GlcNAc-terminated glycodendrimers form defined precipitates with the soluble dimeric receptor of rat natural killer cells, sNKR-P1A

Karel Bezouška[a,*], Vladimír Křen[b], Christoffer Kieburg[c], Thisbe K. Lindhorst[c]

[a]*Department of Biochemistry, Faculty of Science, Charles University Prague, Hlavova 8, CZ-12840 Praha 2, Czech Republic*
[b]*Institute of Microbiology, Laboratory of Biotransformation, Academy of Sciences of Czech Republic, Vídeňská 1083, CZ-14220 Praha 4, Czech Republic*
[c]*Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany*

Received 14 February 1998; revised version received 3 March 1998

Abstract Synthetic GlcNAc-terminated thiourea-bridged glycoclusters were found to be potent inhibitors of binding of the soluble dimeric receptor of rat natural killer cells, sNKR-P1A protein, to its high affinity ligand. Moreover, we have shown here that characteristic precipitation curves can be recorded upon mixing of the GlcNAc glycoclusters with sNKR-P1A. For the $GlcNAc_8$ glycocluster the precipitation curve is biphasic, with high affinity and low affinity precipitates differing in their sensitivity towards GlcNAc-mediated inhibition of precipitation. Quantitative analyses of the precipitates indicate the occurrence of a single sugar binding site per sNKR-P1A subunit, and lead to a model of the most possible spatial arrangements of the glycocluster-receptor lattices. These results provide new tools for further studies on carbohydrate recognition by NKR-P1A.

Key words: Glycodendrimer; Ligand clustering; Natural killer cell; Receptor; Lectin

1. Introduction

Synthetic glycoconjugates are becoming increasingly popular in studies of the carbohydrate binding proteins. These compounds have been traditionally prepared by conjugating mono- or oligosaccharides to lipid or protein carriers resulting in neoglycoconjugates with a wide range of applications [1]. Despite the recent improvements in attachment chemistries allowing full preservation of the natural bond between sugar and the carrier molecule [2,3], classical neoglycoconjugates suffer a number of drawbacks [4]. The heterogenous distribution of ligands complicates unambiguous interpretation of the binding data. Binding results obtained with artificially high valency neoglycoconjugates are difficult to relate to the situations in vivo, and the use of these compounds in therapeutic protocols may result in uncontrollable events due to the occurrence of highly avid interactions. At the same time, the problems with the antigenicity of these compounds increase severely with their molecular size. For these reasons, new synthetic schemes have been designed that afford the preparation of chemically defined multivalent glycoconjugates [4–6]. Four families of such glycoconjugates have emerged: carbohydrate oligomers, multivalent glycopeptido-mimetics, glycan derivatives of calix [4] arenes, and glycodendrimers (tree-like sugar containing molecules, reviewed in [4]), as well as smaller glycoclusters. The synthetic scheme for the preparation of thiourea-bridged glycoclusters and glycodendrimers have been elaborated recently. It involves the reaction of glycosyl isothiocyanates of different configurations with branched and hyperbranched tri-, tetra-, hexa- and octaamines to provide the corresponding multiantennary thiourea-bridged glycoclusters [7].

Here we report on the reactivities of glycoclusters containing three, four, six or eight terminal α-D-mannosyl or β-D-*N*-acetylglucosaminyl residues with the dimeric soluble form of the rat natural killer cell receptor, sNKR-P1A protein [8]. This is a recombinant protein prepared by bacterial expression of the extracellular portion of rat NKR-P1A antigen starting with leucine 66 [8]. It has been shown to be a carbohydrate binding protein (animal lectin [9]) that binds *N*-acetyl-D-glucosamine in its free or conjugated form [8,10,11] in addition to the spectrum of carbohydrates bearing acidic functionalities [12]. We have found that in plate inhibition experiments the low molecular mass GlcNAc-terminated glycoclusters are good ligands for sNKR-P1A with inhibition activities comparable with the classical high affinity neoglycoprotein ligands. Mixing of more concentrated solutions of the glycoclusters with the receptor results in the formation of insoluble precipitates. Highly specific lattices are formed between the $GlcNAc_8$ cluster and sNKR-P1A at ligand concentrations as low as 10^{-8} M. Quantitative analysis of the precipitates allowed us to detect the occurrence of a single carbohydrate binding site per sNKR-P1A subunit, and to suggest the structural patterns that govern these glycocluster-receptor interactions.

2. Materials and methods

$GlcNAc_{23}BSA$ was a gift from Prof. Y.C. Lee, Johns Hopkins University, Baltimore, MD. The synthesis and characterization of glycoclusters used in this study was described previously [7]. Two mM stock solutions were prepared by dissolving individual lyophilized glycoclusters in phosphate buffered saline (PBS, 10 mM sodium phosphate buffer with 0.14 M NaCl and 2 mM KCl, pH 7.4), and adjusting the final concentration on the basis of the determination of total hexoses (Man clusters [13]) and bound hexosamines (GlcNAc clusters [14]). The dimeric soluble form of NKR-P1A protein (sNKR-P1A, previously designated NKR-341) was prepared as described [8]. For testing as inhibitors of sNKR-P1A binding to $GlcNAc_{23}BSA$ (the standard high affinity ligand used in the previous studies [8,11]), the stock solutions of glycoclusters were serially diluted in PBS buffer, and added to the plate inhibition assays [8]. For precipitation assays, 30 μl aliquots of sNKR-P1A (2×10^{-4} M) in PBS were mixed with 30 μl aliquots of glycoclusters serially diluted in PBS

*Corresponding author. Fax +420 (2) 2195-2331.
E-mail: BEZOUSKA@BIOMED.CAS.CZ

Abbreviations: NK, natural killer; sNKR-P1A, soluble natural killer receptor protein-1, isoform A; PBS, phosphate buffered saline; BSA, bovine serum albumin; BCA, bicinchoninic acid

PII S0014-5793(98)00340-8

to twice their final concentrations tested, incubated for 2 h at room temperature, spun for 10 min in microcentrifuge, and the precipitate was washed three times with PBS. The amount of precipitated protein was assayed by bicinchoninic acid (BCA) method [15] and compared to that precipitated by 10% trichloroacetic acid. Additional precipitation and inhibition of precipitation experiments were performed in similar way with 10 μl of ^{125}I-sNKR-P1A (2×10^{-6} M, diluted with unlabeled sNKR-P1A to a specific activity of 10^5 c.p.m./mg protein). Glycoclusters were added in 5 μl at four times their final concentrations tested together with 5 μl of PBS, or (where appropriate) 5 μl of serial dilutions of the monosaccharides. After incubation and spinning as above, the precipitates were washed three times with 1 ml of PBS, and their radioactivity was determined in a gamma counter. In quantitative precipitation experiments the indicated amounts of protein and glycoclusters (see Table 1) were mixed in the given volumes of PBS, incubated overnight at 4°C, and then for 2 h at room temperature. The precipitate was recovered by centrifugation, and washed four times with the appropriate volumes of PBS. Washed precipitates were dried, and used for the determination of protein (BCA assay) [15] and of the bound GlcNAc [14].

3. Results

The structures of the glycoclusters used in this study are shown in Fig. 1. Eight compounds were tested from the collection of glycodendrimers prepared [7] based on the previously reported carbohydrate specificity of sNKR-P1A [8]. GlcNAc was selected as the high affinity monosaccharide ligand (IC_{50} for sNKR-P1A is 2×10^{-7} M), while mannose, the least active monosaccharide inhibitor (IC_{50} 10^{-3} M) was chosen as the negative control. Thus, tri-, tetra-, hexa-, and octavalent glycoclusters with terminal α-D-Man and β-D-GlcNAc residues were employed, for which the respective designation Man_3, Man_4, ..., Man_8, and $GlcNAc_3$, $GlcNAc_4$, ..., $GlcNAc_8$ will be used.

Fig. 2a presents the results obtained when these compounds were tested as inhibitors of binding of sNKR-P1A with the

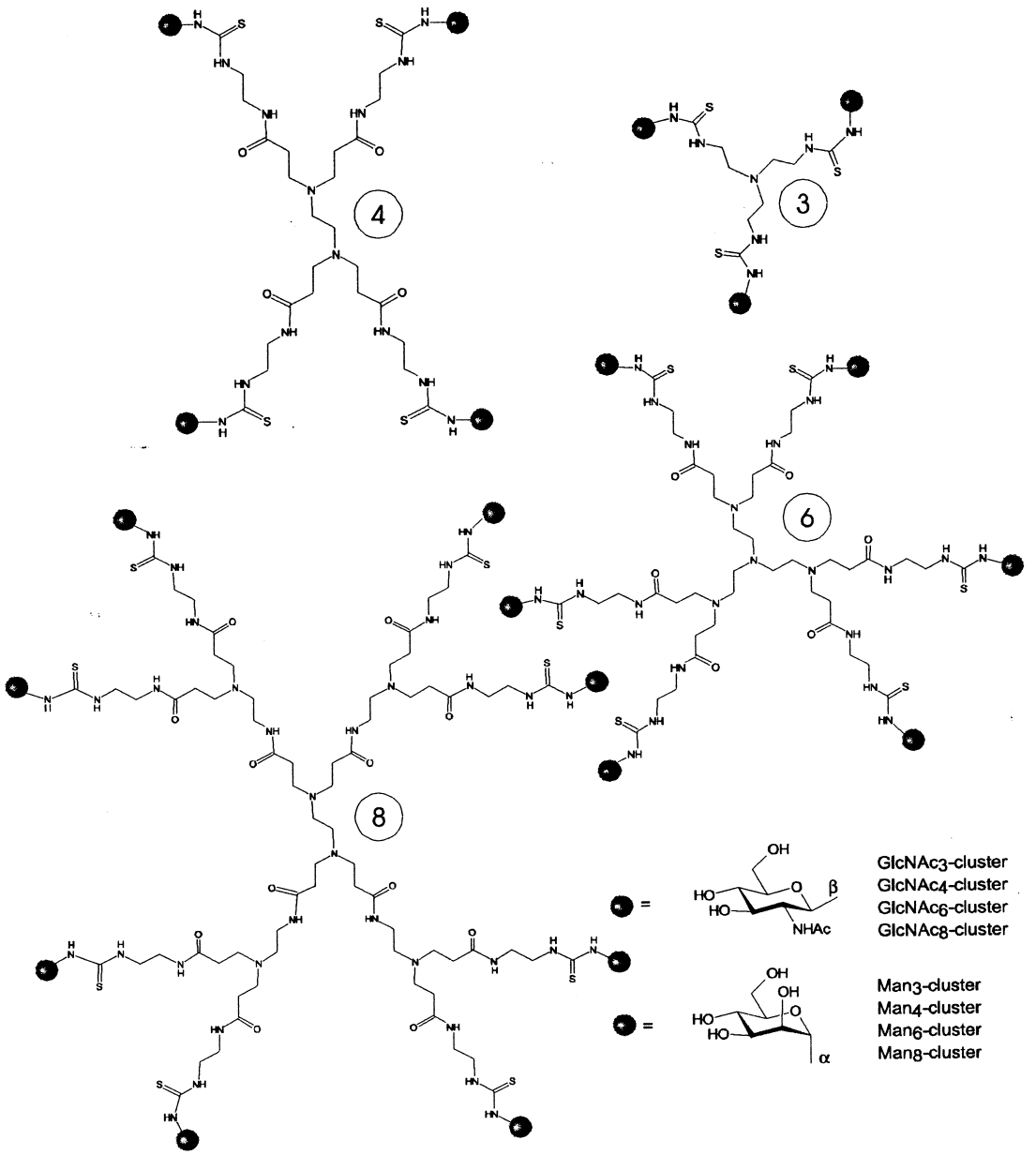

Fig. 1. Structure of the glycoclusters used in this study.

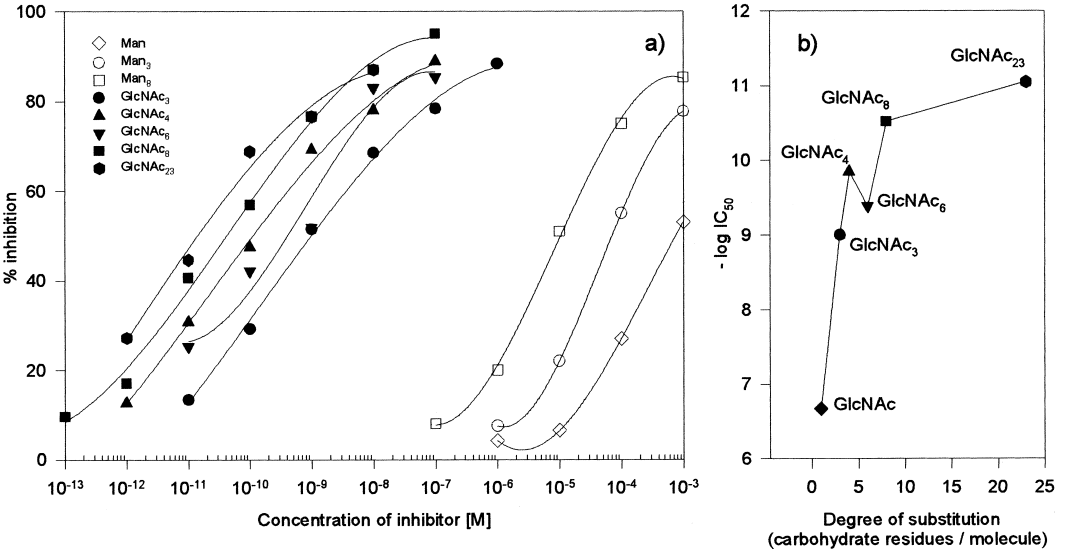

Fig. 2. Evaluation of the activities of selected glycoclusters as inhibitors of sNKR-P1A binding to its high affinity ligand, $GlcNAc_{23}$BSA. Selected glycoclusters (see a, inset) were serially diluted over a range of concentrations, and added to the inhibition assay performed as described in Section 2. $GlcNAc_{23}$ is the soluble high affinity neoglycoprotein ligand, $GlcNAc_{23}$BSA [8]. In b, the IC_{50} values are plotted against the degree of substitution by carbohydrate for the GlcNAc glycoconjugates. Data plotted are average values of duplicate experiments.

high affinity ligand, $GlcNAc_{23}$BSA. It is obvious from the inhibition curves recorded that both $GlcNAc_3$ and $GlcNAc_4$ were quite good inhibitors with IC_{50} of 10^{-9} and 10^{-10} M, respectively. Surprisingly, $GlcNAc_6$ was a worse inhibitor than $GlcNAc_4$ with IC_{50} dropping down to approximately 4×10^{-10} M. However, the inhibition potency of the $GlcNAc_8$ was very high (IC_{50} 4×10^{-11} M), so that it compared favorably with the highly substituted neoglycoprotein ligand $GlcNAc_{23}$BSA (Fig. 2b). Mannose clusters were used as negative controls in these experiments. Although they displayed moderate increase in IC_{50} values with the increased sugar valency, their inhibitory concentrations remained high, mostly in the micromolar range.

In addition to the above tests using solid phase inhibition assays, we wanted to study the interaction of the glycoclusters with sNKR-P1A protein in solution. Since the interaction of certain lectins with multivalent oligosaccharide ligands may results in the formation of saccharide-protein lattices [16–18], we tried to establish the conditions for the formation of insoluble precipitates with our protein. Serially diluted solutions of the glycoclusters were mixed with constant amounts of sNKR-P1A, and the precipitated protein was measured by BCA assay or by gamma counting. The results obtained by these approaches were similar. Data obtained using ^{125}I-sNKR-P1A are shown in Fig. 3a. No significant precipitation occurred with any of the Man clusters at any of the concentrations tested. Only little precipitation could be observed with $GlcNAc_6$ (Fig. 3a). Precipitation with $GlcNAc_3$ and $GlcNAc_4$ followed a standard saturation curve with the maximum at the highest concentrations of the clusters examined (10^{-4} M). However, $GlcNAc_8$ provided a biphasic precipitation curve with two distinct maxima at 10^{-8} and 10^{-4} M concentrations of the glycocluster (see Fig. 3a).

Interestingly, the occurrence of these two concentration regions for precipitation with $GlcNAc_8$ seems to reflect the existence of two qualitatively different forms of the precipitate, as evident from the inhibition of precipitation experiment shown in Fig. 3b. When the formation of precipitate between $GlcNAc_4$ and sNKR-P1A at 10^{-5} M concentration of the glycocluster was inhibited by the addition of the monosaccharide ligand, GlcNAc, the IC_{50} for this inhibition was 2×10^{-4} M. The IC_{50} for the inhibition of the analogous complex between sNKR-P1A and the $GlcNAc_8$ formed at 10^{-5} M concentration of the glycocluster was nearly identical, 3×10^{-4} M. However, when the inhibition of precipitation was performed at the concentration of 10^{-8} M $GlcNAc_8$ added, the IC_{50} was as low as 3×10^{-7} M. No inhibition of precipitation could be achieved with the nonreactive monosaccharide mannose (Fig. 3b, dotted line).

Quantitative analyses of the composition of the glycoden-

Table 1
Determination of the quantitative composition of precipitates formed between the GlcNAc glycoclusters and sNKR-P1A under different experimental conditions

Volume of pptn. mixture (ml)	sNKR-P1A added ($\times10^{-9}$ moles)	Glycocluster added ($\times10^{-9}$ moles)	sNKR-P1A in the ppt. ($\times10^{-9}$ moles)	GlcNAc in the ppt. ($\times10^{-9}$ moles)	Glycocluster in the ppt. ($\times10^{-9}$ moles)	sNKR-P1A subunit to glycocluster ratio (mole/mole)
1	10	$GlcNAc_3$c, 10	3.1 ± 0.3	6.0 ± 0.6	2.0 ± 0.2	3.1 ± 0.7
1	10	$GlcNAc_4$c, 10	4.0 ± 0.2	7.6 ± 1.2	1.9 ± 0.3	4.2 ± 1.0
1	10	$GlcNAc_8$c, 10	4.8 ± 0.3	19.2 ± 2.4	2.4 ± 0.3	4.0 ± 0.8
100	10	$GlcNAc_8$c, 1	4.0 ± 0.2	8.2 ± 0.3	1.0 ± 0.0	8.0 ± 0.4

Abbreviations used: pptn., precipitation; ppt., precipitate. Results shown are the average values ± standard deviations of the triplicate experiments. BCA assay and the assay for total bound GlcNAc ([15] and [14], respectively) were used for the determination of protein and glycocluster concentrations, respectively.

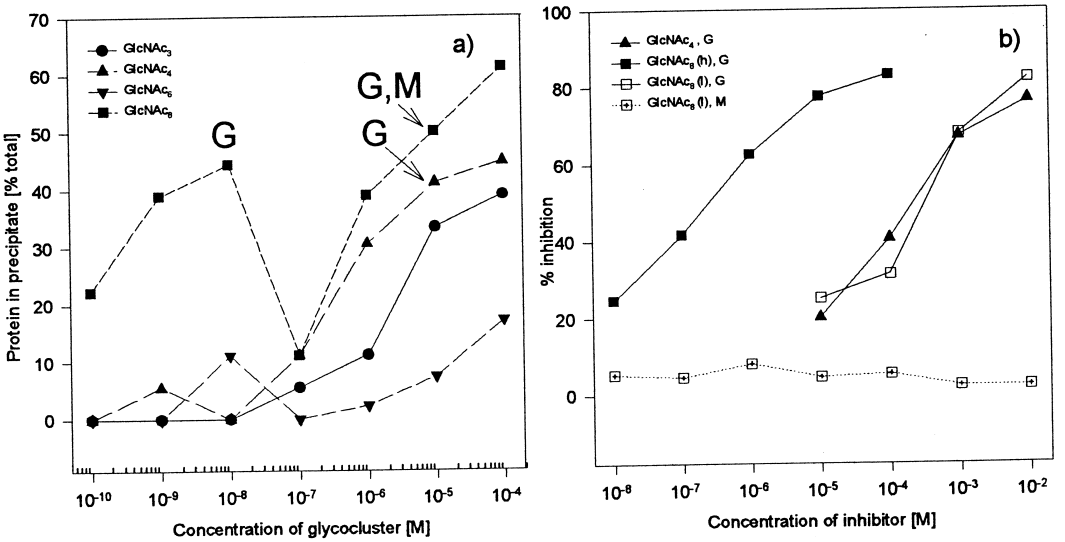

Fig. 3. Precipitation curves for the interaction between GlcNAc glycoclusters and sNKR-P1A protein. In a, a constant concentration of sNKR-P1 was mixed with serially diluted GlcNAc glycoclusters, and the % of total protein recovered in the precipitate was measured as described in Section 2. In b, the complexes marked in a were precipitated in the presence of serially diluted GlcNAc (G) or Man (M) to observe the inhibition of precipitation. $GlcNAc_8$ (h) and $GlcNAc_8$ (l) are abbreviations used for the high affinity and the low affinity $GlcNAc_8$ complexes, respectively. Data plotted are average values of duplicate experiments.

drimer-receptor complexes formed under different experimental conditions were performed by direct estimation of the amounts of total protein (BCA assay) and total bound GlcNAc in the washed precipitates (Table 1). Notably, by calculating the amounts of moles of the monomeric sNKR-P1A subunits bound per one mole of the glycocluster in the precipitate, these analyses allowed us to estimate the number of sugar-combining sites per sNKR-P1A subunit (this subunit contains a single stretch of sequence homologous with that of the carbohydrate-recognition domain of C-type animal lectins [9]). Since 3.1, 4.2, 4.0, and 8.0 moles of sNKR-P1A subunits were calculated to be bound to one mole of $GlcNAc_3$, $GlcNAc_4$, $GlcNAc_8$ (low affinity form of this precipitate), and $GlcNAc_8$ (high affinity form of this precipitate) glycocluster, respectively, (see Table 1), our results are consistent with the existence of a single sugar-combining site per sNKR-P1A subunit. In order to verify experimentally the efficiency of formation of the high affinity GlcNAc precipitate, a precipitation reaction was set in large volume (Table 1, last row). Even under such conditions, the complex between diluted solutions of the receptor and the glycocluster was formed very effectively, as judged from the remarkably high recovery of $GlcNAc_8$ in the precipitate.

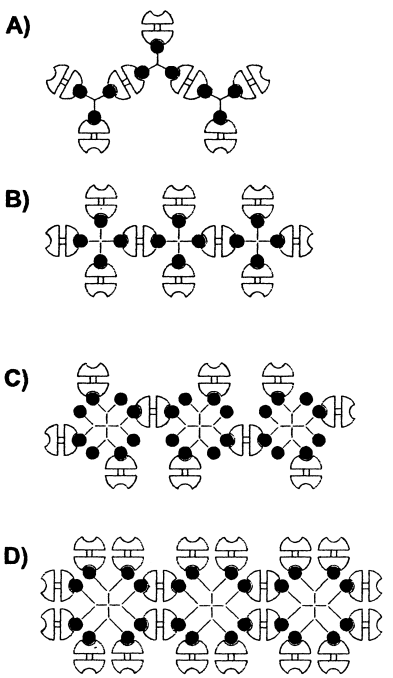

Fig. 4. Suggested structure of the GlcNAc glycocluster: sNKR-P1A lattices formed during the precipitation reactions. The symbols for individual glycoclusters were derived by further simplification of full structures given in Fig. 1, dimeric sNKR-P1A protein is depicted as sectioned globular head with each subunit containing a single binding site for carbohydrate (see Sections 3 and 4). The conditions used for the preparation of precipitates shown under A–D are those described in Table 1. In C and D, precipitates with sNKR-P1A were formed in high and low concentrations of $GlcNAc_8$, respectively, as explained in Table 1.

4. Discussion

We have found here that synthetic, highly branched GlcNAc-terminated glycoclusters, such as $GlcNAc_8$ are potent inhibitors of binding of the sNKR-P1A receptor to its high affinity ligand, $GlcNAc_{23}BSA$. It is obvious from our results that these compounds, despite their low molecular mass, are well comparable with traditional high molecular mass carbohydrate ligands for sNKR-P1A, such as natural glycoproteins [11] or neoglycoproteins [8]. The strong reactivity of these compounds with sNKR-P1A may relate to the degree of conformational freedom and accessibility conferred to the terminal monosaccharide residues; this is assumed to be better in the synthetic glycoclusters than in the neoglycoconjugates. This phenomenon may also explain the somewhat unusual hierarchy of inhibitory activities observed with GlcNAc glycoclusters: While the $GlcNAc_3$ and $GlcNAc_4$

may not possess sufficient density of the presented monosaccharide, linker lengths or linker flexibility, there may be steric reasons for the poor reactivity of the $GlcNAc_6$ glycocluster. On the other hand, the spacing of carbohydrates occurring in the $GlcNAc_8$ seems to be ideal for maximum reactivity with the receptor.

Our observation that the sNKR-P1A protein forms precipitates with the GlcNAc glycoclusters studied here seems to add a number of lectins for which this phenomenon has already been observed [16]. Results of Brewer and colleagues have shown that branched oligosaccharides can form a unique homogenous cross-linked lattices with certain animal and plant lectins [17,18]. A similar complex between galectin-1 and dibranched biantennary oligosaccharides has been examined by protein crystallography [19]. Recently, Gupta and Brewer observed the formation of two distinct complexes between calf spleen galectin-2 and asialofetuin: at high ratios of galectin added to asialofetuin a complex formed contained nine lectin subunits per glycoprotein molecule, while decreasing the concentration of galectin led to the formation of complexes in which only three subunits of galectin per glycoprotein molecule were bound [20]. From the precipitation and inhibition of precipitation data presented here we may assume that two different forms of $GlcNAc_8$ complexes observed by us may reflect a similar situation in which there is double occupancy of the carbohydrate binding sites of sNKR-P1A in the high affinity complexes when compared to their low affinity homologs. This hypothesis is strongly supported by the quantitative analyses of the precipitates (Table 1) that allowed us to establish the occurrence of a single carbohydrate binding site per sNKR-P1A subunit. Altogether our data support a working model (Fig. 4) for the lattices formed during the precipitation of sNKR-P1A protein (composed, as discussed above, of two subunits each bearing a single carbohydrate-combining site) with $GlcNAc_3$ (Fig. 4A), $GlcNAc_4$ (Fig. 4B), and $GlcNAc_8$ performed in higher (Fig. 4C) or lower (Fig. 4D) concentration of this glycocluster. It should be noted that the efficient formation of lattices between the GlcNAc-terminated glycodendrimers and sNKR-P1A was clearly dependent on the highly ordered presentation of the carbohydrate found in the synthetic compounds; no such lattices could be observed with the high affinity neoglycoprotein ligand, $GlcNAc_{23}$BSA.

In conclusion, the possibility to organize the dimeric soluble NKR-P1A protein into higher order lattices should prove invaluable during the ongoing studies on the recognition of carbohydrates by these receptors. In particular, such compounds may aid in the isolation of the natural form of NKR-P1A by their direct precipitation from NK cell membrane lysates, and may play seminal roles in our current efforts to crystallize the soluble NKR-P1A protein. Additional GlcNAc clusters to those described here may now be prepared based on the structural suggestions tailored to the needs of the ongoing crystallization experiments performed with this receptor.

Acknowledgements: This work was supported by a program grant from the Ministry of Education of Czech Republic (program Renaissance of Science at Czech Universities, grant VS96141), by the Grant Agency of Czech Republic (grants No. 303/96/0937, and 203/96/1267), by the Deutsche Forschungsgemeinschaft (DFG), and by the Deutscher Akademischer Austauschdienst (DAAD). The authors wish to thank to Dr. O. Horváth for his help with the preparation of figures.

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