

# NKG2D: Binding Properties for Glycan Ligands, and Mutagenesis Analysis

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**Abstract:** Killer lectin-like receptor NKG2D, which is found on natural killer cells, recognizes MHC class I-related ligands and also interacts with glycan ligands, heparin-conjugated bovine serum albumin (heparin-BSA) and sialyl Lewis X (sLeX) on multi-antennary N-glycans on transferrin secreted by HepG2 cells (HepTF). Using the glutathione-S-transferase-fused extracellular domain (AA 73-216) of NKG2D (rGST-NKG2D) and seven site-directed mutants, we explored in detail the binding of NKG2D to sulfate-containing glycan-BSA and HepTF. rGST-NKG2D binds to sulfate-containing glycan-BSA with  $K_d$  values of 25 nM  $\pm$  15 for  $\lambda$ -carrageenan-BSA, 66  $\pm$  23 nM for fucoidan-BSA, and 1.5  $\pm$  0.5  $\mu$ M for heparan sulfate-BSA. Of the site-directed rGST-NKG2D mutants, Y152A, Q185A, K197A, Y199A, E201A, and N207A reduced binding to these glycans. These results indicate that NKG2D interacts with highly sulfated- and  $\alpha$ 2,3-NeuAc-containing glycans and suggest that the glycan-binding sites on NKG2D are shared between sulfate- and  $\alpha$ 2,3-NeuAc-containing glycans, and might overlap with protein ligand binding sites.

**Keywords:** NKG2D, Glycan ligands, glycosaminoglycan, sialyl lewis X, mutagenesis.

## INTRODUCTION

Natural killer (NK) cells are important in innate immunity and, the cytotoxic activity of NK cells is regulated by the integration of signals produced by missing self and induced self interactions and a shift of the balance in activating and inhibiting cell surface receptors of the immunoglobulin-like and C-type lectin-like superfamilies [1].

Of the C-type lectin-like receptors, natural killer group 2D (NKG2D) is one of the most important activating receptors [2-5]. NKG2D is distantly related to other members of the NKG2 family, NKG2A, B, C, E, and H, which form heterodimers with CD94 and bind to the non-classical major histocompatibility complex (MHC) class I molecules HLA-E in human and Qa-1 in mouse. Activating receptor NKG2D is expressed on all NK cells and most  $\gamma\delta$ T-cell receptor ( $\gamma\delta$ TCR)-positive T cells [6] and is a homodimeric type 2 transmembrane glycoprotein that forms a salt-bridged hexamer with two homodimers of the immunoreceptor tyrosine-based activation motif (ITAM) YINM-containing adaptor molecule DNAX-activating protein of 10 kDa (DAP10) in human [7].

Although the ligands for NKG2D are identified MHC class I related proteins: MHC class I related chain family protein (MIC) A and B [6] and UL 16-binding protein (ULBP) 1-5 [8], its glycan ligands are yet unidentified in human.

We found that fucosyltransferase (*FUT*) 3-transfected K562 cells (K562/*FUT*) that highly express the sialyl Lewis X (sLeX) antigen, NeuAc $\alpha$ 2,3Gal $\beta$ 1,4(Fuc $\alpha$ 1,3)GlcNAc-R, were more susceptible to lysis by NK-derived KHYG cells *in vitro*, and that this susceptibility was suppressed by pre-treatment with anti-sLeX and KHYG cells with anti-CD94 and anti-NKG2D antibodies [9]. Using recombinant glutathione S-transferase (GST)-fused extracellular domains of NKG2D (rGST-NKG2D) and CD94 (rGST-CD94) and their site-directed mutants, we revealed that both rGST-NKG2D and rGST-CD94 can bind to multimeric sLeX-expressing transferrin secreted by HepG2 cells (HepTF) [10] or  $\alpha$ 2,3-NeuAc containing  $\alpha$ 1-acid glycoprotein [11] and heparin [12]. Moreover, NKG2D and CD94 interact with heparin and HepTF [13].

Here, we report that we have further characterized the binding of rGST-NKG2D and its mutants to sulfate-containing glycans and HepTF. We found that rGST-NKG2D binds to glycans with  $K_d$  values. Moreover, mutagenesis suggested that the glycan-binding sites on NKG2D may overlap with protein ligand binding sites.

## MATERIALS AND METHODOLOGY

### Cells and Cell Culture

Human NK-derived KHYG cells from the Japanese Collection of Research Bioresources Cell Bank (JCRB) (Tokyo, Japan) were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) and 0.6 mg/ml L-glutamate (Wako Chemicals Co., Osaka, Japan) in a humidified atmosphere containing

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5% CO<sub>2</sub> at 37°C. Recombinant interleukin (IL)-2 (Shionogi Pharmaceutical Co., Osaka, Japan) was added to the medium to give a final concentration of 100 U/ml. Human hepatoma HepG2 cells (JCRB) were cultured in 10% FBS containing Dulbecco's modified Eagle's medium (DMEM) (Nissui).

### Preparation of rGST-NKG2D and its Site-Directed Mutant Constructs

rGST-NKG2D and its site-directed mutant constructs were prepared as described previously [11, 12]. Briefly, the extracellular domain of NKG2D (coding for AA 73-216, pGEX4T-1/NKG2D) was amplified from KHYG-derived cDNA using primers 5'-CACCATATGGAGTGCTGTATT CCTAAAC-3' (forward) and 5'-TTACACAGTCCTTTGCA TGCA-3' (reverse). Seven site-directed mutant constructs, Y152A, Q185A, K197A, Y199A, E201A, N207A, and C203G, were prepared using the KOD-plus-Mutagenesis kit (Toyobo, Tokyo, Japan), which is based on inverse-PCR. The pGEX4T-1/NKG2D plasmid was used as a PCR template. The forward and reverse primers designed for mutant vectors were shown in Table 1. The recombinant plasmids were transformed into DH5 $\alpha$ , (Takara, Otsu, Japan) and purified, and were confirmed by DNA sequencing.

The recombinant plasmids were re-transformed into Chaperone competent cells pTf16/BL21 (Takara, Otsu, Japan) to express the recombinant protein.

After induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Promega Co., Madison, WI), the cells were harvested and sonicated on ice for 10 s  $\times$  6. The recombinant proteins were purified on a GStrap<sup>TM</sup> FF column (1 ml packed volume; GE Healthcare) according to the manufacturer's instructions and dialyzed against 1 mM dithiothreitol (DTT) in phosphate-buffered saline (PBS). The proteins

were subjected to 10% SDS-PAGE with and without pre-treatment in 1% 2-mercaptoethanol (2-ME) at 95°C for 3 min, and stained with Coomassie brilliant blue. Protein concentrations were determined using advanced protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.

### Preparation of Glycan-Coated Plates

Glycans were conjugated with BSA in the presence of sodium cyanoborohydride and coated on 96-well plates, as reported previously [12]. As a control, plates were treated with 0.1% BSA/PBS. Heparin (porcine intestinal mucosa; Wako), heparan sulfate (porcine intestinal mucosa; Funakoshi, Tokyo, Japan), fucoidan (marine algae; Sigma-Aldrich, St. Louis, MO), and  $\lambda$ -carrageenan (*Kappaphycus cottonii*; Wako) were conjugated with BSA and coated on the plates. HepTF was purified from the supernatant of HepG2 cell culture [10] and directly coated on the plates [11].

### Binding of rGST-NKG2D to Glycan-Coated Plates

To each plate was added rGST-NKG2D (100  $\mu$ l) in TBS-T [TBS-T: Tris-HCl buffer (20 mM, pH 7.4) containing CaCl<sub>2</sub> (10 mM), BSA (0.1%), NaCl (150 mM) and Tween (0.3%)]. Plates were incubated for 2 h at 37°C. The concentrations of rGST-NKG2D used in the binding assay were 0 ~ 0.5 to 1.0  $\mu$ M for fucoidan-BSA,  $\lambda$ -carrageenan-BSA, heparin-BSA, heparan sulfate-BSA, and HepTF. After washing three times with TBS-T, the plates were further incubated for 1 h at room temperature with 100  $\mu$ l 2  $\mu$ g/ml peroxidase (POD)-conjugated anti-GST antibody (Rockland Immunochemicals Inc., Gilbertsville, PA) in TBS-T. Then, after washing three times with TBS-T, the plates were incubated with 100  $\mu$ l tetramethylbenzidine solution (BioFX Lab., Owings Mills, MD) for 5 min at room temperature. The reac-

**Table 1. Specific Primer Sets for Mutant Vectors**

Mutated Amino Acid		Primer Sequences	Length
Y152A	F	5'- TCAG <u>CTC</u> CATTGGATGGGACTAGTA -3'	24
	R	5'- CTCACCAGTTTAAGTAAATCCT-3'	23
Q185A	F	5'- <u>GCG</u> AAGGGAGACTGTGCACTCTATG-3'	25
	R	5'- CATTTC AATTATGT TAGTAGTTGGGTG-3'	29
K197A	F	5'- TTGC <u>AGG</u> CTATATAGAAAAGTGTCAACT-3'	29
	R	5'- AGCTCGAGGCATAGAGTGCACAGTC-3'	25
Y199A	F	5'- AGGC <u>GCT</u> ATAGAAAAGTGTCAACT-3'	25
	R	5'- TTAAGCTCGAGGCATAGAGTG-3'	22
E201A	F	5'- <u>GCA</u> AACTGTTCAACTCCAAATACG-3'	24
	R	5'- TATATAGCCTTTAAAGCTCGAGG-3'	23
N207A	F	5'- <u>GCT</u> ACATACATCTGCATGCATGCAAAGG-3'	28
	R	5'- TGGAGTTGAACAGTTTTCTATATAGCC-3'	27
C203G	F	5'- AAC <u>GGT</u> TCAACTCCAAATACG-3'	21
	R	5'- TTCTATATAGCCTTTAAAGCTCG-3'	23

Underlined nucleotides were mutated nucleotides.

tion was stopped by addition of  $\text{H}_2\text{SO}_4$  (100  $\mu\text{l}$ , 1 M) and absorbance was read at 450 nm with a Model DTX800 plate reader (Beckman Coulter, Fullerton, CA).

$K_d$  values were determined using linear reciprocal plots,  $[M]/\Delta\text{Abs}$  versus  $[M]$ , and calculated from slopes ( $1/B_{\text{max}}$ ) and y intercepts ( $K_d/B_{\text{max}}$ ), where  $[M]$  is the concentration of rGST-NKG2D (MW=43,784 Da) and  $B_{\text{max}}$  is the maximum binding of rGST-NKG2D.

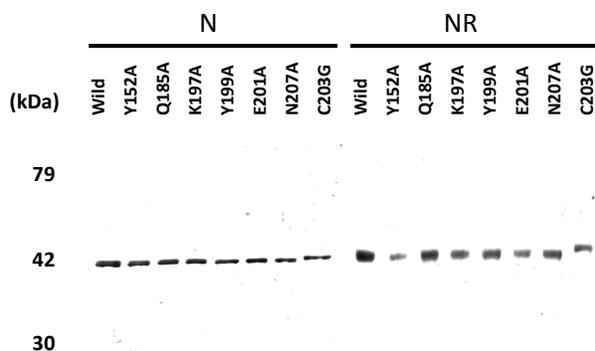
### Binding of Site-Directed Mutant rGST-NKG2D to Glycans

The concentrations of mutant used to determine their binding to glycans were based on the  $K_d$  values of rGST-NKG2D<sub>wild</sub> for each glycan: 20 nM for fucoidan-BSA and  $\lambda$ -carrageenan-BSA, and 230 nM for HepTF, heparin-BSA and heparan sulfate-BSA, respectively. The binding was measured as described above and compared with that of rGST-NKG2D<sub>wild</sub>.

## RESULTS

### Binding of rGST-NKG2D to Sulfate-Containing Glycans

We prepared rGST-NKG2D and its mutants using Chaperon competent cells pTf16/BL21, purifying on a glutathione column. To evaluate the molecular forms of rGST-NKG2D and its mutants, these recombinant proteins were treated with or without 1% 2-ME and subjected to 10% SDS-PAGE. They separated at around 42 kDa under both reducing and non-reducing conditions, indicating that the forms mainly obtained were monomeric (Fig. 1).



**Fig. (1).** SDS-PAGE for rGST-NKG2D and its mutants.

Recombinant proteins purified on a glutathione column were separated on 10% SDS-PAGE with (R) and without (NR) pretreatment with 1% 2-ME at 95°C for 3 min.

We have demonstrated that rGST-NKG2D binds to  $\alpha$ 2,3-NeuAc on multi-antennary N-glycan [11] and heparin-BSA [12]. Binding of rGST-NKG2D to heparin-BSA was suppressed by soluble forms of heparin, heparan sulfate, fucoidan, and  $\lambda$ -carrageenan [12]. To further investigate the binding of NKG2D to these sulfate-containing glycans, we determined the binding of rGST-NKG2D to plates coated with heparan sulfate-BSA, fucoidan-BSA, and  $\lambda$ -carrageenan-BSA (Fig. 2A-C). rGST-NKG2D bound to these glycans in a dose-dependent fashion.

Binding affinities ( $K_d$  values) were calculated using linear reciprocal plots,  $[M]/\Delta\text{Abs}$  versus  $[M]$  (Fig. 2D-F and Table 2). The averaged  $K_d$  values of rGST-NKG2D deter-

mined for  $\lambda$ -carrageenan-BSA, fucoidan-BSA, and heparan sulfate-BSA were  $25 \pm 15$  nM,  $66 \pm 23$  nM and  $1.50 \pm 0.5$   $\mu\text{M}$ , respectively. We re-estimated the  $K_d$  value of rGST-NKG2D for heparin-BSA to be  $0.51 \pm 0.2$   $\mu\text{M}$ . But we could not determine the  $K_d$  value of rGST-NKG2D for Dermatan sulfate-BSA using linear reciprocal plots.

### Binding of Site-Directed Mutant rGST-NKG2D to Sulfate- and NeuAc-Containing Glycans

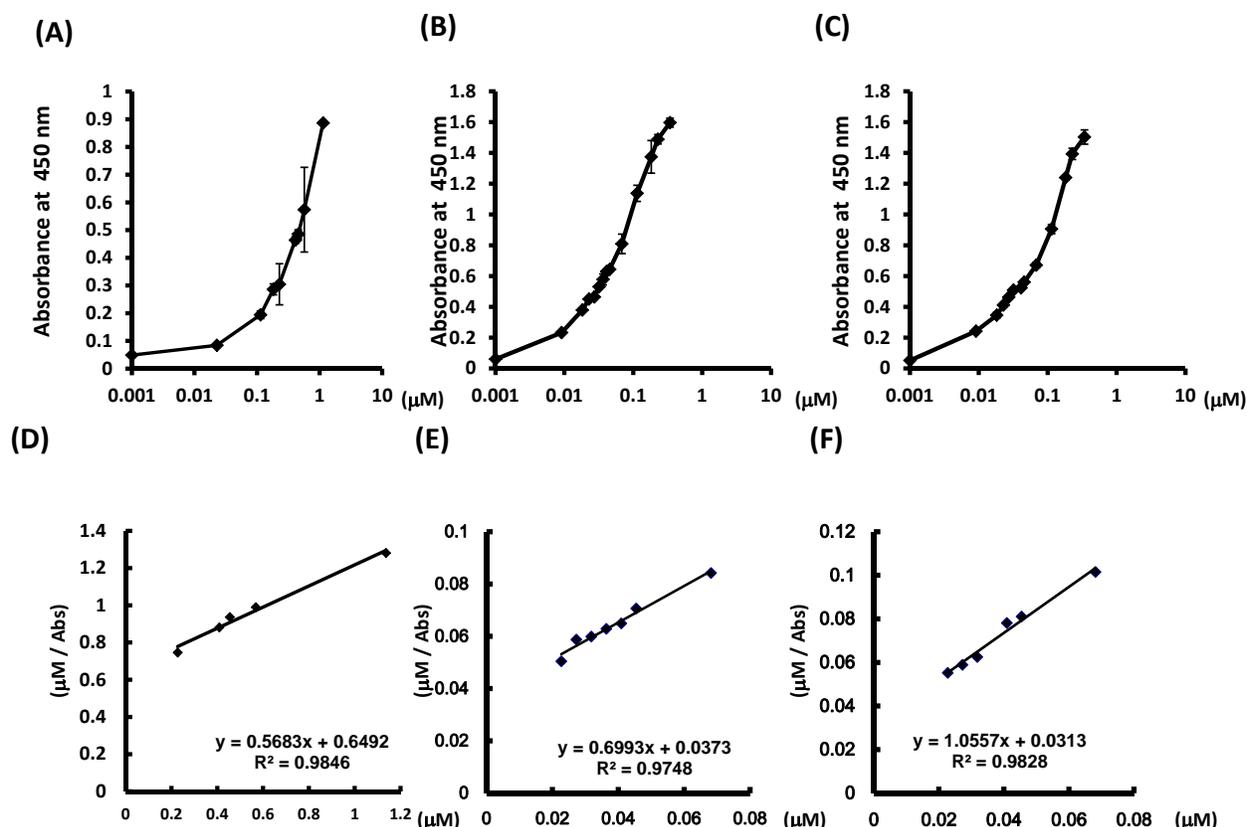
The hydrophobic residues Y152, I182, M184, and Y199 in NKG2D are essential for its recognition of MICA and ULBP3; K197 and E201 in NKG2D are salt-bridged; K150, E185, and N207 in NKG2D are hydrogen-bonded with MICA and ULBP3 [14, 15]; and C203 in NKG2D is essential for disulfide link formation between  $\beta$ -sheets [16]. Therefore, we prepared seven site-directed mutant forms of rGST-NKG2D, Y152A, Q185A, K197A, Y199A, E201A, N207A, and C203G, and compared them with wild-type rGST-NKG2D to clarify binding sites for sulfate- or  $\alpha$ 2,3-NeuAc-containing glycans (Fig. 3). The concentrations of rGST-NKG2D used were based on the  $K_d$  values for each glycan. Mutants Y152A, Q185A, K197A and Y199A significantly and E201A and N207A non-significantly reduced binding to heparin-BSA (Fig. 3A), while mutants Y152A, K197A, Y199A, E201A, N207A, and Q185A significantly reduced binding to heparan sulfate-BSA (Fig. 3B), fucoidan-BSA (Fig. 3C),  $\lambda$ -carrageenan-BSA (Fig. 3D), and HepTF (Fig. 3E). Mutation C203G did not affect the binding to these glycans.

These results indicate that interactions between NKG2D and glycans are mainly hydrophobic and/or hydrogen-bonding interactions, while formation of ionic salt bridges is less important. The binding sites on NKG2D for sulfate- and  $\alpha$ 2,3-NeuAc-containing glycans seem to overlap with protein ligand binding sites.

## DISCUSSION

In this studies, we have demonstrated that rGST-NKG2D can interact with sulfate- and  $\alpha$ 2,3-NeuAc-containing glycans with high binding affinities:  $K_d$  values were  $25 \pm 15$  nM,  $66 \pm 23$  nM,  $0.35 \pm 0.2$   $\mu\text{M}$ ,  $0.51 \pm 0.2$   $\mu\text{M}$  and  $1.5 \pm 0.5$   $\mu\text{M}$  for  $\lambda$ -carrageenan-BSA, fucoidan-BSA, HepTF, heparin-BSA and heparan sulfate-BSA, respectively. NKG2D interacts with distinct and highly polymorphic ligands [17] with high affinities:  $K_d$  values for the human NKG2D homodimer ranged from 0.3 to 0.94  $\mu\text{M}$  for MICA, 0.79  $\mu\text{M}$  for MICB, and 1.1 to 4.0  $\mu\text{M}$  for ULBP1-4; corresponding values for the mouse homodimer ranged from 0.35 to 1.9  $\mu\text{M}$  for RAE-1 $\alpha$ - $\epsilon$ , 14 to 27 nM for H60, and 1.5 to 5.6 nM for MULT1, respectively [15, 18-21]. Although the binding affinities of human and mouse NKG2D for ligands vary widely, the affinities are higher than those of other NK cell receptors and T cell receptors (10 to 100  $\mu\text{M}$ ). The  $K_d$  values of human NKG2D for glycan ligands determined in this and previous studies [13] are comparable with those for protein ligands, suggesting that glycan ligands might function physiologically.

Crystal structures of representative NK receptors have been determined in isolated and ligand-bound forms [22]. The crystal structures of NKG2D alone [23] and in complex with MICA [14], MICB [17], ULBP3 [15], and RAE-1 $\beta$  [18]



**Fig. (2). Binding of rGST-NKG2D to sulfate-containing glycans and Typical linear reciprocal plots analysis.**

(A-C) rGST-NKG2D were added to (A) heparan sulfate-BSA, (B) fucoidan-BSA, and (C) λ-carrageenan-BSA coated plates for 2 h at 37°C and binding was determined using POD-conjugated anti-GST antibody. The results are given as mean ± SD (n=3). (D-F) Typical linear reciprocal plots,  $[M]/\Delta Abs$  vs.  $[M]$ , for binding of rGST-NKG2D to (D) heparan sulfate-BSA, (E) fucoidan-BSA, and (F) λ-carrageenan-BSA.  $K_d$  values were calculated from slopes ( $1/B_{max}$ ) and y intercepts ( $K_d/B_{max}$ ), where  $B_{max}$  is the maximum binding of rGST-NKG2D.

**Table 2.  $K_d$  Values of rGST-NKG2D to Glycans**

	$K_d \pm SD$ ( $\mu M$ )
HepTf	$0.35 \pm 0.2$
Heparin-BSA	$0.51 \pm 0.2$
Heparan sulfate-BSA	$1.50 \pm 0.5$
Dermatan sulfate-BSA	n.d.
Fucoidan-BSA	$0.066 \pm 0.023$
λ-Carrageenan-BSA	$0.025 \pm 0.015$

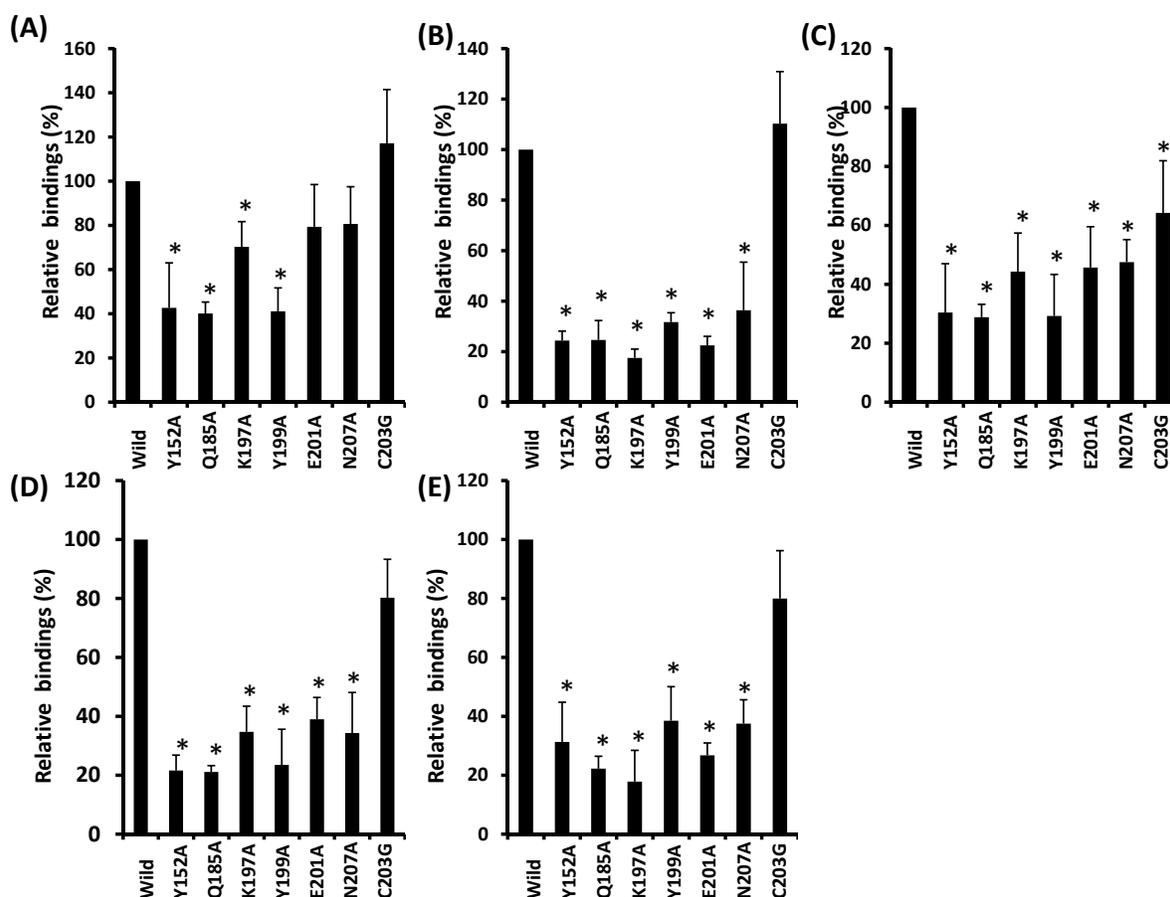
Using linear reciprocal plots,  $[\mu M] / \Delta F$  or Abs versus  $[\mu M]$ , as described in Figs. (2),  $K_d$  values were calculated based on slopes ( $1/B_{max}$ ) and y intercepts ( $K_d/B_{max}$ ), where  $B_{max}$  is the maximum binding of rGST-NKG2D. The results are given as mean ± SD (n=3). n.d.: not determined.

have revealed that the NKG2D homodimer forms a concave surface to interact with the convex monomeric ligand *via* a network of hydrophobic, hydrogen-bonding, and salt-bridging interactions and that each NKG2D monomer predominantly interacts with either the  $\alpha 1$  or the  $\alpha 2$  platform domain of these divergent ligands. This indicates that two symmetric binding sites on the NKG2D homodimer bind to different epitopes on the asymmetric ligand monomer [24,

25]. Each NKG2D subunit forms a hydrophobic patch with MICA [14] and ULBP3 [15], using the same receptor residues (Y152, I182, M184, and Y199) but different ligand residues. Y152, I181, E183, Q185, K186, S195, Y199, T205, and N207 in NKG2D are hydrogen bonded and K197 and E201 or K150 in NKG2D are salt bridged with MICA and/or ULBP3, respectively. At the center of the NKG2D binding sites lie two conserved residues, Y152 and Y199, that constitute the dominant binding-energy hotspots [25]. Two mechanisms have been considered to explain NKG2D's multi-specific ligand recognition: an induced-fit model [15, 26] or a rigid adaptation mechanism [19, 27].

We have revealed the glycan-ligand binding sites on NKG2D using single amino acid-substituted mutants: Y152, Q185, K197, Y199, E201, and N207 are essential for interactions with sulfate- and  $\alpha 2,3$ -NeuAc-containing glycans, with some variation between glycans. Interestingly, the dominant binding-energy hotspots Y152 and Y199 interact with both sulfate- and  $\alpha 2,3$ -NeuAc-containing glycans, suggesting that glycan-binding sites may overlap with protein ligand binding sites to physiologically modulate NKG2D-mediated NK cell cytotoxicity.

We have reported that increased cytotoxicity of IL2-activated KHYG cells against K562/FUT cells is suppressed by pretreatment with anti-sLeX and KHYG cells with anti-



**Fig. (3). Binding of rGST-NKG2D mutant forms to glycans.**

The binding of rGST-NKG2D wild and its mutants to glycan-coated plates: (A) heparin-BSA, (B) heparan sulfate-BSA, (C) fucoidan-BSA, (D) λ-carrageenan-BSA, and (E) HepTF. The relative binding of rGST-NKG2D mutants to each glycan is normalized to the binding of wild-type rGST-NKG2D as 100. The results are given as mean ± SD (n=3) and asterisks (\*) denote statistically significant differences (P<0.05).

CD94 and anti-NKG2D antibodies [9] and also that the other C-type lectin-like receptors CD94, NKG2A, and NKG2C interact with sulfate- and α2,3-NeuAc-containing glycans with high but different affinities [11-13, 28]. Heparin and heparan sulfate interact with the natural cytotoxicity receptors NKp46, NKp44, and NKp30 to induce NK cell cytotoxicity [29-31]. Soluble forms of heparin compete with the sLeX-dependent interactions of tumor cells with L- and P-selectin and inhibit platelet aggregation and vascular-cell adhesion with tumor cells to prevent extravasation and migration of tumor cells [32-34]. NK cell-dependent cytotoxicity is modulated through sulfation and sialylation status of target cells. Further studies are needed to clarify the role of sulfate- and sLeX-containing glycans in tumor surveillance via NK cells.

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