Binding Properties of CD94 to Sulfated Glycans and α2,3-NeuAc-Containing Glycoproteins and Mutagenesis Analysis

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Abstract: Killer lectin-like receptor CD94 on natural killer cells can bind to HLA-E, α2,3-NeuAc-containing glycoproteins, and heparin. Using the glutathione-S-transferase-fused CD94 (rGST-CD94), we investigated its binding to plates coated with α2,3-NeuAc-containing transferrin secreted by HepG2 cells (HepTF) and sulfate-containing glycan-conjugated BSA. rGST-CD94 bound directly to several glycans with similar high affinities: rGST-CD94 bound to heparin-BSA, heparan sulfate-BSA, fucoidan-BSA, λ-carrageenan-BSA, and HepTF with Kd values of 36 nM, 141 nM, 36 nM, 92 nM and 84 nM, respectively. Different mutants of rGST-CD94 were generated by site-directed mutagenesis. Mutants N160A and C166G had reduced binding to sulfated glycans and HepTF, also F114A, L162A, D163A, and E164A showed reduced binding to sulfated glycans with some variability. These results indicated that CD94 interacts with sulfated glycans and α2,3-NeuAc-containing glycoproteins suggesting that glycan binding sites in CD94 partially overlap with the HLA-E binding sites to modulate natural killer cell-dependent cytotoxicity.

Keywords: CD94, killer lectin-like receptors, heparin, sialy Lewis X, mutagenesis.

INTRODUCTION

Natural killer (NK) cells play important roles in innate immunity and in the immune surveillance of malignant transformed cells and viral-infected cells. The cytotoxic activity of NK cells is regulated by the balance of opposing signals transduced through activating and inhibiting cell surface receptors of the immunoglobulin (Ig)-like and C-type lectin-like receptors, and by signal integration and dynamic fine-tuning of NK cell responses [1,2].

Among C-type lectin-like receptors, CD94 forms disulfide-linked heterodimers with either natural killer group 2 (NKG2) A, B, C, E, or H [3-5]. The inhibitory receptors NKG2A and B have two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, which associate with the SH2 domains of the protein tyrosine phosphatases SHP-1 and SHP-2 [6]. The activating receptors NKG2C, E, and H have a positively charged K residue within their transmembrane regions, and associate with a negatively charged D residue in the disulfide-linked homodimer adaptor molecule DNAX-activating protein of 12 kDa (DAP12), which contains the immunoreceptor tyrosine-based activating motif (ITAM) [7]. In human cells, the ligand for CD94/NKG2A, B, C, and E is the non-classical major histocompatibility complex class (MHC)-1b molecule, human leukocyte antigen (HLA)-E, complexed with nonamer leader peptides of other MHC class I members, including HLA-A, B, C, and G [8-10].

The C-type lectin domain (CTLD) fold has a double-loop structure with its N- and C-terminal β strands (β1 and β2) coming close together to form an antiparallel β-sheet. The most conserved CTLD residues (C1-C4) form disulfide bridges: C1 and C4 link β5 and the α1 helix to form the whole domain loop, while C2 and C3 link β3 and β5 to form the long loop region that is involved in Ca2+-dependent carbohydrate binding. The C-type lectin-like receptor CD94 lacks one of two major α helices and most of the conserved Ca2+-binding residues present in other C-type lectins [11-13]. Their glycan ligands have yet to be elucidated.

Mouse melanoma B16-F1 cells transfected with the fucosyltransferase (FUT)-3 gene and overexpressing sialyl Lewis X (sLeX) NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNAc-R, in shorter N-glycans, are more susceptible to lysis by NK cells in vivo, which can be prevented by pretreatment with anti-CD94 and anti-sLeX antibodies [14,15]. Similarly, it was found that FUT 3-transfected human erythroleukemia K562 (K562/FUT) cells selected for high expression of sLeX were more susceptible to lysis by human NK-derived KYHG cells in vitro, and that this susceptibility was suppressed by pretreatment of K562/FUT cells with anti-sLeX, and of KYHG cells with anti-NKG2D and anti-CD94 antibodies [16]. Using recombinant glutathione S-transferase (GST)-fused extracellular domains of NKG2D (AA 73-216) (rGST-NKG2D) and CD94 (AA 32-179) (rGST-CD94), and their site-directed mutants, it was found that rGST-NKG2D and rGST-CD94 can bind to plates coated with sLeX-expressing transferrin secreted by HepG2 cells (HepTF) [17] and heparin-conjugated bovine serum albumin (heparin-BSA) [18]. Moreover, it was found that rGST-NKG2A and rGST-NKG2C bind to heparin-BSA and HepTF with high binding affinities [19].
In the present study, we further characterized the binding of rGST-CD94 and seven site-directed mutants to HepTF and sulfated glycans. We found that rGST-CD94 binds directly to heparin-BSA, heparan sulfate-BSA, fucoidan-BSA, \( \lambda \)-carrageenan-BSA, and HepTF with small \( K_d \) values. Moreover, site-directed mutagenesis analysis revealed that N160 and C166 are essential for binding to HepTF and sulfated glycans, and that F114, L162, D163, and E164 contribute to sulfated glycan binding.

**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), 0.6 mg/ml L-glutamate (Wako Chemicals Co., Osaka, Japan), 100 U/ml penicillin (Banyu Pharmaceutical Co., Tokyo, Japan), and 100 \( \mu \)g/ml streptomycin (Meiji Seika Co., Tokyo, Japan) in a humidified atmosphere containing 5% \( \text{CO}_2 \) at 37 °C. 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 and Mutation of rGST-CD94 Constructs**

rGST-CD94, and its site-directed mutated constructs, were prepared as described previously [17,18]. Briefly, the extracellular domain of CD94 (coding for AA 32-179, pGEX4T-1/CD94) was amplified from KHYG-derived cDNA using 5'-TTGAAGCTTATCCACAAAAATTGA-3' (forward) and 5'-TTAAATGAGCTTGGTTAAGTTCAAATG-3' (reverse). Site-directed mutant constructs of CD94, Q112A, F114A, N160A, L162A, D163A, E164A, and C166G were prepared using the KOD-plus Mutagenesis kit (Toyobo, Tokyo, Japan) and pGEX4T-1/CD94 as the template according to the manufacturer’s instructions. Primers designed for mutant vectors were shown in Table 1.

<table>
<thead>
<tr>
<th>Mutated Amino Acid</th>
<th>Primer Sequences</th>
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<tbody>
<tr>
<td>Q112A</td>
<td>5'-AGTGCACAATTTTACTGAGATGGAC-3'</td>
</tr>
<tr>
<td>F114A</td>
<td>5'-GCAGCTCATCAAATCCAGTTTCATCT-3'</td>
</tr>
<tr>
<td>N160A</td>
<td>5'-GCTGCTTTAGATGAATCCTGTGA-3'</td>
</tr>
<tr>
<td>L162A</td>
<td>5'-GGCATATCTCTGGAAGATATATG-3'</td>
</tr>
<tr>
<td>D163A</td>
<td>5'-GCTGAATCTGGAAGATATATG-3'</td>
</tr>
<tr>
<td>E164A</td>
<td>5'-GCTGCTTTAGATGAATCCTGTGA-3'</td>
</tr>
<tr>
<td>C166G</td>
<td>5'-GGATTCAATCTAAGACCTTCCA-3'</td>
</tr>
</tbody>
</table>

The purified PCR products were ligated into pGEX4T-1 vector (GE Healthcare Bio-Science, Uppsala, Sweden) with Ligation High (Toyobo). The recombinant plasmids were transformed into Chaperone competent cells, pTF16/BL21 (Takara, Otsu, Japan), and positive clones were confirmed by DNA sequencing.

After induction with 1 mM isopropyl-\( \beta \)-D-thiogalactopyranoside (Promega Co., Madison, WI), the cells were harvested and sonicated for 10 s \( \times \) 6 on ice. Recombinant proteins were purified on a GStrap™ FF column (1 ml packed volume; GE Healthcare) according to the manufacturer’s instructions, and dialyzed against 1 mM dithiothreitol (DTT)/PBS. Proteins were separated on 10% SDS-PAGE with and without pretreatment in 1% 2-mercaptoethanol (2-ME) at 95 °C for 3 min and stained with Coomassie brilliant blue. Protein concentrations were determined using the Advanced Protein Assay Reagent (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard.

**Preparation of Glycan-Coated Plates**

Glycans were conjugated with BSA in the presence of sodium cyanoborohydride and coated on 96-well plates according to a previous report [18]. 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. Multimeric sLeX-expressing transferrin secreted by HepG2 cells (HepTF) [20] was purified from culture supernatant and directly coated on the plates [17]. As a control, plates were treated with 1% BSA/PBS.

**Binding of rGST-CD94 to Glycan-BSA-Coated Plates**

To each glycan-coated plate, 100 μl of 0 to 1.2 μM rGST-CD94 in 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM CaCl2, 0.1% BSA, 150 mM NaCl, and 0.3% Tween (TBS-T) was added, and plates were incubated for 1 h at 37 °C. After washing with TBS-T three times, the plates were further incubated with 100 μl of 2 μg/ml peroxidase (POD)-conjugated anti-GST antibody (Rockland Immunochemicals Inc., Gilbertsville, PA) in TBS-T for 1 h at room temperature. Then, after washing with TBS-T three times, the plates were further incubated with 100 μl of tetramethylbenzidine (TMB) solution (BioFX Lab., Owings Mills, MD) for 5 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M H2SO4, and absorbance was read at 450 nm with a Model DTX800 plate reader (Beckman Coulter, Fullerton, CA).

$K_d$ values of rGST-CD94 for glycans were determined using linear reciprocal plots [21], $[M]/ΔAbs$ versus $[M]$, where $[M]$ is the concentration of rGST-CD94 (MW=43,784 Da). $K_d$ values were calculated from slopes $(1/B_{max})$ and $y$ intercepts $(K_d/B_{max})$, where $B_{max}$ is the maximum binding of rGST-CD94.

**Binding of Site-Directed Mutants of rGST-CD94 to Glycans**

To estimate the binding of site-directed mutants of rGST-CD94 to glycans, the concentrations of each mutant used were 22 nM for heparin-BSA, fucoidan-BSA, λ-carrageenan-BSA, and HepTF, and 45 nM for heparan sulfate-BSA. Binding was measured as described above and compared to wild-type rGST-CD94.

**RESULTS**

**Binding of rGST-CD94 to Sulfate- and α2,3-NeuAc-Containing Glycans**

We prepared rGST-CD94 and its mutants using Chaperon competent cells, pTf16/BL21, proteins were purified on a glutathione-column. Recombinant proteins in 1 mM DTT/PBS were separated on 10% SDS-PAGE at around 44 kDa, which was a similar size to those pretreated with 1% 2-ME, indicating that monomeric forms were obtained (Fig. 1). Chaperon proteins and GST were concomitantly detected at around 60 and 32 kDa, respectively.

We previously demonstrated that rGST-CD94 binds to α2,3-NeuAc on multi-antennary glycoproteins [17] and heparin-BSA [18]. Binding of rGST-CD94 to heparin-BSA is suppressed by soluble forms of heparan sulfate, dermatan sulfate, fucoidan, and λ-carrageenan [18]. To investigate the binding of CD94 to sulfated glycans and α2,3-NeuAc containing glycoprotein, we determined the binding of rGST-CD94 to plates coated with these glycans (Fig. 2).

![Fig. 1](image)

**Fig. (1). SDS-PAGE for rGST-CD94 and its mutants.**

rGST-CD94 and its mutants purified on a glutathione column and dialyzed against 1 mM DTT/PBS were separated on 10% SDS-PAGE with (+; left lanes) and without (-; right lanes) pretreatment in 1% 2-ME at 95 °C for 3 min. Bands at around 44 kDa are rGST-CD94.

**Mutagenesis Analysis for the Binding of rGST-CD94 to Glycans**

Residues Q112, F114, N160, L162, D163, and E164 in CD94 are essential for its recognition of HLA-E [22, 23]. Among these amino acids, F114 and N160 in CD94 are homologous to the NeuAc recognition domain of E-selectin [24], and also to the glycan-recognition domain of dectin-1 [25]. C166 in CD94 is essential for disulfide linkage formation between β-sheets [26]. We prepared rGST-CD94 mutants Q112A, F114A, N160A, L162A, D163A, E164A, and C166G, and compared their binding to wild-type rGST-CD94 to clarify the binding sites in CD94 for sulfated glycans and α2,3-NeuAc-containing glycoproteins (Fig. 4). Mutagenesis analysis revealed that CD94 mutants N160A...
Fig. (2). Binding of rGST-CD94 to sulfated glycans and α2,3-NeuAc containing glycoprotein.

rGST-CD94 (0 to 1.2 μM) was added to plates coated with (A) heparin-BSA, (B) heparan sulfate-BSA, (C) fucoidan-BSA, (D) λ-carrageenan-BSA, and (E) HepTF for 1 h at 37 °C, and binding was determined using POD-conjugated anti-GST antibody. The results are given as means ± SD (n=3).

Fig. (3). Linear reciprocal plots for binding of rGST-CD94 to sulfated glycans and α2,3-NeuAc containing glycoprotein.

Linear reciprocal plots, [M]/ΔAbs vs. [M], for binding of rGST-CD94 to (A) heparin-BSA, (B) heparan sulfate-BSA, (C) fucoidan-BSA, (D) λ-carrageenan-BSA, and (E) HepTF as determined in Fig. (2). The results for binding of 11 to 220 nM rGST-CD94 were used, and K_d values were calculated from the slopes (1/B_max) and y-intercepts (K_d/B_max), where [M] and B_max are the concentrations and maximum binding of rGST-CD94, respectively.
and C166G had reduced binding to heparin-BSA (Fig. 4A), heparan sulfate-BSA (Fig. 4B), fucoidan-BSA (Fig. 4C), λ-carrageenan-BSA (Fig. 4D), and HepTF (Fig. 4E), while the effects of F114A, L162A, D163A, and E164A on binding were insignificant in HepTF, and variable among sulfated glycans.

These results suggested that interactions between CD94 and glycans are partly composed of hydrophobic and/or hydrogen bonding interactions, and that the binding sites in CD94 for sulfate- and α2,3-NeuAc-containing glycans partially overlap with their protein ligand HLA-E binding sites.

**DISCUSSION**

In the present study, we found that the rGST-CD94 monomer binds directly to heparin-BSA ($K_d$: 36 nM), heparan sulfate-BSA (141 nM), fucoidan-BSA (36 nM), λ-carrageenan-BSA (92 nM), and HepTF (84 nM) with small $K_d$ values, and also to dermatan sulfate-BSA (value not determined). Previous competitive binding assays revealed that binding of rGST-CD94 to heparin-BSA is not suppressed by keratan sulfate, chondroitin sulfate A, B, and C, hyaluronic acid, mannan, β-glucan, or by either 2-O-, 6-O-, or N-desulfated heparin [18]. We previously found that rGST-NKG2A and rGST-NKG2C also bind to heparin-BSA (20 and 40 nM), heparan sulfate-BSA (185 and 151 nM), fucoidan-BSA (1.35 and 0.27 nM), λ-carrageenan-BSA (16.3 and 6.7 nM), and HepTF (80 and 114 nM), respectively, and that 2-O-sulfate residues in heparin are essential for binding [19].

The binding affinities of CD94/NKG2x to HLA-E/peptide complexes are peptide-dependent, and these interactions have very fast association and dissociation rate constants, although inhibitory receptor CD94/NKG2A binds to HLA-E/peptides with a much lower $K_d$ than stimulatory receptor CD94/NKG2C [27]. Inhibitory receptor CD94/NKG2A and stimulatory receptor CD94/NKG2E display essentially identical affinities for HLA-E/peptide complexes with $K_d$ values ranging from 0.7 to ~20 μM for different peptides, which are uniformly 6-fold stronger than those of CD94/NKG2C, with $K_d$ values ranging from ~4 to >100 μM [28]. CD94, NKG2A, and NKG2C monomers bind to glycan ligands with higher affinities compared to these HLA-E/peptide complexes, suggesting that these glycan ligands compete with protein ligands to modulate NK cell-dependent cytotoxicity.

The crystal structure of the CD94 homodimer revealed that CD94 is quite similar to the classical C-type lectin fold despite lacking one major α-helix and Ca$^{2+}$-binding loop [11]. Based on the structure of NKG2D/MICA, CD94/NKG2A should bind to HLA-E, with CD94 oriented over the α1 helix of HLA-E and the C-terminal part of the peptide, and NKG2A over the α2 helix [23]. A binding study using alanine substitutions of HLA-E loaded with the HLA-

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**Fig. (4). Mutagenesis study for binding of rGST-CD94 to glycans.**

The binding of rGST-CD94 mutants Q112A, F114A, N160A, L162A, D163A, E164A, and C166G to glycan-coated plates, including (A) heparin-BSA, (B) heparan sulfate-BSA, (C) fucoidan-BSA, (D) λ-carrageenan-BSA, and (E) HepTF, was determined as described in the legend of Fig. (2). The concentrations of rGST-CD94 wild-type and mutants used were 22 nM for heparin-BSA, fucoidan-BSA, λ-carrageenan-BSA, and HepTF, and 45 nM for heparan sulfate-BSA. The relative binding of rGST-CD94 mutants to each glycan is represented as 100% of wild-type rGST-CD94. The results are given as means ± SD (n=3), and asterisks denote significance differences (P<0.05).
The crystal structure and alanine scanning have also revealed that loops 2, 3, and 5, and β strands 6 and 7 in CD94, interact broadly with residues 65-89 in the α1 helix of HLA-E, and that Q112, F114, N160, I162, D163, E164, and D168 in CD94, and Q212, R137, and R215 in NKG2A are important for the interaction. However, CD94 is positioned mostly over the α1-helix of HLA-E, and NKG2A over the α2-helix, and CD94 plays a crucial role in interacting with HLA-E while the role of NKG2A is more limited [23, 30]. The crystal structure of CD94/NKG2A with the HLA-E-peptide complex shows that two stretches of CD94 make significant contacts with the HLA-E platform: CD94 Q112, Q113, and F114 are directly adjacent to the interface with NKG2A; and CD94 N160, L162, D163, E164, F114, and L162 bind to a hydrophobic patch composed of HLA-E V76, E173, and F at P8 in the peptide [22]. The interaction between CD94/NKG2A and HLA-E is also driven by charged interactions: an acidic region of CD94 with a basic region on the α1 helix, and a basic region of NKG2A with the α2 helix of HLA-E [30]. The crystal structure of the CD94/NKG2A heterodimer indicated that the dimer interface is dominated by nonpolar interactions that comprise ~2/3 of the interface, and by two salt bridges: E161 and K135 in NKG2A salt-bridged to K64 and D106 in CD94, respectively [23]. F at P8 in the HLA-G leader sequence (VMAPRTVLL) is preferentially recognized by CD94/NKG2A and CD94/NKG2C. A hydrophobic patch on HLA-E composed of I73 and V76, and F at P8 in the peptide which interacts with CD94 L112 and F114, are critical for the interaction between CD94/NKG2 and HLA-E/peptide. Charged interactions between R75 and R79 in HLA-E, and D163 and E164 in CD94, are also important; however, CD94/NKG2A is particularly sensitive to minor changes in the peptide conformation [5, 23].

Amino acids mutated in the present study are shown in a ribbon structure of CD94 (Fig. 5). Mutagenesis analysis of the interaction between CD94 and glycan ligands revealed that binding of rGST-CD94 to sulfated glycans and α2,3-NeuAc-containing glycoprotein was reduced significantly by substitutions of N160A and C166G in CD94, while F114A, L162A, D163A, and E164A affected binding to sulfated glycans with some variability, but not significantly to HepTF. C166 forms a disulfide-linkage between C2 and C3 to stabilize the conformation of the long loop region. The C166G mutation may disturb this conformation to suppress glycan binding. Q112 and F114 in the C-terminal region of loop 3, and N160, L162, D163, and E164 in the β6-sheet, are important for HLA-E binding. The N160A mutation in CD94 suppressed binding to both HepTF and sulfated glycans, and L162A, D163A, and E164A mutations affected binding to sulfated glycans, indicating that glycan binding sites may overlap with HLA-E binding. Further study is needed to clarify the glycan binding site.

Heparan sulfate proteoglycans (HSPGs) are expressed ubiquitously on the surfaces of animal cells and in the extracellular matrix. Low molecular weight and modified heparin species are suggested to inhibit heparanase, mitogenic signaling through competition of binding of growth factors to their receptors, and metastasis by blocking selectin-mediated interactions of tumor cells with platelets, leukocytes, and endothelium [31-33]. Heparanase is implicated in a variety of pathologies, such as tumor growth, angiogenesis, metastasis, inflammation, and glomerular diseases. Heparanase is over-expressed in a variety of malignant tumors and inflammation, and is linked to tumor growth and metastasis through release of growth factors and remodeling of extracellular matrix, suggesting it as a target for anticancer therapy [34, 35]. Tumor metastasis is facilitated by complex formation of tumor cells with P-selectin on platelets and interactions with L-selectin. Heparin inhibits P-selectin-mediated platelet/tumor cell interaction and metastasis in an L-selectin-dependent manner, but does not affect E-selectin. A heparin-derived tetrasaccharide, ΔUA2Sxn1,4GlcNS6St1, 4IdoASxt1,4GlcNS6S, strongly inhibits L- and P-selectin-Ig binding to sLeX-BSA [36]. GlcNS6S in heparin is critical for interactions with P- and L-selectin [37]. P-selectin binds to heparin with a slow off-rate and a $K_d$ of 115 nM according to surface plasmon resonance [38], and 1210 to 580 nM according to quartz crystal microbalance [39].

Our results show that CD94 binds to heparin, heparan sulfate, fucoidan, λ-carrageenan, and HepTF (α2,3-NeuAc on multimeric N-glycans), and suggest that the binding sites for these glycans partially overlap with those for protein ligands to modulate NK-dependent cytotoxicity. Further study is needed to clarify the role of glycans in cancer immunity.
ACKNOWLEDGEMENTS

This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Young Scientists (B), 21790544, 2009, and by the “Open Research Center” Project.

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