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3D Modeling of a Natural Killer Receptor, Siglec-7: Critical Amino Acids for Glycan-Binding and Cell Death-Inducing Activity

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1. Introduction

Siglecs comprise a family of sialic acid-binding Ig-like lectins, expressed mainly on hematopoietic cells (O'Reilly and Paulson 2010; Angata 2006; Crocker, Paulson et al. 2007). More than ten Siglecs of human orgin have been cloned, all of which bind sialoglycans. Structural commonalities include an extracellular N-terminal V-set Ig-like domain, a sialoglycan-binding domain followed by variable numbers of C2-set Ig-like domains, a transmembrane domain, and a cytoplasmic signaling domain. Each member is expressed in a cell-specific manner, e.g., Siglec-1 on macrophages, Sigelc-2 on B cells, Siglec-7 on natural killer cells, and Siglec-9 on myelocytic cells.

Even though Siglecs bind terminal sialic acids on glycoconjugates, each member preferentially binds different oligosaccharide ligands. The nature of a specific sialic acid, its linkage to substituted sugars, and underlying neutral oligosaccharides can all influence Siglec recognition (see Table 1). For instance, Siglec-1 binds a terminal NeuAcα2-3Gal, but not a NeuAcα2-6Gal residue. In contrast, Siglec-2 preferentially binds a terminal NeuAcα2-6Gal residue (Blixt, Collins et al. 2003; Blixt, Han et al. 2008). Siglec-9 binds both of the structures equally. Siglec-7 binds tumor-associated glycans such as so-called "melanoma antigen" (disialyl glycan; NeuAcα2-8NeuAcα2-3Gal) and the branched α2-6sialyl glycan (Galβ1-3[NeuAcα2-6]GlcNAc) (Yamaji, Teranishi et al. 2002; Miyazaki, Ohmori et al. 2004). The binding of Siglec-7 to unique sialoglycans may be associated with tumor recognition by NK cells. In this context, it is notable that antibody-crosslinking of Siglec-7 on NK cells attenuates the cytotoxicity of NK cells against FcγR$^+$ P815 murine mastocytoma cells (Nicoll,

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Avril et al. 2003). The inhibitory signal of Siglec-7 is transduced by its cytoplasmic signaling domain containing immune receptor tyrosine-based inhibitory motifs (ITIMs) (Ikehara, Ikehara et al. 2004; Yamaji, Mitsuki et al. 2005), which have been described as suppression motifs for a variety of immunocytes.

2. The 3D mapping of critical amino acids for glycan-binding and inibitory activity

We first developed assays for glycan-binding activity and immunosuppressive activity. To identify influential amino acids, we constructed a series of mutants and tested for their activities. Amino acids of significance were mapped on a 3D model of Siglec-7.

2.1 The mapping of important amino acids for recognizing tumor-associated glycans

2.1.1 Overexpression of Siglec-7 on cultured cells

We used two types of cells for overexpressing Siglec-7: hematopoietic U937 monocytic leukemia cells and Chinese hamster ovary (CHO) cells. Each type of cell line was transfected with Siglec-7 cDNA and then stable transformants were isolated (U937-WT7 or CHO-WT7). Mock transfection cells were also prepared (U937-Mock or CHO-Mock). Expression levels of Siglec-7 on the transformants were estimated by immunoblotting and/or flow cytometry using specific antibodies against Siglec-7 (Yamaji, Mitsuki et al. 2005). Prior to binding assays, the cells were treated with sialidase to remove endogenous sialic acid.

We also prepared Siglec-7 ligand-expressing cells, i.e., human erythroleukemia K562 cells were transfected with cDNA of α2-8sialyltransferase, ST8SiaVI, which biosynthesizes NeuAcα2-8NeuAcα2-3Gal epitope, a preferred ligand of Siglec-7. The isolated stable transformants (K562-ST) were examined for their epitope expression by flow cytometry using S2-566 antibody, which recognizes the disialyl epitope.

2.1.2 Binding activity of Siglec-7 expressed on U937 cells

Siglec-7 binds to melanoma antigen (α2-8disialyl epitope: NeuAcα2-8NeuAcα2-3). We therefore used NeuAcα2-8NeuAc-polyacrylamide (disialo-PAA) as a probe for characterizing

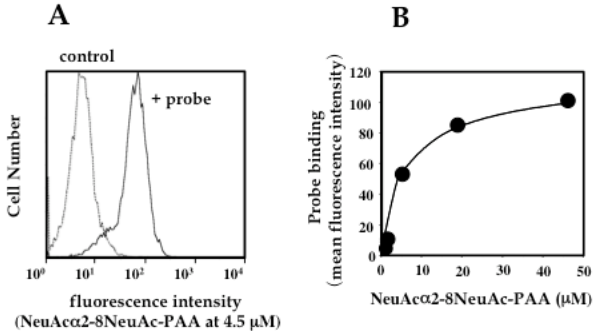

Fig. 1. Binding of a disialyl probe (NeuAcα2-8NeuAc-PAA) to U937-WT7 cells, which overexpress Siglec-7. U937-WT7 cells were incubated with biotinylated NeuAcα2-8NeuAc-PAA. After the cells were incubated with FITC-streptavidin, fluorescnce intensity was detected by flow cytometry (A). Mean fluorescnce intensity increases in a saturable manner in a range of 1-45 μM probe concentration (B).

glycan-binding activity of Siglec-7 on U937-WT7 cells (Fig. 1A). The probe bound to U937-WT7 cells in a saturable manner showing a less than 10 μM of the K_d value (Fig 1B)(unpublished data, Yamaji et al.).
To examine whether Siglec-7 mediates cellular binding, U937-WT7 cells were co-cultured with K562-ST cells, which express α2,8disialyl epitopes. U937-WT7 cells formed aggregates with K562-ST (Fig. 2A) but not with K562-Mock cells (Fig. 2B), suggesting that the cell-cell interaction depends on α2,8disialyl epitopes on K562-ST cells. In this model system Siglec-7 binds α2,8disialyl epitopes on the target cells in a trans-acting manner.

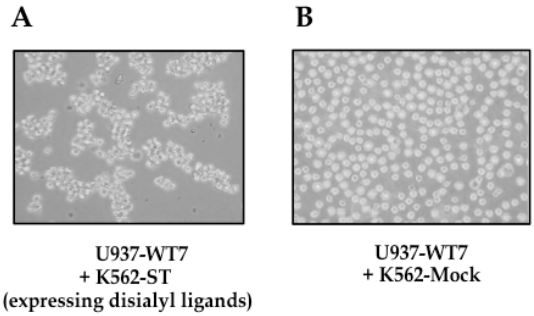

Fig. 2. U937-WT7 cells form aggregates with K562-ST cells (A), which express α2,8disialyl epitopes, but not with K562-Mock cells (B).

Siglec-7 functions as a negative regulator in various immuno-responses. Upon activation of Siglec-7, cytoplasmic immune receptor tyrosine-based inhibitory motifs (ITIMs) are phosphorylated and transduce the inhibitory signal. Co-cultivation of U937-WT cells with K562-ST cells induced tyrosine phosphorylation of Siglec-7, whereas that with K562-mock cells did not (data not shown), suggesting that interaction of Siglec-7 with ligands on opposing cells transduces inhibitory signalling (unpublished data, Yamaji et al.).

2.1.3 Binding specificity of Siglec-7 expressed on CHO cells

To characterize the glycan-binding specificity of Siglec-7, a high-throughput sensitive assay was developed (Yamaji, Nakamura et al. 2003). For the assay we prepared a streptavidin-based neoglycoprotein as a glycoprobe (Hashimoto, Suzuki et al. 1998). Briefly, streptavidin was coupled with oligosaccharides by reductive amination (Mahoney and Schnaar 1994). The synthsized oligosaccharyl streptavidin was mixed with biotinylated BSA, yielding a ''polymer'' that carries 10-11 molecules of oligosaccharyl streptavidin with more than 100 oligosaccharides (Fig. 3). The multivalency of oligosaccharide ligands increased binding "avidity" of the probe (O'Reilly and Paulson 2010). Radioiodination of biotinylated BSA before mixing gave a radioiodinated glycoprobe. The binding specificity of Siglec-7 on CHO-WT7 cells was examined by utilizing a set of glycoprobes, such as GD3-, GM1-, GD1a-, GT1b-, LSTa-, LSTb-, and LSTc-polymers (Table 1).
GD3-polymer bound to CHO-WT7 cells more effectively than GD1a-polymers (Fig. 4A), suggesting that α2-8disialyl epitopes (NeuAc α2-8 NeuAc α2-3Gal) of the GD3-polymer were more potent ligands than the terminal α2-3-linked sialyl residues of the GD1a-polymer. The GT1b-polymer bound well to CHO-WT7 cells because GT1b contains the α2-8disialyl residue attached to the internal galactose. LSTb-polymer containing the branched α2-6sialyl residue (Galβ1-3[NeuAcα2-6]GlcNAc) also bound well to CHO-WT7 cells. LSTa-,

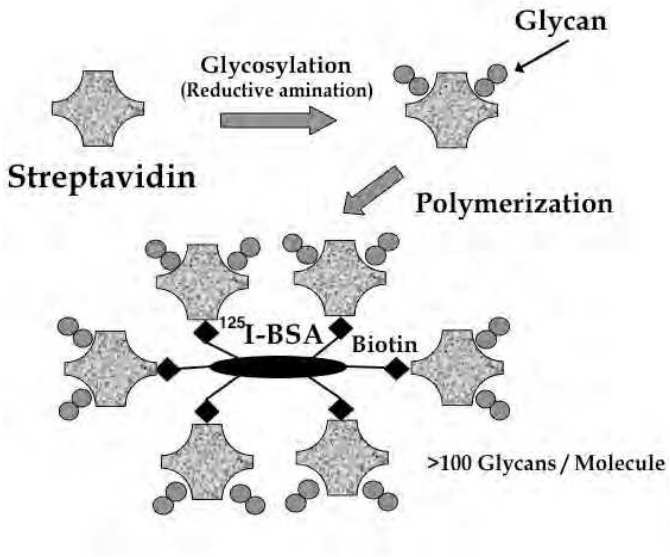

Fig. 3. Preparation of a streptavidin-based glyco-probe.

	Glycochain structures	Siglec-7	Siglec-9
GD3[a]	Siaα2-8Siaα2-3Galβ1-4Glc	++	-
GM1[a]	Galβ1-3GalNAcβ1-4[Siaα2-3]Galβ1-4Glc	-	-
GD1a[a]	Siaα2-3Galβ1-3GalNAcβ1-4[Siaα2-3]Galβ1-4Glc	±	++
GT1b[a]	Siaα2-3Galβ1-3GalNAcβ1-4[Siaα2-8Siaα2-3]Galβ1-4Glc	++	++
LSTa	Siaα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	-	+
LSTb	Galβ1-3[Siaα2-6]GlcNAcβ1-3Galβ1-4Glc	++	-
LSTc	Siaα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	±	++

[a] The nomenclature is based on that of Svennerholm (*J. Neurochem.* **10**, 613, 1963).

Table 1. Glycan structures of oligosaccharides and their recognition by Siglecs.

and LSTc-polymers containing α2-3/6-linked sialyl residue did not bind to the cells. The apparent K_d and B_{max} values of the GD3-polymer to CHO-WT7 cells were about 10 nM and 70 fmol/2 x 10^4 cells, respectively. Next we examined the binding specificity of Siglec-9 (Fig. 4B), which has the highest sequence similarity to Siglec-7 (83% identity) among all Siglecs reported. CHO-WT9 bound poorly to GD3- and LSTb-polymers, but did bind well to GD1a-, LSTa-, GT1b-, and LSTc-polymers, suggesting that Siglec-9 recognizes a terminal NeuAcα2-3(or 6) Gal residue and its binding specificity is distinguished from that of Siglec-7 (Fig. 4).

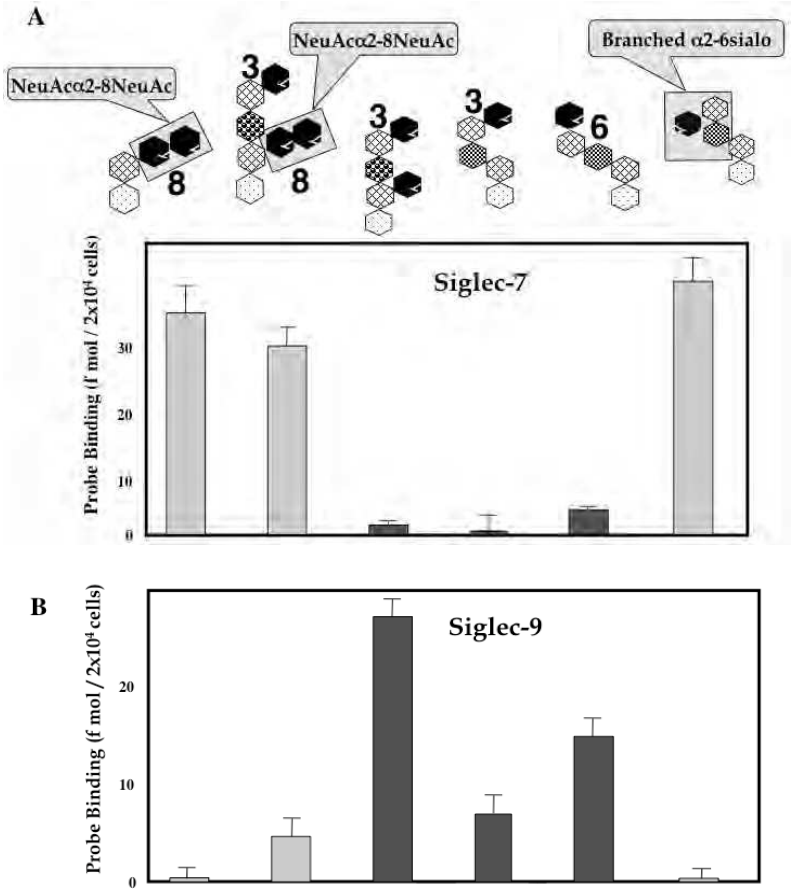

Fig. 4. The glycan-binding specificity of Siglec-7 (A) and Siglec-9 (B).

2.1.4 Mapping of critical amino acids for recognizing tumor-associated glycans

The differences in glycan-binding specificity of the two Siglecs appeared to be attributable to sequence differences in the glycan-binding V-set domains. To identify amino acid(s) responsible for these specificity differences, we prepared a series of shuffling chimeras in the V-set domain between Siglecs-7 and -9. We eventually found that substitution of a small region, Asn70-Lys75, of Siglec-7 with the equivalent region of Siglec-9, Ala66-Asp71 (6 a.a. chimera) resulted in the loss of Siglec-7-like binding specificity and the acquisition of the Siglec-9-like binding property (Fig. 5), suggesting that only the six amino acid sequence in the glycan-binding domain is important for determining the binding specificity of Siglec-7 and -9. None of single amino acid mutants changed binding specificity, suggesting that all of the six amino acids, or possibly a certain combination among these amino acids, is responsible for Siglec-7-like binding specificity (Yamaji, Teranishi et al. 2002).

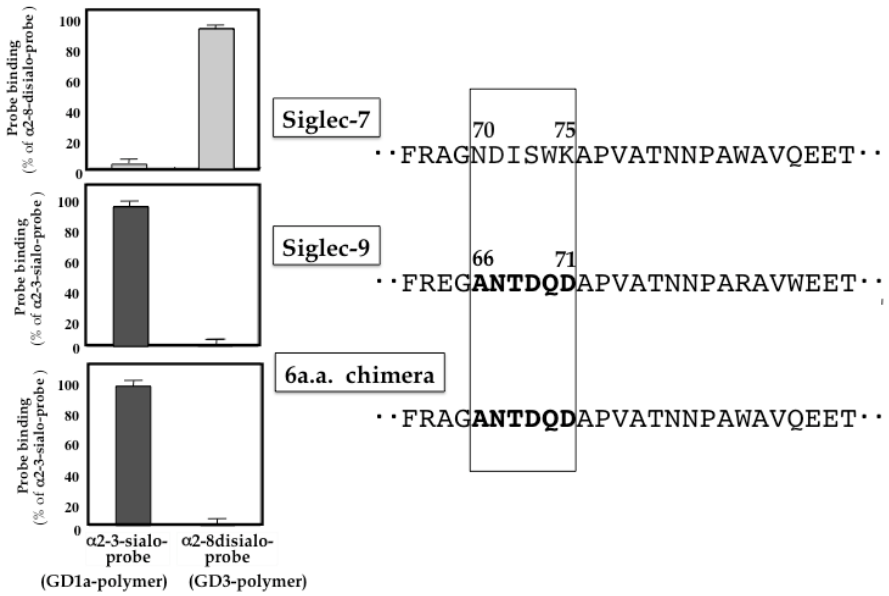

Fig. 5. Siglec-7 binds well to α2-8disialo-probe (Siaα2-8Siaα2-3Gal) whereas Siglec-9 does α2-3sialo-probe (Siaα2-3Gal). Replacement of six amino acids (Asn70-Lys75) of Siglec-7 results in Siglec-9-like binding specificity (6 a.a. chimera).

2.1.5 The 3D mapping of the six amino acid in Siglec-7 V-set domain

To date, crystal structures of Siglec domains have been reported including mouse Siglec-1 V-set domain, human Siglec-5 V-set plus C2-set domain and human Siglec-7 V-set domain (Fig. 6). (Alphey, Attrill et al. 2003; Dimasi, Moretta et al. 2004; Attrill, Imamura et al. 2006; Attrill, Takazawa et al. 2006)

Structural information on Siglecs is thus accumulating, and the basis for the recognition of a terminal sialic acid is becoming established. The interaction is highlighted by a conserved arginine residue that forms a crucial salt bridge with the sialic acid carboxylate.

Crystal structures of Siglec-7 V-set domain have been reported so far with or without sialosides. The structure of unliganded Siglec-7 V-set domain is an Ig-like β-sandwich fold formed by two β-sheets (strands A'GFCC' and ABED) (Fig. 7, left) (Alphey, Attrill et al. 2003). The conserved Arg124, which is the key ligand-binding residue, is solvent-exposed and is located on the center of A'GFCC' face.

Six amino acid residues (Asn70 to Lys75), which determine the binding preference for disialo-glycans (Yamaji, Teranishi et al. 2002), are located on the tip of the C-C' loop. The crystal structure of Siglec-7 V-set domain in complex with α(2-8)disialylated glycan reveals how this important family of lectins binds the structurally diverse sialosides (Fig. 7, right). The terminal sialic acid is the major determinants of ligand binding, making several hydrogen bonds with the protein. A key salt bridge is formed between the Arg124 guanidinium group and the terminal sialic acid carboxylate. The C-C' loop, a region implicated in ligand-binding specificity, undergoes a drastic conformational shift, allowing

it to interact with the underlying neutral glycan core. The ligand-induced conformational change observed in the C-C′ loop may be characterstic of Siglec-7.

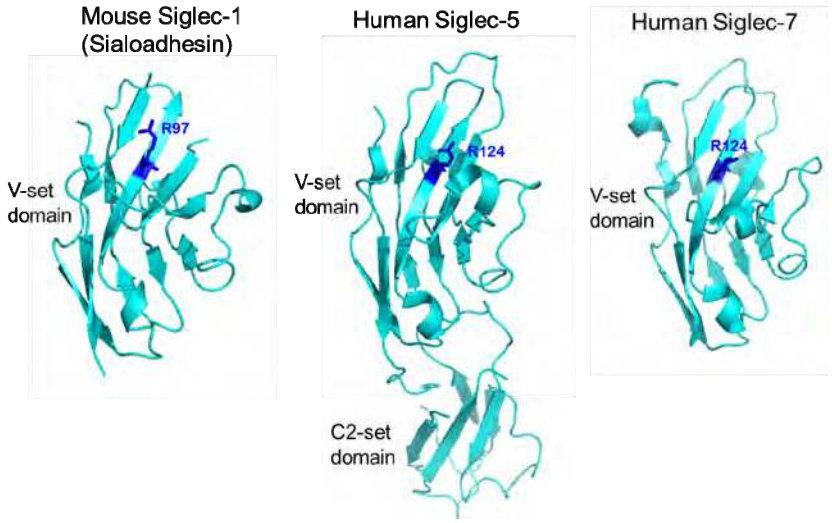

Fig. 6. Crystal structures of Siglec domains reported to date. Mouse Siglec-1 (left, PDB code 1QFO), human Siglec-5 (center, PDB code 2ZG1) and human Siglec-7 (right, PDB code 1O7V) are shown with ribbon representation. Conserved arginine residue is highlighted in dark blue.

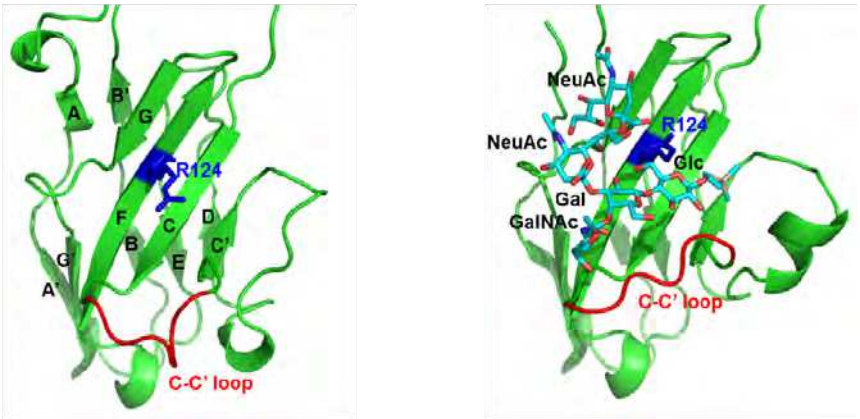

Fig. 7. Dynamic conformational shift of the C-C′ loop of Siglec-7 at the binding pocket. Crystal structures of unliganded (left) (Alphey, Attrill et al. 2003) and liganded (right) (Attrill, Imamura et al. 2006). Siglec-7 V-set domains are shown in ribbon diagrams. In both structures, a conserved Arg124 residue is highlighted in blue with stick representation. Six amino acid residues, Asn70 to Lys75 in the C-C′ loop, are colored in red. Disialylated GT1b glycan is shown with stick representation (right).

2.2 The 3D mapping of critical amino acids for cell death-inducing activity

2.2.1 Cell death-inducing activity of Siglec-7

To analyze effects of Siglec-7 on a cellular function, Siglec-7 on U937-WT7 cells was ligated with an $F(ab')_2$ fragment of anti-Siglec-7. The ligation of Siglec-7 with the specific antibody, 13-3-D, increased cell death at 30-50% (Fig. 8A). Control $F(ab')_2$ fragments of irrelevant antibody showed a subtle effect on the cell death under our experimental conditions. Figure 6B shows a graph of percentage of Annexin V-positive cells in the Annexin V-positive window in Figure 8B. The cell death of Siglec-7-expressing cells was time-dependent and dose-dependent of incubation with the $F(ab')_2$ fragment. A pancaspase inhibitor, Z-VAD-FMK, did not inhibited Siglec-7-dependent cell death, suggesting that caspases are not involved in the cell death. In addition, we could not detect DNA ladder formation in the cell death process (Mitsuki, Nara et al. 2010).

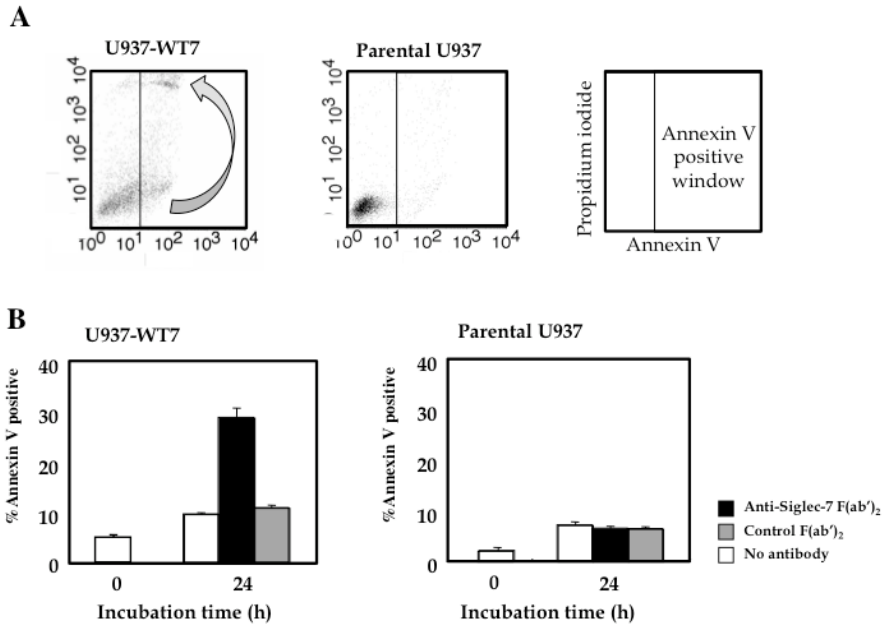

Fig. 8. Ligation of Siglec-7 elicits death of U937-WT7.

2.2.2 Morphological changes of death-induced U937-WT7

Electron microscopy revealed that ligation of Siglec-7 induced some chromatin condensation in the nucleus, but did not induce nuclear condensation and fragmentation (Fig. 9). It was noted that Siglec-7 ligation induced a number of vacuoles, which were associated with "ribosome-like granules", suggesting that the vacuoles are derived from rough endoplasmic reticulum. Neither nuclear condensation nor fragmentation was detected in the cell death, suggesting that Siglec-7 induces non-apoptotic cell death.

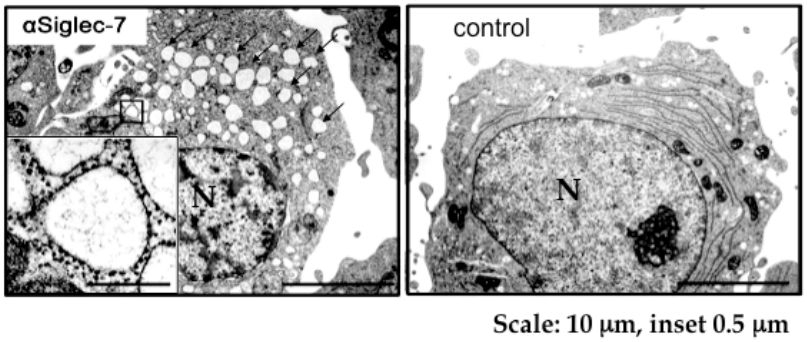

Fig. 9. Electron micrograph of U937-WT cells, which are treated with (αSiglec-7) or (control) an F(ab')$_2$ fragment of anti-Siglec-7. Siglec-7 ligation induces some chromatin condensation and formation of vacuoles with "ribosome-like granules"(inset).

2.2.3 Mapping of important amino acids for inducing cell death

To test the involvement of ITIM for signaling, we deleted the cytoplasmic portion of Siglec-7 and expressed it in U937 cells (U937-7Δcytosol). Unexpectedly, U937-7Δcytosol without cytosolic domain elicited cell death (Fig. 10A), indicating that cell death does not involve

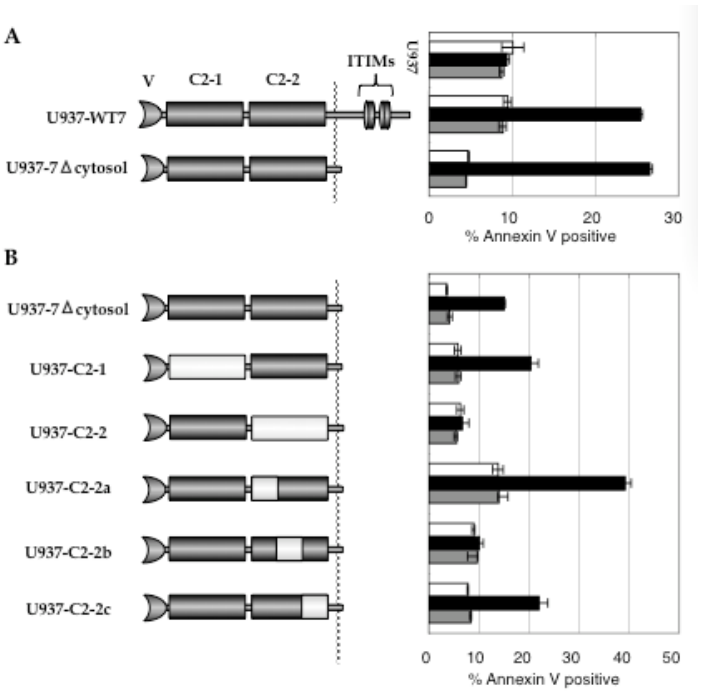

Fig. 10. Shuffling chimeras in C-set domains between Siglec-7 (gray bar) and –9 (white bar). Replacement of C2-2 or C2-2b domain abolishes cell death-inducing activity.

ITIMs. Since U937-WT9 did not show cell death, we prepared a series of domain shuffling chimeras between Siglecs-7 and -9 to identify domain(s) responsible for the cell death.
Each domain of U937-7Δcytosol was replaced with the corresponding domain of Siglec-9. Assays for death inducing activity of the chimeras revealed that replacement of the membrane-proximal C2-set domain abolished cell death activity (U937-C2-2), indicating that C2-2 domain is important for the cell death (Fig. 10B)(Mitsuki, Nara et al. 2010). To narrow down the region responsible for cell death, we prepared additional chimeric mutants. The C2-2 domain was tentatively divided into three portions; C2-2a, C2-2b, and C2-2c regions. When C2-2b region was replaced with the corresponding region of Siglec-9, cell death was completely abolished (U937-C2-2b) (Fig. 10B). Neither replacement of C2-2a nor C2-2c had any effect. These results suggest that the C2-2b region contributes most to cell death.
Six amino acids differ between Siglec-7 and -9 in the C2-2b region, prompting us to prepare single amino acid mutants using U937-7Δcytosol cDNA as a parental construct. Five of six possible mutants were established as stable cell lines. Four of the five mutants (W288L, T289S, S292G, and L304G) showed marked decreases in cell death activity (Fig. 11), suggesting that the four amino acids are critical for inducing the cell death.

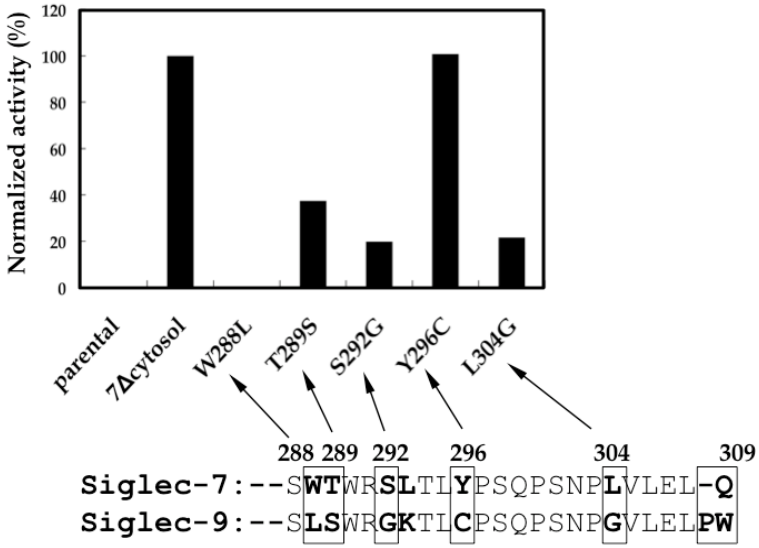

Fig. 11. Single amino acid mutants of Siglec-7 and their cell death-inducing activity. Four mutants (W288L, T289S, S292G, and L304G) show marked decrease in the cell death activity.

2.2.4 The 3D mapping of critical amino acids for cell death-inducing activity

Although structural information on the V-set domain is available for some of Siglecs, little is known about the Siglec C2 domains. We therefore built models of C2-2 domains of Siglec-7 and -9 (Fig. 12). A homology search based on Smith-Waterman algorithm was performed using SSearch (Smith and Waterman 1981) to identify sequences that are homologous with the human Siglec-7 C2-2 domain. The second immunoglobulin domain of human paladin (PDB code 2dm3) was selected as a template among those with low *E* values. The positions of the cysteine residues are conserved between Siglec-7/-9 C2-2 domains and second Ig domain of paladin. The qualities of the resultant protein structures were checked using the Procheck program (Laskowski, MacArthur et al. 1993), which gives Ramachandran plots and a quantitative distribution of the geometric parameters within the allowed conformational space. Importantly, the model of Siglec-7 C2-2 domain suggests that the four amino acid residues (L304, W288, T289 and S292) were proximal to one another (Fig. 12). The close location of these four residues tempted us to speculate that they would be involved in interactions with other adjacent molecules to transduce the death signal.

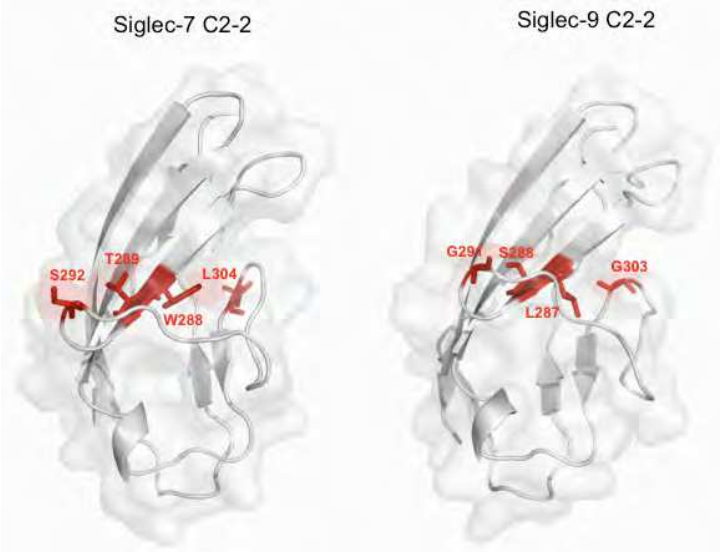

Fig. 12. Computer-assisted homology modeling of the C2-2 domains of Siglec-7 and -9. The models are shown by space filling (transparent) and ribbon representation. Homology modeling was performed using MODELLER version 9.4 software (Sali and Blundell 1993; Fiser, Do et al. 2000; Marti-Renom, Stuart et al. 2000). The four amino acids in Siglec-7 (L304, W288, T289 and S292), which are responsible for the cell death activity, are shown in red with stick representation. The corresponding residues in Siglec-9 (G303, L287, S288 and G291) are also shown in red.

3. Conclusion

We have demonstrated that Siglec-7 may mediate non-apoptotic cell death by signal transduction after binding to tumor-associated glycans such as "melanoma antigen" (α2-8disialyl epitopes). In particular, we have identified amino acid residues responsible for the activity. These residues are mapped on a 3D-structure of Siglec-7 and their functions are discussed from a structural point of view.

Six amino acid residues (Asn70 to Lys75), responsible for the binding preference for α2-8disialyl epitope, are located on the tip of the C-C′ loop and undergoes a drastic conformational shift allowing it to interact with the underlying neutral glycan core. This dynamic comformational change may determine the ligand-binding specificity of Siglec-7. Thus our biochemical data in conjunction with the reported crystallographic data are of great value in understanding the structure-function relationships of Siglecs and other sialoside-binding proteins.

Four amino acid residues (L304, W288, T289 and S292) in the Siglec-7 C2-2 domain, responsible for eliciting cell death, are proximal to one another. It is tempting to speculate that these residues are crucial for interacting with other adjacent molecules to transduce the death signal. These structural models would be useful for developing structure-guided inhibitors or activators of Siglec-7 on NK cells.

4. Acknowledgements

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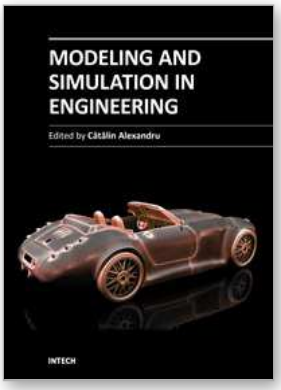

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This book provides an open platform to establish and share knowledge developed by scholars, scientists, and engineers from all over the world, about various applications of the modeling and simulation in the design process of products, in various engineering fields. The book consists of 12 chapters arranged in two sections (3D Modeling and Virtual Prototyping), reflecting the multidimensionality of applications related to modeling and simulation. Some of the most recent modeling and simulation techniques, as well as some of the most accurate and sophisticated software in treating complex systems, are applied. All the original contributions in this book are jointed by the basic principle of a successful modeling and simulation process: as complex as necessary, and as simple as possible. The idea is to manipulate the simplifying assumptions in a way that reduces the complexity of the model (in order to make a real-time simulation), but without altering the precision of the results.

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