Unlike Natural Killer (NK) p30, Natural Cytotoxicity Receptor NKp44 Binds to Multimeric α 2,3-NeuNAc-Containing *N*-Glycans

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Natural cytotoxicity receptor 2 (NCR2 or natural killer (NK)p44) and NCR3 (NKp30) bind to heparin and heparin sulfate; however, other natural ligands have yet to be identified. We previously reported that NCR1 (NKp46) can bind to multimeric NeuNAc-containing *N*-glycans and sulfated glycans. In this study, we investigated whether NKp44 and NKp30 can bind to NeuNAc-containing glycans using their common recombinant extracellular domain tagged with 6×His (NKp44-H6 and NKp30-H6). NKp44-H6, but not NKp30-H6, bound multimeric sialyl Lewis X expressing transferrin secreted by HepG2 cells (HepTF) with a K_d of 420 nM. Competitive and direct binding assays revealed that NKp44-H6 mainly recognizes α 2,3-NeuNAc residues on non-reducing ends of *N*-glycans on HepTF. Moreover, site-directed mutants of NKp44-H6, such as R47Q, R55Q, R92Q, R95Q, K103Q, and R106Q, had reduced binding to α 2,3-sialylated *N*-glycans. These results suggest that NKp44 binds to α 2,3-sialylated *N*-glycans through ionic interactions, and that these binding sites might have some overlap with heparin binding sites.

Key words natural killer p44; natural killer p30; sialyl Lewis X; natural cytotoxicity receptor; mutagenesis

Natural killer (NK) cells play important roles in natural immunity against transformed and virally infected cells. Natural cytotoxicity receptors (NCRs) NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337) belong to the immunoglobulin (Ig) superfamily. Although not homologous, they are characterized by extracellular Ig-like domains, transmembrane regions containing positively-charged amino acids, and short cytoplasmic tails lacking immunoreceptor tyrosine-based activating motifs (ITAM).^{1–3} Through positively-charged residues, NCRs associate with a negatively-charged Asp in transmembrane regions of different ITAM-containing signaling proteins, thereby triggering NK cytotoxicity against target cells having low expression of major histocompatibility complex (MHC) class I molecules.

NKp44 has a single extracellular IgV-like domain (a transmembrane segment in which Lys associates with Asp in DNAX-activating protein [DAP] 12) and a cytoplasmic domain⁴; like NKp30, it associates with CD3 ζ . In contrast, NKp46 consists of two IgC2-like domains in the extracellular portion—distal (D1) and proximal (D2), a transmembrane region in which Arg interacts with Asp in CD3 ζ and Fc ϵ R1 γ , and a short cytoplasmic tail.⁵ NKp44 is absent on NK cell surfaces in freshly isolated peripheral lymphocytes and is induced by *in vitro* stimulation with interleukin (IL)-2,⁶ whereas NKp46^{7,8} and NKp30⁹ are expressed on both resting and activated NK cells. NCRs interact with adaptor molecules on the cell surface and are involved in intracellular signaling. Selective cross-talk among these different NCRs amplifies activating signals.¹⁰

Like NKp46D2-Ig, NKp44-Ig binds to influenza virus hemagglutinin and Sendai virus hemagglutinin-neuraminidase via α 2,6-*N*-acetylneuraminic acid (NeuNAc) on NKp44; however, tumor cells do not interact via NeuNAc.^{11–13} NKp44 can also bind to Bacillus Calmette-Guerin (BCG)¹⁴ and envelope glycoproteins from the West Nile and Dengue viruses.¹⁵ Microarray and surface plasmon resonance analysis have indicated that NKp44 binds to highly charged IdoA2SGlcNS6S

heparin oligosaccharides with a binding pattern that differs from that of NKp46 and NKp30 (which display similar binding patterns, although NKp46 binding affinity is one order of magnitude lower than NKp30).¹⁶ Heparin epitopes recognized by NKp44 differ from those recognized by NKp46 or NKp30, whereas epitopes recognized by NKp46 and NKp30 overlap.¹⁷ However, natural ligands for NKp44 remain to be elucidated.

We previously reported that fucosyltransferase (*FUT*)-3 gene-transfected K562 (K562/*FUT*) cells that express high levels of the sialyl Lewis X (sLeX) antigen (NeuNAc α 2,3-Gal β 1,4(Fuca1,3)GlcNAc-R) are more susceptible to lysis by IL-2-activated KHYG cells,¹⁸ and that glutathione *S*-transferase-fused extracellular domains of C-type lectin-like receptors (rGST-NKG2D, rGST-CD94, rGST-NKG2A, and rGST-NKG2C) can directly bind to heparin, highly sulfated glycans, and multimeric α 2,3-NeuNAc-containing *N*-glycans.^{19–22} In addition, we have clarified that recombinant extracellular domains (AA22–192) of NKp46-H6 can also bind to sulfate- and α 2,3-NeuNAc-containing glycans, and that basic amino acids in NKp46 are essential for glycan binding.²³

In the present report, we prepared NKp44-H6 and NKp30-H6, created site-directed mutants using an *Escherichia coli* expression system, and measured the binding of NKp44-H6 and NKp30-H6 to sLeX or α 2,3-NeuNAc containing multi-antennary *N*-glycans on transferrin secreted by HepG2 cells (HepTF). Moreover, we investigated these glycan binding sites in NKp44-H6 using site-directed mutants R47Q, K53Q, R55Q, H88Q, R92Q, R95Q, K103Q, and R106Q.

MATERIALS AND METHODS

Preparation of NKp44-H6 and NKp30-H6 Constructs
NKp44-H6 and NKp30-H6 constructs were prepared using pET-22b/BL (DE3) (TaKaRa, Otsu, Japan) according to the manufacturer's instructions and a previous report.¹⁰ Briefly, the extracellular domains of NKp44 (AA22–192) (pET-NKp44) and NKp30 (AA1–135) were amplified from cDNA in human

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Fig. 1. SDS-PAGE for NKp44-H6, NKp30-H6 and Mutants

NKp44-H6 and its mutants (A) and NKp30-H6 (B) purified on a Ni-sepharose column were separated by 12% SDS-PAGE with (+) and without (-) pretreatment with 1% 2-ME at 95°C for 3 min.

NK-derived KHYG cells (Japanese Collection of Research Bioresources Cell Bank [JCRB], Tokyo, Japan) according to the manufacturer's instructions, using PrimeSTAR HS DNA polymerase (Takara) and the following specific primers: forward, 5'-AAGAATTCTCAATCCAAGGCTCAGTACTTC-3', and reverse, 5'-AAGAATTCATGGCAATGGGGGCTGCAGGGC-CAGG-3' for NKp44; and forward, 5'-AAGAATTCTATGGCC-TGGATGCTGTTGCT-3', and reverse, 5'-AAGAATTCATCC-CTAGCTGAGGATGTTCTTTCT-3' for NKp30. Site-directed mutant constructs of pET-NKp44 (R47Q, K53Q, R55Q, H88Q, R92Q, R95Q, K103Q, and R106Q) were prepared using the KOD-plus Mutation kit (Toyobo, Tokyo, Japan), with pET-NKp44 as the template, according to the manufacturer's instructions. Primers used for site-directed mutagenesis were: 5'-GTCACCAGCTCCAAGCCC-3' and 5'-TAACTGGATGCACACAAGTG-3' (R47Q); 5'-CCCAGGACGATGGCTTGG-3' and 5'-CTGGGAGCTGGTGACTAACCTG-3' (K53Q); 5'-CAGACGATGGCTTGGACCTCT-3' and 5'-GGGCTTGGAGCTGGTGACTAA-3' (R55Q); 5'-GGACAGTACTGTGTAGAATCTA-3' and 5'-TGAGTCTTCCTCTCTCAGATC-3' (H88Q); 5'-CAGATCTACCGCCCTTCTGA-3' and 5'-ACACCAAGTAATGTCCTGAGTCTT-3' (R92Q); 5'-CCA-GCCTTCTGACAACCTGT-3' and 5'-TAGATTCTACACCA-GTAATGTCCT-3' (R95Q); 5'-TCTCTCAGTCCGTCAGATTCTATC-3' and 5'-CAGAGTTGTCAGAAGGGCG-3' (K103Q); and 5'-CAGTTCTATCTGGTGGTATCTCC-3' and 5'-GACG-GACTTAGAGACAGAGTTGT-3' (R106Q). The purified polymerase chain reaction (PCR) products were ligated into

the pET-22b vector (Takara) with Ligation High (Toyobo). The recombinant plasmids were transformed into Chaperone competent cells pET-22b/BL (DE3) (Takara), and positive clones were confirmed by DNA sequencing using a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Refolding of Recombinant Proteins NKp44-H6, NKp30-H6 and their mutants were refolded according to a previous report.¹⁰ After induction with 1 mM isopropyl- β -D-thiogalactopyranoside (Promega Co., Madison, WI, U.S.A.) at 25°C for 5 h, cells were harvested and sonicated for 10 s $\times$ 6 on ice. Inclusion bodies were collected by centrifugation and treated with 6.0 M urea and 20 mM dithiothreitol (DTT) in phosphate buffered saline (PBS) for 2 h at 37°C. The supernatant was applied to a Ni-Sepharose column (1 mL of bed volume) (GE Healthcare Bio-Science, Uppsala, Sweden) according to the manufacturer's instructions. The column was washed stepwise with 10 mL of 2.0 M urea followed by 3 mL each of 1.5 M, 1.25 M, and 1.0 M urea in 40 mM imidazole/PBS. NKp44-H6 and NKp30-H6 were eluted with 4 mL of 500 mM imidazole in 1.0 M urea/PBS. The eluate was treated with a 5 mM glutathione mixture (reduced:oxidized forms=10:1) in PBS for 2 h at 25°C and dialyzed against PBS. After centrifugation, refolded proteins were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with and without pretreatment in 1% 2-mercaptoethanol (2-ME) at 95°C for 3 min, and then stained with Coomassie brilliant blue. Protein concentrations were determined using the Advanced Protein Assay Reagent (Bio-Rad, Hercules, CA,

Fig. 2. Binding of NKp44-H6 and NKp30-H6 to Heparin-BSA and NeuNAc-Containing *N*-Glycans

NKp44-H6 and NKp30-H6 were placed on (A,C) heparin-BSA and (B,D) HepTF-coated plates for 2 h at 37°C. Binding was determined using POD-conjugated anti-6×His antibody. Mean ± S.D. ($n=3$).

U.S.A.), with bovine serum albumin (BSA) as the standard.

Preparation of Glycan-Coated Plates Glycan-coated 96-well plates were prepared according to previous reports.^{19,20} Briefly, 300 mg of heparin (porcine intestinal mucosa) (Wako Chemicals, Osaka, Japan) was conjugated with BSA (30 mg) in the presence of sodium cyanoborohydride (8 mg) in 1.5 mL PBS (pH 8.0) at 37°C for 2 d. Wells were incubated with heparin-BSA (100 μ L, 50 μ g/mL) overnight at 37°C and then blocked with 350 μ L of 0.1% BSA/PBS for 2 h at 37°C.

Glycoproteins, including multimeric sLeX-expressing TF (HepTF) purified from culture media of human hepatoma-derived HepG2 cells (JCRB),^{19,24} human TF (NorTF) (Nacalai Tesque, Kyoto, Japan), and human α 1-acid glycoprotein (AGP) (Sigma-Aldrich, Chicago, IL, U.S.A.), were directly coated onto the plates. The plates were further coated with 0.1% BSA/PBS. Desialylated HepTF-coated plates were obtained by treatment with 1 U/mL α 2,3/6/8 neuraminidase (Seikagaku Co., Tokyo, Japan) in 100 mM MES (pH 6.0) for 2 d at 37°C. Control plates were coated with 0.1% BSA/PBS.

Binding of NKp44-H6 and NKp30-H6 to Glycan-Coated Plates Binding of NKp44-H6 and NKp30-H6 to glycan-coated plates was determined according to a pre-

vious report.²³ Briefly, 100 μ L of 0–50 nM heparin-BSA, 0–700 nM NKp44-H6 glycoprotein, 0–500 nM heparin-BSA, or 0–2000 nM NKp30-H6 glycoprotein, in PBS (pH 7.4) containing 0.1% BSA and 0.3% Tween-20 (PBS-T), were incubated on heparin-BSA and HepTF-coated plates for 2 h at 37°C. After three washes with PBS-T, the plates were further incubated with 100 μ L of 0.1 μ g/mL peroxidase (POD)-conjugated anti-6×His antibody (Wako) in PBS-T for 1 h at room temperature. After three washes with PBS-T, the plates were incubated with 100 μ L of tetramethylbenzidine (TMB) solution (BioFX Lab., Owings Mills, MD, U.S.A.) for 5 min at room temperature. After the addition of 100 μ L of 1 M H_2SO_4 , absorbance was read at 450 nm using a Model DTX800 plate reader (Beckman Coulter, Fullerton, CA, U.S.A.).

K_d values were determined using linear reciprocal plots of $[M]/\Delta Abs$ versus $[M]$ and calculating from the slopes ($1/B_{max}$) and y-intercepts (K_d/B_{max}), where $[M]$ is the concentration of NKp44-H6 or NKp30-H6, and B_{max} is the maximum binding of NKp44-H6 or NKp30-H6.

The binding of NKp44-H6 (400 nM) to HepTF-coated plates was also determined in the presence of 0, 10, and 100 mM soluble monosaccharides of NeuNAc, *N*-acetylglucosamine

Fig. 3. NKp44-H6 Binding to Neuraminidase-Treated HepTF and Competition with Monosaccharides

(A) NKp44-H6 (400 nM) binding to plates coated with *N*-glycans, including HepTF, desialylated HepTF (desHepTF), NorTF, and AGP. Binding determined as previously described. (B) NKp44-H6 (400 nM) binding in the presence of 0, 10 and 100 mM of the following monosaccharides: galactose, *N*-acetylglucosamine, fucose, *N*-acetylneuraminic acid, mannose. The results are given as mean $\pm$ S.D. ($n=3$) and asterisks (*) denote statistically significant differences ($p<0.05$) against 0 mM.

(GlcNAc), galactose (Gal), fucose (Fuc) and mannose (Man) in NKp44-H6 solution, and their binding to the plates was determined as described above.

Binding of Site-Directed Mutants of NKp44-H6 to Glycans Binding of site-directed mutants of NKp44-H6 (R47Q, K53Q, R55Q, H88Q, R92Q, R95Q, K103Q, and R106Q) to Heparin-BSA and HepTF was determined as described above. NKp44-H6 wild-type and mutant concentrations used were based on K_d values for each glycan: 10 nM for heparin-BSA and 100 nM for HepTF. The binding was measured as described above and normalized to that of NKp44-H6 wild-type.

Statistical Analysis Data are expressed as means $\pm$ S.D. The results were considered to be statistically significant if the value of p was <0.05 . Differences between two groups were compared using Student's *t*-test.

RESULTS

Binding of NKp44-H6 and NKp30-H6 to Heparin-BSA It has been reported that NKp46D2-Ig, NKp44-Ig, and NKp30-Ig bind to heparin; however, the sialylation and glycosylation status of NCRs affects their ligand recognition.^{11,12} Therefore, we prepared NKp44-H6 and its mutants using an *E. coli* expression system. The proteins were then refolded by stepwise dilution of guanidine and separated on 12% SDS-PAGE with or without 1% 2-ME pretreatment. In reducing and non-reducing conditions, NKp44-H6 and NKp30-H6 wild-type and their mutants separated at similar molecular weights of around 25 kDa [NKp44-H6] and 18 kDa [NKp30-H6], indicating that the refolded proteins were monomeric (Fig. 1).

We confirmed binding of NKp44-H6 and NKp30-H6 to heparin-BSA-coated plates using POD-conjugated anti-6 $\times$ His antibody (Fig. 2). NKp44-H6 (0–50 nM) and NKp30-H6 (0–500 nM) bound to heparin-BSA in a dose-dependent man-

ner (Figs. 2A,B). K_d values for heparin-BSA binding were 23 nM (NKp44-H6) and 260 nM (NKp30-H6), as determined by linear reciprocal plots (data not shown), indicating that NKp44-H6 bound to heparin-BSA with one order of magnitude affinity higher than NKp30-H6.

We previously demonstrated that NKp46-H6 binds not only to heparin-BSA, but also to HepTF.²³ HepTF expresses multivalent sLeX in di- to tetra-antennary *N*-glycans.²⁴ To confirm the direct binding of NKp44-H6 and NKp30-H6 to HepTF *N*-glycans, we assessed binding to plates coated with HepTF (Figs. 2C,D). NKp44-H6 (0–700 nM) bound to HepTF in a dose-dependent manner (Fig. 2C) with a K_d of 420 nM ($R^2=0.9641$) by linear reciprocal plots (data not shown), which was one order of magnitude higher than that for heparin-BSA. On the other hand, higher doses of NKp30-H6 (0–2000 nM) did not bind to HepTF-coated plates (Fig. 2D).

Binding of NKp44-H6 to HepTF Depends on Multimeric Neuraminic Acid HepTF expresses multivalent sLeX in di- to tetra-antennary *N*-glycans, NorTF has α 2,6-NeuNAc in di-antennary *N*-glycans,²⁵ and human AGP has α 2,6-NeuNAc in di- to tetra-antennary *N*-glycans.^{26,27} To confirm the direct binding of NKp44-H6 to these *N*-glycans, we determined the binding to plates coated with HepTF, NorTF, AGP, and desialylated HepTF. NKp44-H6 did not bind to HepTF-coated plates after treatment with α 2,3/6/8 neuraminidase (Fig. 3A). In addition, NKp44-H6 did not bind to the α 2,6-linked NeuNAc-containing AGP- or to the α 2,3- and α 2,6-linked NeuNAc-containing NorTF-coated plates. These results indicate that α 2,3-linked, but not α 2,6-linked, NeuNAc on multi-antennary *N*-glycan is critical for NKp44 binding.

We have reported previously that HepTF contains multi-antennary *N*-glycans with α 2,3-linked NeuAc and α 1,3-linked Fuc²⁵ and it consists of *N*-acetylglucosamine (GlcNAc), mannose, galactose, Fucose and *N*-acetylneuraminic acid (NeuNAc). We further estimated their binding to HepTF in the presence of excess monosaccharides including NeuAc, Fuc, GlcNAc, Gal, and Man (Fig. 3B). NeuNAc competition significantly affected NKp44-H6 binding to HepTF in a dose-dependent manner, but GlcNAc and galactose monosaccharides slightly suppressed NKp44-H6 binding to HepTF. These results indicate that NKp44-H6 recognizes mainly the NeuNAc residues on multi-antennary *N*-glycans of HepTF.

Binding of Site-Direct Mutants of NKp44-H6 To determine the glycan ligand binding sites on NKp44, we prepared site-direct mutants R47Q, K53Q, R55Q, H88Q, R92Q, R95Q, K103Q, and R106Q (Fig. 4A), and determined their binding to glycan-coated plates (Figs. 4B,C). The eight single Q-substituted NKp44-H6 mutants had restrained binding to heparin-BSA (Fig. 4B) and HepTF (Fig. 4C), suggesting that NKp44-H6 binds to sulfate- and α 2,3-NeuNAc-containing glycans through ionic interactions.

DISCUSSION

In the present study, we clarified the binding properties of NKp44-H6 and NKp30-H6 using direct and competitive binding assays. NKp44-H6 recognized not only heparin-BSA, but also HepTF. In contrast, NKp30-H6 recognized only heparin-BSA. Furthermore, competitive and direct binding assays also revealed that NKp44-H6 mainly bound multimeric α 2,3-NeuNAc-containing *N*-glycans.

Fig. 4. Binding of Site-Directed NKp44-H6 Mutants to Heparin-BSA and NeuNAc-Containing *N*-Glycans

(A) Ribbon structure of NKp44 denoting mutation sites, obtained from the Protein Data Base (PDB) # 1hkf. Basic amino acid is dark gray, and acidic amino acid is gray. Binding of NKp44-H6 mutants to (B) heparin-BSA and (C) HepTF, normalized to NKp44-H6 wild-type binding. Mutant concentrations: 13 nM (heparin-BSA) and 400 nM (HepTF). Mean $\pm$ S.D. ($n=3$). * $p<0.05$.

NKp44-H6 mutations (R47Q, K53Q, and R55Q in the sequence of ⁴⁷RLVTSSKPR, and H88Q, R92Q, R95Q, K103Q, and R106Q in ⁸⁸HYWCRIYRPSDNSVSKSVR) significantly restrained binding to sulfate- and α 2,3-NeuNAc-containing glycans with some different patterns between heparin-BSA and HepTF. These results indicate that ionic interactions of basic amino acids in NKp44 are important for binding to these glycans. The crystal structure shows that the NKp44-Ig domain forms a saddle-shaped dimer, where a charged surface groove constituted by R47, H88, R92, and R106 protrudes from the core structure in each subunit.^{28,29} The crystal structure of Siglec-1 in complex with 3'-sialyllactose has confirmed that R97 plays a critical role in sialylated glycan recognition.³⁰ This R97 has a close structural equivalent in NKp44 R92 that could potentially mediate binding to sialylated glycans.³¹ Basic amino acids in the surface groove of NKp44 are also involved in its binding to heparin sulfate and tumor cells,

but not to influenza virus-infected cells.³²

Simultaneously, Q4 (K133Q, R136Q, H139Q, and R142Q) or Q4+K146T-substituted NKp46 mutants in ¹³³KEGRSSHVQRGYGK have markedly reduced binding to heparin sulfate and tumor cells, but not to influenza virus-infected cells.³³ We previously demonstrated that each single basic amino acid-substituted NKp46-H6 had restrained binding to heparin-BSA and HepTF.²³ NKp30 has a similar basic amino acid sequence, ⁶⁴KEVRNGTPEFRGRLAPLASSRFLH (EMBL accession number AJ223253).³⁴ Heparin epitopes recognized by NKp44 do not overlap with NKp46 and NKp30, while the binding affinities of NKp46 to heparin are one order of magnitude lower than those of NKp30 and NKp44.^{16,17} We also reported that NKp44-H6 interacts with various sulfate residues with one or two orders of magnitude higher binding affinity than NKp46-H6, although the K_d value of NKp44-H6 for HepTF (420 nM) was similar to that of NKp46-H6

(520 nm).²³⁾ The spatial distribution of basic amino acids in NCRs may contribute to these differences. The broad binding spectra and high binding affinities of NKp44 may be caused by two regions of basic amino acid sequences in that protein.

In the present study, we revealed that NKp44 can interact with multimeric α 2,3-NeuNAc-containing *N*-glycans, and that these glycans interact with basic amino acids in NKp44. When considered together with previous reports, these results suggest that the high expression of α 2,3-NeuNAc residues on tumor cell surfaces plays an important role in NK cell cancer immunosurveillance.

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