Membrane-Associated Heparan Sulfate Proteoglycans Are Involved in the Recognition of Cellular Targets by NKp30 and NKp46[1]

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Lysis of virus-infected and tumor cells by NK cells is mediated via natural cytotoxicity receptors (NCRs). We have recently shown that the NKp44 and NKp46 NCRs, but not the NKp30, recognize viral hemagglutinins. In this study we explored the nature of the cellular ligands recognized by the NKp30 and NKp46 NCRs. We demonstrate that target cell surface heparan sulfate proteoglycans (HSPGs) are recognized by NKp30 and NKp46 and that 6-*O*-sulfation and *N*-acetylation state of the glucose building unit affect this recognition and lysis by NK cells. Tumor cells expressing cell surface heparanase, CHO cells lacking membranal heparan sulfate and glypican-1-suppressed pancreatic cancer cells manifest reduced recognition by NKp30 and NKp46 and are lysed to a lesser extent by NK cells. Our results are the first clue for the identity of the ligands for NKp30 and NKp46. Whether the ligands are particular HSPGs, unusual heparan sulfate epitopes, or a complex of HSPGs and either other protein or lipid moieties remains to be further explored. ***The Journal of Immunology,* 2004, 173: 2392–2401.**

Natural killer cells destroy virus-infected and transformed cells, apparently without prior Ag stimulation (1, 2). The interaction between NK cells and their targets is mediated via a complex array of NK inhibitory and activating receptors (3–7). Heavily implicated in this interplay are inhibitory receptors of the NK cell surface, whose ligands are polymorphic and nonpolymorphic MHC class I molecules (3–7). With regard to activation, some NK cells express activation receptors specific for MHC class I molecules homologous to various NK inhibitory receptors (3–7). Yet, three novel lysis receptors, expressed mainly on human NK cells, were recently identified. These natural cytotoxicity receptors (NCRs)[4] include the NKp30, NKp44, and NKp46 molecules (3, 5). All of them are capable of mediating direct killing of tumor and virus-infected cells and are specific for non-MHC ligands. The NCRs are highly NK specific, and NKp30 and NKp46 precisely mark NK cells, whether resting or activated, whereas NKp44 is expressed by activated NK cells (3, 5).

We have recently shown that the NKp44 and NKp46 proteins, but not the NKp30, recognize the hemagglutinin (HA) of influenza virus and the HA-neuraminidase of Sendai virus (8–10). The recognition of the HA and HA-neuraminidase requires the sialylation of NKp44 and NKp46 oligosaccharides and the binding of these NCRs to HAs is required for the lysis of virus-infected cells by NK (9, 10). We have further shown that this recognition is restricted to one of the three glycans found on the second extracellular domain of NKp46 (11). However, the cellular ligands recognized by the NCRs are still elusive.

Cantoni et al. (12) published the three-dimensional structure of NKp44 and noted that the positively charged inner surface of a prominent groove may constitute a binding site for anionic ligands, possibly sugars. Thus, binding of NCRs to their cellular ligands might involve carbohydrate structures on the putative ligands. The binding of other NK activation and inhibitory receptors to their ligands does involve oligosaccharides. For example, mouse Ly-49 family of C-type lectin-like NK receptors include inhibitory and activating receptors that recognize different MHC class I molecules on target cells (13). Recently, a co-crystal of Ly-49A and its H-2D^d ligand indicated a possible different role for the two *N*-linked glycosylations present on the H-2D^d (13, 14).

Human NK cells also possess a family of C-type lectin-like receptors consisting of heterodimers formed from CD94 and one of the NKG2 family members (15, 16). Both CD94-NKG2A and CD94-NKG2C heterodimeric receptors bind to human HLA-E, but inhibit or activate NK function, respectively (15, 16). Over-expression of sialyl Lewis X (SLe^X) oligosaccharides by tumor cells lead to attack by NK of these tumor cells due to recognition of SLe^X by the CD94 NK cell receptor (17). Yet, the evolvement of tumor cells expressing higher levels of SLe^X, which is a ligand for E-selectin, is probably due to the related enhancement of their metastatic potential. Aberrations in cell surface carbohydrates are often associated with malignant transformation and other pathological conditions (18–20) and specific oligosaccharide structures have been identified on tumors and serve as diagnostic markers for malignant phenotypes (21–23). Thus, it is possible that the tumor-expressed cellular ligands for NCRs involve modified glycosylation.

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[4] Abbreviations used in this paper: NCR, natural cytotoxicity receptor; HA, hemagglutinin; HSPG, heparan sulfate proteoglycan; LIR, leukocyte Ig-like receptor; MFI, mean fluorescence intensity; GAG, glycosaminoglycan; LMW, low molecular weight; PAA, polyacrylamide; FGF, fibroblast growth factor.

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In the present study, we first screened different carbohydrate structures for effect on binding of recombinant NKp30- and NKp46-fusion Igs to tumor cells. We identified 6-*O*-sulfo-*N*-acetyllactosamine (6-*O*-sulfo-LacNAc), with emphasis on the 6-*O*-sulfo-*N*-acetylglucosamine, as a potent inhibitor of this binding. The 6-*O*-sulfo-*N*-acetylglucosamine is a building unit of heparin/heparan sulfate, which also inhibited the binding of NKp30- and NKp46-fusion Igs to tumor cells. We further determined that target cell membrane-associated heparan sulfate proteoglycans (HSPGs) are recognized by NKp30- and NKp46-fusion Igs. Finally, we showed that tumor membrane HSPGs are involved in lysis of target tumor cells by NK.

Materials and Methods

Cells

Cell lines used in this work were as follows. PC-3 is a human prostate adenocarcinoma derived from bone metastasis that is prostate-specific antigen-negative and androgen insensitive from American Type Culture Collection (ATCC, Manassas, VA; catalogue no. CRL-1435). Human melanoma cell line 1106 is established from a recurrent metastatic lesion and expressed no HLA-I Ags (24). HeLa is a human cervical adenocarcinoma (ATCC catalogue no. CCL-2). Eb-SP is Eb murine T lymphoma transfected with cDNA encoding for chimeric heparanase composed of the human heparanase and the chicken heparanase signal peptide. A functional heparanase is localized on the surface of Eb-SP cells (25). PANC-1 is a human pancreatic ductal carcinoma (ATCC catalogue no. CRL-1469) overexpressing glypican-1 (26). GAS6 are PANC-1 cells stably transfected with full-length glypican-1 antisense construct, thus markedly reducing their glypican-1 expression at both the RNA and protein levels; sham-PANC-1 are control-transfected PANC-1 cells, retaining high levels of glypican-1 (26). Wild-type Chinese hamster ovary (CHO) K1 cells and the mutant derivatives CHO pgsA-745 and CHO pgsD-677 were kindly supplied by Dr. J. Esko (University of California at San Diego, La Jolla, CA) and have been characterized in detail elsewhere (27). NK cells (lines and clones) were isolated from peripheral blood lymphocyte using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec, Bergisch Gladbach, Germany). The NK cells were kept in culture as previously described (9).

Carbohydrates and Glyc-PAA library

Glyc-PAAs are carbohydrate conjugates in which Glyc is the oligosaccharide part and polyacrylamide (PAA) is a soluble carrier of 30 kDa. The content of oligosaccharides in the conjugates is 20 mol%. Thus, on the average, each fifth unit of the PAA polymer is conjugated to oligosaccharide (28). As an example, the oligosaccharide content for *N*-acetyllactosamine (LacNAc) is 1.05 μmol LacNAc/mg Glyc-PAA. A library of 35 different Glyc-PAAs containing carbohydrate ligands for siglecs, galectins, selectins, and other lectins was used for initial screen and further identification, namely: 3′,6-*O*-Su$_2$-Lacβ; 6$^{(GlcNAc)}$-*O*-Su-SiaLe^X; Neu5Acα2–3Galβ1–3GlcNAc; Neu5Acα2–6Galβ1–4Glc; 3′-*O*-Su-Galβ1–3(Fucα1–4)GlcNAcβ; Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ; Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAcβ; Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAcβ; Fucα1–2Galβ1–4(Fucα1–3)GlcNAcβ; 6′-SiaLacNAcβ; Galβ1–4GlcNAcβ1–6GalNAcα; 6-*O*-Su-Lac; 6-*O*-Su-LacNAc; Neu5Acα2–3Galβ1–3(6-Su)GlcNAc; Neu5Acα2–3Galβ1–3(6-Su)GalNAcα; GlcNAcβ1–3(GlcNAcβ1–6)GalNAcα; Galα1–3Galβ1–4GlcNAcβ; 3′-*O*-Su-Galβ1–4(Fucα1–3)GlcNAcβ; Galβ1–4(Fucα1–3)GlcNAcβ; Neu5Acα2–3Galβ1–4GlcNAcβ; 4′,6′-*O*-Su$_2$-LacNAcβ; 3′-*O*-Su-Galβ1–3GalNAcα; 6′-*O*-Su-LacNAcβ; Neu5Acα2–8Neu5Acα2; 3′-*O*-Su-Galβ1–3GlcNAcβ; 3′-*O*-Su-LacNAcβ; 3-*O*-Su-GalNAcβ; α-D-Man; 6-H_2PO_3Manα; GalNAcβ1–4GlcNAcβ; Galα1–3Galβ1–4Glc; Lac; LacNAc; Galβ1–3GalNAcα; Galβ1–3GalNAcβ; GalNAcα1–3GalNAcβ; GlcNAcβ1–4GlcNAcβ; 6$^{(Gal)}$-*O*-Su-SiaLe^X.

Glycosaminoglycans (GAGs)

Low m.w. (LMW) heparin (H-3400), heparan sulfate (H-9902), hyaluronic acid (H-5388), chondroitin sulfate A (C-8529), and chondroitin sulfate C (C-4384) were purchased from Sigma-Aldrich, St. Louis, MO. Species of heparin that are modified in *N*-sulfation, *O*-sulfation, and acetylation were described (29).

Ig fusion proteins

The generation of NKp30-Ig, NKp46-Ig, CD99-Ig and leukocyte Ig-like receptor (LIR)1-Ig fusion protein was previously described (9, 10, 30). To generate the NKp46D2-Ig, truncated fusion protein in COS cells residues 101–235 domain 2 (D2) of the mature NKp46 protein were PCR amplified, and the PCR-generated fragment was cloned into a mammalian expression vector containing the Fc portion of human IgG1, as previously described (11). To allow expression of NKp46D2-Ig, which lacks its original leader peptide sequence, we added a methionine start codon and cloned the PCR-amplified fragment of NKp46D2 in frame with the leader peptide of CD5. Sequencing of the construct revealed that all cDNAs were in frame with the human Fc genomic DNA and were identical with the reported sequences. COS-7 cells were transiently transfected with the construct using FuGENE6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions, and supernatants were collected and purified on a protein G column. SDS-PAGE analysis revealed that all Ig fusion proteins were ~95% pure and of the proper molecular mass. For the production of NKp30-Ig and NKp46D2-Ig in CHO cells, the corresponding PCR fragments containing kozak sequence and leader sequence of CD5 were cloned into pcDNA3.1 Ig vector. 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 serum-free CHO SFM II medium (Invitrogen Life Technologies, Paisley, U.K.) 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 (11).

Flow cytometry

Cells were incubated with 10 μg of the various fusion Igs for 2 h at 4°C, washed and stained with FITC-conjugated F(ab′)$_2$ goat anti-human-IgG Fcγ (109-096-098; Jackson ImmunoResearch Laboratories, West Grove, PA). Staining and washing buffer consisted of 0.5% (w/v) BSA and 0.05% sodium azide in PBS. Staining of CHO and mutant CHO cells was carried with 2% FCS instead of BSA in the different buffers. Propidium iodide was added before reading for exclusion of dead cells. Flow cytometry was performed using a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). Data files were acquired and analyzed using BD CellQuest 3.3 software. Fluorescence data were acquired using logarithmic amplification and reported fluorescence intensity units represent conversion of channel values according to the logarithmic scale (range 10^0 to 10^4). Results are presented either as staining histograms (*x*-axis represents fluorescence intensity and *y*-axis represents cell counts) or as the geometric mean fluorescence intensity (MFI) of the stained populations. Geometric mean of the fluorescence intensities is recommended by the manufacturer for comparing relative fluorescence intensities between logarithmically acquired samples. For most binding inhibition experiments, 10 μg of fusion Ig were premixed with the carbohydrate (Glyc-PAA or GAG) and added to cells for staining as previous. In some experiments, cells were preincubated with the carbohydrates for 90 min at 4°C, washed twice and stained with fusion Ig as described. In all experiments, each sample was stained twice in different wells. When results are presented as MFI, average MFI ($\pm$SD) of the duplicate staining is brought to show consistency of staining procedure.

Glycosidases and treatment of cells

Tumor cells (10^6) were washed twice in PBS, resuspended in 1-ml reaction buffer alone (mock treatment) or reaction buffer containing one of the following GAG-degrading enzymes (Sigma-Aldrich): keratanase (1 U/ml, K-2876), heparin lyase I (1.5 U/ml, H-2519), and heparin lyase III (1.25 U/ml, H-8891). Reaction buffer consisted of 1% (w/v) BSA, 1 μg/ml leupeptin, and 10 U/ml aprotinin in PBS. Cells were incubated with enzyme for 60 min at 37°C, washed twice with PBS and stained with fusion Igs as previously discussed.

Cytotoxicity assays and mouse anti-NCR serum

The cytotoxic activity of NK lines against the various targets was assessed in 5-h 35 S-release assays and in 4-h Eu release time-resolved fluorescence assays, as previously described (31, 32). In experiments in which carbohydrates were included, NK cells were first mixed with the carbohydrates and then added to target cells. In all experiments shown, the spontaneous release was <25% of maximal release. Each point represents the average of duplicate/triplicate values. The range of the duplicates/triplicates was within 5% of their mean. Anti-NKp30, anti-NKp46, and control serums were produced as described (9, 10). For inhibition of lysis, the anti-NKp30 and anti-NKp46 serums were used at 1/100 dilution, a concentration at which binding was saturated, as measured by flow cytometry.

Results

*Binding of NKp30- and NKp46-fusion Igs to tumor cells is inhibited by 6-*O*-sulfo-LacNAc in which 6-*O*-sulfation and* N*-acetylation of the glucose are involved*

We and others published that NKp30-Ig and NKp46-Ig bind to cellular ligands expressed on tumor cells (3, 9, 10, 33). We further showed that the membrane-proximal domain (NKp46D2), but not membrane-distal domain (NKp46D1), of NKp46 retained the binding of NKp46 to cellular and viral ligands (11). Thus, in this study we used the NKp46D2-Ig. To study the effect of glycosylation on the binding of NKp30-Ig and NKp46-Ig to tumor cells, we screened a library of 35 different Glyc-PAAs containing carbohydrate ligands for siglecs, galectins, selectins, and other lectins. Glyc-PAAs were mixed with Ig fusion proteins, and staining of tumor cells with the Ig fusion protein was measured. One Glyc-PAA, in which the saccharide moiety was 6-*O*-sulfo-LacNAc, inhibited binding of NKp30-Ig and NKp46D2-Ig to HeLa cells (Fig. 1*A*). Similar reaction was observed when other human tumor cell lines, prostate cancer PC-3 and 1106 melanoma, were assessed (Fig. 1*C* and data not shown). Titration of 6-*O*-sulfo-LacNAc concentrations, needed for inhibition of binding, showed that inhibition (20%) can be observed already at 15 μM and saturation is reached at 900 μM (60–70% inhibition). Preincubation of the cells with 6-*O*-sulfo-LacNAc-PAA, followed by wash and application of the fusion proteins did not affect the binding (data not shown). This excluded a setting in which target cell receptor for 6-*O*-sulfo-LacNAc-like moiety recognized this structure on NKp30 and NKp46 oligosaccharides.

LIR1 is expressed by NK cells, recognize a broad range of MHC class I molecules on target cells and transduces an inhibitory signal (34). LIR1 was chosen as a control because the overall structures of LIR1 and NKp46 are very similar, with LIR1 displaying a root mean-square difference from NKp46 of 1.9 Å (35). LIR1-Ig binds to HeLa and PC-3 cells but not to 1106 melanoma cells. Binding of LIR1-Ig to HeLa and PC-3 cells was not inhibited by 6-*O*-sulfo-LacNAc (Fig. 1*A* and data not shown). Thus, inhibition was specific to NKp30 and NKp46 binding. We further dissected the contribution of the different glucose modifications to the inhibition of binding. Fig. 1, *B* and *C*, shows that removal of either the sulfate, *N*-acetyl or both from the *N*-acetylglucosamine abolished the effect on binding of NKp46D2-Ig to HeLa and PC-3 cells. Similar results were obtained for NKp30-Ig (data not shown). We further studied the effect of sulfation of galactose in LacNAc-PAA on the binding of Ig fusion proteins. 3′-*O*-sulfo-LacNAc and 4′,6′-di-*O*-sulfo-LacNAc did not inhibit binding of NKp30-Ig or NKp46-Ig, whereas 6′-*O*-sulfo-LacNAc manifested inconsistent inhibition phenotype of up to 25% reduction in binding (data not shown).

Blocking experiments with mAbs that specifically recognize the 6-*O*-sulfo-LacNAc moiety (36) did not inhibit the binding NKp30-Ig and NKp46-Ig to tumor cells. In addition, 6-*O*-sulfotransferase-transfected cells expressing high levels of 6-*O*-sulfo-LacNAc moiety within the saccharides of their membrane glycoproteins (36), were not

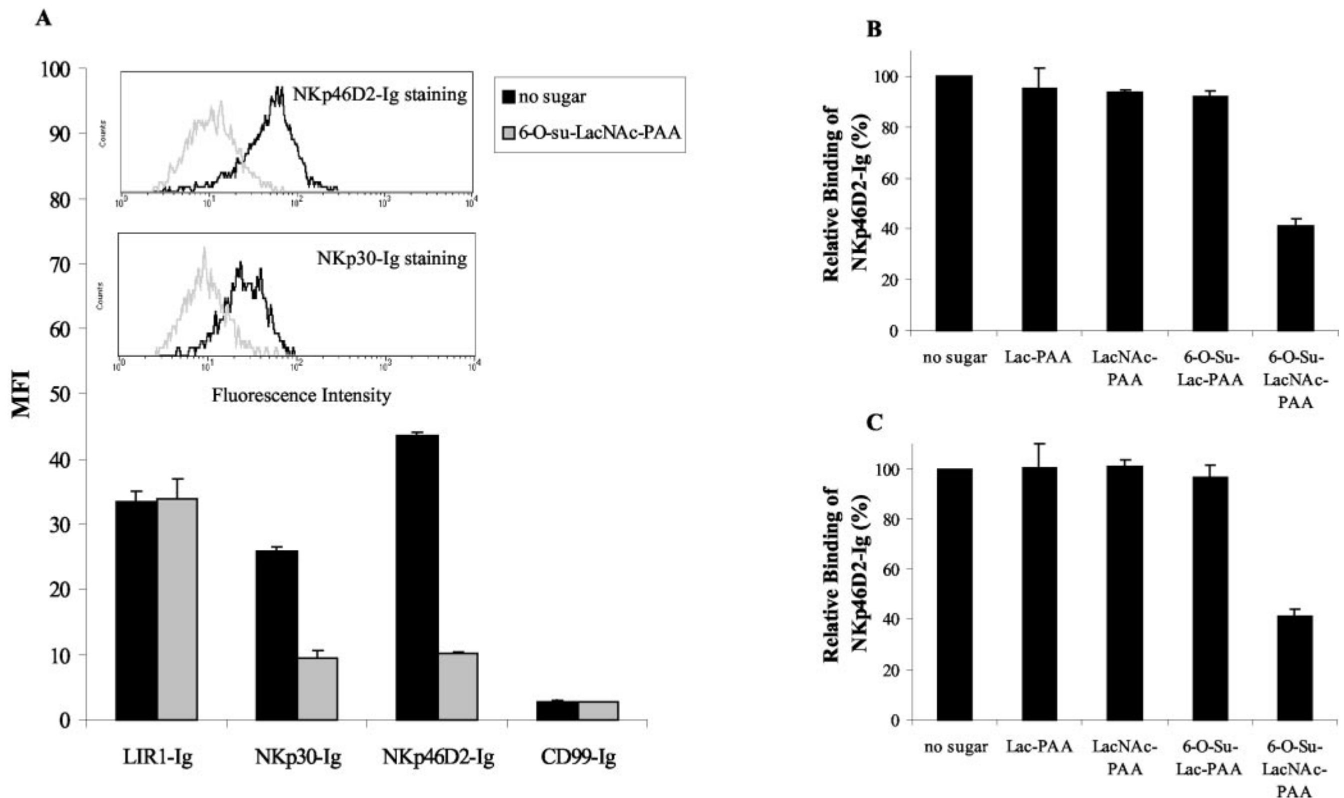

FIGURE 1. Effect of 6-*O*-sulfo-LacNAc-PAA on binding of NKp30- and NKp46-fusion Igs to tumor cells. Ten micrograms of fusion Ig were premixed with different Glyc-PAAs and cells were added for 2 h at 4°C (100 μl of total volume, final concentration of the disaccharides is 0.45 mM). After incubation, cells were washed and incubated with FITC anti-Fc second Ab. Propidium iodide was added to exclude dead cells. *A*, Staining of HeLa cells is shown. Results are presented as MFI (as described in *Materials and Methods*). *Inset* shows primary FACS histograms of the effect of 6-*O*-sulfo-LacNAc-PAA on binding of NKp30- and NKp46-fusion Igs to HeLa cells. *B* and *C*, Staining of HeLa and PC-3 cells, respectively. Results are presented as percentage of binding as compared with staining of cells with NKp46D2-Ig alone without Glyc-PAA mix. All results are from one representative experiment of three and are the average of two different samples assayed in the same experiment. Error bars (±SD) of the duplicate, indicating the consistency of the staining procedure.

stain positively with NKp30, 46-fusion proteins as compared with parental mock-transfected cells (data not shown). Therefore, 6-*O*-sulfo-LacNAc is not the correct carbohydrate moiety recognized by NKp30 and NKp46. This also explains the relatively high concentrations (450 μM) of 6-*O*-sulfo-LacNAc needed to inhibit NKp30-Ig and NKp46D2-Ig binding by 50%. To summarize, whether cell membrane-associated oligosaccharides are involved in the binding of NKp30 and NKp46 to their cellular ligands, 6-*O*-sulfo-*N*-acetylglucosamine is likely to be a keystone of these oligosaccharides.

Binding of NKp30- and NKp46-fusion Igs to tumor cells is inhibited by heparin/heparan sulfate, and O-*sulfation, and* N-*acetylation are involved*

N-acetylglucosamine was a component in about half of the Glyc-PAAs that we screened and four Glyc-PAAs contained 6-*O*-sulfo-*N*-acetylglucosamine (6-*O*-Su-LacNAc, 6-Su-3′SiaLe^{C}, 6-Su-3′SLN, and 6$^{(GlcNAc)}$-Su-SLe^{X}). Only the 6-*O*-sulfo-LacNAc inhibited binding of NKp30 and NKp46 (Fig. 1 and data not shown). Yet, 6-*O*-sulfo-LacNAc was not the precise structure recognized. Therefore, the nature of the sulfated saccharide involved in binding of NKp30 and NKp46 to tumor cells was further evaluated by determining the possible role of GAGs containing 6-*O*-sulfo-*N*-acetylglucosamine in various variable epitopes/domains (37, 38). HeLa cells were incubated with LMW heparin (10 μg/ml) and either NKp30-Ig, NKp46D2-Ig, or LIR1-Ig. All three fusion proteins bound well to HeLa cells and heparin inhibited the binding of NKp30-Ig and NKp46D2-Ig, but not the binding of LIR1-Ig (Fig. 2*A*). We also tested killer Ig-like receptor 2 domain long tail 1, KIR2DL1, which assumes overall structure very similar to that found for NKp46 (35). KIR2DL1-Ig (30) binding to HLA-Cw4-transfected 721.221 cells (39) was not inhibited by heparin (data not shown). Chondroitin sulfate A did not inhibit the binding of any of the three fusion proteins (Fig. 2*A*). Preincubation of the cells with LMW heparin, followed by wash and application of the fusion proteins did not affect the binding (data not shown). Similar phenotype was observed when PC-3 prostate cancer and 1106 melanoma were assessed (Fig. 2, *B2* and *B3*). The specific role of heparin/heparan sulfate in inhibition of NKp46-fusion Ig binding to all three tumor cell lines is further shown in Fig. 2*B*. Incremental concentrations of chondroitin sulfate A, chondroitin sulfate C, and hyaluronic acid up to 10 μg/ml did not inhibit binding of NKp46D2-Ig. In contrast, LMW heparin and heparan sulfate in concentrations of 0.1 μg/ml (~0.03 μM) inhibited binding of these fusion proteins. Similar results were obtained for NKp30-Ig (data not shown). de Fougerolles et al. (40) reported that the membrane-proximal domain of CD19, CD19-domain 3 (D3), recognizes stromal cell-associated heparan sulfate. The other two extracellular domains of CD19 did not recognize heparan sulfate. CD19

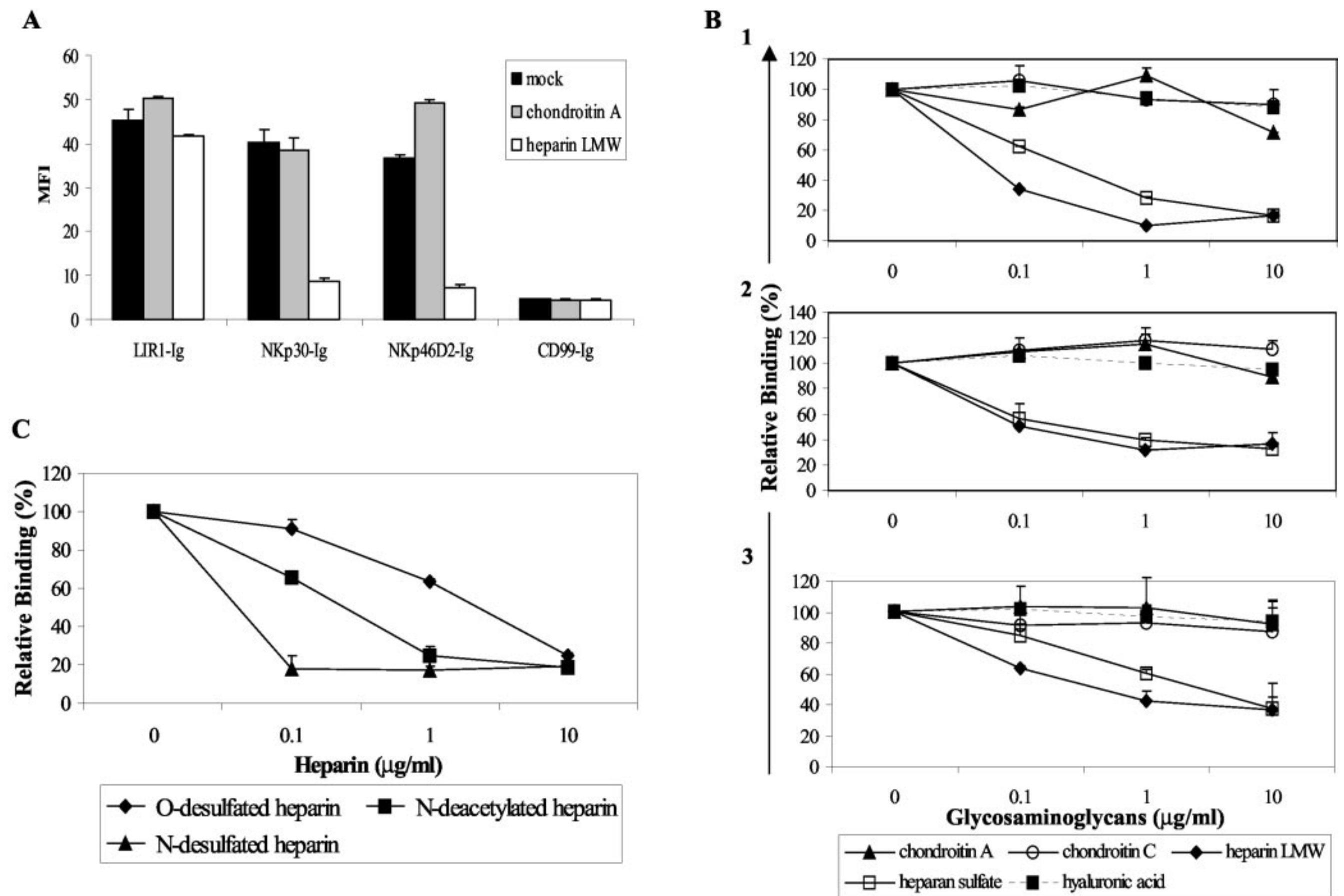

FIGURE 2. Effect of heparin/heparan sulfate on binding of NKp30- and NKp46-fusion Igs to tumor cells. Ten micrograms of fusion Ig were premixed with different GAGs and 10^5 cells were then added for 2 h at 4°C (100 μl of total volume, final concentration of GAGs-proteoglycans is 0.1 to 10 μg/ml). Heparan sulfate concentration of 0.1 μg/ml is ~0.03 μM. After incubation, cells were washed and incubated with FITC anti-Fc second Ab. Propidium iodide was added to exclude dead cells. *A*, Staining of HeLa cells. Concentrations of LMW heparin and chondroitin sulfate A are 10 μg/ml. Results are presented as MFI. *B1–B3*, Effect of titrated concentration of different GAGs. Staining of HeLa, PC-3, and 1106 cells, respectively, is with NKp46D2-Ig. *C*, Effect of modified heparin. Staining of HeLa cells. Results are presented as a percentage of binding as compared with staining of cells with NKp46D2-Ig alone without GAG or proteoglycan mix. Results are from one representative experiment of two (for *A*, *B2*, *B3*, and *C*) and of five (*B2*). Results are the average of two different samples assayed in the same experiment. Error bars (±SD) are indicated.

is a B cell-specific membrane protein that amplifies positive signaling. Similar to our observations with NKp30-Ig and NKp46D2-Ig, binding of CD19-D3-Ig to stromal cell lines was inhibited by soluble heparan sulfate in concentrations of 0.1 μg/ml and higher (40).

We next examined the influence of variations in *O*-sulfation and *N*-acetylation of heparin on its capacity to inhibit the binding of NKp30- and NKp46-fusion Igs to tumor cells. *N*-desulfation of heparin resulted in 100% removal of the *N*-sulfate groups whereas *O*-desulfation removed 99% of the *O*-linked sulfates (29). *N*-desulfated heparin was a potent inhibitor of NKp46D2-Ig binding whereas *O*-desulfation of heparin reduced significantly the observed inhibition (Fig. 2*C*). We also tested *N*-deacetylated heparin, in which all *N*-acetyl groups were replaced by *N*-hexanoyl. This modification also reduced the capacity of heparin to inhibit binding of NKp46D2-Ig (Fig. 2*C*). Similar results were obtained for NKp30-Ig (data not shown).

NKp30- and NKp46-fusion Igs bind to heparan sulfate on tumor cells

We next determined the involvement of cell membrane-associated GAGs in the binding of NKp30 and NKp46 to their cellular ligands. 6-*O*-sulfo-*N*-acetylglucosamine is a component of keratan sulfate and heparin/heparan sulfate, but not of chondroitin sulfate and dermatan sulfate. Therefore, we treated tumor cells with 1) heparin lyase III, which efficiently degrades heparan sulfate with broad specificity, and 2) heparin lyase I, which is selective in cleaving highly sulfated regions in heparan sulfate. Neither, however, degrade keratan sulfate or chondroitin sulfates A to E (41). We also treated tumor cells with keratanase, which efficiently degrades keratan sulfate, but not other GAGs. Treatment of HeLa, PC-3, and 1106 cells with heparin lyase I or lyase III, but not with keratanase, reduced the binding of NKp30-Ig and NKp46D2-Ig by 50–70% (Fig. 3*A*). We studied the specificity of heparin lyase treatment on binding of LIR1-Ig to HeLa cells. Both heparin lyase I and lyase III treatments did not reduce the binding of LIR1-Ig to HeLa cells (Fig. 3*A1*). Heparan sulfates are mostly attached to the core protein by *O*-linked glycoside bonds, whereas keratan sulfates are mostly attached by *N*-linked bonds (42). In accordance, treatment of tumor cells with a blocker of *O*-glycosylation, α-benzyl-GalNAc, significantly reduced the binding of NKp30- and NKp46-fusion Igs (data not shown).

We further stained Eb-SP and Eb T lymphoma cells which express or fail to express a functional heparanase on their cell surface, respectively (25). Staining of Eb-SP with NKp46D2-Ig was reduced by 50% as compared with parental Eb or mock-transfected

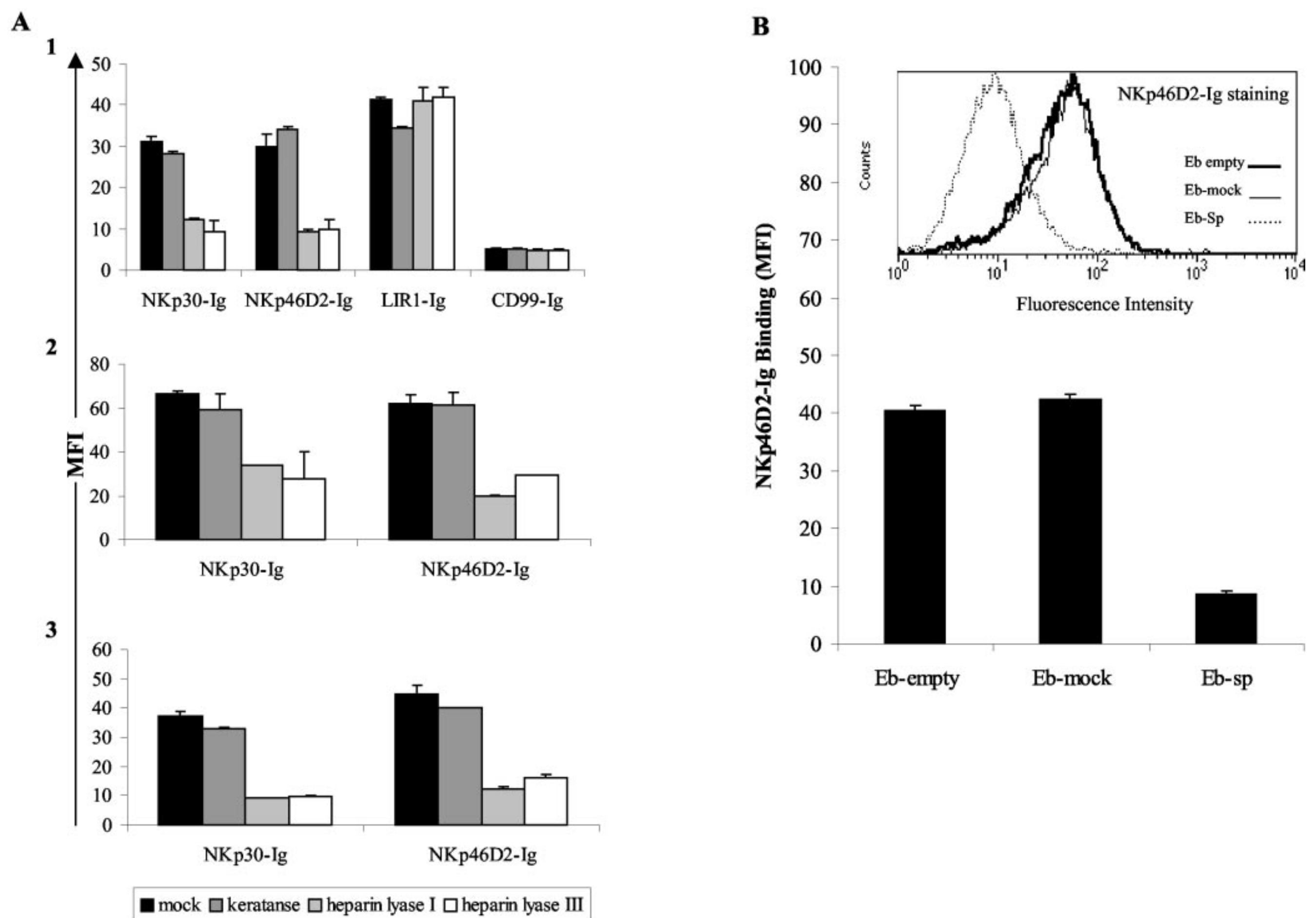

FIGURE 3. Effect of heparan sulfate-degrading enzymes on binding of NKp30- and NKp46-fusion Igs to tumor cells. HeLa, PC-3, and 1106 (*A1–A3*) cells were incubated in reaction buffer alone (mock treatment) or reaction buffer containing a GAG-degrading enzyme. After incubation, cells were washed and stained with fusion Igs. *B*, Staining with NKp46D2-Ig of Eb lymphoma, mock-transfected Eb and heparanase-transfected Eb cells that express a functional heparanase on the cell surface (Eb-SP). *Inset* shows primary FACS histogram overlay from the same experiment. Results are from one representative experiment of five (*A1*) and of two (*A2*, *A3*, and *B*). MFI results are the average of two different samples assayed in the same experiment with error bars (±SD).

Eb cells (Fig. 3*B*), and staining with NKp30-Ig revealed the same phenotype (data not shown). These results indicate that NKp30-Ig and NKp46D2-Ig bind to cell membrane-associated heparan sulfate. An alternative interpretation is that the binding is to a cell surface molecule associated with heparan sulfate, but we believe that this possibility is excluded by the observation that soluble heparan sulfate directly inhibits the binding of NKp30- and NKp46-fusion Igs to tumor cells (Fig. 2).

To further demonstrate the interaction of NKp30- and NKp46-fusion Igs with cell surface heparan sulfate we stained CHO mutants defecting in GAG synthesis. Staining of CHO pgsA-745 cells, which have no heparan sulfate and chondroitin sulfate, with NKp46D2-Ig and NKp30-Ig was reduced by 50 and 70%, respectively, as compared with parental CHO-K1 cells (Fig. 4*A*). Similar reduced staining pattern was observed for CHO pgsD-677 (Fig. 4*A*). The latter result is most revealing because CHO pgsD-677 have no heparan sulfate but produce chondroitin sulfate that accumulates to levels two to three times higher than in wild-type cells (27).

Membrane-associated HSPGs are mostly divided into two families: glypicans, which are attached to the plasma membrane via GPI anchors, and syndecans, which are transmembrane proteins (43). We examined the involvement of GPI-anchored proteins in the binding of NKp30- and NKp46-fusion Igs to tumor cells. Treatment of cells with D-mannosamine inhibits GPI incorporation into GPI-anchored proteins, thus converting GPI-anchored membranal proteins to nonpolarized secreted proteins (44). For HeLa and PC-3 cells, such inhibition reduces binding of NKp30-Ig and NKp46D2-Ig by 2- to 4-fold (Fig. 4, *B1* and *B2*). Yet, staining of D-mannosamine-treated 1106 cells was marginally affected as compared with nontreated cells (Fig. 4*B3*). These results indicate that glypicans might be involved in binding of NKp46D2-Ig and NKp30-Ig in various, but not all, tumors.

To better assess the glypicans involvement we studied the following model: PANC-1 is a human pancreatic ductal carcinoma over-expressing glypican-1 (26). GAS6 are PANC-1 cells stably transfected with full-length glypican-1 antisense construct, thus markedly reducing their glypican-1 expression; sham-PANC-1 are control-transfected PANC-1 cells, retaining high levels of glypican-1 (26). We stained sham-PANC-1 and GAS6 with NKp30-Ig, NKp46D2-Ig. Fig. 4*C* shows that staining of GAS6 was markedly reduced as compared with sham-PANC-1 cells (in MFI, 53 to 27

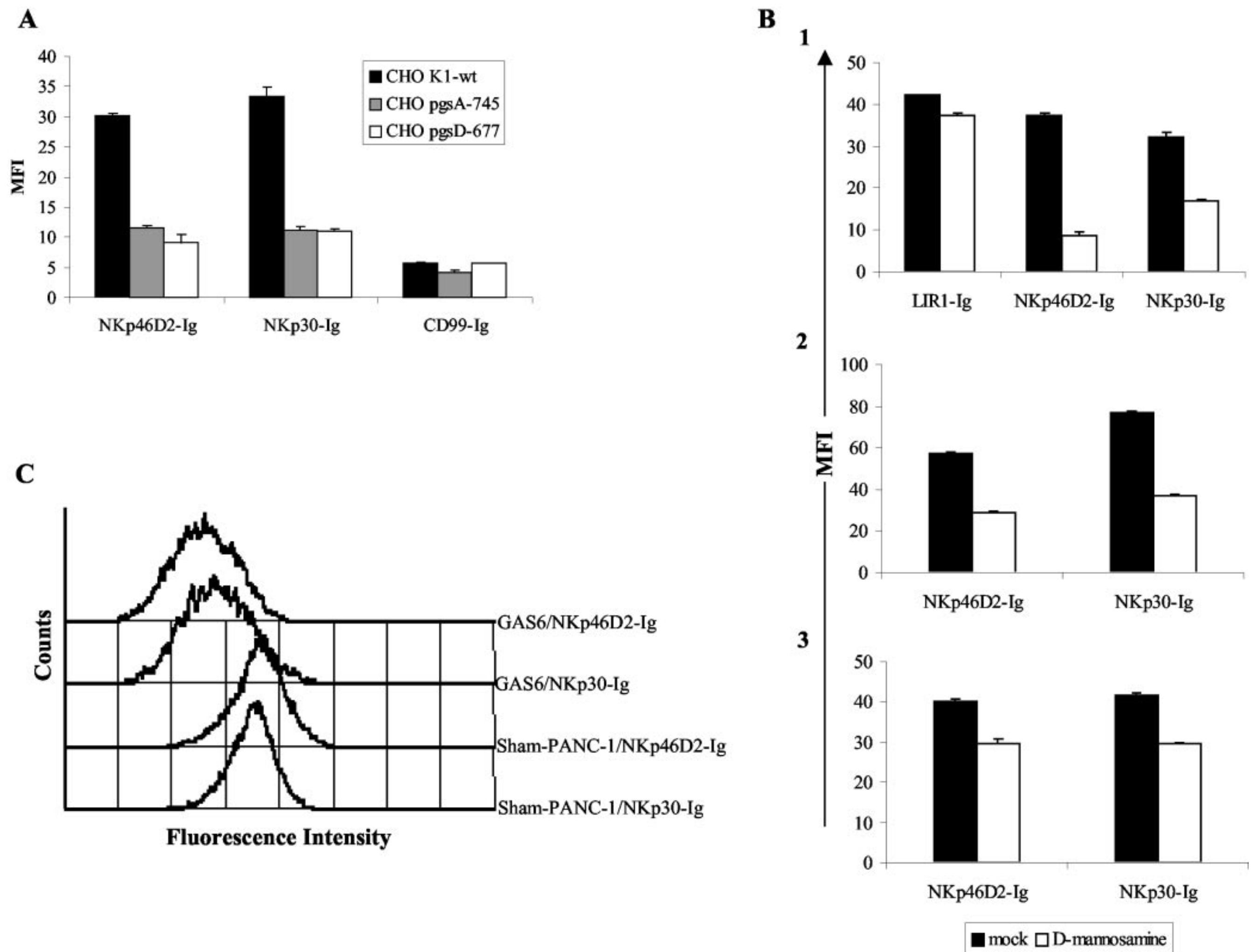

FIGURE 4. Effect of heparan sulfate deficiency, D-mannosamine treatment, and glypican-1 suppression on binding of NKp30- and NKp46-fusion Igs to tumor cells. *A*, Staining of parental CHO-K1, heparan sulfate-negative and chondroitin sulfate-negative CHO pgsA-745, and heparan sulfate-negative and chondroitin sulfate high-positive CHO pgsD-677 by Ig fusion proteins. *B1*, *B2*, and *B3*, Staining with fusion Igs of HeLa, PC-3, and 1106 cells, respectively, pretreated with 40 mM D-mannosamine overnight. *C*, Staining with fusion Igs of sham and GAS-6 cells. Results are presented as MFI. Results are from one representative experiment of four (*A* and *B1*) and of two (*B2*, *B3*, and *C*) and are the average of two different samples assayed in the same experiment. Error bars are ±SD.

and 61 to 20 for NKp30-Ig and NKp46D2-Ig, respectively). Both cells showed dull but similar staining with LIR1-Ig. In parallel we stained the cells with anti-heparan sulfate Ab HS4E4 (37). Results paralleled to the NKp30-Ig and NKp46D2-Ig staining: MFI was 103 and 32 for sham-PANC-1 and GAS6, respectively. The reduced staining of GAS6 with the anti-heparan sulfate Ab is expected, because in contrast to the high levels of glypican-1 in sham-transfected, the other glypicans are not expressed in PANC-1 cells (45). Therefore, a major portion of the PANC-1 membranal heparan sulfate is conjugated to glypican-1. The parallel reduction in staining with NKp30-Ig and NK46D2-Ig, combined with the other results illustrated in Figs. 2 to 4 indicate that the heparan sulfate conjugated to glypican-1 is involved in the recognition of PANC-1 by NKp30- and NKp46-fusion Igs. The remaining membranal heparan sulfate might be attributed to expression of syndecan-1 (46), and syndecans 2–4 expression was not assessed.

*Effect on NK cytotoxicity by 6-*O*-sulfo-LacNAc-PAA and heparan sulfate*

To test the role of NKp30 and NKp46 recognition of carbohydrates in NK lysis, we studied the lysis of HeLa cells by NK in the presence of Glyc-PAA. Presence of 6-*O*-sulfo-LacNAc-PAA, but not LacNAc-PAA, reduced the lysis of HeLa cells by third (Fig. 5*A*). HeLa cells lysis by NK is mediated by NKp46 because specific anti-NKp46 serum, produced as described (9), reduced HeLa lysis by 2-fold (data not shown). The 6-*O*-sulfo-LacNAc concentration that caused significant reduction of lysis was 900 μM (Fig. 5*A*). This relatively high concentration of 6-*O*-sulfo-LacNAc needed for partial inhibition of lysis is reasonable because 6-*O*-sulfo-LacNAc is not the correct ligand of NKp30 and NKp46. This is also in the concentration range that reduced the binding of NKp30- and NKp46-fusion Igs to HeLa and other tumor cells (Fig. 1). However, when we applied LMW heparin to block NK lysis, a

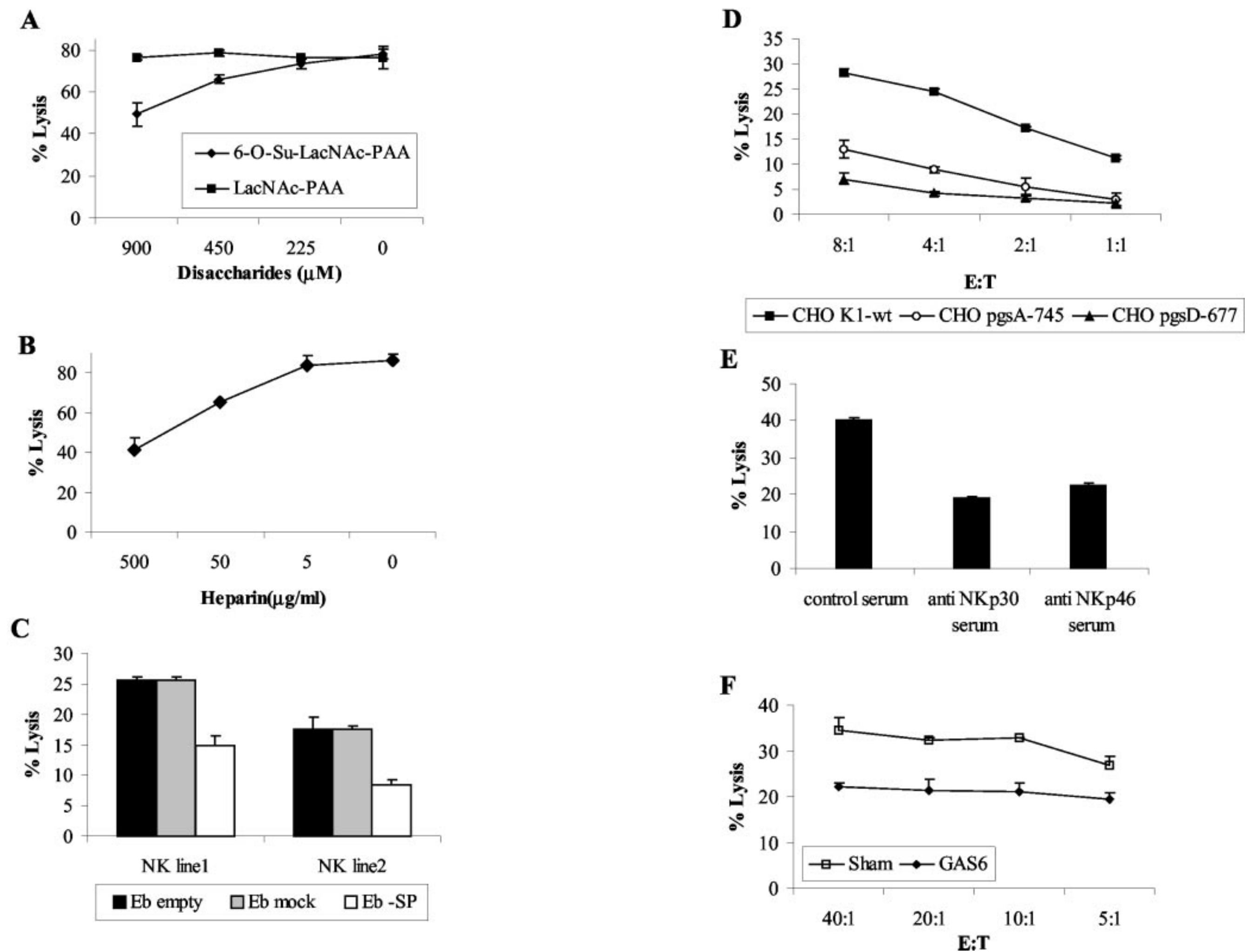

FIGURE 5. Effect of 6-*O*-sulfo-*N*-acetylglucosamine, target cell surface heparanase and heparan sulfate deficiency on lysis by primary NK lines. *A*, Primary NK line cells were mixed with incremented amounts of Glyc-PAAs and added to Eu-labeled target cells for a 4 h lysis assay. Final concentrations of the disaccharides ranged from 0.225 to 0.9 mM. E:T ratio is 50:1. Results are from one representative experiment of four. *B*, Inhibition of NK lysis of HeLa cells by heparin. Primary NK line cells were mixed with incremented amounts of heparin LMW and added to Eu-labeled target cells for a 4 h lysis assay. Results are from one representative experiment of two. *C*, Lysis by primary NK lines of Eb, Eb-mock transfected, and transfected Eb cells-expressing a functional cell surface heparanase (Eb-SP). E:T ratio is 20:1. Two NK lines are representative of seven primary NK lines assayed in different experiments. *D*, Lysis by primary NK line of CHO and GAG-deficient CHO cells. E:T ratio ranged from 1:1 to 8:1. Representative of three experiments (*E*) NK cells were incubated with control serum, anti-NKp30 or anti-NKp46 serum (1/100 dilution) for 1 h on ice. NK cells were then washed and incubated with tumor cells at 12:1 E:T ratio. Representative of two experiments. *F*, Lysis by primary NK line of sham-PANC-1 and GAS6 cells. Representative of four experiments. All results are the average of a duplicate/triplicate assayed in the same experiment and error bars are shown ($\pm$SD).

significant reduction in lysis of HeLa cells by NK was observed only at concentrations of 50 μg/ml and higher (Fig. 5*B*). This result (studied with comparable heparin concentrations) is in agreement with previous publications on heparin-mediated inhibition of NK lysis (47, 48). Yet, contrary to 6-*O*-sulfo-LacNAc-PAA, the heparin concentration needed for significant inhibition of lysis is at least 10-fold higher than the concentration used for inhibition of NKp30-Ig and NKp46D2-Ig binding (Figs. 1 and 2). A possible explanation is the multitude of heparin functions that can augment cytotoxicity despite the masking of NKp30 and NKp46. For example, heparin efficiently potentiates the lytic activity of perforin (49). Indeed, opposite effects of heparin on NK activity, which are time- and concentration-dependent, were reported by Wasik and Gorski (48). Hence, a concentration-dependent balance between lysis augmenting activities of heparin and masking of NKp30 and NKp46 can result in suppression of lysis in relatively high concentrations of heparin.

To better study the effect of target membrane-associated HSPGs on lysis by NK, we compared the lysis of Eb and Eb-SP by NK cells. Eb-SP lysis by NK cells was reduced 2-fold as compared with parental Eb or Eb-mock cells (Fig. 5*C*). Thus, reduction in binding of NKp30- and NKp46-fusion Igs to tumor cells expressing cell surface functional heparanase is correlated with the reduced lysis of these cells by NK (Figs. 3*B* and 5*C* and data not shown). Similarly, CHO mutant cells lacking cell surface heparan sulfate, independently from chondroitin sulfate production (CHO pgsA-745 and CHO pgsD-677), were lysed to a lesser extent as compared with parental CHO-K1 cells (Fig. 5*D*). The 2-fold reduction in lysis, observed for mutant CHO cells paralleled with the 2-fold reduction in lysis of parental CHO-K1 cells in the presence of anti-NKp30 or anti-NKp46 serums (Fig. 5, *D* and *E*). Neither serum was able to inhibit the residual lysis of the mutant CHO cells. Finally, GAS6 cells lacking glypican-1 were less sensitive to NK killing as compared with parental sham-PANC-1 cells over-expressing glypican-1 (Fig. 5*F*). Therefore, presence of membranal heparan sulfate was correlated with NKp30- and NKp46-mediated lysis of tumor by NK cells.

Discussion

This report shows that carbohydrates on target cells are involved in the binding of NK cytotoxicity receptors, NKp30 and NKp46, to their cellular ligands. We initially picked the 6-*O*-sulfo-LacNAc from a carbohydrate library and further emphasized the significance of 6-*O*-sulfo-*N*-acetylglucosamine in target cell carbohydrate structures for binding of NKp30- and NKp46-fusion Igs (Fig. 1). Because 6-*O*-sulfo-LacNAc was not the exact structure recognized, we further determined that heparin/heparan sulfate can inhibit the binding of these fusion proteins (Fig. 2). Efficient Inhibition was observed even at 0.1 μg/ml (~0.03 μM) of heparan sulfate (Fig. 2). In correlation with our observations for 6-*O*-sulfo-*N*-acetylglucosamine, *O*-sulfation and *N*-acetylation, but not *N*-sulfation, of heparin were imperative for its efficient blocking (Fig. 2*C*). We further demonstrated that cell surface HSPGs are recognized by NKp30 and NKp46 and that cells lacking heparan sulfate, but not chondroitin sulfate, were recognized to a lesser extent by NKp30 and NKp46 (Figs. 3 and 4*A*). For PANC-1 cells we demonstrated the involvement of a particular HSPG, glypican-1, in recognition of tumor cells by NKp30 and NKp46 and this recognition paralleled membranal heparan sulfate expression (Fig. 4*C* and *Results*). As GPI-anchored proteins, glypicans are probably sorted into membranal lipid rafts (50). Confocal microscopy studies with NKp30-Ig and NKp46-Ig revealed that their cellular ligands are aggregated on the cell membrane in a pattern reminiscent of a lipid rafts pattern (described for PC-3, A. Porgador, unpublished observations). Yet, involvement of heparan sulfate on glypicans is probably not used for all tumors as shown for 1106 melanoma cells (Fig. 4*B3*). As tumors do not have to express or over-express glypicans, heparan sulfate chains on other classical HSPGs, the syndecans, might be involved in the recognition by NKp30 and NKp46. Additionally, other membranal proteins on tumor cells, reported to be modified by sulfated GAG chains, such as the CD44 (51) or chemokine receptor CXCR4 (52), might be involved.

Moretta et al. (33) discussed that although the cellular ligands for NCRs appear to be expressed on both normal and tumor cells, it is possible that their expression could be modified by cellular stress, cell activation, or tumor transformation. Indeed, HSPGs (both glypicans and syndecans) of transformed cells were reported to be either over-expressed or modified in their GAG content (42, 45, 46, 53, 54).

In accordance with our binding inhibition results (Figs. 1–4), NK lysis of tumor cells was reduced in the presence of 6-*O*-sulfo-LacNAc-PAA and tumor cells expressing cell surface heparanase were lysed to a lesser extent as compared with parental cells (Fig. 5, *A* and *C*). Similarly, failure to produce heparan sulfate resulted in reduced NKp30- and NKp46-mediated lysis by NK, independently from the levels of cell surface chondroitin sulfate (Fig. 5, *D* and *E*). Involvement of HSPGs in NK lysis was shown for glypican-1 expressed by PANC-1 cells, as specific suppression of its expression reduced tumor cell lysis by NK cells (Fig. 5*F*). Presence of LMW heparin reduced NK lysis only when high heparin concentrations were applied (Fig. 5*B*). This probably reflects the complex contradictory effects of heparin on lytic potential of NK cells (48). Activation of lysis by NK cells is also affected by NK receptors other than NCRs. The 2B4 receptor is considered a co-receptor that functions through the simultaneous engagement of triggering receptors such as the NKp46 (3, 5). The natural ligand of 2B4 is CD48, a GPI-anchored protein that bind to heparin/heparan sulfate (55). An intriguing scenario is that engagement of target-expressed CD48 and HSPGs in lipid rafts of target tumor cells causes engagement of 2B4 and either NKp30 or NKp46 on the NK membrane, followed by NK activation signaling.

We previously reported that the NKp44 and NKp46 proteins, but not the NKp30, recognize viral HAs and that this recognition requires the sialylation of NKp44 and NKp46 oligosaccharides (9, 10). However, the direct involvement of carbohydrates in binding of NKp30 and NKp46 to their cellular ligands is probably restricted to carbohydrates expressed on target cells and not to the oligosaccharides of NKp30 and NKp46. First, desialylation of NKp46-Ig reduces its binding to viral HAs, but not to cellular ligands (9, 10) and similarly for NKp30-Ig (data not shown). Secondly, the absence of the whole oligosaccharide side chain in each of the three glycosylation sites of NKp46D2 does not reduce its binding to cellular ligands (11). Thirdly, in this study, preincubation of target cells with either 6-*O*-sulfo-*N*-acetylglucosamine-based Glyc-PAA or heparin/heparan sulfate, followed by a wash of the unbound carbohydrate excess, did not inhibit the binding of NKp30- and NKp46-fusion Igs. Thus, these carbohydrates are associated with the cellular ligands and are recognized by NKp30 and NKp46 and not vice versa.

Lysis experiments based on mAb-mediated masking of NCRs revealed that different tumor cells appear to lack certain NCR ligands and that the ligands for NKp30, NKp44, and NKp46 are probably different (33). Our results indicate that NKp30 and NKp46 have similar recognition pattern with regard to carbohydrate structures on target cells. These observations are not mutually exclusive and several models can be brought forward that also suggest the possible role of HSPGs in recognition of tumors by NCRs: 1) The model of heparin/heparan sulfate interaction with

growth factors and growth factor receptors. The most characterized model is the binding of fibroblast growth factor (FGF) to FGFR. Both FGF and FGFR contain heparin-binding sequences (56). Two crystal structures of FGF, FGFR, and a heparin decasaccharide complex reveal that heparin makes numerous contacts with both FGF and FGFR, additional to the contacts between FGF and FGFR (57, 58). From experiments with cells deficient in HSPG synthesis or sulfation, or cells pretreated with heparan sulfate-degrading enzymes, it is clear that interaction between FGF and FGFR involves membrane-associated HSPGs acting as coreceptors (59). It is possible that the cellular ligands for NKp30 and NKp46 are different, yet both contain heparin/heparan sulfate-binding sequences and the heparan sulfate GAG of target cell membrane-associated HSPG, acting as a "co-ligand," stabilizes the binding between the NCR and its specific cellular ligand. Recognition of stromal cells by CD19, a coreceptor of the B cell receptor, was shown to involve HSPGs expressed by the stromal cells (40). 2) The model of interaction of HSPG with a lipid-binding protein. The binding of membrane-associated HSPG to lipid-binding proteins, e.g., apolipoprotein E or annexins, promotes their activities (56). It is possible that NKp30 and NKp46 bind to different target cell-membranal lipid moieties and the association with membranal HSPG augments this binding. Despite intensive biochemical efforts, we and others have been unable to identify a specific protein entity to account for a cellular ligand for NCR, thus favoring the likelihood of cellular ligands involving carbohydrates, lipids or both.

A third model in which the cell surface HSPGs are the primary cellular ligands and not just augment binding of NCRs to yet unknown ligands. Cell surface HSPGs belong primarily to two families of molecules, the syndecans and glypicans, containing >10 different members (43). A possible interpretation of our findings is that both GAG side chains and the core protein of the HSPG contribute to the recognition by NKp30 and NKp46. The difference between the cellular ligands of NKp30 and NKp46, observed from lysis assays, is correlated with NKp30 and NKp46 recognition of distinct HSPGs, differing in the core protein sequences that contribute to the binding. Yet, our results with the PANC-1 and glypican-1 hardly fit within this model. Heparan sulfate exhibits a considerable number of unique overlapping sequences with peculiar sulfation profiles and these sequences are recognized by specific complementary proteins (42). A more intriguing scenario is that the tumor-modified heparan sulfate can express separate unique carbohydrate epitopes recognized distinctively by NKp30 or NKp46 and rarely or not expressed on membranal HS of normal cells. A recent observation indicated that membranal expression of heparin/HS-degrading endosulfatase (HSulf-1) is evident for normal, but not cancerous, cells (60). Membranal HS-degrading endosulfatase diminish HSPGs sulfation and its absence on cancer cells leads to higher HSPG sulfation correlated with better signaling through growth factor receptors involving HSPGs as coreceptors (60). In particular, the sulfation state of *N*-acetylglucosamine residues on HSPGs is influencing signaling by heparin/heparan sulfate binding growth factors and corresponding receptors (61). If unusual heparan sulfate epitopes on cancer cell membranes lead to better signaling through growth factor receptors, it might be that NK-expressed NCRs evolved to recognize and penalize the uncommon epitope-expressing transformed cells. If so, this recognition can be considered as "transformed cell pattern recognition" by NK cells, similar to pattern recognition of infectious agents used by innate immunity in which NKs play a central role.

Identification of the cellular ligands recognized by NCRs is one of the major problems remains to be solved in the NK field. Our results are the first clue of the identity of these ligands. Further research is needed to determine whether these ligands are particular HSPGs or a complex of HSPGs with other protein or lipid moieties.

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