Youth and Sports of the Czech Republic.

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