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Preparation of soluble isotopically labeled NKp30, a human natural cytotoxicity receptor, for structural studies using NMR

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ABSTRACT

Using a codon-optimized gene fragment, we report remarkable yields for extracellular domain of human NK cell receptor (NKp30ex) when produced on M9 minimal medium, even with low (2 g/L) glucose concentration. The yields were identical using media containing $^{15}NH_4Cl$ or $^{15}NH_4Cl$ in combination with all-^{13}C–D-glucose allowing to produce homogenous soluble monomeric NKp30 in several formats needed for advanced NMR studies. Our optimized protocol now allows to produce routinely 10 mg batches of these NKp30ex proteins per 1 L of M9 production medium in four working days. The purity and identity of the produced proteins were checked by SDS–PAGE, MALDI MS peptide mapping, and high resolution ion cyclotron resonance MS. Analytical ultracentrifugation confirmed the monomeric status of the produced proteins. Long-term stability of the produced protein proved to be very good allowing its use for NMR studies using elevated temperatures. These studies should reveal further details of the interaction of NKp30 with several of its ligands including target cell surface proteins and heparin-derived oligosaccharides.

Introduction

Natural killer (NK)[1] cells represent a specialized lymphoid population with a potent cytolytic activity against tumor, virally infected or stressed cells [1]. The ability to recognize these targets efficiently requires a cooperation between inhibitory and activation receptors at the surface of NK cells [2]. The former category involves receptors specific for MHC class I alleles or alternative missing-self molecules inactivating NK cells through recruitment and activation of SHP-1 and SHP-2 phosphatases [3–6]. Activation receptors of human NK cells involve NKG2D (CD314), a type II membrane protein characterized by a lectin-like domain [7,8], natural cytotoxicity receptors (NCR) NKp46 (CD335 or NCR1), NKp44 (CD336 or NCR2), and NKp30 (CD337 or NCR3) [9–12], and a broad range of coreceptors including 2B4, NTB-A, DNAM-1, NKp80 and other cell surface molecules [13,14]. These may, depending on the state of NK cell and target cell context, enter individually or together in numerous interactions with their ligands forming the "receptor zipper" as a core of the immune activation synapse at NK cell – target cell interface [1,14].

NKp30 is a specific surface marker of human NK cells, and a member of the CD28 family of leukocyte surface receptors (which also includes CTLA-4 and PD-1 lymphocyte inhibition antigens) characterized by a signal peptide (missing in the mature receptor), a single N-terminal immunoglobulin (Ig) domain, a transmembrane domain, and a short intracellular peptide [15]. Charged residues in the transmembrane region of these receptors allow them to interact with the signaling ζ–γT-cell receptor chains [16]. Ligands for NKp30 have been difficult to identify [17]. Recently, however, NKp30 has been shown to bind the tumor cell surface protein B7–H6 [18], the nuclear factor BAT3 [19], and tegument pp65 protein of human cytomegalovirus [20]. Practical use of the above findings in tumor therapies have been made using a NKp30–Ig fusion protein able to inhibit the growth of prostate cancer cell line *in vivo*, including a complete tumor removal in 50% of treated mice [21].

While the three dimensional structure of NKp46 and NKp44 has been solved early in the last decade [22,23], only in 2011 two papers reported the structure of NKp30 [24], and its complex with B7–H6 ligand [15]. However, the mode of recognition of two other

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[1] *Abbreviations used:* CD, cluster of differentiation; DTT, ditihiothreitol; NKp30ex, extracellular IgV domain of NKp30; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Ig, immunoglobulin; IgV, immunoglobulin variable domain; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI, matrix-assisted laser desorption and ionization; MS, mass spectrometry; MWCO, molecular weight cut-off; NK, natural killer; NKp30, natural killer protein 30; NMR, nuclear magnetic resonance.

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ligands, BAT3 and pp65, remains unknown. Indeed, even the three dimensional structure of pp65 and BAT3 themselves has not been solved except of the short N-terminal ubiquitin-like domain of BAT3 (PDB code 4DFW and 4EEW). Moreover, the molecular details of somewhat controversial interactions with heparan sulfate epitopes suggested possible target structures in tumor cell plasma membranes, but these have not been clarified [25–28]. Furthermore, it would appear interesting to complement the static picture of NKp30 – B7–H6 interaction [15] by more dynamic data obtained using advanced NMR techniques. All these studies require considerable amounts of stable soluble NKp30 protein, not only in the unlabeled but also in the ^{15}N and $^{15}N/^{13}C$ uniformly labeled form. Previously, only brief description of procedures for the production of recombinant NKp30 were published but not in isotopically labeled form [15,24].

Here we describe the production of uniformly labeled NKp30 for NMR investigations using the synthetic codon optimized gene coding for the extracellular Ig domain of this receptor. We achieved a high level of production in M9 minimal medium even with a low (2 g/L) glucose concentration, and simplified the folding and purification protocol. This allowed us to produce routinely 10 mg batches of monomeric soluble NKp30 in unlabeled, ^{15}N-labeled or $^{15}N/^{13}C$-double labeled formats.

Materials and methods

Materials, bacterial strains, plasmids and culture media

$^{15}NH_4Cl$ and all-^{13}C-labeled glucose were purchased from Cambridge Isotope Laboratories, Cambridge, MA, USA. *Escherichia coli* strains BL21-Gold, BL21-Gold (DE3), BL21-Gold (DE3)pLys, and BL21-CodonPlus® RIPL purchased from Stratagene were used for protein production. These were grown under constant shaking (200 RPM) in glass tubes or shaker flasks using the media described below. LB: Luria Bertani medium, 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl pH 7.5 [29]; PBTB: phosphate buffered terrific broth, 12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L KH_2PO_4, 2.2 g/L K_2HPO_4 pH 7.0 [30]; M9G2 and M9G4: M9 minimal medium with 2 or 4 g/L D-glucose, respectively, 17.2 g/L $Na_2HPO_4{\cdot}12H_2O$, 3 g/L KH_2PO_4, 2.5 g/L NaCl, 1 g/L NH_4Cl, 14.7 mg/L $CaCl_2{\cdot}2H_2O$, 245.5 mg/L $MgSO_4{\cdot}7H_2O$, 100 mg/L thiamine [29]. M9 minimal medium in which NH_4Cl was substituted for $^{15}NH_4Cl$ was designated M9G2N, and M9 minimal medium in which NH_4Cl was substituted for $^{15}NH_4Cl$, and D-glucose for all-^{13}C–D-glucose, respectively, was designated M9G2NC. All media were supplemented with 50 mg/L kanamycin to assure the maintenance of expression pET30a plasmid, or with additional antibiotics (tetracyclin at 10 mg/L, or chloramphenicol at 50 mg/L) when recommended by bacterial strain supplier. All other chemicals were of the highest commercially available purity from Sigma or other indicated suppliers.

Construction of codon-optimized protein expression vector pET30aNKp30ex

The codon-optimized NKp30 expression vector was purchased from Generay Biotechnology, Shanghai, China (http://www.generay.co.cn). The vector was constructed by inserting the DNA fragment coding for the extracellular region of NKp30 (residues Leu19–Gly135) into NdeI and XhoI restriction sites of pET30a expression vector (Novagene); ATG triplet of NdeI created the initiation Met, and a single stop codon (TAA) was inserted just before the XhoI site. For expression in *E. coli*, the DNA insert coding for NKp30ex was extensively codon optimized by Generay for codon usage bias, GC content, CpG dinucleotides content, mRNA secondary structure, cryptic splicing sites, premature polyA sites, internal chi sites and ribosomal binding sites, negative CpG islands, RNA instability motif (ARE), repeat sequences (direct repeat, reverse repeat, and Dyad repeat), and the presence of restriction sites that may interfere with cloning. The optimized sequence was deposited into GenBank/EMBL/DDBJ database under the accession number JX436478.

Expression trials in rich and minimal medium

For the initial selection of a bacterial strain for protein production, *E. coli* was transformed with the expression plasmid pET30aNKp30ex, and individual colonies were obtained on LB plates with kanamycin and other antibiotics suggested by strains supplier. Three well separated colonies were transferred into 1 ml of LB medium plus antibiotics, and overnight stationary cultures were grown at 37 °C. For test induction experiments, 10 μl of stationary culture was inoculated into 1 ml of fresh LB medium, shaken in 13 ml test tubes for 3 h at 37 °C, induced with 1 mM IPTG, and shaken for another 3 h. The entire culture was transferred to plastic 1.5 ml microtubes, spun for 10 min at 18,000*g* in a microfuge, and the medium was discarded. The bacterial pellet was resuspended in 160 μl of SDS–PAGE sample buffer with 100 mM DTT [29], boiled for 5 min, briefly sonicated to reduce the viscosity of the solution, and spun again for 10 min as above. Five microliter of this supernatant was analyzed by SDS–PAGE using 18.5% polyacrylamide gels stained with Coomassie Brilliant Blue R250 (required to resolve well small size NKp30ex – Ref. [29]). Optimization of protein production (temperature, medium, IPTG concentration) was performed as above; when induced at 28 °C, the protein was produced for 16 h.

Large scale expression, isolation of inclusion bodies, protein folding and purification

For large scale protein production, 6–8 bacterial colonies were inoculated into 20 ml of medium with antibiotics, and shaken (200 RPM) overnight at 37 °C to produce the starter culture. Four to six 1 L flasks containing 200 ml of medium were inoculated with 2 ml of the starter culture each, and shaken at 200 RPM and 37 °C for 3–4 h until the O.D. at 550 nm reached 0.5–0.6. The production of protein was induced with 0.1 mM IPTG, and shaking continued under identical conditions (37 °C) for additional 4 h. Cells were harvested, and inclusion bodies were prepared and their purity was analyzed by SDS–PAGE [31,32]. Briefly, harvested bacteria were suspended on ice in 2 × 25 ml of sucrose buffer (50 mM Tris–HCl pH 8.0 with 25% (w/v) sucrose, 1 mM EDTA, 1 mM NaN_3, and 10 mM DTT) with protease inhibitors (1 mM PMSF and 1 μM leupeptin) using sonication, and frozen at −20 °C. Suspension was thawed, supplemented with $MgCl_2$, DNase I, and RNase (final concentrations 10 mM, 30, and 10 μg/ml, respectively), and sonicated 4 times for 30 s. Freezing, thawing, and sonication was repeated once. Inclusion bodies were sedimented at 20,000*g* for 20 min at 4 °C, and the supernatant was discarded. Inclusion bodies were resuspended in 2 × 25 ml of Triton buffer (50 mM Tris–HCl pH 8.0 with 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM NaN_3, and 1 mM DTT), sonicated and spun as above. The wash in Triton buffer was repeated once followed by another wash in Triton-free buffer (50 mM Tris–HCl pH 8.0 with 100 mM NaCl, 1 mM EDTA, 1 mM NaN_3, and 1 mM DTT). Inclusion bodies were kept at −80 °C until further use. The folding of NKp30ex proceeded according to the published protocol [15] with the following modifications: inclusion bodies from individual batches (0.8–1.2 L of production medium) were dissolved in 10 ml denaturing buffer (8 M urea with 50 mM Tris–HCl pH 8.0 and 10 mM DTT), and the protein content was determined using Bradford assay (BioRad). Five hundred milliliter of refolding buffer (Ref. [15] – 0.4 M L-arginine, 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 3 mM reduced glutathione, 0.9 mM oxidized glutathione, 1 mM