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Siglec-15: an immune system Siglec conserved throughout vertebrate evolution

Takashi Angata[1], Yukako Tabuchi, Kazunori Nakamura, and Mitsuru Nakamura

Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan

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Siglecs are vertebrate cell-surface receptors that recognize sialylated glycans. Here we have identified and characterized a novel Siglec, named Siglec-15. Siglec-15 is a type-I transmembrane protein consisting of: (i) two immunoglobulin (Ig)-like domains, (ii) a transmembrane domain containing a lysine residue, and (iii) a short cytoplasmic tail. Siglec-15 is expressed on macrophages and/or dendritic cells of human spleen and lymph nodes. We show that the extracellular domain of Siglec-15 preferentially recognizes the Neu5Acα2−6GalNAcα− structure. Siglec-15 associates with the activating adaptor proteins DNAX activation protein (DAP)12 and DAP10 via its lysine residue in the transmembrane domain, implying that it functions as an activating signaling molecule. Siglec-15 is the second human Siglec identified to have an activating signaling potential; unlike Siglec-14, however, it does not have an inhibitory counterpart. Orthologs of Siglec-15 are present not only in mammals but also in other branches of vertebrates; in contrast, no other known Siglec expressed in the immune system has been conserved throughout vertebrate evolution. Thus, Siglec-15 probably plays a conserved, regulatory role in the immune system of vertebrates.

Key words: DAP12/dendritic cells/macrophages/sialyl Tn/Siglec

Introduction

Cell-surface lectins of vertebrates are involved in many physiological processes, such as glycoprotein turnover, cellular trafficking, and pathogen recognition (Gabius et al. 2002). The Siglec family is the largest known group of vertebrate lectins that recognize sialylated glycans (Varki and Angata 2006; Crocker et al. 2007). Most Siglecs are expressed on cells of the immune system, with the notable exception of Siglec-4 (also known as myelin-associated glycoprotein or MAG), which is expressed exclusively in the nervous system. To date, MAG/Siglec-4 is the only Siglec known to be conserved throughout vertebrate evolution (Lehmann et al. 2004); the others show lesser and varying degrees of conservation (Angata 2006; Varki and Angata 2006). In particular, cluster of differentiation (CD)33-related Siglecs, a subfamily of mammalian Siglecs with higher mutual sequence similarity, show marked inter-species differences even within the same order of mammals (Angata et al. 2004). Although the biological functions of Siglecs expressed in the immune system are not yet fully understood, in vitro and in vivo analyses suggest that these lectins are involved in coupling glycan recognition to immunological regulation.

Most of the mammalian Siglecs that are expressed in the immune system have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain, and these Siglecs have been shown to regulate negatively the cells that express them (O'Keefe et al. 1996; Otipoby et al. 1996; Sato et al. 1996; Nitschke et al. 1997; Mingari et al. 2001; Ulyanova et al. 2001; Nutku et al. 2003; von Gunten et al. 2005). Recent discoveries of the Siglecs that associate with the activating adaptor molecule DAP12, which has an immunoreceptor tyrosine-based activating motif (ITAM), have shed light on a subgroup of Siglecs that have an activating signaling potential (Angata et al. 2006; Blasius et al. 2006). The presence of both activating and inhibitory members within a family is a feature that is also found in other immunity-related cell-surface receptor families such as killer cell immunoglobulin (Ig)-like receptors of primates (Vilches and Parham 2002), leukocyte Ig-like receptors of primates (Martin et al. 2002), and the Ly49 family of rodents (Yokoyama and Plougastel 2003). Although the functional significance of the presence of counteracting members within a family is not fully understood, it has been proposed that such members act cooperatively to fine-tune cellular responses (Lanier 2001), or that the activating members function to counteract pathogens that exploit their inhibitory counterparts (Arase and Lanier 2004). Identification of the full variety of the Siglec family and analysis of their basic characteristics, such as ligand preference and signaling properties, would lead to a better understanding of the biological functions of this family as a whole.

In this study, we report the identification and characterization of a novel Siglec, named Siglec-15. We demonstrate that Siglec-15 is expressed on the cells in the immune system, can recognize sialylated ligands, and can interact with the activating adaptor molecules DAP12 and DAP10 via a lysine residue in the transmembrane domain. The presence of a Siglec-15 ortholog in fish suggests that the ancestry of Siglec-15 pre-dates the diversification of vertebrates. These results thus suggest that Siglec-15 plays conserved role in the vertebrate immune system.

Results

Cloning of human Siglec-15 and its orthologs

We previously reported the presence of a genomic DNA segment that may encode a Siglec-like protein, which we

[1]To whom correspondence should be addressed; Tel: +81-29-861-9460; Fax: +81-29-861-3191; e-mail: takashi-angata@aist.go.jp

tentatively named X (Angata et al. 2001). A recent study of the human transcriptome (Ota et al. 2004) revealed that this genomic segment is actually transcribed to yield a messenger RNA (mRNA) encoding a putative transmembrane protein, termed "CD33L3" (GenBank accession number: AK172835). We obtained an equivalent complementary DNA (cDNA) clone from the IMAGE consortium (IMAGE ID 6280187). Sequencing of this IMAGE consortium clone and several other clones obtained by reverse transcription–polymerase chain reaction (RT–PCR) revealed that the IMAGE clone encodes the longest isoform of the protein, which we named "Siglec-15" (GenBank accession number: AY971516), on the basis that it recognizes glycans that contain sialic acid (see *Siglec-15 preferentially binds sialyl Tn*).

Unlike other Siglecs, Siglec-15 contains an even number of cysteine residues in each of its first and second Ig-like domains (Figure 1 and Supplementary Figure 1). The cysteine residues on β-strands B (C64) and F (C142) are likely to form one disulfide bond, and those in loops C–C′ (C95) and C′–D (C104) may form another (Supplementary Figure 2).

The *SIGLEC15* gene is also unusual in several aspects compared with other Siglec genes. For example, two exons (versus 1 or 0 in other Siglec genes) precede the exon encoding the first Ig-like domain; the exon encoding the second Ig-like domain also encodes the transmembrane domain (in other Siglec genes, the exon encoding the transmembrane domain and that encoding the preceding Ig-like domain are separate); and it is extremely GC-rich (approximately 65%).

We also cloned its mouse ortholog (mouse Siglec-15; GenBank accession number: AY971517) and its putative zebrafish ortholog (zebrafish Siglec-like protein 1; GenBank accession number: DQ471941). Alignment of the amino acid sequences of these two proteins and human Siglec-15 (Figure 2) revealed that the arrangement of cysteine residues is conserved among them. In addition, the presence of a conserved lysine residue in the transmembrane domain of these proteins suggests that this residue is functionally important (see *Siglec-15 can associate with DAP12 and DAP10*).

Molecular phylogenetic analysis of the N-terminal region of human Siglecs revealed that Siglec-15 does not belong to the CD33-related Siglec subfamily (Figure 3). The chromosomal location of the human *SIGLEC15* gene (18q12.3) also differs from that of the CD33-related Siglec genes (19q13.3–13.4). Thus, Siglec-15 does not have a particularly close phylogenetic

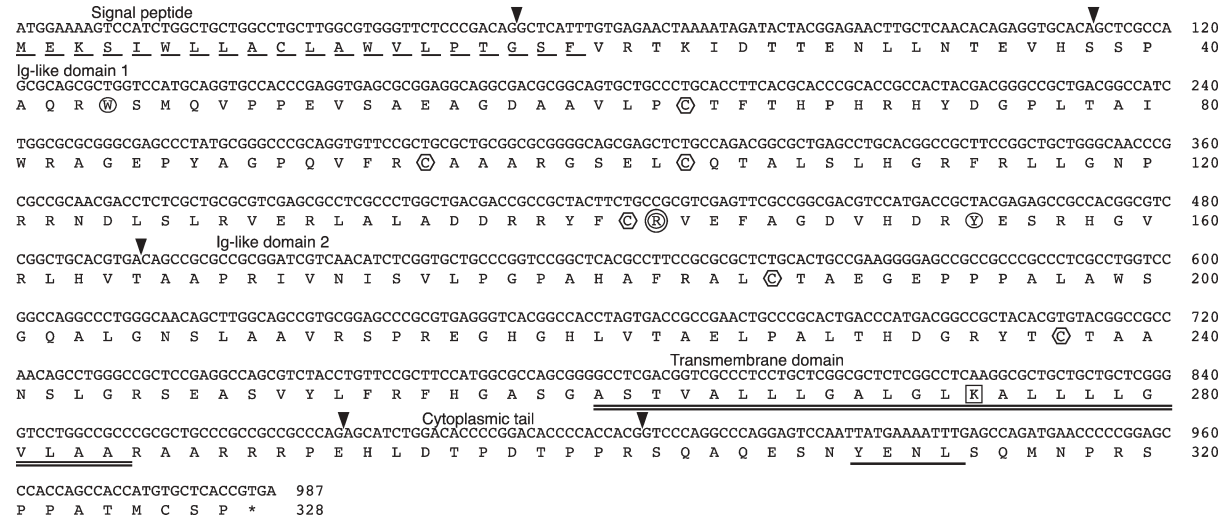

Fig. 1. Nucleotide and amino acid sequences of human Siglec-15. The ORF of human Siglec-15 cDNA and its theoretical translation are shown, along with the following features. Double circle, "essential" arginine residue involved in sialic acid recognition; circle, conserved aromatic amino acid residue; square, lysine residue in the transmembrane domain; hexagon, conserved cysteine residue; dotted underline, signal peptide; double underline, transmembrane domain; underline, putative endocytosis motif; arrowhead, exon junction. Note that the first and second Ig-like domains of human Siglec-15 contain four and two cysteine residues, respectively, whereas most other Siglecs contain three cysteine residues in both the first and the second Ig-like domains.

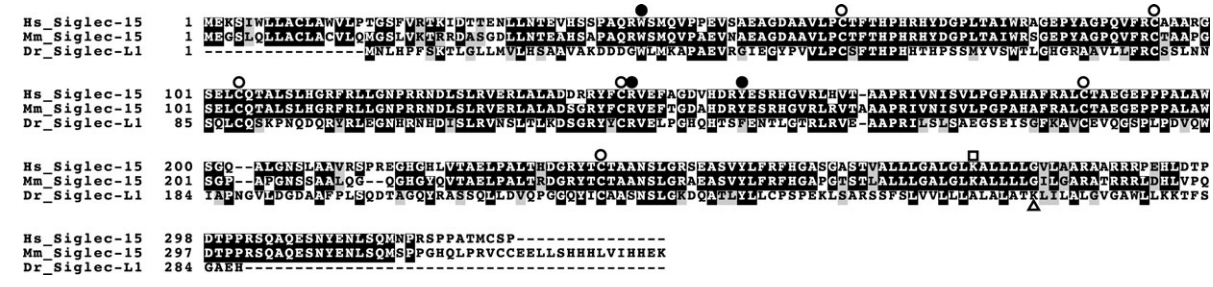

Fig. 2. Alignment of the amino acid sequences of human and mouse Siglec-15, and zebrafish Siglec-like protein 1. Some conserved features are shown above the alignment. Filled circle, residue involved in sialic acid recognition; open circle, cysteine residue; square, conserved lysine residue in the transmembrane domain. The lysine residue in zebrafish Siglec-like protein 1 does not completely align with that in human and mouse Siglec-15, and is marked with a triangle. Hs_Siglec-15 and Mm_Siglec-15 denote human and mouse Siglec-15, respectively. Dr_Siglec-L1 denotes zebrafish Siglec-like protein 1.

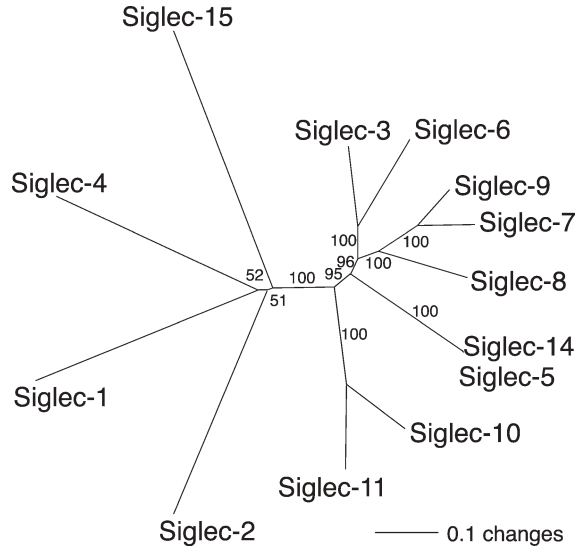

Fig. 3. Molecular phylogenetic tree based on the N-terminal region of human Siglecs. The amino acid sequences of the first two Ig-like domains of all human Siglecs were aligned and used to reconstruct a molecular phylogenetic tree using the neighbor-joining method. The number by each internode represents the percentage of bootstrap support. Siglec-XII was omitted from the alignment, because it has two V-set domains and, therefore, does not align with other Siglecs (Angata et al. 2001). Similarly, Siglec-13 was omitted, because it is not found in humans (Angata et al. 2004). CD33-related Siglecs form a cluster, and Siglec-15 does not belong to this cluster.

relationship with the CD33-related Siglecs. We therefore consider "CD33L3" to be a misnomer, and propose that the name "Siglec-15" be used instead.

Siglec-15 is expressed in macrophages and/or dendritic cells of spleen and lymph nodes

The expression of the Siglec-15 transcript in various human tissues was analyzed by real-time PCR. We observed a relatively high level of Siglec-15 mRNA in spleen, testis, and small intestine among the tissues examined, with highest expression in spleen (data not shown). To test whether Siglec-15 protein is expressed in spleen, we developed a rabbit polyclonal antibody against Siglec-15 and used it to probe tissue sections by immunohistochemical methods.

A preliminary analysis of human spleen sections using anti-Siglec-15 antibody showed that a very small number of splenocytes express Siglec-15 (data not shown). To determine the cell type(s) expressing Siglec-15, we performed double staining of spleen tissue sections with the anti-Siglec-15 antibody and antibodies against lineage markers such as CD3 (T-cells), CD79α (B-cells), CD68 (myeloid cells), and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) (macrophages/dendritic cells). Lymph node sections were also probed, because lymph node is another major secondary lymphoid organ. Siglec-15-positive cells were found in the marginal zone of spleen and in the paracortex and medullary sinus of lymph nodes. In both spleen (Figure 4A) and lymph nodes (Figure 4B), most Siglec-15-positive cells also expressed DC-SIGN. Conversely, less than a half of the DC-SIGN-positive cells in the lymph nodes and spleen were Siglec-15 positive, and the proportion of Siglec-15-positive cells within the DC-SIGN-positive cells differed among cell clusters. These results strongly suggest that Siglec-15 is

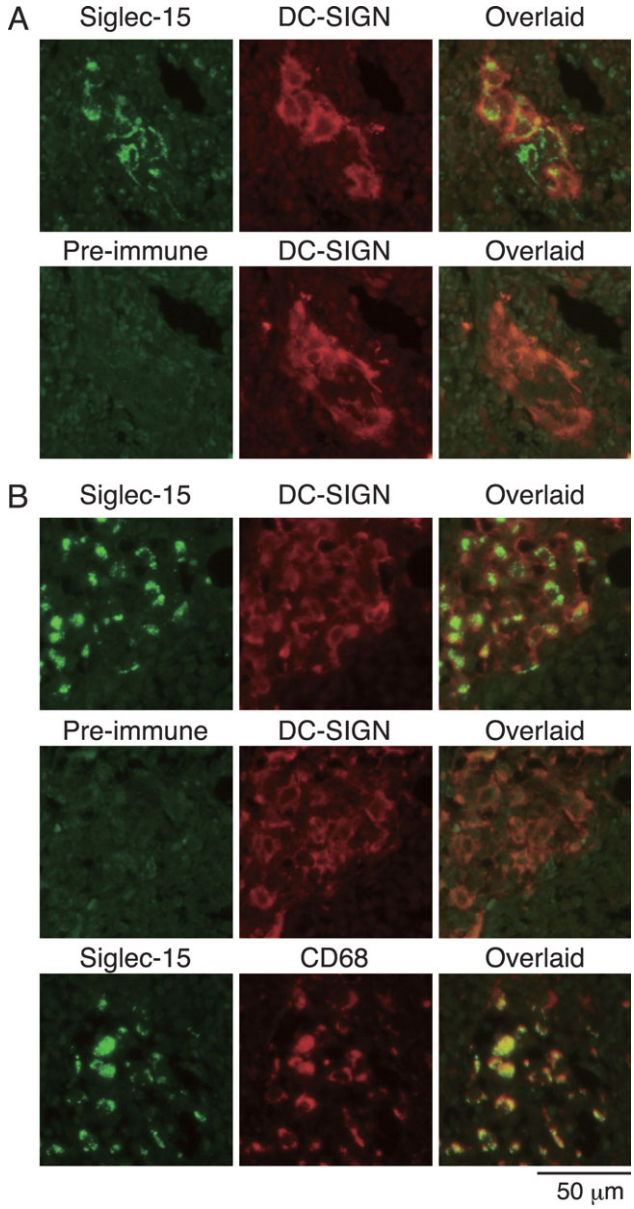

Fig. 4. Expression of human Siglec-15 in macrophages/dendritic cells of spleen and lymph nodes. Serial sections of spleen (**A**) and lymph nodes (**B**) were immunostained with antibodies against Siglec-15 and a lineage marker (DC-SIGN or CD68). The IgG fraction from pre-immune rabbit serum was used as a control for Siglec-15 antibody. Siglec-15-positive cells were localized in the marginal zone of spleen and in the paracortex/medullary sinus of lymph nodes. In both spleen and lymph nodes, most of the Siglec-15-positive cells were also positive for DC-SIGN. The subcellular localization of Siglec-15 was predominantly intracellular, as its overlap with CD68 suggests (B, bottom panel).

expressed in a subset of macrophages and/or dendritic cells. The subcellular localization of Siglec-15 appeared to be predominantly intracellular, because the localization of Siglec-15 overlapped with that of CD68 (Figure 4B, bottom panel), which is known to be localized intracellularly (Saito et al. 1991).

Siglec-15 preferentially binds sialyl Tn

To test whether Siglec-15 recognizes sialylated glycans, we first examined whether human erythrocytes bind to COS

cells expressing human Siglec-15 in a sialic acid-dependent manner. We did not detect binding of erythrocytes to COS-7 cells transfected with human Siglec-15 (data not shown). In contrast, we observed robust erythrocyte binding to COS-7 cells expressing mouse Siglec-15, and this binding diminished when the erythrocytes were pre-treated with sialidase (data not shown).

To determine the glycan structures that are preferentially recognized by Siglec-15, we prepared recombinant fusion proteins consisting of the extracellular domain of Siglec-15 and the fragment crystallizable (Fc)-portion of human IgG. We immobilized recombinant proteins on 96-well plates and tested whether they bound to synthetic polymer probes that were multiply substituted with sialylated oligosaccharides, as described previously (Patel et al. 1999; Angata and Varki 2000). Among the probes tested, human Siglec-15 showed preferential binding to a Neu5Acα2–6GalNAcα– (sialyl Tn) structure (Figure 5). Mouse Siglec-15 showed binding to Neu5Acα2-3Galβ1-4Glc (3′-sialyllactose) in addition to sialyl Tn. Overall, mouse Siglec-15 showed more robust binding compared with human Siglec-15.

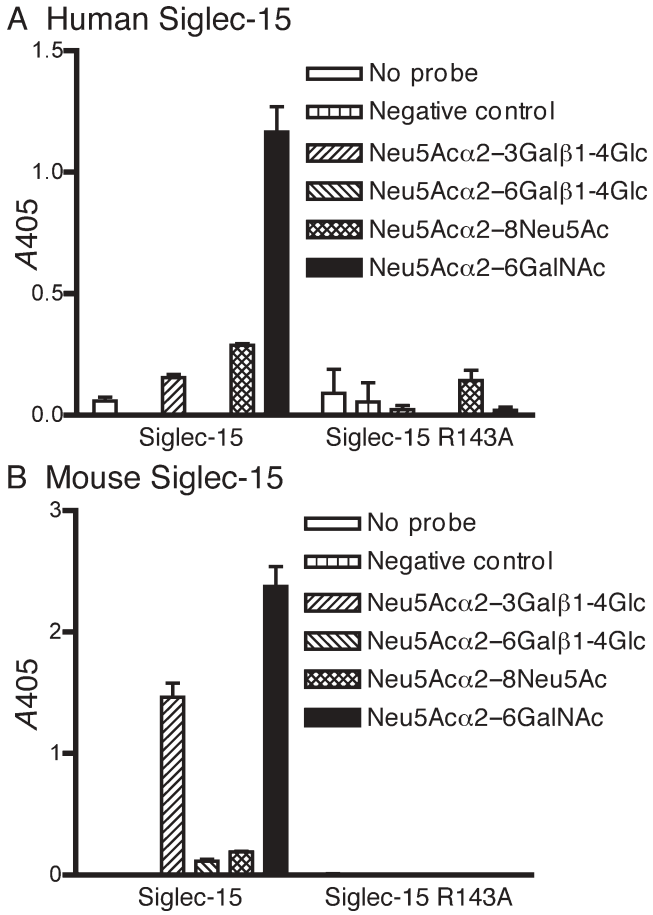

Fig. 5. Glycan binding preference of human and mouse Siglec-15. Human (**A**) and mouse (**B**) Siglec-15–Fc recombinant fusion proteins were immobilized on the wells of 96-well plates, and their binding to synthetic polymer probes multiply substituted with oligosaccharides was analyzed as described in the *Materials and methods* section. Both human and mouse Siglec-15 recognized Neu5Acα2–6GalNAc most strongly, and mouse Siglec-15 also recognized Neu5Acα2–3Galβ1-4Glc. These interactions were dependent on the "essential arginine" residue, because introduction of an Arg-to-Ala mutation (R143A) at the arginine residue almost completely abrogated binding of the probes to these proteins. The error bar represents standard deviation.

The introduction of an alanine mutation at an arginine residue (R143) that is conserved among all functional Siglecs and referred to as the "essential arginine" resulted in almost complete loss of the glycan-binding ability of Siglec-15 (Figure 5). These results suggest that the basic mode of glycan recognition by Siglec-15 is similar to that used by other Siglecs. Of note, two aromatic amino acid residues that are conserved among most Siglecs—one located near the N-terminus and one located near the essential arginine residue—also appear to be conserved in Siglec-15 (Figure 1 and Supplementary Figure 1).

Siglec-15 can associate with DAP12 and DAP10

The presence of a conserved lysine residue in the transmembrane domain of Siglec-15 (Figure 2) implies that this residue is under some functional constraint. Some activating adaptor proteins, such as DAP12, DAP10, and Fc receptor γ subunit (FcRγ), are known to associate with proteins that have a positively charged amino acid residue in the transmembrane domain. To test whether Siglec-15 can associate with DAP12, DAP10, and/or FcRγ, we transfected 293T cells with constructs expressing human Siglec-15 and FLAG-tagged DAP12, DAP10, or FcRγ, and examined whether Siglec-15 associates with these molecules.

As shown in the Figure 6, DAP12 and DAP10 co-immunoprecipitated with human Siglec-15. Remarkably, the association between DAP12 and Siglec-15 was more prominent, even though DAP12 was expressed at a lower level than DAP10 or FcRγ (Figure 6, lower panel).

Mutation of the conserved lysine residue of Siglec-15 to alanine completely abrogated the associations between Siglec-15 and DAP12 or DAP10, demonstrating that these interactions are dependent on this lysine residue. The association between Siglec-15 and FcRγ was minimal, and the Lys-to-Ala mutation of Siglec-15 did not disrupt this interaction. Essentially, the same results were obtained with mouse Siglec-15 (data not shown).

These results demonstrate that the lysine residue in the transmembrane domain of Siglec-15 is involved in the specific

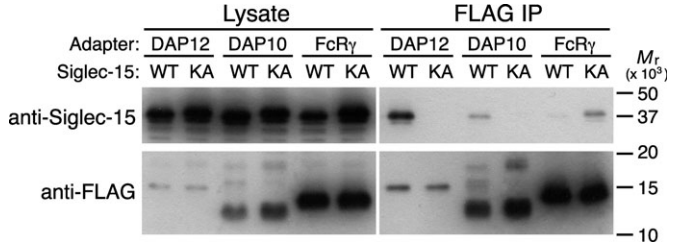

Fig. 6. Association of Siglec-15 with DAP12 and DAP10. The ability of Siglec-15 to associate with the activating adaptor proteins DAP12, DAP10, and FcRγ was analyzed by a co-immunoprecipitation assay. FLAG-tagged adaptor proteins and human Siglec-15 (wild type or K274A mutant) were co-expressed on 293T cells. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, western blotting, and probing with anti-Siglec-15 or anti-FLAG antibody. Equivalent levels of Siglec-15 wild type (WT) and K274A mutant (KA) were detected in the cell lysate, but FLAG-DAP12 and FLAG-DAP10 immunoprecipitates contained only wild-type Siglec-15. The association between Siglec-15 and FcRγ was minimal, and was not abrogated by the K274A mutation.

interaction of this Siglec with the activating adaptor molecules DAP12 and DAP10.

Discussion

In this report, we have described the identification and characterization of a novel Siglec, namely Siglec-15. In addition to the human and mouse Siglec-15 and the putative zebrafish ortholog of Siglec-15 (Siglec-like protein 1) that we cloned, we identified in GenBank some cDNA sequences of putative Siglec-15 orthologs from other vertebrate branches, such as expressed sequence tag clones from amphibians (GenBank accession number: CX474419 and CX474420, from *Xenopus tropicalis*) and from cartilaginous fish (GenBank accession number: CV652407, from *Squalus acanthias*). A Siglec-15 gene is present in the chicken genome, although its cDNA has not been isolated. Thus, Siglec-15 is present in all major branches of vertebrates. The only other Siglec that is conserved in all vertebrates is MAG/Siglec-4 (Lehmann et al. 2004), which is expressed exclusively in the nervous system.

The extent of amino acid sequence identity in the extracellular domain between human Siglec-15 and zebrafish Siglec-like protein 1 (approximately 40%) is even higher than that between human and pufferfish CD4 (approximately 20%) (Suetake et al. 2004), an essential protein that belongs to the Ig superfamily and is expressed on helper T cells. This high degree of conservation implies conserved function and suggests that the extracellular domain of Siglec-15 may recognize conserved ligand(s). Identification of the natural ligand of Siglec-15 would help to understand the biological functions of this Siglec. Although the zebrafish Siglec-like protein 1 did not show strong binding to the glycan probes tested (data not shown), it is possible that the glycan structure preferred by zebrafish Siglec-like protein 1 was not represented in the probe set that we used.

The positions of cysteine residues in Siglec-15 are unique among Siglecs. On the one hand, a disulfide bond between cysteine residues on β-strands B and E is a common structural motif in the Siglec family (May et al. 1998; Attrill et al. 2006), but this bond is absent in Siglec-15. On the other hand, the cysteine residues on β-strands B (C64) and F (C142) of Siglec-15 align with those in other members of the Ig superfamily (Supplementary Figure 2), and thus are likely to form a disulfide bond. Based on this finding, we speculate that Siglec-15 may be evolutionarily situated between the Siglecs and other Ig superfamily proteins; in other words, Siglec-15 is an ancestral Siglec.

Human and mouse Siglec-15 both associate with DAP12 and DAP10 via a lysine residue in the transmembrane domain. There is also a lysine residue in the transmembrane domain of zebrafish Siglec-like protein 1, which suggests that this molecule may also interact with adaptor molecules that have an ITAM. It has been reported that genes expressing ITAM-bearing adaptor molecules, such as DAP10, DAP12, FcRγ, and CD3ζ, are present in the pufferfish genome (Guselnikov et al. 2003). These observations are consistent with our hypothesis that the function of Siglec-15 is immunity related, and has been conserved throughout vertebrate evolution. However, expression of Siglec-15 in the immune system of non-mammalian vertebrates has not been verified, and a careful study would be required to prove it.

An immunity-related activating receptor is often paired with an inhibitory counterpart that shows significant sequence similarity. For example, we previously reported that human Siglec-5 (inhibitory) and Siglec-14 (activating) are paired receptors that have undergone concerted evolution (Angata et al. 2006). It has been proposed that an activating member of the Ly49 family might have derived from an inhibitory member in order to counter a viral pathogen that exploits the inhibitory member (Arase et al. 2002). In contrast, there is no inhibitory counterpart of Siglec-15 in mammals. One possible explanation is that the inhibitory counterpart of Siglec-15 in mammals might have been lost during evolution. In this regard, we cloned another Siglec-like molecule (Siglec-like protein 2; GenBank accession number: DQ471942) from zebrafish that resembles Siglec-15. Siglec-like proteins 1 and 2 are encoded by genes that lie in a head-to-head orientation on the zebrafish genome. Although we did not test whether Siglec-like protein 2 has an inhibitory function, this protein has a cytoplasmic domain with multiple tyrosine residues, one of which (Y285) is in a sequence motif (MVYANV) that is similar to ITIM ([I/L/V]xYxx[L/V]).

What is the selective pressure underlying the conservation of Siglec-15 in vertebrates? It is possible that this conservation has been driven (at least partly) by selective pressure exerted by some pathogens. Alternatively, Siglec-15 might have been conserved because of an essential function to recognize endogenous ligand(s) in, for example, tumor immune surveillance. The sialyl Tn structure is synthesized by ST6GalNAc I (Ikehara et al. 1999; Marcos et al. 2004) and is often expressed by malignant tumors (e.g. stomach, colon, pancreas, breast, and ovarian cancers; Hakomori 2001). Its expression on tumors is a consequence of aberrant or incomplete glycosylation of *O*-glycans (Brockhausen 2003, 2006), a phenomenon that often accompanies malignant transformation. If Siglec-15 on macrophages/dendritic cells recognizes tumor cells that express glycoproteins (e.g. mucins) bearing sialyl Tn or related glycan structures, then a cell-to-cell interaction initiated by such a recognition event may affect the behavior of the macrophages/dendritic cells that express Siglec-15 and/or that of the tumor cells. It has been shown that tumor-derived mucins up-regulate prostaglandin secretion from macrophages by binding to the macrophage scavenger receptor (Inaba et al. 2003), although this binding is not dependent on a glycan structure, and does not control tumor growth but rather helps. To test whether Siglec-15 is involved in the immune surveillance of tumors, it will be essential to determine the outcome of the interaction between cells expressing Siglec-15 and those expressing its ligands.

Unexpectedly, Siglec-15 was found to be localized inside macrophages/dendritic cells, rather than on the cell surface. There are examples of transmembrane immune receptors that are predominantly localized inside cells, such as some members of the Toll-like receptor (TLR) family (TLRs-7, -8, and -9; Wagner 2004) and CD68 (Saito et al. 1991). Further studies are required to identify the subcellular compartment in which Siglec-15 is localized, and to determine whether Siglec-15 functions within that compartment or translocates to the cell surface on some cue. For example, CD68 is known to translocate to the plasma membrane upon macrophage activation (Ramprasad et al. 1996), and Siglec-15 may undergo a similar process. Of note, the cytoplasmic tail of

Siglec-15 contains a sequence motif (YENL) that conforms to the consensus endocytosis motif, YxxØ (where Ø stands for amino acid residue with a bulky hydrophobic side chain; Bonifacino and Traub 2003). From an evolutionary point of view, the sequestration of important immune receptors within cells may be a strategy to protect them from exploitation by pathogens. This hypothesis can be tested by comparative analysis of the subcellular localizations and degrees of conservation of immune-related receptors.

In conclusion, we have identified a highly conserved Siglec that may play an essential role in the immune system. Its activating signaling potential and unique preference for glycan recognition implies that Siglec-15 may be involved in the immune surveillance of tumors. Its biological functions are the subject of our future studies.

Materials and methods

Molecular cloning of human Siglec-15 and its orthologs

A BLAST search based on the predicted partial sequence of human X (Angata et al. 2001) revealed the presence of a full-length cDNA clone encoding this molecule (GenBank accession number: AK172835) in an encyclopedic collection of human full-length cDNAs (Ota et al. 2004). Because this clone was not freely available, we obtained an alternative cDNA clone from the IMAGE consortium (IMAGE ID 6280187). DNA sequencing of the IMAGE clone and other independent clones obtained using RT–PCR revealed that the IMAGE clone contained a full-length cDNA. Its sequence similarity to known Siglecs and its ability to recognize sialylated glycans justified its inclusion in the Siglec family. Following the convention for Siglec nomenclature (Crocker et al. 1998; Varki and Angata 2006), we named the molecule Siglec-15.

The mouse ortholog of human Siglec-15 was cloned by RT-PCR. In brief, we synthesized first-strand cDNA from poly(A)$^+$ RNA from the thymus of a C57BL/6 mouse by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The open reading frame (ORF) was amplified by nested-PCR using Phusion DNA polymerase (Finnzymes, Epsoo, Finland) with the following primers: MmSigX F2 (5′-ggtgaccaggctgggtagag 3′) and MmSigX R2 (5′-ggtccccttggatgttgcc-3′) for the first round; and MmSigX F1 (5′-tgcaggcgtcagagcATGG-3′) and MmSigX R1 (5′-ccaggtctagcctggtactgtcc-3′) for the second round. The reaction mixture contained 3% (v/v) dimethylsulfoxide or 1 M betaine, without which no specific DNA amplification was observed. The DNA fragment was cloned into the *Eco*RV site of pBluescript (Stratagene, La Jolla, CA).

The zebrafish ortholog of Siglec-15 (Siglec-like protein 1) and its closely related paralog (Siglec-like protein 2) were cloned as follows. A TBLASTN search of the zebrafish genome sequence with the human Siglec-15 sequence as a query revealed the presence of four genomic DNA segments (two pairs of two putative exons) resembling the human Siglec-15 gene sequence. We designed primers for rapid amplification of cDNA ends (RACE) based on these putative exon sequences (Supplementary Table I). Total RNA was prepared from whole internal organs of a zebrafish by using RNeasy Mini (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA) in accordance with the manufacturer's instructions. Both 5′- and 3′-RACE were performed with the same kit, and the obtained clones, which collectively covered the entire cDNA, were sequenced. A separate preparation of first-strand cDNA was generated from the total mRNA using Superscript II reverse transcriptase (Invitrogen), and the ORF of each gene was cloned by nested-PCR using Phusion DNA polymerase (Finnzymes) and the following primers: DrL1-F2 (5′-acgcggggagatctgcaATGAACC-3′) and DrL1-R1 (5′-atcTTAGTGCTCCGCTCCACTAAAC-3′) in the first round, and DrL1-Expr F (5′-taatctagagccaccATGAACCTGCATCCCTTCAG-3′; *Xba*I site underlined) and DrL1-R1 in the second round of the amplification of the Siglec-like protein 1 ORF; and DrL2-F2 (5′-ggtgagattccagcacaatcagact-3′) and DrL2-R1 (5′-atcTCACTTTCCACCGGTGCTG-3′) in the first round, and DrL2-Expr F (5′-taatctagagccaccATGCAAGGCTTTGTGTTTGG-3′; *Xba*I site underlined) and DrL2-R1 in the second round of the amplification of the Siglec-like protein 2 ORF. The DNA fragments were purified, digested with *Xba*I, cloned into the *Xba*I–*Eco*RV sites of pcDNA3.1(−) (Invitrogen), and sequenced.

Preparation of expression constructs

Various Siglec expression constructs were prepared as follows.

Full-length protein expression constructs. The ORF of human Siglec-15 was amplified by PCR using the above-mentioned plasmid, containing the full-length cDNA as a template, Phusion DNA polymerase (Finnzymes), and the primer pair HsSigX Expr F (5′-ccctctagactcgaggccaccATGGAAAAGTCCATCTGGCTGCTG-3′; *Xba*I + *Xho*I sites underlined) and HsSigX R1 (5′-gatgttggtggctgagggac-3′). The PCR product was digested with *Xho*I and cloned into the *Xho*I–*Eco*RV sites of pcDNA3.1(−), and the insert sequence was confirmed. The resulting construct was named hSiglec-15/pcDNA. An expression construct for a human Siglec-15 K274A point mutant was prepared by using a QuickChange II Site-directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer's instructions. The hSiglec-15/pcDNA was used as a template for PCR with the primers HsSigX K274A S (5′-CTCGGCGCTCTCGGCTTCgccGCGCTGCTGCTGCTC-3′; Ala codon indicated with lower case letters) and HsSigX K274A AS (complementary to HsSigX K274A S). The resulting construct was named hSiglec-15 (K274A)/pcDNA. Expression constructs for full-length mouse Siglec-15 proteins (wild type and K273A mutant) were prepared in a manner similar to their human Siglec-15 counterparts.

Soluble Siglec–Fc fusion protein expression construct. The extracellular domain-coding segment (containing two Ig-like domains) of human Siglec-15 cDNA was amplified by PCR using the plasmid containing full-length cDNA as a template, Phusion DNA polymerase (Finnzymes), and the primer pair HsSigX Expr F (see *Full-length protein expression constructs*) and HsSigX Fc R (5′-atcCCCGCTGGCGCCATGGAAGCGGAAC-3′; *Eco*RV half-site underlined). The PCR product was digested with *Xba*I and cloned into the *Xba*I–*Eco*RV sites of EK–Fc/pcDNA3.1(−) (Angata et al. 2002), and the insert sequence was confirmed. The resulting construct was named hSiglec-15–Fc/pcDNA. An expression construct for an

hSiglec-15–Fc R143A point mutant was prepared by using a QuickChange II Site-directed Mutagenesis Kit. The hSiglec-15–Fc/pcDNA was used as a template for PCR with the primers HsSigX R143A S (5′-GCC GCTACTTCTGCgccGT CGAGTTCGCCG-3′; Ala codon indicated with lower case letters) and HsSigX R143A AS (complementary to HsSigX R143A S). The resulting construct was named hSiglec-15–Fc (R143A)/pcDNA. Expression constructs for mouse Siglec-15–Fc fusion proteins (wild type and R143A mutant) were prepared in a manner similar to their human Siglec-15 counterparts.

Preparation of Siglec–Fc fusion proteins

Recombinant Siglec–Fc proteins were prepared as described previously (Angata et al. 2002, 2006).

Glycan-binding analysis

Binding analysis using recombinant Siglec–Fc proteins and biotinylated polyacrylamide probes multiply substituted with sialylated oligosaccharides (PAA-Bio; Glycotech, Rockville, MD) was performed as described previously (Patel et al. 1999; Angata and Varki 2000; Angata et al. 2006). Binding between each Siglec–ligand pair was tested in triplicate wells.

Preparation of a specific polyclonal antibody

Recombinant human Siglec-15–Fc protein (3 mg) was digested with EK Max (Invitrogen), and the Fc part was removed by adsorption to protein A–Sepharose (GE Healthcare, Amersham, UK). The flow-through containing the extracellular domain of human Siglec-15 was used to immunize a rabbit by a local contractor (Tanpaku Seisei Kogyo, Gunma, Japan). Blood was collected a week after the fourth boost, and the serum was prepared by a standard method. A polyclonal antibody preparation specific to human Siglec-15 was prepared from a portion (5 mL) of the antiserum as follows. The antiserum was first mixed with human Siglec-15–Fc conjugated to Affigel-15 (Bio-Rad, Hercules, CA) to enrich for Siglec-15-reactive antibodies, and then subtracted against Siglec-6–Fc conjugated to Affigel-15 to remove any antibodies that cross-reacted with the Fc part. Enzyme-linked immunosorbent assay against various human Siglec–Fc proteins confirmed that the antibody preparation was specific to Siglec-15 (data not shown).

Preparation of C-terminal FLAG-tagged human DAP10 and FcRγ expression constructs

DAP10 cDNA was amplified by PCR from first-strand cDNA prepared from the THP-1 human monocytic cell line with the primers DAP10 F1 (5′-ccccagtccaccATGATCC-3′) and DAP10 R1 (5′-tcaaaggtccaagctgcagg-3′), and the product was purified by agarose gel electrophoresis. The purified product was subjected to nested-PCR with the primers DAP10 Expr F (5′-ccc<u>ctcgag</u>ccaccATGATCCATCTGGGTC-3′; *Xho*I site underlined) and DAP10 R FLAG (5′-ggggaattc TCA*CTTGTCATCGTCGTCCTTGTAGTC*GCCCCTGCCTGG CATGTTG-3′; *Eco*RI site underlined, complementary FLAG tag-coding sequence italicized), digested with *Xho*I and *Eco*RI, and cloned into the *Xho*I–*Eco*RI sites of pcDNA3.1(−). The resulting plasmid was sequence-verified and named DAP10-FLAG/pcDNA.

FcRγ cDNA was amplified similarly by PCR from first-strand cDNA prepared from THP-1 cells with the primers FcRg F1 (5′-tctccagcccaagATGATTCC-3′) and FcRg R1 (5′-cgcatctattctaaagCTACTGTGGTG-3′), and the product was purified by agarose gel electrophoresis. The purified product was subjected to nested-PCR with the primers FcRg Expr F (5′-ccc<u>ctcgag</u>ccaccATGATTCCAGCAGTGGTCTTG-3′; *Xho*I site underlined) and FcRg R FLAG (5′-ggggaattCTA *CTTGTCATCGTCGTCCTTGTAGTC*TGTGGTGGTTTCTCA TGCTT-3′; *Eco*RI site underlined, complementary FLAG tag-coding sequence italicized), digested with *Xho*I and *Eco*RI, and cloned into the *Xho*I–*Eco*RI sites of pcDNA3.1(−). The resulting plasmid was sequence verified, and named as FcRγ-FLAG/pcDNA.

Co-immunoprecipitation of human Siglec-15 and FLAG-tagged adaptor molecules

DAP12-FLAG/pcDNA was prepared as described previously (Angata et al. 2006). DAP12-FLAG/pcDNA, DAP10-FLAG/pcDNA, or FcRγ-FLAG/pcDNA was transfected into 293T cells, either singly or in combination with hSiglec-15/pcDNA or hSiglec-15 (K274A)/pcDNA, using Lipofect-AMINE 2000 reagent (Invitrogen). Immunoprecipitation of FLAG-tagged proteins and detection of human Siglec-15 and FLAG tag were done as described previously (Angata et al. 2006).

Immunohistochemical analysis of human Siglec-15 expression in spleen and lymph nodes

Formalin-fixed, paraffin-embedded human spleen and lymph node tissue slides were purchased from SuperBioChips (Seoul, South Korea). The slides were de-paraffinized and re-hydrated by a standard protocol, and then subjected to mild antigen retrieval in 10 mM citrate buffer, pH 6.0, using a microwave oven. The slides were incubated with 3% (v/v) goat serum and 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature, and then with rabbit anti-Siglec-15 antibody (10 μg/mL) or control rabbit IgG (10 μg/mL), along with mouse monoclonal anti-DC-SIGN (10 μg/mL; clone 120507, R&D Systems, Minneapolis, MN) or anti-CD68 (10 μg/mL; clone KP1, DAKO) antibody in 3% (v/v) goat serum with 1% (w/v) BSA in PBS at 4°C overnight in a humidified chamber. The slides were washed with PBS, and incubated with AlexaFluor 488-conjugated goat anti-rabbit IgG and AlexaFluor 546-conjugated goat anti-mouse IgG (10 μg/mL each; Invitrogen) in 3% (v/v) goat serum with 1% (w/v) BSA in PBS at room temperature for 1 h. The slides were washed with PBS, drained, mounted with FluorSave (CalBiochem, Darmstadt, Germany), and observed under an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan). Photomicrographs were taken with a digital camera (DP71, Olympus) attached to the microscope.

Molecular phylogenetic analysis

The amino acid sequences of the first two Ig-like domains of human Siglecs were aligned with Clustal X (Jeanmougin et al. 1998). Phylogenetic trees were constructed by using the neighbor-joining method (Saitou and Nei 1987).

Supplementary data

Supplementary data are available at Glycobiology online (http://glycob.oxfordjournals.org/).

Conflict of interest statement

None declared.

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Abbreviations

BSA, bovine serum albumin; CD, cluster of differentiation; cDNA, complementary DNA; DAP, DNAX activation protein; DC-SIGN, dendritic cell-specific ICAM-3 grabbing non-integrin; Fc, fragment crystallizable; FcRγ, Fc receptor γ subunit; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAG, myelin-associated glycoprotein; mRNA, messenger RNA; Neu5Acα2–6GalNAcα–, sialyl Tn; ORF, open reading frame; PBS, phosphate-buffered saline; RT–PCR, reverse transcription–polymerase chain reaction; TLR, Toll-like receptor.

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