Expression, crystallization and X-ray diffraction analysis of a complex between B7-H6, a tumor cell ligand for the natural cytotoxicity receptor NKp30, and an inhibitory antibody

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Natural killer (NK) cells are essential components of the innate immune response to tumors and viral infections. In humans, the activating natural cytotoxicity receptor NKp30 plays a major role in NK cell-mediated tumor cell lysis. NKp30 recognizes the cell-surface protein B7-H6, which is expressed on tumor, but not healthy, cells. A mouse monoclonal antibody (17B1.3) against human B7-H6 has been developed ($K_d = 0.2 \mu\text{M}$) to investigate NKp30-mediated NK cell activation and to target tumors expressing B7-H6. Surprisingly, 17B1.3 blocks NK cell activation without interfering with the binding of B7-H6 to NKp30. Understanding the inhibitory mechanism of this antibody will require knowing the structure of 17B1.3 bound to B7-H6. The antigen-binding fragment (Fab) of 17B1.3 was expressed by *in vitro* folding from bacterial inclusion bodies. The extracellular domain of B7-H6 was produced by secretion from baculovirus-infected insect cells. Crystals of the Fab 17B1.3–B7-H6 complex grown by macro-seeding diffracted to 2.5 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 89.6$, $b = 138.0$, $c = 171.4$ Å, $\alpha = \beta = \gamma = 90^\circ$. Comparison of the Fab 17B1.3–B7-H6 structure with the known NKp30–B7-H6 structure will elucidate the inhibitory mechanism of 17B1.3.

1. Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system that participate in the elimination of malignantly transformed and virally infected cells (Di Santo, 2008; Vivier *et al.*, 2008, 2011). The cytolytic activity of NK cells is regulated by positive-signaling activating receptors (resulting in target cell lysis) and negative-signaling inhibitory receptors (preventing lysis) (Lanier, 2008; Long *et al.*, 2013). It is the dynamic interplay between these signals that ultimately determines the outcome of NK cell–target cell encounters. The dominant signal received by an NK cell is inhibitory and is provided by the interaction of inhibitory receptors with normal levels of major histocompatibility complex class I (MHC-I) molecules. If MHC-I expression is reduced by tumorigenic or infectious processes, this inhibitory signal is attenuated and the NK cell undergoes activation. Inhibitory receptors specific for MHC-I include the killer immunoglobulin-like receptors (KIRs) in humans and the C-type lectin-like Ly49 receptors in rodents (Li & Mariuzza, 2014).

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Table 1
Macromolecule-production information.

17B1.3 Fab	
Source organism	<i>Mus musculus</i>
Expression vector	pET-26b
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	
Heavy chain of Fab	QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNM-HWVKTPGQGLEWIGAIYPGNGDTSYNQKFKG-KATLTADKSSSTAYMQLSSLTSEDSAVYYCAR-EGLGALLRDLYYWGGQTSVTVSSAKTTAPSVY-PLAPVCGDGTGSSVTLGCLVKGYFPEPVLTWN-SGSLSSGVHTFPAVLQSDLYTLSSSVTTSST-WPSQSITCNVAHPASSTKVDKKILE
Light chain of Fab	QIVLTQSPALMASPGEKVTMTCSASSSVSYMYWY-QQKPRSSPKPWIYLTSLNLSAGVPAFCGSGSG-TSYSLTISSEAEADAATYCCQWSSNPLTFGA-GTKLELKRADAAPTVISIFPPSSEQLTSGGASV-VCFLLNFPYKIDINVKWKIDGSEKQNGVLNSWT-DQSKDSTYSMSSTLTTLTKDEYERHNSYTCEA-THKTSTSPIVKSFNRNECLE
B7-H6	
Source organism	<i>Homo sapiens</i>
Expression vector	pAcGP67-B
Expression host	Sf9 insect cells
Complete amino-acid sequence of the construct produced	ADLKVEMMAGGTQITPLNDNVTIFCNIFYSQPLN-ITSMGITWFWKSLTFDKEVKVFEFFGDHGEAF-RPGAIVSPWRLKSGDASLRPLGQLEEAAGEYR-CEVVVTPKAAQGTQVQLVVASPASRLLLDQVG-MKENEDKYMCESSGFYPEAINITWEKQTQKFP-HPIEISEDVITGPTIKNMDGTFNVTSCCLKLNS-QSEDPGTVYQCVRHASLHTPLRSNFTLTAAR-HSLSETEKTDNFSAHHHHHH

In addition to an absent inhibitory signal, activating signals are also required for NK cell triggering and tumor cell lysis (Lanier, 2008; Vivier *et al.*, 2011; Long *et al.*, 2013). These signals are delivered by diverse activating receptors, including NKG2D, DNAM-1, 2B4 and the natural cytotoxicity receptors (NCRs). Ligands for activating receptors comprise both MHC-like and non-MHC molecules. The NCR family includes Nkp30, Nkp44 and Nkp46. These very potent activating receptors are type I transmembrane glycoproteins comprising one (Nkp30 and Nkp44) or two (Nkp46) immunoglobulin-like extracellular domains (Li & Mariuzza, 2014).

Recently, Nkp30 has been shown to recognize the tumor-cell surface protein B7-H6 (Brandt *et al.*, 2009). We previously determined the structure of Nkp30 bound to B7-H6 (Li *et al.*, 2011). B7-H6 is a member of the B7 family, which includes ligands (B7-1 and B7-2) for the T-cell co-stimulatory receptor CD28 and the co-inhibitory receptor CTLA-4, as well as ligands (PD-L1 and PD-L2) for the T-cell co-inhibitory receptor PD-1 (Zou & Chen, 2008). Similar to other B7 family members, the extracellular portion of B7-H6 consists of a V-like and a C-like domain, with the V-like domain distal from the membrane (Li *et al.*, 2011). Importantly, B7-H6 was not detected in normal human tissues, but was selectively expressed on a variety of human tumor cell lines, including T and B lymphomas, melanomas and carcinomas, as well as on primary blood tumor cells (Brandt *et al.*, 2009; Kaifu *et al.*, 2011). The interaction of B7-H6 on tumor cells with Nkp30 on NK cells led to interferon- γ production and tumor cell killing. Thus, B7-H6 functions as a tumor-induced self-molecule that alerts innate immunity to cellular transformation (Brandt *et al.*,

2009). Furthermore, B7-H6 was expressed on CD14⁺CD16⁺ proinflammatory monocytes in patients with sepsis conditions, and was correlated with increased mortality (Matta *et al.*, 2013). A soluble form of B7-H6 was also produced by activated monocytes, which may serve as a decoy molecule to block Nkp30-dependent cytotoxicity. Collectively, these findings indicate that B7-H6 is not only involved in tumor immunosurveillance but also participates in the inflammatory response to infections.

To investigate Nkp30-mediated NK cell activation, as well as to target tumors expressing B7-H6, Matta *et al.* (2013) isolated a set of four mouse monoclonal antibodies directed against human B7-H6. Although all four antibodies blocked the activation of NK cells expressing Nkp30 by tumor cells or monocytes expressing B7-H6, they appeared to do so through different mechanisms. Two of them (Az20 and 1849) directly blocked the binding of B7-H6 to Nkp30 (Matta *et al.*, 2013). This indicates that these antibodies bind to sites on B7-H6 that at least partially overlap the binding site for Nkp30, thus explaining their inhibitory activity. By contrast, two other antibodies (17B1.3 and 4E5.5) did not block the interaction of B7-H6 with Nkp30, implying that they employ a different mechanism to prevent Nkp30-dependent cell activation. Here, we report the expression of the antigen-binding fragment (Fab) of antibody 17B1.3 and B7-H6, and crystallization of the Fab 17B1.3–B7-H6 complex. Structural analysis of this complex should reveal the relation between the binding sites on B7-H6 for Nkp30 and 17B1.3, and thereby elucidate the inhibitory mechanism of this antibody.

2. Materials and methods

2.1. Macromolecule production

Antibody molecules are composed of light (L) and heavy (H) polypeptide chains, each having variable (V) and constant (C) regions. To produce recombinant Fab 17B1.3, DNA fragments encoding the V_L and C_L domains of the L chain (residues 1–218) and the V_H and C_{H1} domains of the H chain (residues 1–212) were cloned into the expression vector pET-26b (Novagen) (Table 1). The V_LC_L and V_HC_{H1} DNA sequences were optimized for expression in *Escherichia coli* and synthesized chemically (GenScript). To obtain inclusion bodies for *in vitro* folding of Fab 17B1.3, *E. coli* BL21(DE3) cells were separately transformed with the pET-26b-L and pET-26-H chain plasmids. Bacteria expressing the V_LC_L and V_HC_{H1} chains were grown separately at 37°C in LB medium to an absorbance of 0.6–0.8 at 600 nm and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside. After incubation for 3 h, the bacteria were harvested separately by centrifugation and resuspended in 50 mM Tris–HCl pH 8.0 containing 5% Triton X-100. The bacteria were disrupted by sonication. Following centrifugation, the supernatants were discarded and the pellets were washed three times with 50 mM Tris–HCl pH 8.0, 5% Triton X-100 and twice with 50 mM Tris–HCl pH 8.0. Inclusion bodies were dissolved in 8 M urea, 50 mM Tris–HCl pH 8.0, 10 mM DTT. For *in vitro* folding, the V_LC_L and V_HC_{H1}

Table 2
Crystallization.

Method	Sitting/hanging-drop vapor diffusion with macro-seeding
Plate type	EasyXtal (15-well)
Temperature (K)	300
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	10 mM MES pH 6.0, 10 mM NaCl
Composition of reservoir solution	
Condition F2	20% PEG 1000, 100 mM potassium phosphate monobasic/sodium phosphate dibasic pH 6.2, 200 mM NaCl
Condition D10	10% PEG 8000, 100 mM imidazole-HCl pH 8.0, 200 mM calcium acetate
Optimized D10	9% PEG 8000, 100 mM Tris-HCl pH 8.0, 200 mM lithium sulfate
Volume and ratio of drop	2 µl, 1:1 ratio of protein:reservoir solution
Volume of reservoir (µl)	500

inclusion bodies were mixed in a 1:1 molar ratio and diluted by dropwise addition to ice-cold folding buffer consisting of 0.8 M L-arginine-HCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 3.7 mM cystamine-HCl, 6.6 mM 2-mercaptoethylamine-HCl to a final protein concentration of 40 mg l⁻¹. After 72 h at 4°C, correctly folded Fab 17B1.3 was separated from aggregates using a Superdex 75 10/300 GL column (GE Healthcare). Further purification was performed by Mono S cation-exchange chromatography followed by a second gel-filtration step using a Superdex 200 10/300 GL column.

The extracellular portion of B7-H6 (residues 1–240) was fused to the gp67 secretion signal sequence of baculovirus expression vector pAcGP67-B (BD Biosciences) with a C-terminal His₆ tag and expressed in Sf9 insect cells (Invitrogen) (Table 1). To generate recombinant baculovirus, this construct was transfected into Sf9 cells together with Baculo-

Gold Linearized DNA (BD Biosciences). The cells were incubated at 27°C and the supernatant was collected after 4 d. This supernatant was used for the production of soluble B7-H6. In a typical preparation, 1000 ml Sf9 cells at 1.2×10^6 cells ml⁻¹ were inoculated with 10 ml recombinant baculovirus at 1.0×10^8 pfu per cell. Supernatants were harvested 3 d post-infection. After concentration and dialysis against PBS, the supernatants were loaded onto a HisTrap Ni²⁺-NTA column (GE Healthcare) for affinity purification. Recombinant B7-H6 was eluted with an imidazole gradient and further purified using sequential Superdex 200 10/300 GL and Mono Q columns. Final yields were typically ~3 mg per litre of Sf9 cells.

The affinity of Fab 17B1.3 for B7-H6 was measured by surface plasmon resonance (SPR) under equilibrium binding conditions using a BIAcore T100 biosensor (GE Healthcare). 1800 resonance units (RU) of B7-H6 were immobilized on a CM5 sensor chip by random amine coupling. Solutions containing different concentrations of Fab 17B1.3 were injected sequentially over flow cells immobilized with B7-H6 or buffer as a blank. Injections of Fab 17B1.3 were stopped when the SPR signals reached a plateau. The dissociation constant (K_d) was determined by fitting the equilibrium data with a 1:1 binding model using the *BIAevaluation* 4.1 software (GE Healthcare).

Figure 1
Gel-filtration profile of the Fab 17B1.3–B7-H6 complex. The Superdex 200 10/300 GL column was equilibrated with PBS. The positions at which unbound Fab 17B1.3 and B7-H6 eluted are marked by arrows. Molecular-weight standards (kDa) eluted at the indicated volumes (V_e). The inset shows SDS-PAGE analysis (Coomassie Blue staining) of dissolved crystals of the Fab 17B1.3–B7-H6 complex. Bands corresponding to the Fab and B7-H6 are indicated. Molecular-weight markers are labeled in kDa.

Figure 2
Crystals of the Fab 17B1.3–B7-H6 complex. The upper panel shows crystals grown in Wizard I and II condition F2 (Table 2). The lower panel shows crystals grown in optimized condition D10 that were used for data collection. The largest crystal in the field is a side view of a thick plate.

2.2. Crystallization

For crystallization of the Fab 17B1.3–B7-H6 complex, Fab 17B1.3 (10 mg ml⁻¹) was mixed with B7-H6 (10 mg ml⁻¹) in a 1:1 molar ratio. The complex was purified using a Superdex 200 10/300 GL column pre-equilibrated in PBS (Fig. 1) and was then dialyzed against 10 mM MES pH 6.0 containing 10 mM NaCl and concentrated to 10 mg ml⁻¹. Initial crystallization conditions were screened at room temperature by the sitting-drop vapor-diffusion method using a Mosquito robot (TTP Labtech) and the crystallization screen kits Wizard I and II and Wizard III and IV (Emerald Bio). Rod-like crystals (Fig. 2, upper panel) appeared in droplets with reservoir solution consisting of 20% (w/v) polyethylene glycol (PEG) 1000, 100 mM potassium phosphate monobasic/sodium phosphate dibasic pH 6.2, 200 mM NaCl (condition F2 from Wizard I and II; Table 2). These crystals diffracted to no better than 5 Å resolution using an in-house X-ray source and were not pursued further. Thin plate-shaped crystals grew using 10% (w/v) PEG 8000, 100 mM imidazole–HCl pH 8.0, 200 mM calcium acetate (condition D10 from Wizard I and II; Table 2). Condition D10 was optimized to 9% (w/v) PEG 8000, 100 mM Tris–HCl pH 8.0, 200 mM lithium sulfate to obtain thicker plates (Fig. 2, lower panel). Diffraction-quality crystals were

Table 3

Data collection and processing.

Values in parentheses are for the highest resolution shell.

Diffraction source	Beamline X29A, NSLS
Wavelength (Å)	1.075
Temperature (K)	100
Detector	ADSC CCD
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	89.6, 138.0, 171.4
α , β , γ (°)	90, 90, 90
Resolution range (Å)	50–2.5 (2.59–2.50)
Total No. of reflections	1071629
No. of unique reflections	74555
Completeness (%)	99.9 (99.8)
Multiplicity	14.3 (13.3)
$\langle I/\sigma(I) \rangle$	22.1 (3.4)
$R_{\text{merge}}^{\dagger}$	14.3 (78.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl) \rangle$ is the average intensity of that reflection.

grown by macro-seeding using the sitting-drop vapor-diffusion method.

2.3. Data collection and processing

For data collection, crystals of the Fab 17B1.3–B7-H6 complex from optimized condition D10 were transferred to a cryoprotectant solution consisting of mother liquor containing 30% (v/v) glycerol before flash-cooling in liquid nitrogen. X-ray diffraction data to 2.5 Å resolution were recorded on beamline X29 of the Brookhaven National Synchrotron Light Source (NSLS) using an ADSC CCD detector. The data set consisted of 360 images collected with a 1° oscillation range at a wavelength of 1.075 Å and a crystal-to-detector distance of 300 mm. These data were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997). A summary of the diffraction data statistics is shown in Table 3.

3. Results and discussion

We expressed recombinant Fab 17B1.3 by *in vitro* folding from inclusion bodies produced in *E. coli*. This method has significant advantages over the conventional method for Fab preparation involving papain digestion of whole IgG molecules from hybridomas or transfected mammalian cells (for example, CHO or HEK293). Not only are *E. coli*-produced Fabs not glycosylated, but genetic truncation of the H chain at the C-terminus of the C_H1 domain avoids the problem of ‘ragged ends’ resulting from imprecise papain cleavage of IgG at the flexible hinge connecting C_H1 and C_H2. The greater homogeneity of *E. coli*-produced Fabs facilitates their crystallization. However, a disadvantage of the *in vitro* folding method is low efficiency. In a typical preparation, 80 mg of inclusion bodies (40 mg of each chain) were required to obtain 1 mg of soluble, correctly folded Fab 17B1.3. To increase our yield of inclusion bodies (and therefore Fab), we used synthetic V_LC_L and V_HC_H1 DNA sequences that were codon-optimized for expression in *E. coli*. These optimized genes each produced ~180 mg of inclusion bodies per litre of bacterial culture.

Figure 3

SPR analysis of the binding of Fab 17B1.3 to B7-H6. In the upper panel, Fab 17B1.3 at concentrations of 0.08, 0.16, 0.31, 0.63, 1.25, 2.5 and 5.0 μM was injected over immobilized B7-H6 (1800 RU). The lower panel shows the fitting curve for equilibrium binding that resulted in a K_d of 0.2 μM.

We also attempted to express B7-H6 by *in vitro* folding from inclusion bodies. However, no correctly folded material could be obtained, possibly because folding of this protein requires glycosylation (B7-H6 contains six potential N-linked glycosylation sites). Accordingly, we produced recombinant B7-H6 by secretion from baculovirus-infected insect cells and purified ~3 mg of B7-H6 per litre of culture.

To confirm that recombinant Fab 17B1.3 and B7-H6 were both functional before undertaking co-crystallization trials, we measured their affinity by SPR. Under equilibrium binding conditions, a K_d of 0.2 μ M was obtained (Fig. 3), which is typical for antigen–antibody interactions (Sundberg & Mariuzza, 2002).

Initial hits from the crystallization screen were found in two conditions from Wizard I and II (F2 and D10; Table 2). Optimization of one of these conditions (D10), combined with macro-seeding, produced diffraction-quality crystals of the Fab 17B1.3–B7-H6 complex belonging to space group $P2_12_12_1$ (Table 3). The presence of both Fab 17B1.3 and B7-H6 was confirmed by SDS–PAGE analysis of dissolved crystals (Fig. 1, inset). The calculated molecular weights of B7-H6 and Fab 17B1.3 are 27.78 and 48.58 kDa, respectively. The ‘fuzziness’ of the B7-H6 band compared with that of the Fab is consistent with glycosylation. Diffraction data were collected to 2.5 Å resolution at 100 K. According to the Matthews coefficient (Matthews, 1968), the asymmetric unit contains three complex molecules with 46.9% solvent content. Structure determination is under way. The Fab 17B1.3–B7-H6 complex should elucidate the mechanism by which antibody 17B1.3, which does not interfere with the binding of B7-H6 to NKp30, nevertheless blocks NK cell activation (Matta *et al.*, 2013).

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