Natural Killer Group 2A (NKG2A) and Natural Killer Group 2C (NKG2C) Bind to Sulfated Glycans and α2,3‐NeuAc‐containing Glycoproteins

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Killer lectin‐like receptors on natural killer (NK) cells mediate cytotoxicity through glycans on target cells. We prepared recombinant glutathione S‐transferase‐fused extracellular lectin‐like domains (AA 94‐231) of natural killer group 2A (NKG2A) (rGST‐NKG2A) and NKG2C (rGST‐NKG2C) and determined the binding of these receptors to plates coated with heparin‐conjugated bovine serum albumin (heparin‐BSA) and glycoproteins. rGST‐NKG2A and rGST‐NKG2C directly bound to heparin‐BSA with Kd values of 20 and 40 nM, respectively. Binding of rGST‐NKG2A and rGST‐NKG2C to heparin‐BSA was suppressed in the presence of soluble heparin, heparan sulfate, fucoidan, λ‐carrageenan, and dextran sulfate. 2‐O‐Sulfate residues in heparin were essential for the binding of rGST‐NKG2A and rGST‐NKG2C. Moreover, rGST‐NKG2A and rGST‐NKG2C bound to multimeric sialyl Lewis X expressing transferrin secreted by HepG2 cells with Kd values of 80 and 114 nM, respectively. This is the first report showing that NKG2A and NKG2C bind to heparin and α2,3‐NeuAc‐containing glycoproteins.

Key words natural killer group 2A; natural killer group 2C; heparin; natural killer cell; sialyl Lewis X; binding affinity

Natural killer (NK) cell cytotoxicity is regulated by the action of a large number of distinct inhibitory and activating receptors that bind to the major histocompatibility complex (MHC) on the surface of target cells. Two major types of MHC class I‐binding receptors are expressed on human NK cells: immunoglobulin (Ig) superfamily molecules, including killer cell Ig‐like receptors (KIRs) and leukocyte Ig‐like receptors (LIRs), and heterodimers of the C‐type lectin‐like molecules CD94 and natural killer group 2 (NKG2), including NKG2A/B, NKG2C, and NKG2E/H.1,2 NKG2B and NKG2H are alternatively spliced variants of NKG2A and NKG2E, respectively. NKG2F has no C‐type lectin‐like domain and does not associate with CD94.3 NKG2D, which is distantly related to other NKG2 proteins, forms a homodimer and associates with the DNA‐activating proteins DAP10 or DAP12 to induce NK cell cytotoxicity.

The NKG2 receptors are closely related, as evidenced by the 81‐94% identity in their C‐type lectin‐like extracellular domains, and form disulfide‐linked heterodimers with CD94.4 The CD94/NKG2A heterodimer is an inhibitory receptor due to the two immunoreceptor tyrosine‐based inhibitory motifs (ITIM) in the cytoplasmic tail of NKG2A.5–7 In contrast, CD94/NKG2C and CD94/NKG2E heterodimers function as activating receptors due to the association of positively charged Lys residues in the transmembrane regions of NKG2C and NKG2E with Asp residues of the immunoreceptor tyrosine‐based activating motif (ITAM) of adaptor molecule DAP12.8

Human leukocyte antigen (HLA)‐E serves as the ligand for CD94/NKG2A, CD94/NKG2C, and CD94/NKG2E. HLA‐E is a non‐classical MHC class Ib molecule loaded with non‐amer leader peptides derived from the signal sequences of MHC class Ia, HLA‐A, –B, –C, or –G proteins.9–11 Both the inhibitory CD94/NKG2A and activating CD94/NKG2C recognize the same nonamer peptide/HLA‐E complex, although CD94/NKG2A has a higher binding affinity than CD94/NKG2C.12

These C‐type lectin‐like receptors lack most of the conserved Ca2+‐binding region, and their glycan ligands have yet to be elucidated.14 However, of the C‐type lectin‐like receptors on NK cells, several have been reported to recognize sulfated glycans: mouse Ly‐49A binds to fucoidan15–18 and osteoclast inhibitory lectin (OCIL, lectin‐like transcript 1: LLT1) binds to fucoidan, λ‐carrageenan, and dextran sulfate.19 Preclinical and clinical studies suggest that heparin and low molecular weight heparins are effective in preventing tumor growth and metastasis. The molecular mechanisms of the anti‐tumor effects of heparin are complicated and largely unclarified. Hypotheses include competition with the binding of growth factors to heparan sulfate proteoglycans, inhibition of hepanase, and inhibition of interaction with L‐ and P‐selectins on vascular endothelial cells and platelets.20,21

We demonstrated that K562 cells transfected with the fucosyltransferase 3 gene that highly express the sialyl Lewis X (sLeX) antigen, NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc‐R, are more susceptible to in vitro lysis by KHYG cells. This susceptibility is suppressed by pretreatment of K562 cells with anti‐sLeX and KHYG cells with anti‐CD94 and anti‐NKG2D.22 In previous reports,23,24 we showed that recombinant glutathione‐S‐transferase‐fused extracellular domains of NKG2D (rGST‐NKG2D; AA 73‐216) and CD94 (rGST‐CD94; AA 68‐179) directly bind to multimeric sLeX‐expressing transferrin secreted by HepG2 cells (HepTF) and α2,3‐ NeuAc‐remodeled human α2‐acid glycoprotein (AGP), heparin/heparan sulfate, and sulfate‐containing glycans, suggesting that glycans could modulate NK‐cell‐dependent cytotoxicity via activating or inhibitory NK cell receptors.

Here, we report that recombinant GST‐fused extracellular domains (AA 94‐231) of NKG2A (rGST‐NKG2A) and NKG2C (rGST‐NKG2C) bind to heparin and sulfate‐containing glycans, and that this interaction is suppressed in the presence of soluble heparin, heparan sulfate, fucoidan, λ‐carrageenan, and dextran sulfate.25,26 We demonstrated that K562 cells transfected with the fucosyltransferase 3 gene that highly express the sialyl Lewis X (sLeX) antigen, NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc‐R, are more susceptible to in vitro lysis by KHYG cells. This susceptibility is suppressed by pretreatment of K562 cells with anti‐sLeX and KHYG cells with anti‐CD94 and anti‐NKG2D.22 In previous reports,23,24 we showed that recombinant glutathione‐S‐transferase‐fused extracellular domains of NKG2D (rGST‐NKG2D; AA 73‐216) and CD94 (rGST‐CD94; AA 68‐179) directly bind to multimeric sLeX‐expressing transferrin secreted by HepG2 cells (HepTF) and α2,3‐ NeuAc‐remodeled human α2‐acid glycoprotein (AGP), heparin/heparan sulfate, and sulfate‐containing glycans, suggesting that glycans could modulate NK‐cell‐dependent cytotoxicity via activating or inhibitory NK cell receptors.

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ränge nan, and dextran sulfate. Moreover, rGST-NKG2A and rGST-NKG2C bind to sLeX or α2,3-NeuAc, but not to α2,6-NeuAc containing glycoproteins.

**MATERIALS AND METHODS**

**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, U.S.A.) and 0.6 mg/ml L-glutamate (Wako Pure Chemicals Co., Osaka, Japan) in a humidified atmosphere containing 5% CO₂ at 37°C. For culture of KHYG cells, recombinant interleukin-2 (Shionogi Pharmaceutical Co., Osaka, Japan) was added to the medium at 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-NKG2A and rGST-NKG2C** The genes corresponding to the extracellular domains of NKG2A and NKG2C (coding for AA 94-231 in each case) were amplified from KHYG-derived cDNA using the following primers: 5’-CACCTCTACATTAATACAGAGGCACAA-CAA-3’ (forward) and 5’-CTAAAGCTTATGGCTTACAATGAT-3’ (reverse) for NKG2A, and 5’-CACCACTCCTTCTCCTGGAGCAGAA-3’ (forward) and 5’-CTAAAGCTTATGGCTTACAAATGAT-3’ (reverse) for NKG2C. Purified polymerase chain reaction (PCR) products were ligated into the pGEX4T-1 vector (GE Healthcare) according to the manufacturer’s instructions. Plasmid DNA was purified from competent cells pT16/BL21 (Takara, Otsu, Japan) and positive clones were confirmed by DNA sequencing.

**Assay for rGST-NKG2A and rGST-NKG2C Binding to Glycan-Coated Plates** Binding of rGST-NKG2A and rGST-NKG2C to glycan-coated plates was determined according to previously reported methods.²³,²⁴ Briefly, sulfated glycan-coated plates were further treated with 1 U/ml α2,3/6/8 neuraminidase (Seikagaku Co., Tokyo, Japan) in 100 mm MES (pH 6.0) for 2 d at 37°C. Binding of rGST-NKG2A and rGST-NKG2C on the plates was determined using peroxidase (POD)-conjugated anti-GST antibody (Rockland Immunochemicals Inc., Gilbertsville, PA, U.S.A.) and tetramethylbenzidine (TMB) (BioFX Laboratories, Owings Mills, MD, U.S.A.).

The binding affinities (Kₛ values) of rGST-NKG2A and rGST-NKG2C for glycan were determined using linear reciprocal plots of [M]/[Abs] versus [M], where [M] is the concentration of rGST-NKG2A (molecular weight (MW)=43516 Da) or rGST-NKG2C (MW=43784 Da). Kₛ values were calculated from the slope (1/Bₘₐₓ) and y intercept (Kₛ/Bₘₐₓ), where Bₘₐₓ represents the maximum binding of rGST-NKG2A or rGST-NKG2C.

**Competition by Sulfate-Containing Glycans for Binding to Heparin-BSA** Competition by soluble forms of glycans was determined by evaluating the binding of rGST-NKG2A and rGST-NKG2C (each 50 nm) to heparin-BSA-coated plates in the presence of 0, 1, 10, and 100 μg/ml of heparin (porcine intestinal mucosa; Wako); heparan sulfate (porcine intestinal mucosa; Funakoshi, Tokyo, Japan); dermatan sulfate (porcine intestinal mucosa; MP Biomedicals, Solon, OH, U.S.A.); keratan sulfate (bovine cornea; Seikagaku); chondroitin sulfate A (whale cartilage; Wako), B (pig skin; Wako), and C (shark cartilage; Wako); hyaluronic acid (pig skin; Seikagaku); mannan (yeast; Nacalai Tesque); fucoidan (marine algae; Sigma-Aldrich); and carrageenan (Kappaphycus cottonii; Wako); dextran sulfate (Wako); and selectively desulfated heparin. Selectively desulfated heparin, including 2-O-, 6-O-, and N-desulfated heparin, was prepared according to the previous report.²⁴

**RESULTS**

rGST-NKG2A and rGST-NKG2C Bind to Heparin-BSA To evaluate the molecular forms of rGST-NKG2A and rGST-NKG2C, these proteins were purified on a glutathione column, treated with or without 1% 2-ME, and subjected to 10% SDS-PAGE. These recombinant proteins formed bands under non-reducing conditions at around 43 kDa, similar to reduced proteins, indicating that monomeric forms were mainly obtained (Fig. 1).

To ascertain whether NKG2A and NKG2C bind to sulfate-containing glycans, we determined the binding of rGST-NKG2A and rGST-NKG2C to heparin-BSA-coated plates using POD-conjugated anti-GST (Fig. 2). Both rGST-NKG2A and rGST-NKG2C bound to heparin-BSA in a dose-dependent fashion (Figs. 2A, B). The Kₛ values of rGST-NKG2A and rGST-NKG2C for heparin-BSA obtained from linear reciprocal plots of [M]/[Abs] versus [M] were 20 nm (R²=0.9922) and 40 nm (R²=0.9875) (Figs. 2C, D), respectively. These results indicated that NKG2A and NKG2C bind to heparin with significantly high affinity.

**Competition of rGST-NKG2A and rGST-NKG2C for Binding to Heparin-BSA** To investigate whether NKG2A and NKG2C bind to sulfate-containing glycans, the binding of rGST-NKG2A and rGST-NKG2C (each 50 nm) to heparin-BSA-coated plates was assayed in the presence of 0, 1, 10, and 100 μg/ml of various soluble glycans (Fig. 3). The
binding of rGST-NKG2A (Fig. 3A) and rGST-NKG2C (Fig. 3B) to heparin-BSA-coated plates was significantly suppressed by sulfated glycans in the order of fucoidan, β-carrageenan, and dextran sulfate heparin and heparan sulfate. Furthermore, the binding of rGST-NKG2A and rGST-NKG2C to HepTF-coated plates was significantly suppressed by heparin (data not shown). Even at the highest concentration tested (100 μg/ml), chondroitin sulfate A, B, and C, dermatan sulfate, keratan sulfate, hyaluronic acid, and mannan did not affect the binding of rGST-NKG2A and rGST-NKG2C to heparin-BSA. $K_d$ values of rGST-NKG2A and rGST-NKG2C were 212 nM ($R^2=0.9755$) and 164 nM ($R^2=0.9846$) for heparan sulfate-BSA and 1.35 nM ($R^2=0.9990$) and 0.27 nM ($R^2=0.9980$) for fucoidan-BSA, respectively.

Next, we investigated the binding of NKG2A and NKG2C to selectively desulfated heparin (Fig. 4). rGST-NKG2A and rGST-NKG2C were incubated in the presence of soluble 2-O-, 6-O-, and N-desulfated heparin on heparin-BSA-coated plates to determine whether sulfate residues in heparin are essential for their binding. Binding of rGST-NKG2A and rGST-NKG2C (each 50 nM) to heparin-BSA was suppressed by soluble 6-O- and N-desulfated heparin, but not by 2-O-desulfated heparin at the highest concentration (100 μg/ml) (Figs. 4A, B). These results indicated that 2-O-sulfate residues in heparin are essential for the binding of rGST-NKG2A and rGST-NKG2C.

**Binding of rGST-NKG2A and rGST-NKG2C to NeuAc-Containing Glycoproteins** To clarify whether NKG2A and NKG2C can bind to NeuAc-containing glycoproteins, the binding of rGST-NKG2A and rGST-NKG2C to HepTF, NorTF, AGP, and desialylated HepTF was investigated (Fig. 5). Hep-TF expresses multivalent sLeX in di- to tetra-antennary $N$-glycans, while NorTF has α2,6-NeuAc in di-antennary $N$-glycans, and human AGP has α2,6-NeuAc in di- to tetra-antennary $N$-glycans. rGST-NKG2A and rGST-NKG2C (each 200 nM) bound directly to HepTF-coated plates but less to NorTF and AGP, and desialylated HepTF restrained the binding (Figs. 5A, B). $K_d$ values of rGST-NKG2A and rGST-NKG2C for HepTF were determined to be 80 ($R^2=0.9903$) and 114 nm ($R^2=0.9514$), respectively (Figs. 5C, D). These results revealed that NKG2A and NKG2C bind to multimeric α2,3-NeuAc-containing gly-
coproteins.

DISCUSSION

In the present report, we clarified that rGST-NKG2A and rGST-NKG2C bind to heparin-BSA with similarly high affinities. The heparin binding of rGST-NKG2A and rGST-NKG2C was significantly suppressed by sulfated glycans in the order of fucoidan, l-carrageenan, and dextran sulfate, but not by chondroitin sulfate A, B, and C, dermatan sulfate, keratan sulfate, hyaluronic acid, or mannan. Moreover, a competition binding assay revealed that 2-O-sulfate residues in heparin are essential for the binding of rGST-NKG2A and rGST-NKG2C to heparin.

*Kd values of rGST-NKG2A and rGST-NKG2C for heparin-BSA were one or two orders of magnitude smaller than those of rGST-NKG2D (1.11 μM) and rGST-CD94 (1.45 μM), and compatible to those of natural cytotoxicity receptors (NCRs). The extracellular domain sequences of NKG2A and...
NKG2C are 92% identical; however, the binding affinity of CD94/NKG2A for HLA-E is six- to eight-fold higher than that of CD94/NKG2C. Alterations in the heterodimer interface between NKG2A and NKG2C may impact binding affinity. HLA-E mutation analysis indicates that CD94/NKG2A and CD94/NKG2C bind to the top of the HLA-E α1/α2 domain. The interaction of an acidic region on CD94 with a basic region on the α1 helix of HLA-E, and a basic patch on NKG2A with an acidic region on the α2 helix of HLA-E, are also important for the binding of CD94/NKG2A to HLA-E.

Several amino acid residues in NKG2A are essential for recognition of HLA-E. In a preliminary study, we prepared rGST-NKG2A mutants R137A, P171A, S172A, K199A, Q212A, R215A, and K217A, which are residues responsible for NKG2A recognition of HLA-E, and determined the binding of these mutants to heparin-BSA. Binding of rGST-NKG2A mutants was restrained by substitutions P171A, K199A, and R215A, suggesting that the heparin binding sites of NKG2A may partially overlap with the HLA-E binding sites (data not shown). Basic amino acids in NKp44 and in NKp46 are involved in their respective binding to heparin/heparan sulfate. On the other hand, heparin binding is suppressed by NKG2D mutants Y199A and Y152A, and CD94 mutants F114A and N160A. These results suggest that NKG2A and NKG2C recognize heparin mainly by ionic interactions similar to NCRs, while NKG2D and CD94 bind to heparin by hydrophobic interactions. Further study is needed to clarify whether the interactions of CD94/NKG2A and CD94/NKG2C with sulfated glycans and α2,3-NeuAc-containing glycoproteins can modulate cellular signaling and are relevant to natural immunity in cancer progression and metastasis.

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