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NKR-P1A Protein, an Activating Receptor of Rat Natural Killer Cells, Binds to the Chitobiose Core of Uncompletely Glycosylated N-linked Glycans, and to Linear Chitooligomers

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NKR-P1 represent a family of activating receptors in rodent natural killer cells related to C-type animal lectins. We identify here the elements involved in the reactivity of the major receptor of rat, NKR-P1A, with N-linked oligosaccharides of glycoproteins. Plate inhibition assays with isolated, structurally defined N-glycans as inhibitors of binding of NKR-P1A to $GlcNAc_{16}$-BSA revealed that the removal of both the external sialic acids and the penultimate galactose residues resulted in attaining of significant inhibitory activities. Surprisingly, additional plate inhibition and glycoprotein overlay experiments brought evidence that the core chitobiose, depending on its substitution, can *per se* support the interaction with NKR-P1A. In a series of linear chitooligomers (n = 2–7), the inhibitory activities reached a maximum for the chitotetraose. The ability of NKR-P1 to recognize both the periphery and the core region of complex type oligosaccharides may define its dual specificity towards carbohydrate components of eukaryotic (e.g., tumor) cell surfaces, but also reflect an evolutionarily conserved reactivity with microbial saccharides important in immune recognition and signaling functions.

Natural killer (NK) cells represent a distinct class of lymphocytes that do not express clonotypic antigen-specific receptors characteristic for T- and B-lymphocytes [1], but display at their surface a group of antigens related to C-type animal lectins [2,3]. These receptors, through their association with the key enzymes involved in intracellular signaling, regulate a balance between activating and inhibitory signals controling the outcome of many processes occuring in NK cells [2,4].

Because of the evolutionarily distant relation of these receptors to most animal lectins [5,6], their ability to bind carbohydrates as well as calcium had to be verified experimentally. It has been indeed found for at least three members of this family, namely NKR-P1, CD69 and Ly-49, that they bound both calcium and carbohydrates with high affinity [2,7-9]. Detailed study of the binding specificity of rat NKR-P1 protein, a prototype molecule of the whole family, showed that this protein can bind to a multitude of neutral as well as charged oligosaccharide sequences related to blood group, ganglio and glycosaminoglycan structures [7,10].

Since the simplest carbohydrate ligands for NKR-P1 are GalNAc and GlcNAc [7], and in the view of the published data showing that underglycosylated oligosaccharides related to N-linked glycans of glycoproteins might be involved in tumorigenesis, metastasis and NK sensitivity of certain malignant cells [11], we have been interested to investigate the reactivity of soluble NKR-P1 proteins with this group of potential carbohydrate ligands [12]. Here we show that the removal of external sialic acid and galactose residues results in acquisition of reactivity of NKR-P1 towards the internal carbohydrate sequences of N-linked glycans. This binding activity is due to the interaction with the external GlcNAc branches as well as with the chitobiose core. This hitherto unreported reactivities of NKR-P1 may be critical for the role of this receptor in evolutionarily conserved recognition and signaling processes.

MATERIALS AND METHODS

Materials. $GlcNAc_{16}$BSA, jack bean β-galactosidase and jack bean β-N-acetylglucosaminidase were from Sigma. Enzymes peptide: N-glycosidase F, α1-2,3 mannosidase and α1-6 mannosidase were

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from New England BioLabs, acylamino acid peptidase was from Boehringer-Mannheim. Other reagents were of analytical grade.

Preparation and analysis of glycoproteins. Egg white glycoproteins ovalbumin and ovomucoid were purified by HPLC as described previously [13], and their homogeneity verified by SDS-PAGE, and by performing 15 cycles of automated Edman degradation of their N-termini directly from PVDF blotted samples (Protein Sequencer LF3600, Beckman). In the case of ovalbumin, the N-terminus of which is blocked by acetylation [14], enzymatic deblocking by aminoacyl aminotransferase was performed [15].

Preparation and analysis of oligosaccharides. Preparation of oligosaccharides G2 [Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2-Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc], N2 [GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc], and M2 [Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc] was described previously [16]. Oligosaccharides M6 [Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc] and M3 [Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc] were prepared from oligosaccharide M2 by digestion with α1-2,3 mannosidase and α1-6 mannosidase, respectively, and repurified by HPLC. In a similar way, oligosaccharide 2d [Manβ1-4GlcNAcβ1-4GlcNAc] was prepared by the combined digestion of the two above enzymes. The preparation of linear chitooligomers 2, 3, 4, 5, 6 and 7 (all containing β1-4 anomeric linkages) and disaccharides 2e [GlcNAcβ1-6GlcNAc], 2a [Glcβ1-4GlcNAcβ1-4GlcNAc] and 2b [Galβ1-4GlcNAcβ1-4GlcNAc] has been published [17]. Oligosaccharide 2c [Glcα1-4GlcNAcβ1-4GlcNAc] was prepared by the transglucosylation of chitobiose using thermophylic cyclodextrineglucanotransferase (CGTase) from *Bacillus stearothermophilus* (kind gift of Hayashibara Co., Okayama, Japan) [18]. Chitobiose (160 mg, 0.37 mmol) and soluble dextran (300 mg) was dissolved in Na-acetate buffer (2 ml, 50 mM, pH 6.0), 5 % $CaCl_2$ solution (50 μl) and 140 U (100 μl) of CGTase were added and the reaction mixture was incubated at 37 °C for 6 days. Then the reaction was stopped by heating, and the product was separated by gel filtration (column 90 $\times$ 2.5 cm) on Toyopearl WH40S (Toyo Soda). The yield of the title compound **2c** was 23.5 %. CGTase is known to attach a αGlc*p* unit stereo- and regioselectively to terminal sugars of the *gluco*- configuration in various compounds [19,20]. 1H NMR, 13C NMR and ESI-MS were used to confirm the anticipated structures of the above oligosaccharides.

Preparation of recombinant soluble forms of NKR-P1A. The dimeric (NKR-341) and the monomeric (NKR-391) soluble forms of NKR-P1 were prepared as described previously [7], and characterized by SDS electrophoresis under reducing and nonreducing conditions, HPLC gel filtration analysis (Superdex 200 HR column, Pharmacia), and by performing 12 cycles of automated Edman degradation (Protein Sequencer LF3600, Beckman). The refolded proteins were stored at 4 °C in a storage buffer containing 10 mM Hepes pH 7.5 with 150 mM NaCl, 1 mM $CaCl_2$ and 5 % glycerol at protein concentration of 1 mg/ml. Proteins were radioiodinated from this stock solutions using carrier-free $Na^{125}I$ (Amersham) and Iodogen protocol (Pierce).

Overlay on PVDF blots with natural glycoproteins. 40 μl of aqueous solutions of ovalbumin or ovomucoid (both at 10 mg/ml) was pipetted into a set of triplicate tubes, and treated with buffer only (samples N) or with a mixture of jack bean β-galactosidase (5 U) and β-N-acetylhexosaminidase (5 U - samples E) or denatured and treated with peptide:N-glycosidase (100 U, samples G) as recommended by the enzyme manufacturers. After an overnight incubation at 37 °C, protein samples were resolved on duplicate 10 % polyacrylamide gels and electrotransferred onto PVDF sheets (Immobilone P, Millipore). One membrane was stained for protein by Coomassie Brilliant Blue R-250 (and used also for N-terminal sequencing - see above), and the duplicate membrane was blocked in 5 % BSA in PBS, overlayed with radioiodinated NKR-P1 protein diluted in 5 % BSA in PBS, (10^6 cpm/ml, specific activity approx. 10^7 cpm/ug protein) for 1h at room temperature, washed three times 1 min, dried, and exposed onto Kodak XAR-5 film with intensifying screens. Aliquots of the original samples were also dialysed extensively against 1 % acetic acid, and used for the determination of bound hexosamines [21], and for mass spectro-metry. Positive ion MALDI mass spectra were measured on a Bruker BIFLEX time of flight mass spectrometer (Bruker-Franzen, Bremen, Germany) in linear mode. Typically, 50 shots were summed into a single mass spectrum, and average masses of $[M+H]^+$ and $[M+2H]^{2+}$ ions of BSA were used for external calibration. 2,5-dihydroxybenzoic acid was used as the matrix.

Quantitative binding and inhibition assays. These assays were performed essentially as described previously [7]. Oligosaccharide inhibitors tested were quantified by monosaccharide compositional analysis, and than kept frozen at -20 C as concentrated (10^{-2} M) solutions in assay buffer (PBS with 5% BSA). The samples were then serially diluted in assay buffer, and mixed with the solution of

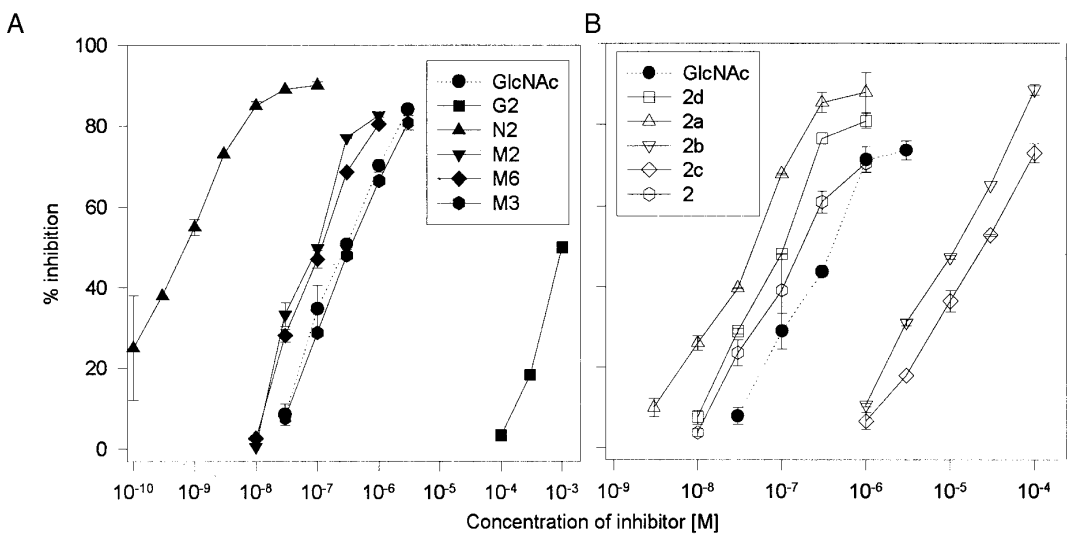

FIG. 1. Evaluation of potencies of oligosaccharides related to biantennary structures (1a) and to the mannosyl chitobiose core (1a, 1b) of N-linked glycans to inhibit binding of soluble dimeric NKR-P1A to its high affinity ligand. See Materials and Methods for the details of this binding assay, and for the structures of individual oligosaccharides tested.

radiolabelled soluble NKR-P1 [7] immediately before each inhibition assay.

RESULTS AND DISCUSSION

Our initial study on the reactivity of NKR-P1A with defined N-linked oligosaccharides revealed a complex inhibition pattern [12]. To investigate this complexity, we used a series of neutral structurally defined oligosaccharides subjected to sequential exoglycosidase treatments (Fig. 1a). We observed that the galactosylated biantennary oligosaccharide G2 is an extremely poor inhibitor (IC_{50} around 10^{-3} M), but it became a good ligand after the subsequent removal of the penultimate β-D-galactosyl residues (see oligosaccharide N2, IC_{50} approx. 10^{-9} M). Interestingly, however, further degradation of the oligosaccharide chain resulted in a series of mannose-terminated oligosaccharides M2, M6 and M3, the IC_{50} values of which did not drop suddenly to zero, but were gradually approaching the level of inhibition typical for GlcNAc (approx. 3×10^{-7} M, Fig. 1a). Thus, despite notable loss of binding after the removal of peripheral GlcNAc branches, the resulting oligosaccharides could still inhibit binding of NKR-P1A.

To assess the relevance of this "residual" binding in a more complex context of the entire glycoprotein, we performed overlay experiments with two model glycoproteins, ovalbumin (bearing a single site of glycosylation at Asn 293) and ovomucoid (with four sites of N-glycosylation, Asn 34, 77, 93, and 99), subjected to various glycosidase treatments. Significant loss of binding was observed after the digestion with exoglycosidases (Fig. 2, compare lanes N and H), concomitant with the measurable (by mass spectrometry) decrease in molecular masses of these glycoproteins, and decrease in bound hexosamines (see Fig. 2 legend). In contrast, a complete abolishion of binding was observed after the complete deglycosylation of these glycoproteins by peptide: N-glycosidase F (Fig. 2, lanes G) proved by the expected reduction in their molecular masses (as shown by SDS electrophoresis mobility shifts, and by mass spectrometry, see Fig. 2 and legend to Fig. 2).

To investigate further the specificity of interaction of NKR-P1A with the core region of N-linked oligosaccharide chains, we performed inhibition assays with a second series of structurally defined oligosaccharides. We have found that the naturally occuring trisaccharide 2d was a good ligand for NKR-P1A (IC_{50} of about 10^{-7} M), although even higher affinity could be achieved with a closely related trisaccharide 2a (IC_{50} approx. 3×10^{-8} M) in which the β 1-4 linked mannose was substituted by similarly linked glucose. The chitobiose core *per se* (oligosaccharide 2) is slightly more active than GlcNAc, with IC_{50} of 1 and 3×10^{-7} M, respectively. Moreover, the affinity of this interaction towards oligosaccharides resembling the naturally occuring structures is clearly demonstrated by poor inhibitory activities of oligosaccharides 2b and 2 c containing the terminal galactosyl $\beta 1 \rightarrow 4$, and glucosyl $\alpha 1 \rightarrow 4$ residues, respectively (both with IC_{50} around 10^{-5} M).

Among various other substitutions of the core chitobiose tested, the extension by the addition of GlcNAc was of particular interest. We first established that, in order to achieve optimal binding, such GlcNAc oligo-

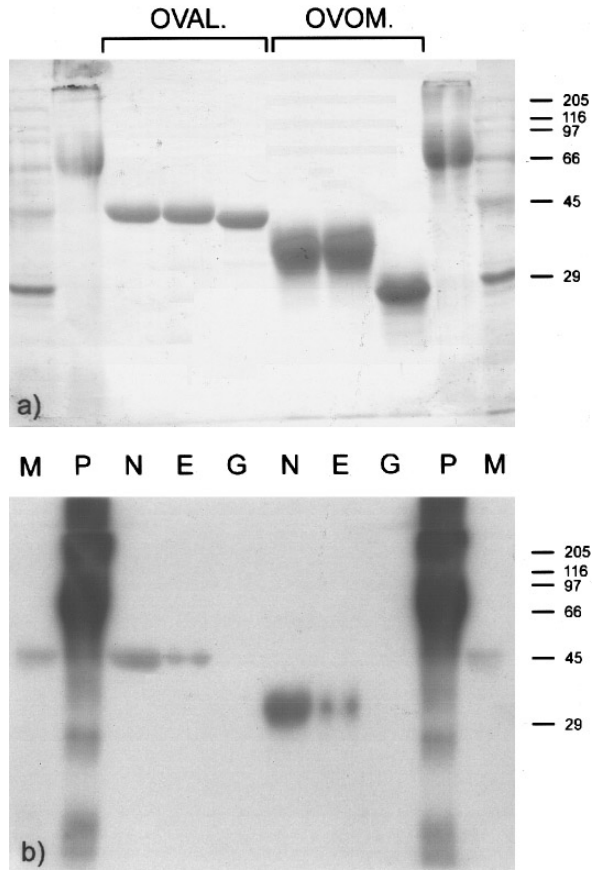

FIG. 2. Binding of soluble dimeric NKR-P1A protein to natural glycoproteins. Egg white glycoproteins ovalbumin (oval.) and ovomucoid (ovom.) either native (N), or treated with the exoglycosidases (E) or with N-glycanase (G), were separated on polyacrylamide gels and electrotransferred onto PVDF membranes. Membrane was stained with Coomassie Brilliant Blue R-250 (a), or blocked, overlayed with radioiodinated NKR-341 protein, washed, and binding detected by autoradiography (b) as described under Materials and Methods. Protein markers (M) included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa), 1 μg of each per lane. $GlcNAc_{16}BSA$ lanes (P) were also included as positive controls. The precise molecular masses of the glycoprotein ligands determined by mass spectrometry were 44851, 44078 and 43303 for native, exoglycosidase-treated and N-glycanase treated ovalbumin, respectively (the theoretical molecular mass for ovalbumin devoid of carbohydrates is 42750). These values for ovomucoid were 29260, 27910, and 20128, respectively (theoretical molecular mass 20098). The content of bound hexosamines for native ovalbumin and exoglycosidases-treated ovalbumin was 3.87 and 2.14 mol/mol of protein, respectively, and these values for ovomucoid were 15.41 and 10.5 mol/mol protein, respectively.

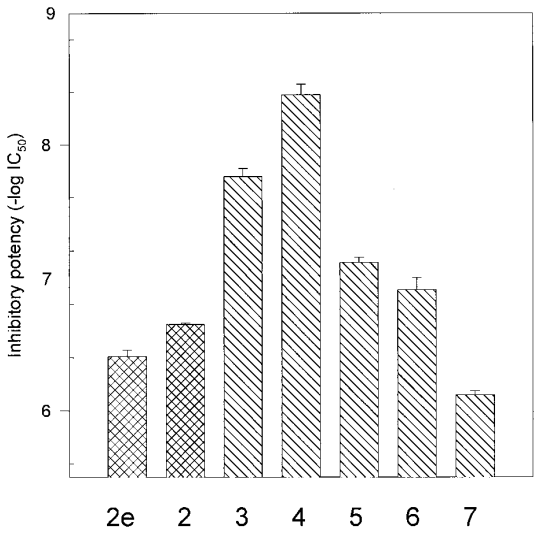

FIG. 3. Inhibition of NKR-P1 binding to $GlcNAc_{16}BSA$ with linear chitooligomers. Inhibition experiments were performed as described in Materials and methods, and in reference 7. Inhibitory potencies (-log IC_{50}) shown are the mean ± range calculated from two independent experiments performed with the dimeric (NKR-341) protein; the above differences could not be observed with the monomeric (NKR-391) protein, for which IC_{50} were around 2×10^{-6} M for all the compounds tested with the exception of chitotetraose ($IC_{50} = 1 \times 10^{-6}$ M) and oligosaccharide 2e, which was not tested with the monomeric protein.

mers must possess β1→4 linkages, as evident from the notable drop in the inhibitory activity of the disaccharide GlcNAcβ1-6GlcNAc when compared to the reference chitobiose (Fig. 3). Additional extension in the linear chitooligomer series resulted in a gradual increase in inhibitory potency with the maximal value being achieved for the chitotetraose (IC_{50} of 5×10^{-9}). Upon further extension of length the inhibitory activity decreased. Moreover, this effect could only be seen for the dimeric form of the protein, since similar experiment performed with the monomeric form showed a uniform inhibitory potency with IC_{50} values being all around that recorded for a simple GlcNAc inhibition (approx. 10^{-6} M, see ref. 7).

In conclusion, we have demonstrated that a major activating receptor of rat natural killer cells, NKR-P1A protein, reacts with neutral degalactosylated oligosaccharides related to N-linked glycans of glycoproteins. Part of this reactivity is towards the peripheral GlcNAc branches, but a significant portion seems to be mediated by the substituted chitobiose core. This reactivity may be of physiological importance since it can be demonstrated in the context of the entire glycoprotein molecule. Moreover, its significance may extend beyond the recognition of incompletely glycosylated N-linked oligosaccharide chains [11], since the extended linear chitooligomer chains would be encountered by NKR-P1A as a part of the demonstrated protective function of NK cells against microbial infections [22]. The ability of the dimeric form of NKR-P1A to interact with defined linear chitooligomers such as chitotetraose may become extremely useful during ligand interaction and ligand cocrystallization studies currently performed with this receptor.

It is worth to note that analogous carbohydrate motif, i.e. modified chitopentaoses (lipochitin oligosaccharides) occur as NOD factors triggering differentiation of root tissues of leguminous plants and thus forming symbiotic nodules with rhizobia [23]. Recently, studies in vertebrate embryogenesis in Zebrafish have also shown that protein DG42 produces similar chitooligomers that play important role in embryonic development (e.g. tail formation) [24]. Our findings with NKR-P1 protein together with the above findings can lead to a hypothesis that derivatives of chitopentaose are presumably important signaling factors found at various stages of phylogenesis.

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