Altered glycosylation of recombinant NKp30 hampers binding to heparan sulfate: a lesson for the use of recombinant immunoreceptors as an immunological tool

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NKp30 is a natural cytotoxicity receptor expressed by human NK cells and involved in NK lytic activity. We previously published that membranal heparan sulfate serves as a coligand for human NKp30. In the present study, we complement our results by showing direct binding of recombinant NKp30 to immobilized heparin. The heparan sulfate epitope(s) on target tumor cells and the heparin epitope(s) recognized by NKp30 share similar characteristics. Warren and colleagues (Warren HS, Jones AL, Freeman C, Bettadapura J, Parish CR. 2005. Evidence that the cellular ligand for the human NK cell activation receptor NKp30 is not a heparan sulfate glycosaminoglycan. J Immunol. 175:207–212) published that NKp30 does not bind to membranal heparan sulfate on target cells and that heparan sulfate is not involved in NKp30-mediated lysis. In the current study, we examine the binding of six different recombinant NKp30s to membranal heparan sulfate and conclude that NKp30 does interact with membranal heparan sulfate. Yet, two of the six recombinant NKp30s, including the commercially available recombinant NKp30 (employed by Warren et al.) did not show heparan sulfate-dependent binding. We demonstrate that this is due to an altered glycosylation of these two recombinant NKp30s. Upon removal of its N-linked glycans, heparan sulfate-dependent binding to tumor cells and direct binding to heparin were restored. Overall, our results emphasize the importance of proper glycosylation for analysis of NKp30 binding to its ligand and that membranal heparan sulfate could serve as a coligand for NKp30. At the cellular level, soluble heparan sulfate enhanced the secretion of IFNγ by NK-92 natural killer cells activated with anti-NKp30 monoclonal antibody. We discuss the involvement of heparan sulfate binding to NKp30 in NKp30-mediated activation of NK cells.

Keywords: glycosylation/heparan sulfate/natural killer/NCR/NKP30

Introduction

Recombinant receptor-immunoglobulin Fc (receptor-Ig) chimeras have become imperative for basic and applied scientific research in various fields. In particular, the utilization of immunoreceptor-Ig chimeras for the elucidation of the CD28/CTLA4 and B7 interactions was reported 15 years ago (Linsley, Brady, Grosmaire et al. 1991; Linsley, Brady, Ürnes et al. 1991). When compared to antiligand mAbs, the receptor-Ig chimera approach could present advantages for studies involving unknown ligands, receptors that recognize a multitude of ligands and agonist studies. For example, individual NK cells express diverse combinations of cell surface receptors, including members of the killer cell immunoglobulin (Ig)-like receptor (KIR) family that interact with different human leukocyte antigen (HLA) class I alleles. Therefore, it is often difficult to assign defined ligand specificities to any given KIR on an NK cell. This difficulty can be circumvented by using an assay that detects direct binding of soluble forms of individual KIR to transfected cell lines that express single HLA class I allotypes (Winter and Long 2000). Another approach is the employment of NKG2D-Ig to analyze the expression of the whole array of ligands to the NK activating receptor, NKG2D, on target cells (Cerwenka and Lanier 2003).

The utilization of recombinant proteins including receptor-Ig molecules for scientific research and biotechnology industry is associated with the concern for proper glycosylation (Jenkins et al. 1996). The importance of the posttranslational modification of proteins with N- or O-linked oligosaccharides is well documented by their implication in numerous biological phenomena (Gabius 2006; Rudd, Elliott et al. 2001). Membranal receptors are essentially glycoproteins. Apart from the 3D-domain structure that governs its decoration with glycans, the tissue or cell type that synthesizes a glycoprotein also plays an important role in the phenomenon of microheterogeneity of protein glycans (Rudd and Dwek 1997). Among the eukaryotic cell lines often used in research and biotechnology industry for the production of recombinant glycoproteins are CHO, BHK-21, NSO, COS, 293T, and recently the PerC6 cell line (Lewis et al. 2006; Werner et al. 1998). The recombinant glycoproteins produced by the pharmaceutical industry are analyzed regularly for their glycan content in accordance with FDA regulations. However, insufficient analysis of glycan content is performed for recombinant glycoproteins produced solely for research purposes. This is correct for commercially and laboratory-produced reagents.
Recognition of heparan sulfate by NKp30

It is clear that different glycosylation patterns of recombinant glycoproteins produced in different conditions or cell lines can lead to discrepancies in the observed functional results.

NKp30 is a natural cytotoxicity receptor expressed by NK cells and involved in NK cytotoxicity (Pende et al. 1999). Two studies were published with contradictory results with regard to NKp30-putative ligands recognized by recombinant human NKp30-Ig (rNKp30-Ig) glycoprotein. In the paper by Bloushtain et al. (2004), rNKp30-Ig recognized heparan sulfate (HS), while the paper by Warren et al. (2005) clearly showed that rNKp30-Ig did not recognize HS. We aimed to understand this biochemical discrepancy. We compared the rNKp30-Ig produced by these two studies as well as three other NKp30-Igs and recombinant NKp30-ILZ trimeric protein. Four out of six rNKp30s recognized HS. One rNKp30 that did not recognize HS was the rNKp30-Ig produced in NSO cells that manifested a multitude of high molecular weight N-glycoforms. Enzymatic removal of this excessive N-glycosylation restored HS recognition by the NSO-produced rNKp30-Ig. 293T-produced NKp30-Ig manifested an excess of complexed glycans bearing sialic acid, and removal of its N-glycans restored its direct binding to heparin. Therefore, we show that rNKp30 does bind to heparin/HS and that proper glycosylation of rNKp30 is essential for that binding.

Materials and methods

Cells

Cell lines used in this work are as follows: HeLa—a human cervical adenocarcinoma line (ATCC no. CCL-2), HEK293—a human embryonal kidney line (CRL-1573), CHO K1—a Chinese hamster ovarian line (CCL-61). The CHO-mutant derivative CHO pgsA-745 has been characterized in detail elsewhere (Esko 1991). A549 is a human lung carcinoma line (CCL-185). COS-7 is an African green monkey kidney fibroblast-like line expressing the SV40 large T antigen (CRL-1651). The 293T line was derived from SV40 large T antigen-transfected HEK293 cells (Rio et al. 1985). NK-92 (CRL-2407) is a human natural killer lymphoma.

Ig fusion proteins

The generation of NKp30-Ig in COS and CHO was previously described (Arnon et al. 2001; Bloushtain et al. 2004). In brief for COS-NKp30-Ig: the sequence encoding the extracellular portions of NKp30 (accession number AJ223153, protein id CAB54004) was amplified by PCR from cDNA isolated from human NK clones. The PCR-generated fragment was cloned into a mammalian expression vector containing the Fc portion of human IgG1 (Mandelboim et al. 1999; Arnon et al. 2001). COS-7 cells were transiently transfected with the plasmid containing the NKp30-Ig cDNA, and supernatants were collected and purified on a Poros 20 protein G column in the High Pressure Perfusion Chromatography Station, BioCAD (PerSeptive Biosystems Inc., Framingham, MA). In brief for CHO-NKp30-Ig, the corresponding PCR fragments encoding the extracellular portions of NKp30, Kozak consensus sequence, and leader sequence of CD5 were cloned into a pcDNA3.1-Ig vector (Bloushtain et al. 2004). CHO cells were transfected with these expression vectors, and G418-selected clones were screened for highest protein production. Recloned high producer clones were grown in BIO-CHO-1 medium (Biological Industries, Kibbutz Beit Haemek, Israel), and supernatants were collected daily and purified on protein G columns using FPLC. Production of Ig fusion proteins in COS and CHO cells did not affect their binding phenotype to tumor cells (Arnon et al. 2004). Sodiumdodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis revealed that all Ig fusion proteins were approximately 95% pure and of the proper molecular mass. A549 cells were stably transfected using FuGene 6 (Roche Diagnostics, Mannheim, Germany) with the above-described CD5 leader/NKp30 ectodomain/hIgG1 cDNA subclone into a modified pHBAPr-1-neo expression vector (Momburg et al. 1996) and selected with 1.0 mg/mL G-418 sulfate (Sigma-Aldrich, Taufkirchen, Germany). NKp30-Ig was purified from the culture supernatants stably transfected A549 cells using protein A Sepharose beads (Amersham BioSciences, Freiburg, Germany). 293T cells were transiently transfected with the NKp30-Ig cDNA subclone into the vertebrate expression vector pMT2+ncs (a gift of Dr. J. Neeffjes, NKI, Amsterdam, The Netherlands) using the calcium phosphate method. NKp30-Ig fusion protein was purifed using protein-A agarose beads (Merck BioSciences, Darmstadt, Germany). The production of the ILZ fusion proteins was previously described (Stark et al. 2005). NSO-produced NKp30-Ig was purchased from R&D Systems, Minneapolis, MN (batchs HG001 and HG002). The generation of leukocyte Ig-like receptor (LIR)-1 Ig fusion protein was previously described (Achdout et al. 2003).

Glycosaminoglycans, selectively desulfated heparins, and heparin fragments

Heparin (H-3400, H-4784), HS (H-9902) and chondroitin sulfate C (C-4384) were purchased from Sigma-Aldrich, St. Louis, MO. Selectively desulfated heparins were described previously (Ricard-Blum et al. 2004). Briefly, heparin initially contained 97.7% of the N-sulfate groups, 89.3% of the 2-O-sulfate groups, and 92.4% of the 6-O-sulfate groups. De-N-sulfated/re-N-acetylated heparin contained 90.5% of the 2-O-sulfate groups, 85.3% of the 6-O-sulfates, and a very low quantity of the remaining N-sulfate groups (2.4%). De-2-O-sulfated heparin contained 80.2% of the 6-O-sulfate groups, 91.4% of the N-sulfate groups, and a residual 2.2% of the 2-O-sulfates. De-O-sulfated heparin contained 98.2% of the N-sulfate groups, 54.7% of the 2-O-sulfate groups, and 4.2% of 6-O-sulfate groups. The selectively desulfated heparins were not subject to any depolymerization procedures so their average molecular weight is the same as the native heparin (approximately 15 kDa). Heparin fragments (6, 8, 10, 12, 14, 16-mers) were described previously (Goger et al. 2002).

Separation of NKp30-Ig by SDS-PAGE gel

Ten microgram of each protein were mixed with protein sample loading buffer. Samples designated as (+β-ME) were further mixed with β-mercaptoethanol (final concentration 2%) and denatured at 95°C for 5 min. Samples designated as (-β-ME) were loaded without a denaturation step. All samples were loaded on a 10% acrylamide gel and run in a Bio-RAD protein electrophoresis apparatus at 100 V for approximately 2 h. Then the gel was stained with Coomassie Blue.

Western blots

Ten microgram of each protein were loaded on 10% acrylamide gel. Gel was run as described above. The polypeptides were
transferred to a nitrocellulose blotting membrane (Sartorius AG, Gottingen, Germany) in a BIO-RAD blotting apparatus at a 200 V for 1 h. The membrane was blocked with 1% BSA, 0.05% Na-Azide, 0.5% Tween in PBS for 1 h at RT. Incubation with V for 1 h. The membrane was blocked with 1% BSA, 0.05% Tween 20 (HBS buffer), at a flow rate of 10 \(\mu\)L/min. For activating the chip, EDC/NHS amine coupling procedure was used according to BIACore protocol (www.biacore.com). Ten \(\mu\)g/mL neutravidin (Pierce, Rockford, IL) in 10 mM acetate (pH = 4) was immobilized to give 2000RU on each of the FC’s. Immobilization was followed by blocking the free active groups with 1 M ethanolamine. Following neutravidin binding, Heparin-biotin (10 \(\mu\)g/mL) was injected in 10 mM sodium acetate buffer pH 3 to give 20RU on Fc2; Fc1 served as a control and was subtracted from the responses obtained from the Fc with bound heparin. Kinetic measurements were performed at a flow rate of 20 \(\mu\)L/min in reduced-salt HBS buffer (30 mM NaCl) at 25°C. Different anlyte concentrations (for CHO-NKp30-Ig with or without PNGase: 0, 23, 47, 94, 187, 375, and 750 nM; for 293-NKp30-Ig with PNGase 0, 31.25, 62.5, 125, 250, 500, and 1000 \(\mu\)M; for 293-NKp30-Ig without PNGase 0, 93.75, 187.5, 375, 750, 1500, 3000, and 6000 nM) were injected, each followed by regeneration of the surface using 10 mM NaOH. Data were analyzed using 1:1 Langmuir binding model. The \(\chi^2\) values were less than 2.

**Flow cytometry and antibodies**

Cells were incubated with indicated micrograms of the various fusion-Igs for 2 h at 4°C, washed, and stained with APC-conjugated-F(ab')2 goat-anti-human-IgG-Fc (109-136-098, Jackson Immuno Research, West Grove, PA). In FM’s lab, cells were incubated with 1 \(\mu\)g NKp30-Ig for 45 min at 4°C, followed by phycoerythrin-conjugated F(ab')2 goat-anti-human IgG Fc\(\gamma\) fragment-specific secondary antibodies (Dianova, Hamburg, Germany). Staining and washing buffer consisted of 0.5% (w/v) BSA and 0.05% sodium azide in PBS. Propidium iodide (PI) was added prior to reading for exclusion of dead cells. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and fluorescence data were acquired using logarithmic amplification. Data files were acquired and analyzed using BD CELLQuestTM 3.3 software. Fluorescence data were acquired using logarithmic amplification and reported fluorescence intensity units represent conversion of channel values according to the logaritmic scale (range 10^6 to 10^8). Results are shown either as staining histograms or overlays. In all experiments in AP’s laboratory, each sample was stained twice in different wells. The staining of cells with the ILZ fusion proteins was previously described (Stark et al. 2005). The anti-HS 10E4 was from USBiological (Swampscott, MA). Cells were treated with heparinase I or III (Sigma-Aldrich, Taufkirchen, Germany) using 1.5 mU/mL PBS supplemented with 1% BSA per 2 \(\times\) 10^6 cells for 60 min at 37°C followed by two washes with ice-cold buffer.

**BIACore analysis**

BIACORE 3000 (BIACORE AB, Uppsala, Sweden) was used for studying the interactions between heparin and recombinant NKp30. Data processing was done using BIACevaluation software 4.1. The running buffer for the immobilization process was 10 mM Hepes buffer pH = 7.4, 150 mM NaCl, and 0.005% Tween 20 (HBS buffer), at a flow rate of 10 \(\mu\)L/min. For experiments described in Figures 2 and 3, heparin-biotin (Sigma) was immobilized on a streptavidin (SA) chip (BIACORE AB); heparin-biotin (20 \(\mu\)g/mL) was injected in 0.1 M sodium acetate buffer pH = 3. The immobilization response ranged from 20 to 80 resonance units (RU) in experiments involving different chips. For regeneration of surface we employed 2 M NaCl with 20 mM NaOH. Kinetic measurements were performed at a flow rate of 20 \(\mu\)L/min in HBS buffer at 25°C. An empty flow cell was used as a control and its measurement was subtracted from the heparin flow cell. For inhibition experiments, NKp30-Ig were premixed with the indicated \(\mu\)g/mL of selectively mono-desulfated heparins for 1 h at room temperature prior to injection.

Experiments described in Figure 7 were carried out with a sensor chip CM5, at 25°C. Running buffer was HBS and the flow rate was 10 \(\mu\)L/min. For activating the chip, EDC/NHS amine coupling procedure was used according to BIACore protocol (www.biacore.com). Ten \(\mu\)g/mL neutravidin (Pierce, Rockford, IL) in 10 mM acetate (pH = 4) was immobilized to give 2000RU on each of the FC’s. Immobilization was followed by blocking the free active groups with 1 M ethanolamine. Following neutravidin binding, Heparin-biotin (10 \(\mu\)g/mL) was injected in 10 mM sodium acetate buffer pH 3 to give 20RU on Fc2; Fc1 served as a control and was subtracted from the responses obtained from the Fc with bound heparin. Kinetic measurements were performed at a flow rate of 20 \(\mu\)L/min in reduced-salt HBS buffer (30 mM NaCl) at 25°C. Different anlyte concentrations (for CHO-NKp30-Ig with or without PNGase: 0, 23, 47, 94, 187, 375, and 750 nM; for 293-NKp30-Ig with PNGase 0, 31.25, 62.5, 125, 250, 500, and 1000 \(\mu\)M; for 293-NKp30-Ig without PNGase 0, 93.75, 187.5, 375, 750, 1500, 3000, and 6000 nM) were injected, each followed by regeneration of the surface using 10 mM NaOH. Data were analyzed using 1:1 Langmuir binding model. The \(\chi^2\) values were less than 2.

**HPLC analysis of N-linked glycans**

The various NKp30-Ig recombinant proteins were separated on a SDS–PAGE as specified above. N-Linked glycans were released from gel slices by incubation with PNGase F (Kuster et al. 1997). Labeling, high-performance liquid chromatography (HPLC), and simultaneous exoglycosidase sequencing of the released glycan pool were performed as described (Rudd, Colominas et al. 2001). Exoglycosidases were used at the following concentrations: *Arthrobacter ureafaciens* sialidase (ABS, EC 3.2.1.18), 1–2 U/mL; Bovine testes β-galactosidase (BTG, EC 3.2.1.23), 2 U/mL; Bovine kidney β-fucosidase (BKF, EC 3.2.1.51) 1 U/mL; and 1–2 U/mL *Streptococcus pneumoniae* α-hexosaminidase (SPH, EC 3.2.1.30). All exoglycosidase enzymes were purchased from Prozyme, San Leandro, CA.

**Matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometry of released glycans**

After removing traces of acrylamide and Coomassie stain from the SDS–PAGE gel bands by means of washing glycosidases through a Micropure-EZ centrifugal filter device (Millipore, Bedford, MA), underivatized glycans were purified using a Nafion 117 membrane (Aldrich Chemical Co. Ltd., Poole, Dorset, UK). The glycans were analyzed by MALDI mass spectrometry in positive ion mode with a Bruker REFLEX-IV instrument (Bruker Daltonics, Bremen, Germany). A nitrogen laser VSL-337ND (Laser Science, Inc., Franklin, MA) with an emission wavelength at 337 nm and 4 ns pulse duration was used. Samples (0.3 \(\mu\)L in water) were mixed with a saturated solution of 2,5-dihydroxybenzoic acid on the MALDI target and allowed to dry at room temperature. Each sample was then recrystallized from ethanol. The MALDI instrument was calibrated with dextran oligomers. Monoisotopic masses of the [M+Na]^+ ions were within 0.1 mass units of the calculated values.

**Treatment of NKp30 with PNGase F**

NKp30-Ig from different cells (35 \(\mu\)g in 75 \(\mu\)L PBS) was treated with 5 U of PNGase F (Roche, Cat. No. 116365185001). The
NKp30-Ig protein was incubated with PNGase F for 4 days at 4°C.

*Anti-NKp30 activation of NK-92*

U-bottom 96-well plates were precoated with 0.5 µg/mL anti-NKp30 mAb (R&D Systems, MAB18491) for 3 h at 37°C (diluted with PBS, final volume 100 µL). After 3 h at 37°C, wells were washed three times with 200 µL PBS before cells were added. While incubating the wells with the Ab, NK-92 cells were preincubated in the experimental culture medium (supplemented with 20 U/mL human rIL-2 instead of 100 U/mL human rIL-2) and supplemented with HS or with chondroitin sulfate A (as control) for 2.5 h at 37°C. A total of 4 × 10^4 NK-92 cells/well in 200 µL medium were incubated for 24 h at 37°C. IFNγ concentrations in the supernatants were then assayed by standard ELISA according to the manufacturer’s instructions (BioLegend, San Diego, CA).

**Results**

**HS-dependent binding of recombinant NKp30 to tumor cells**

We previously reported that membranal HS on tumor cells serves as a ligand or coligand for the binding of NKp30 (Bloushtain et al. 2004). We showed that recombinant NKp30-Ig, produced in CHO cells, binds tumor cells in an HS-dependent manner (Bloushtain et al. 2004). Warren et al. published that recombinant NKp30-Ig, produced in CHO, COS, and A549, and NKp30-ILZ trimer) manifested HS-dependent binding to tumor cells (Figure 1). To investigate whether the HS-dependent binding to tumor cells correlates with direct binding of recombinant NKp30 to heparin to tumor cells (Figure 1E). Thus, we confirm that NSO-NKp30-Ig does not bind in an HS-dependent fashion to tumor cells (Bloushtain et al. 2004).

We further compared CHO-NKp30-Ig binding to tumor cells and to immobilized heparin by testing the ability of selectively desulfated heparins to inhibit binding of CHO-NKp30-Ig to:

### Table I. Recombinant NKp30s used in this study

<table>
<thead>
<tr>
<th>Producer lab</th>
<th>Producing cells</th>
<th>Transfection method</th>
<th>Protein sequence</th>
<th>HS-dependent binding to tumors</th>
<th>Direct binding to heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>CHO</td>
<td>Constant</td>
<td>CD5 leader-CAB54004-Fc hlgG1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>OM</td>
<td>COS</td>
<td>Constant</td>
<td>CD5 leader-CAB54004-Fc hlgG1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FM</td>
<td>A549</td>
<td>Constant</td>
<td>CD5 leader-CAB54004-Fc hlgG1</td>
<td>Yes</td>
<td>Not tested</td>
</tr>
<tr>
<td>FM, AP</td>
<td>293T</td>
<td>Transient</td>
<td>CD5 leader-CAB54004-Fc hlgG1</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CW</td>
<td>293T</td>
<td>Transient</td>
<td>lgk leader-Prot. CAB54004-ILZ-His tag</td>
<td>Yes</td>
<td>Not tested</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>NSO</td>
<td>Constant</td>
<td>CD33 leader-CAA75065-Fc hlgG1</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

To complete the study we purchased NSO-produced NKp30-Ig from R&D Systems. We studied both batches that R&D Systems produced—the HGD01 and the newer batch HGD02. HGD02 was used by Warren et al. for the studies described in (Bloushtain et al. 2004) and manifested nonHS-dependent binding to tumor cells. Similarly, HGD02 was shown by Dr. Warren to have the same nonHS-dependent binding to tumor cells (Dr. H. Warren, Australian National University, Canberra, Australia, personal communication). Indeed, mixing NSO-NKp30-Ig with soluble heparin did not inhibit its binding to tumor cells (Figure 1E). Thus, we confirm that NSO-NKp30-Ig does not bind in an HS-dependent fashion to tumor cells (Bloushtain et al. 2004).

Representative examples from various staining experiments performed are shown in Figure 1. Assays to test HS-dependent binding of rNKp30s to tumor cells were: (i) staining with rNKp30s of CHO-pgsA-745 cells that lack HS expression as compared to parental HS-positive CHO cells, (ii) staining of heparinase-treated and nontreated cells, and (iii) staining of tumor cells in the presence of heparin/HS or chondroitin sulfate. Parallel experiments were performed independently in two different laboratories (AP and FM). For CHO-NKp30-Ig, COS-NKp30-Ig, A549-NKp30-Ig, and NKp30-ILZ results were as follows (Figure 1 and data not shown): (i) rNKp30s stained CHO-pgsA-745 cells to a lesser extent compared to parental, HS positive, CHO cells; (ii) rNKp30s stained heparinase-treated CHO-K1 cells to a lesser extent compared to nontreated cells; and (iii) binding of rNKp30s to tumor cells was inhibited by heparin/HS, but not by chondroitin sulfate, while heparin/HS did not affect LIR1-Ig binding to the tumor cells. Therefore, four of the five rNKp30s tested (three NKp30-Igs, produced in CHO, COS, and A549, and NKp30-ILZ trimer) manifested HS-dependent binding to tumor cells (Figure 1). 293T-NKp30-Ig did not manifest consistent HS-dependent binding to tumor cells (Figure 1B).

To investigate whether the HS-dependent binding to tumor cells correlates with direct binding of recombinant NKp30 to heparin, we examined in vitro binding of the rNKp30s to their purified putative ligands on a solid matrix. We studied the kinetics of rNKp30 binding to heparin using the BIAcore analysis system. Biotinylated heparin was immobilized on a streptavidin chip, and different rNKp30s concentrations were injected over the heparin-immobilized surface. We compared CHO-produced-NKp30-Ig and NSO-produced-NKp30-Ig. Clear binding of the CHO-NKp30-Ig analyte to immobilized heparin at 200 to 700 nM concentrations is shown in Figure 2A. The NSO-NKp30-Ig analyte showed no binding at a concentration of 700 nM. Therefore, direct binding to heparin was correlated with HS-dependent binding to tumor cells: CHO-NKp30-Ig interacted with HS on tumor cell membranes and directly bound to heparin, while NSO-NKp30-Ig showed no binding either to membranal HS or to heparin.
Fig. 1. (A) Immunofluorescence staining of CHO-K1 (left) and the HS-deficient CHO mutant pgsA-745 (right) using NKp30-Ig produced in A549 cells or the anti-HS mAb 10E4, respectively (dotted lines, second Ab control; black lines, untreated cells; gray lines, cell treated with heparinase III). (B) Immunofluorescence staining of CHO-K1 (left) and the HS-deficient CHO mutant pgsA-745 (right) using NKp30-Ig fusion proteins produced in CHO cells (black), COS-7 cells (dark gray), and 293T cells (light gray). Dotted lines mark second Ab control. (C) Immunofluorescence staining of CHO-K1 (left) and the HS-deficient CHO mutant pgsA-745 (right) using NKp30-Ig fusion protein (black) or secondary reagents only (gray). (D) Staining of untreated (black) or heparinase I-treated (gray) HEK293 cells with NKp30-ILZ. Dotted lines mark secondary reagents only. (E) Staining of HeLa cells with 4 µg of rNKp30-Igs and LIR1-Ig in the presence of HS, chondroitin sulfate C (CS-C) or no glycosaminoglycan (control). Concentrations of glycosaminoglycans are 1 µg/mL. Results are presented as overlay of primary histograms.

(i) HeLa tumor cells as measured by flow cytometry (Figure 2B). HeLa cells were incubated with CHO-NKp30-Ig mixed with selectively desulfated heparins, or normally sulfated heparin and HS for comparison, followed by fluorophor-labeled anti-human Fc.

(ii) Immobilized heparin as measured by BIAcore (Figure 2C). Desulfated heparins were incubated for 1 h with CHO-NKp30-Ig before injection over immobilized heparin. The inhibitory capacity of selectively desulfated heparins was compared to that of normally sulfated heparin.

In both experimental setups, a strong inhibition of CHO-NKp30-Ig binding (either to HeLa cells or to immobilized heparin) induced by a particular desulfated heparin indicated that
Recognition of heparin sulfate by NKp30

The removed sulfate groups were not involved in the heparin/HS interaction and vice versa. Results from the effects of desulfated heparins on direct CHO-NKp30-Ig binding to heparin were compatible with the results obtained for their binding to tumor cells (Figure 2B, C); N-Sulfation effectively contributed to the interaction of CHO-NKp30-Ig with either tumor cells or immobilized heparin. The contribution of 6-O- and 2-O-sulfation was also considerable, but less noteworthy than N-sulfation. N-Acetylation of the N-desulfated heparin did not improve the inhibitory activity significantly; thus, N-acetyl groups might not contribute to the studied interactions. The normally sulfated heparin and the mono-desulfated heparins were of the same size (approximately 15 kDa). Therefore, the results of the competition assays (Figure 2B, C) were not affected by the size of the heparins.

We then tested the minimal length of heparin fragments that could inhibit binding of NKp30 to tumor cells. We tested 6- to 16-mers of normally sulfated heparin. Six-mers did not inhibit NKp30-Ig binding while eight-mers manifested a small degree of inhibition. Ten- to sixteen-mers showed increased inhibition in correlation with the size of the heparin fragments (Figure 2D).

Heparin/HS epitopes recognized by NKp30 and NKp46 are cross-reactive, yet differ from epitopes recognized by other heparin-binding receptors

We previously published that NKp46 and NKp30 recognize heparin/HS (Bloushtain et al. 2004; Zilka et al. 2005). NKp44, an additional member of the NCR family, also recognizes heparin/HS epitopes (Hershkovitz et al. 2007). To reveal whether the three NCRs share crossreactive or different epitopes on heparin/HS, a series of experiments were conducted using the BIAcore system. The experimental approach was based on the study by Rauchenberger et al. (2003). Biotinylated heparin was immobilized on a streptavidin chip as described in the Materials and methods section, and saturating concentrations per each NCR-Ig were predetermined (10 µM, 1 µM, and 8 µM for NKp30, NKp44, and NKp46D2, respectively). Each NCR-Ig (CHO-NKp30-Ig, NKp44-Ig, and NKp46D2-Ig) was injected at saturating concentration and volume of 60 µL at a rate of 20 µL/min. The first injection was immediately followed (without regeneration) by a second injection (at the above conditions) with the same or with different NCR-Ig. Under saturating NCR-Ig concentrations, successive injections without regeneration of the same NCR-Ig should not result in additional RU increase due to full binding site occupancy after the first injection. Indeed, two consecutive injections at saturating concentrations of the same NCR-Ig did not give any noteworthy rise in RU (Figure 3A1, B1, and C1), indicating a stable equilibrium reached by the first injection.

When different NCR-Igs are injected in the first and second injections (under saturating conditions), an additional increase in the binding signal following the second injection could occur if the second protein recognized different epitopes. Yet, the signal observed after the second injection is the result of the combined association of the second protein and dissociation of the first one, which complicates interpretation of the results. $K_{on}$ and $K_{off}$ of the different NCR-Igs are depicted in Figure 5A.
Fig. 3. Binding of NCR-Ig to immobilized heparin after injecting saturated concentrations of different NCR-Igs. Biotin-tagged-heparin was immobilized on a BIAcore streptavidin chip. NKp30-Ig (A), NKp44-Ig (B), and NKp46D2-Ig (C) were injected (60 µL at a flow rate of 20 µL/min) over the heparin in pretested saturating concentrations (10 µM, 1 µM, and 8 µM, respectively). This was immediately followed by an additional injection (60 µL at a flow rate of 20 µL/min) of the same NCR-Ig (left panels) or different NCR-Igs (middle and right panels) in saturating concentrations. Arrows indicate the time point of the additional injection. BIAcore analysis results are expressed in resonance units (RU) against time. Curves in all panels show specific binding after subtraction of background binding to the control flow cell (no heparin immobilized).

of the supplemental data. Successive injections of saturating concentrations of NKp30-Ig after NKp46D2-Ig (Figure 3C2) and NKp46D2-Ig after NKp30-Ig (Figure 3A2) did not result in significant alteration of RU. A plausible interpretation for these results is that NKp30 and NKp46 share a crossreactive epitope on heparin. $K_{on}$ of NKp30-Ig and NKp46D2-Ig are similar ($5.58 \times 10^3$ and $7.23 \times 10^3$, respectively), while $K_{off}$ of NKp30-Ig is one log lower ($2.65 \times 10^{-4}$ versus $2.96 \times 10^{-3}$). The slight increase observed in Figure 3A2 might be attributed to this difference. When NKp44-Ig was injected after NKp30-Ig or NKp46D2-Ig, in both cases there was a significant rise in RU (Figure 3A3, C3), suggesting that a complete occupancy of heparin binding sites by NKp30 or NKp46 does not interfere with binding of NKp44. Injection of NKp46D2-Ig or NKp30-Ig after NKp44-Ig (Figure 3B2, B3) resulted in reduction of RU, which can be indicative of a higher frequency of epitopes recognized by NKp44 compared to those recognized by NKp30 and NKp46. Alternatively, although NKp44 recognizes a different epitope than NKp30 and NKp46, it still interferes with the binding of the other two. This can be explained if the different epitopes are close, so that binding of NKp44 first can hinder the other sites.

To summarize, a plausible interpretation of the results is as follows. Binding NKp30 first blocked the ability to bind NKp46 and vice versa, while binding either NKp30 or NKp46 did not block binding of NKp44. Therefore, we could assume that heparin epitope(s) recognized by NKp44 differ from those recognized by NKp30 or NKp46. Moreover, it seems likely that the epitopes recognized by NKp30 and NKp46 are overlapping.

**Binding differences between NSO- or 293T-NKp30-Ig and other recombinant NKp30-Igs are due to altered glycosylation**

NSO-NKp30-Ig did not show HS-dependent binding to tumor cells while other rNKp30s did (Figure 1, Bloushtain et al. 2004; Warren et al. 2005). Consistently, CHO-NKp30-Ig showed direct binding to immobilized heparin while NSO-NKp30-Ig did not (Figure 2). We aimed to determine the reason for this phenotypic discrepancy between the different recombinant NKp30-Igs. The amino acid (AA) composition of the different NKp30-Igs is detailed in Figure 4A. For all of the rNKp30-Igs, the NKp30 ectodomain and Fc sequences were identical. Minor AA sequence differences are located within the N-terminus and NKp30-Ig fusion segment (Figure 4A). The NKp30-ILZ fusion protein includes an Igκ leader, the NKp30 ectodomain (accession number CAB54004), and isoleucine-zipper sequences. This construct also manifested the HS-dependent binding phenotype (Figure 1). To summarize, the small AA sequence differences
between the rNKp30-Igs were unlikely to account for any consensus heparin/HS-binding motifs (Hileman et al. 1998).

We then assessed the quality of CHO-, COS-, and NSO-NKp30-Igs on SDS-PAGE in the presence or absence of β-mercaptoethanol (Figure 4B). The results show a similar pattern for CHO- and COS-NKp30-Ig, with no significant degradation and mostly as a dimeric form (lanes without β-mercaptoethanol). In contrast, the NSO-NKp30-Ig batches were of low quality, showing partial degradation (more extensive in the HGD01 batch) and a higher monomeric fraction (more significant in the HGD02 batch). Western blot of NSO-NKp30-Ig (HGD01) and CHO-NKp30-Ig with anti-NKp30 mAb (AF1849, R&D Systems) shows that the additional bands in the NSO-NKp30-Ig lane are indeed degraded or diversely glycosylated forms of NKp30 (Figure 4C). A monomeric versus dimeric state of the Ab-like fusion protein could play a significant role in low-affinity interactions as observed for the rNKp30–heparin interaction (Figure 2B). A549-NKp30-Ig migrated similarly to COS- and CHO-NKp30-Ig (data not shown). The AA-based predicted MW of the different NKp30-Igs is nearly identical (42.0 to 42.8 kDa, without the leader sequence). Therefore, the higher molecular weight of the NSO-NKp30-Ig compared to the CHO-NKp30-Ig was likely due to different glycosylation.

We therefore investigated the glycosylation of the various rNKp30-Igs. The NKp30 ectodomain has two predicted N-glycosylation sites (N42 and N121) and the Fc part contains an additional conserved N-glycosylation site. O-Glycosylation sites are not predicted in the sequence of the NKp30 ectodomain or the Fc part. The NP-HPLC profiles of glycans released from NKp30-Igs are shown in Figure 5 (and in Figures 1–3 in the supplemental data). The NKp30-Igs produced in COS and CHO cell lines had a relatively small variety of glycans that include sialic acid (estimated 30.31% and 54.48%, respectively). The 293T-produced NKp30-Igs had a wider variety of glycans that include sialic acid and the overall quantity of glycans that include sialic acid was 50.31%. The size of the sialylated glycans in the 293T-produced NKp30-Igs was relatively larger (compared to COS and CHO) indicating the presence of a higher amount of sialic acid per glycan. The A549-produced NKp30-Ig manifested a sialylated glycan profile in the range between CHO and COS to 293T and the quantity of glycans that included sialic acid 41.95% (Figure 4 in supplemental data and data not shown). A549-NKp30-Ig did bind in an HS-dependent manner (Figure 1A) but to a lesser extent than CHO- and COS-NKp30-Ig (Figure 1B, E, and data not shown); this is correlated with the presence of some relatively larger sialylated glycans in A549-NKp30-Ig, similar to those manifested by 293T-NKp30-Ig that did not bind in an HS-dependent manner (Figure 1B).

The NSO-NKp30-Ig showed a totally different glycan profile. It had an even wider variety of glycans that includes terminal sialic acids, but also a variety of additional α-linked galactose and probably additional fucoses as can be seen from the MALDI-TOF analysis. The mass spectrometric data of asialylated glycans detected from the different NKp30-Ig samples are listed in Figure 4 in the supplemental data. For the NSO-NKp30-Ig, the quantity of large glycans (above asialylated biantennary glycans including tri- and tetra-antennary glycans) reached a total of 86.13% of its total glycan pool. Of this, 41.83% contained sialic acid. The excessive glycosylation and the presence of α-linked galactose are rather understandable since NSO is derived from a mouse myeloma; thus, NSO-derived recombinant proteins contain a considerable fraction of murine-specific glycan motifs such as Galα1, 3Galβ1, 4GlcNAc (Baker et al. 2001).

To further verify the involvement of glycosylation in the HS-binding differences, we treated NSO- and CHO-NKp30-Ig with PNGase F, which removes the N-glycans from glycoproteins. PNGase F-treated NSO-NKp30-Ig and PNGase F-treated CHO-NKp30-Ig migrated similarly, in contrast to the nontreated proteins (Figure 6A). Thus, the N-glycosylation was indeed the reason for the difference in the apparent MW of NSO-NKp30-Igs compared to the other NKp30-Igs. We then stained HeLa cells with PNGase-treated and nontreated CHO- and NSO-NKp30-Ig. In general, the NSO-NKp30-Ig manifested very low binding to tumor cells when compared in the same staining con-
manifested HS-dependent binding to tumor cells, compared to nontreated NSO-NKp30-Ig (Figures 6B2 and 1E). Tumor binding by PNGase F-treated NSO-NKp30-Ig was low compared to F-treated CHO-NKp30-Ig (Figure 6B1, B3). This could stem from the poor quality of the commercial NSO sample (Figure 4B), or from changes in conformation due to different glycosylation during the final folding of the protein in the ER-Golgi (Molinari, 2007). To summarize, the aberrant glycosylation of NSO-NKp30-Ig seems to suppress its HS-dependent binding to tumor cells. This could be due to the relatively large size of the sialylated glycans on the NSO-NKp30-Ig possibly interfering with noncovalent interactions between amino acids of the NSO-NKp30-Ig fusion protein and HS.

**PNGase F treatment of 293T-NKp30-Ig induces its direct binding to heparin**

The limited source of commercial NSO-NKp30-Ig and its poor quality motivated further study of the direct interaction between heparin and deglycosylated NKp30 with the 293T-NKp30-Ig, and CHO-NKp30-Ig proteins. 293T-NKp30-Ig was prepared from the same construct by two different groups (FM and AP laboratories) and did not manifest HS-dependent binding to tumor cells (Table I, Figure 1B, and the inset in Figure 7A). To estimate the affinity between heparin and recombinant NKp30s, we injected increasing concentrations of PNGase F-treated and nontreated recombinant NKp30s over immobilized heparin (Figure 7). No binding was observed for 293T-NKp30-Ig (Figure 7A) and this could not be attributed to the poor quality of the fusion protein (data not shown) or to poor staining (Figure 7A, inset). Rather, it was correlated with the existence of complexed glycans involving sialic acids (Figure 5D). Indeed, for PNGase F-treated 293T-NKp30-Ig, results show specific binding and receptor limitation according to our test. For nontreated and PNGase F-treated CHO-NKp30-Ig, results show specific binding and $K_p$ was 4.75 $\times$ 10$^{-8}$ M ($\chi^2 = 1.61$). There was no mass transfer limitation according to our test. For nontreated and PNGase F-treated CHO-NKp30-Ig, results show specific binding and $K_p$ was 4.75 $\times$ 10$^{-8}$ M ($\chi^2 = 0.445$) and 1.28 $\times$ 10$^{-8}$ M ($\chi^2 = 1.96$), respectively (Figure 7C, D). Therefore, PNGase F-treatment of 293T-NKp30-Ig induced binding to heparin yet did not restore it to the level manifested by CHO-NKp30-Ig. This could be attributed to nonreversible glycans structure-dictated changes in protein folding that take place in the ER-Golgi (Molinari, 2007).

Binding of the dimer NKp30-Ig to its ligand is expected to be in the ratio of 1:2; each NKp30-Ig binds to two sites on one heparin molecule. Yet, the $K_p$ values for CHO-NKp30-Ig calculated by the 1.1 Langmuir and steady-state models were similar (4.75 $\times$ 10$^{-8}$ M and 4.08 $\times$ 10$^{-8}$ M, respectively; Figure 5 in supplement data for the steady state model). Moreover, calculating $K_p$ using the 1.1 model showed good fit between the calculated and experimental results; thus, it can be concluded that one molecule of NKp30-Ig binds only one molecule of heparin. The $K_D$ value calculated for the heparin/HS binding site in NKp30 is in the range of $K_D$ reported for heparin/HS binding sites of other proteins (e.g., 39 nM for FGFR2, 4 $\mu$M for the major binding site of Tenacin-C, 2 $\mu$M for endostatin, and 3.2 $\mu$M for FGFR1) (Ibrahim et al. 2004; Jang et al. 2004; Ricard-Blum et al. 2004).
Recognition of heparan sulfate by NKp30

Fig. 6. Effect of rNKp30-Ig glycosylation on HS-dependent binding to tumor cells. NSO-NKp30-Ig and CHO-NKp30-Ig were pretreated with PNGase F for 3 days in PBS before being used for FACS staining of HeLa cells in the presence of HS or chondroitin sulfate C (CS-C). (A) Coomassie staining of NKp30-Igs after treatment with PNGase F (+) or mock treatment (−). (B) Primary FACS histogram overlay of PNGase treated/mock-treated NSO- and CHO-NKp30-Ig staining of HeLa cells (B1 and B3). Staining of PNGase-treated NSO- and CHO-NKp30-Ig in the presence of HS, CS, or with no GAG (B2 and B4) and PNGase-treated NKp30-Igs (30 µg/mL, 3 µg/staining) were premixed with HS, CS-C (2 µg/mL), or with no GAG. Then, 10⁵ HeLa cells were added for 2 h at 4°C. HS concentration of 2 µg/mL is approximately 0.6 M. After incubation, cells were washed and incubated with APC-anti-Fc second antibody. PI was added to exclude dead cells. Results are presented as overlay of primary histograms. Results are representative of 3 independent experiments.

Elevated secretion of IFNγ in the presence of HS by NK activated with anti-NKp30 mAb

Lastly, we tested whether HS might influence NK cell activation through NKp30. We employed human natural killer cell line NK-92 that expresses functional NKp30. NK-92 cells were plated in wells that were precoated with an anti-NKp30 mAb. For some of the samples, the experimental culture medium was additionally supplemented with glycosaminoglycans (GAGs), either HS, heparin or chondroitin sulfate A. Twenty-four hours later, IFNγ concentration in the supernatants was assayed by standard ELISA (Figure 8). Incubation of NK-92 cells with anti-NKp30 mAb resulted in IFNγ secretion while resting cells did not secret detectable IFNγ. Significant enhancement of IFNγ secretion was observed for mAb-activated NK-92 cells cocultured with HS or heparin, but not with chondroitin sulfate A (Figure 8). However, anti-NKp30 activation was imperative, since coculturing with HS without anti-NKp30 did not induce IFNγ secretion by NK-92 cells (Figure 8).

Discussion

In the current study we (i) established that four different recombinant NKp30s do bind tumor cells in an HS-dependent manner (Figure 1, Table I), (ii) complemented these results by showing that rNKp30 (CHO-NKp30-Ig) interacts directly with heparin (Figures 2, 7), (iii) demonstrated similar characteristics of HS-dependent tumor binding and heparin binding of rNKp30 (Figure 2), (iv) established that the HS/heparin-dependent binding phenotype of NSO-NKp30-Ig and 293T-NKp30-Ig is obscured by their altered glycosylation (Figures 4–7), and (v) showed that soluble HS enhanced the secretion of IFNγ by NK-92 natural killer cells activated with anti-NKp30 monoclonal antibody (Figure 8). Ligands to NCRs may be expressed primarily as a consequence of cellular stress, activation, viral infection, or tumor transformation (Moretta et al. 2001). The interaction of NKp30 with HS epitopes on HSPGs can facilitate binding of the NKp30 to other, hitherto elusive, cellular ligands: for example, the HS epitopes can serve as coligands for NKp30 in a way similar to the heparin/HS interaction with growth factors and growth factor receptors or with lipid-binding proteins (Sasisekharan and Venkataraman 2000; Ornitz and Itoh 2001; Capila and Linhardt 2002). In this case, the HS epitopes recognized by NKp30 can serve as coligands for NKp30 in a way similar to the heparin/HS interaction with growth factors and growth factor receptors or with lipid-binding proteins (Sasisekharan and Venkataraman 2000; Ornitz and Itoh 2001; Capila and Linhardt 2002). In this case, the HS epitopes recognized by NKp30, NKp44, and NKp46 (Zilka et al. 2005; Hershkovitz et al. 2007) could be similar or identical for the three NCRs, while the primary cellular ligands could differ, which would explain the functional observations indicating different ligands for different NCRs (Moretta et al. 2000; Moretta and Moretta 2004). Binding of NKp30 and NKp46 to HS/heparin has similar characteristics (Figure 2B–D for NKp30 and (Zilka et al. 2005) for NKp46; Figure 3 for both). This indicates that the HS/heparin epitope recognized by NKp30 and NKp46 are crossreactive and, thus, NKp30 and NKp46 should have additional cellular ligand(s) that are different for the two NCRs. The HS/heparin epitope recognized by NKp44 could be different from the NKp30/46-recognized HS/heparin epitopes (Figure 3).

Untreated NSO-NKp30-Ig did not bind to tumor cells in an HS-dependent fashion (Figure 1E and Warren et al. 2005). Yet, removal of the excessive N-glycosylation for NSO-NKp30-Ig partially restored HS-dependent binding to tumor cells (Figure 6) as was observed for the other rNKp30s (Figure 1). Similarly, removal of the glycosylation for 293T-NKp30-Ig...
Fig. 7. Kinetics of NKp30-Ig/heparin interactions with and without PNGase F treatment. Panels show dose-dependent binding to heparin of mock-treated 293T-NKp30-Ig (panel A), PNGase F-treated 293T-NKp30-Ig (B), mock-treated CHO-NKp30-Ig (C), and PNGase F-treated CHO-NKp30-Ig (D). Fusion-Igs were injected over the heparin-immobilized surface at increasing concentrations (see the Materials and methods section). Mass transfer limitation was not observed. Curves in all panels show specific binding after subtraction of background binding to the control flow cell (no heparin immobilized). $K_{on}$ and $K_{off}$ rates: $5.58 \times 10^3$ and $2.65 \times 10^{-4}$ (mock-treated CHO-NKp30-Ig), $4.19 \times 10^3$ and $5.36 \times 10^{-5}$ (PNGase F-treated CHO-NKp30-Ig), and $0.335 \times 10^3$ and $8.19 \times 10^{-4}$ (PNGase F-treated 293T-NKp30-Ig). The results from the BIACore analysis are expressed in resonance units (RU) against time. Inset in panel A: Staining of HeLa cells with 4 µg of 293T-NKp30-Ig in the presence of heparin, chondroitin sulfate C (CS-C), or no glycosaminoglycan (control). Concentrations of glycosaminoglycans are 1 µg/mL. Results are presented as overlay of primary histograms.

partially restored direct binding to heparin (Figure 7). The non-complete restoration of HS-binding following deglycosylation could be attributed to different folding status caused by glycan structure-dictated changes in protein folding that take place in the ER-Golgi (Molinari, 2007). Indeed, comparison of deglycosylated CHO- and 293T-NKp30-Ig by nonreducing SDS–PAGE revealed that fraction of 293T-NKp30-Ig migrated differently compared to CHO-NKp30-Ig, thus possibly indicating a different folding (Figure 6 in the supplemental data).

That altered glycosylation could interfere with ligand binding of a glycoprotein is well established (Kaneko et al. 2006; Krapp et al. 2003). Interestingly, 293T-produced NKp46D2 and NKp44 retained their HS-dependent binding (Zilka et al. 2005; and data not shown). This could be attributed to the location of the glycans in the molecules; both N-glycosylation sites of NKp30 are located within the NKp30 domain, while all glycosylation sites of NKp44 are located in the NKp44 hinge region and not within the NKp44 domain. For NKp46, O- and N-glycosylation sites are located in the hinge region and only one O-glycosylation site is located within the NKp46 domain-2. We showed that the HS/heparin binding site is located within the NKp44 and NKp46D2 domains (Zilka et al. 2005; Hershkovitz et al. 2007). Therefore, the presence of altered glycosylation in the hinge region (and only short O-glycans in one site within the NKp46D2 domain) could have less interference on HS/heparin binding of the domain. The excessive glycosylation observed in NSO, but not in CHO and COS, is partially correlated with the origin of the cells. CHO and COS cell lines were derived from cell cultures of normal or embryonic cells (see the Materials and methods section), while the NSO cell line was derived from a mouse myeloma evolved in vivo (Galfre and Milstein 1982). Aberrant N-glycosylation is associated with mouse and human cancers. This involves the appearance of tri- and tetra-antennary N-glycoforms due to enhanced β1-6 GlcNAc branching of the N-glycans. Indeed, the NSO-NKp30-Ig glycoforms consist mostly of high-molecular weight glycoforms that fit the sequences of tri- and tetra-antennary glycoforms (Figure 4 in supplemental data and data not shown). Yet, the glycans of the A549 line, derived from human lung carcinoma, do not manifest the excessive glycosylation observed for NSO. We have already shown that subtle differences in the glycosylation of NKp46-Ig, due to the identity of the mammalian cell line chosen for its
production, have an imperative effect on the binding to its physiological ligand (Arnon et al. 2004); COS-NKp46-Ig, but not CHO-NKp46-Ig, interacts with H1 type influenza hemagglutinin. This is due to the impaired (2,6)-sialyltransferase activity in CHO cells and the preference of H1-influenza hemagglutinin to the Neu5Ac-(2,6)-Gal linkage (Arnon et al. 2004). Therefore, the differences between the glycosylation of NSO-NKp30-Ig and 293T-NKp30-Ig versus other NKp30-Igs could account for their binding difference as we directly showed with the PNGase-f exposed NSO- and 293T-NKp30-Ig (Figures 5–7 and Figure 4 in supplemental data).

Although A549-NKp30-Ig and NKp30-ILZ showed heparinase-dependent binding to 293 cells, the lysis of 293 by NK-92 cells was not influenced by inclusion of heparin, HS, and chondroitin sulfate (SH and CW, unpublished). Also, CHO-K1 and pgsA-745 were killed with equal efficiency by NK-92 (KIR-deficient) and polyclonal NK cells while the lysis could be blocked by anti-NKp30 antibodies (SH and CW, unpublished). The observation that HS-deficiency or reduction on target cells did not affect NKp30-dependent lysis is in accordance with a previous study (Warren et al. 2005). However, in the study by Bloushtain et al. (2004), primary NK lines from different donors manifested suppressed lysis of HS-deficient or HS-reduced tumor cells. Therefore, there is an apparent discrepancy between the HS-dependent binding results of the rNKp30s and the HS-independent NKp30-mediated lysis of target cells observed for NK-92 and some primary NK lines. Two plausible, nonmutually exclusive explanations could account for these contradicting observations:

(i) HS as a coligand for NKp30 is not involved in NKp30-mediated lysis for most NK cells. Yet, it could be involved in other functions mediated by NKp30. NK-92 cells incubated with anti-NKp30 mAb and HS secreted significantly more IFNγ than NK-92 cells incubated with anti-NKp30 mAb alone or anti-NKp30 and chondroitin sulfate. In addition, incubation of NK-92 cells only with HS did not enhance IFNγ secretion (Figure 8). These results indicate that for NK-92 cells, HS could interact with their native NKp30 as a coligand for induction of IFNγ secretion. Indeed, it was observed for NK–DC interactions that NKG2D and NCR recognition of their respective ligands is a prerequisite for IFNγ production but not for enhanced cytolysis (Draghi et al. 2007). Similarly, NCR–HS interaction could promote NK activation through IFNγ production but not through cytolysis enhancement.

(ii) The presence of NK inhibitory receptors that recognize HS as a coligand could explain the inconsistent results with regard to HS-NCR-dependent cytolysis. Interestingly, a consensus motif for heparin/HS binding is present in certain KIR family members. For KIR2DL4 we showed that this motif does interact with HS (AP, unpublished results). NK-92 expresses both NKp30 and KIR2DL4. If KIR2DL4 does inhibit NK-92 lysis, then the observation that NK-92 lyse to the same extent as parental and HS-deficient target cells, could represent the balance of the suppression of both activation and inhibition signals.

To summarize, membranal HS is involved in a variety of biological interactions; HS composition and sequence varied among different tissues and cells, and between normal and transformed cells (Sasisekharan and Venkataraman 2000; Capila and Linhardt 2002; Dennissen et al. 2002; Lai et al. 2003). To date, HS/heparin has been reported to serve as a coligand for numerous protein or lipid receptors that probably recognize different epitopes on HS/heparin. For example, FGFR2 ligand and FGFR2 receptor have higher affinity for hypersulfated HS/heparin epitopes. This interaction is important for the proliferation of FGFR2-expressing tumor cells. Indeed, tumor cells were reported to express hypersulfated membranal HS (Lai et al. 2003). In view of our findings, it is plausible that certain different epitopes of membranal HS expressed by target cells could serve as coligands for fine tuning of the function of NKp30 and other NK receptors. Further exploration is needed to better understand these interactions and their role in the NK function.

**Supplementary Data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org

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**Conflict of interest statement**

None declared.

**Abbreviations**

GAGs, glycosaminoglycans; GU, glucose units; HLA, human leukocyte antigen; HPLC, high-performance liquid chromatography; HS, heparan sulfate; Ig, immunoglobulin; KIR, killer
cell immunoglobulin (Ig)-like receptor; LIR, leukocyte Ig-like receptor; NKR, natural cytotoxicity receptor; PI, propidium iodide; PNGase F, N-glycopeptidase F; RU, resonance units; SDS-PAGE, sodiumdodecyl sulfate–polyacrylamide gel electrophoresis.

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