New I-type lectins of the CD33-related siglec subgroup identified through genomics

Paul R. Crocker and Jiquan Zhang

Wellcome Trust Biocentre, Division of Cell Biology and Immunology, School of Life Sciences, Dundee University, Dow Street, Dundee DD1 5EH, U.K.

Abstract

Siglecs are sialic-acid-binding proteins of the Ig superfamily that are involved in cell–cell interactions and signalling. In recent years, several novel siglecs that are highly related to CD33/Siglec-2 have been identified through genomics and functional screens. In addition to their distinct sialic-acid-binding properties, most of these novel siglecs bear tyrosine-based signalling motifs that are typically found in inhibitory receptors of the immune system. The restricted expression patterns of CD33-related siglecs in the haemopoietic and immune systems suggests that they are involved in regulating leucocyte activation during inflammatory and immune responses.

Introduction

The siglecs are sialic-acid-binding members of the Ig superfamily [1] that belong to the I-type family of animal lectins [2]. The original members of the siglec family were sialoadhesin (siglec-1), a macrophage adhesion molecule [3], CD22 (siglec-2), a B-cell inhibitory receptor [4], CD33 (siglec-3), a marker of myeloid cells [5] and myelin-associated glycoprotein (MAG; siglec-4), which is expressed on glial cells in the nervous system [6]. These proteins share approx. 25–30% sequence similarity within their extracellular regions. In recent years several novel human, ape and mouse siglec family members have been identified through genomic analyses. All novel siglecs are highly related to CD33 and to each other, with an approx. 50–80% sequence similarity (Figures 1 and 2). They are therefore described collectively as ‘CD33-related siglecs’ and clearly form a separate subgroup from sialoadhesin, CD22 and MAG, both from functional and evolutionary perspectives. Genes encoding eight genuine human

1To whom correspondence should be addressed (e-mail p.r.crocker@dundee.ac.uk).
CD33-related siglecs have been identified and characterized, whereas only five have been identified in the mouse genome [7]. Two of the five murine siglecs still remain to be characterized at the protein level. One siglec-like gene and up to 16 CD33-related siglec pseudogenes have also been identified in humans and two pseudogenes are present in mice [7].

All siglecs are type 1 membrane proteins that possess a characteristic N-terminal sialic acid binding V-set Ig domain, and between one (CD33) and 16 (sialoadhesin) C2 domains that project the sugar-binding site away from the plasma membrane (Figure 1). The cytosolic tails of siglecs are quite variable in sequence and length, although most CD33-related siglecs share regions of high sequence similarity surrounding two conserved tyrosine motifs that are implicated in signalling functions (Figure 2). The genes that encode CD33-related siglecs are clustered on human chromosome 19q13.3–4 or in a syntenic region on mouse chromosome 7p. They appear to have evolved relatively recently by a process involving extensive gene duplication and exon shuffling; however, the

Figure 1 Human and mouse CD33-related siglecs. Full-length forms of all known human and mouse CD33-related siglecs are shown. Individual Ig-like domains are shown as circles. The sialic-acid-binding domain of each protein is shaded. Lines connecting Ig-like domains represent 'linker' regions encoded by separate exons. Hexagons in the cytoplasmic tails represent tyrosine-based motifs. Those shaded in black correspond to immunoreceptor tyrosine-based inhibitory (ITIM) motifs and those in grey correspond to immunoreceptor tyrosine-based switch (ITSM)-like motifs. Potential orthologues are indicated.
repertoire of genes encoding human and mouse CD33-related siglecs appears to be only partially overlapping, which suggests a rapid evolutionary process that differs among mammalian species [7]. This is illustrated by the recent finding that siglec-L1/S2V, a recently-characterized siglec-like protein with two tandem V-set Ig domains, has undergone an inactivating mutation in humans, yet it is still functional in chimpanzees [8]. This chapter focuses on the recently characterized CD33-related siglecs, most of which were identified through genomic studies.

Figure 2 Alignment of sialic-acid-binding V-set domain of all human and mouse CD33-related siglecs (A) and of the cytoplasmic tail C-terminal regions of all human and mouse ITIM-containing CD33-related siglecs (B). (A) Residues that are identical in more than 50% of proteins are shaded in black and those that are similar are shaded in grey. The conserved cysteine residues that are typical of siglecs are indicated by asterisks and residues that are important for sialic-acid-dependent binding are indicated by arrowheads. Predicted β-strands (based on the sialoadhesin V-set domain structure) are shown. (B) Residues that are identical in more than 50% of proteins are shaded in black and those that are similar are shaded in grey. The positions of the ITIMs and ITSM-like motifs are shown.
Evolutionary success of the Ig domain and its adaptation to sialic acid recognition

After the initial sequencing of the human genome, approx. 40% of the predicted proteins could be assigned to one of 12 broad families of 'InterPro' entries based on the presence of certain structural domains or motifs. The most populous InterPro entry was found to be the Ig domain, with 765 predicted proteins carrying at least one Ig domain [9]. This was in striking contrast with Drosophila melanogaster and Caenorhabditis elegans whose proteomes contain far fewer predicted proteins with Ig domains. The dramatic increase in Ig domain-containing proteins during animal evolution correlates with a parallel increase in the number of proteins associated with extracellular recognition events, especially those involved in host immunity [9].

The Ig domain contains two anti-parallel β-sheets that are usually linked by a characteristic disulphide bond and stabilized by a hydrophobic interior. Its evolutionary success in extracellular recognition events is likely to be due to several factors. It has been suggested that Ig domains can display a diversity of sequences that is greater than can be achieved with other protein superfamilies [10]. Sequence diversity can be displayed both on the external faces of the β-sheets as well as on the loops that connect the β-strands. In addition, Ig domains are often encoded by single exons with phase-I splice junctions that allow exon duplication and domain shuffling, leading to gene expansion and additional diversity. The Ig domain is also inherently resistant to proteolytic attack, and is therefore able to withstand the potentially harsh conditions in the extracellular milieu.

It is well known that V-set domains of many Ig molecules can bind to diverse carbohydrate structures including sialic acid; however, it is very unlikely that the ancestral V-set domain of siglecs evolved from an Ig gene. While Igs bind carbohydrate antigens via residues that are located on the hypervariable loop regions, siglecs bind sialylated glycans using residues located on the A, F and G β-strands in a manner that is more similar to that seen for other members of the Ig superfamily involved in protein–protein interactions [11–13]. Interestingly, one of the closest structural matches to the Sn V-set domain is the P0 adhesion molecule that is found in myelin. P0 is a single V-set-containing transmembrane protein that has been proposed to represent a typical primordial Ig-like domain involved in homophilic protein–protein interactions [10,14]. It is therefore likely that the siglec family evolved from an ancestral ‘P0-like’ gene and then, through mutation and selection, acquired the ability to interact specifically with sialic acids. A similar process could have occurred independently for the P0-related protein CD83 expressed by dendritic cells [15]. This receptor has a single V-set domain that can bind cells in a sialic-acid-dependent manner [15] yet has none of the typical features of siglec V-set domains.

If the above scenario is correct, siglecs would have appeared subsequently to the evolution of sialic acid biosynthesis pathways. Sialic acids are present at high levels in deuterostome-lineage animals such as starfish, but are absent or expressed at low levels in the protostome-lineage species C. elegans and D. melanogaster [16]. Genome-wide searches of C. elegans and D. melanogaster failed to uncover obvious siglec homologues or sequences encoding known
enzymes of the sialic acid biosynthesis pathway [16]; it is not known at present if siglecs are present in species such as starfish. MAG (siglec-4) has been identified immunochemically in a wide variety of higher non-mammalian species including fish, frogs, birds, snakes and lizards [17,18] and ‘expressed sequence tag’ (EST) database searches indicate the presence of transcribed siglec-like sequences in the African clawed frog *Xenopus laevis* and the zebrafish *Danio rerio* [7] (P.R. Crocker, unpublished work). The future availability of complete genome projects for both vertebrate and invertebrate species will be of great interest in understanding the evolutionary pathways of siglecs.

The V-set domains of siglecs are particularly well adapted for sialic acid recognition. Long before they were known to be carbohydrate-binding proteins, Williams et al. [10] pointed out that MAG and CD33 (the first members of the siglec family to be cloned and sequenced) had an unusual arrangement of three cysteine residues in the N-terminal V-set Ig domain. These are completely conserved in all siglecs (Figure 2A) and were predicted [14] to give rise to a disulphide bond within the ABED β-sheet rather than between the ABED and GFCC’ β-sheets. The crystal structure of the V-set domain of sialoadhesin complexed with 3′ sialyllactose confirmed this arrangement [13]. Moreover, it showed how the intrasheet disulphide bond resulted in an increased separation between the β-sheets and exposure of two normally buried tryptophan residues on β-strands A and G [13]. These residues make important hydrophobic contacts with the N-acetyl and glycerol moieties of the sialic acid molecule. In addition, a highly conserved arginine residue on the F β-strand (Figure 2A) forms a critical salt bridge with the carboxylate group of sialic acid [13]. This template for sialic acid recognition is shared by all known siglecs and provides a platform onto which additional specificity for sialic acid types and linkages to adjacent sugars can be built. The variable C–C′ loop (Figure 2A), for example, may be important for determining fine specificity, as demonstrated recently for the human siglecs hSiglec-7 and hSiglec-9 [19].

Naturally occurring mutations in the critical arginine residue have been found in a human siglec-like gene that is designated siglec-L1/S2V [8,20]. This unusual molecule contains two tandem V-set domains with Arg→Cys and Arg→Gln mutations. Remarkably, approx. 50% of the human and mouse siglec pseudogenes have mutations in the codon that encodes the critical arginine residue [7]. Phylogenetic analyses suggest that these are likely to have been independent events that could have functionally inactivated the proteins [7]. A lack of positive selection pressure could then lead to additional mutations and eventual inactivation. If correct, this interpretation would support the idea that sialic acid recognition is essential in mediating the biological functions of CD33-related siglecs and maintaining them as functional genes within populations.

**Identification of novel siglecs through genomics**

The creation of extensive databases containing random sequences from cDNA and genomic clones, together with powerful search engines, has enabled facile detection of new gene family members. hSiglec-5, -7, -8 and -10 were identified as CD33-related sequences in the Human Genome Science’s EST database,
which contains more than 1 million ESTs derived from approx. 700 cDNA libraries [21–25]. hSiglec-10 was also obtained from random sequencing of a dendritic cell cDNA library [26], from public genome databases [27] and from searches of Incyte’s EST database which is derived from 995 libraries with more than 4 million clones [28]. Genomic and EST databases were used to identify hSiglec-6, -7, -9 and -11 [16,29–33], as well as the murine siglecs, mSiglec-E, -F, -G and -H [7,34] (P.R. Crocker and J. Zhang, unpublished work). In all cases, examination of the predicted open reading frames revealed the presence of the conserved amino acid residues that are important for sialic acid recognition and, where tested, sialic acid binding has also been demonstrated experimentally. Several CD33-related sequences were identified in functional screens. Siglec-6 cDNA was isolated as a low-affinity leptin-binding protein in an expression cloning strategy and was shown subsequently to bind sialic acids [35]. Siglec-7 was the target in a screen for antigens on human natural killer (NK) cells that modulate killing activity and designated p75/AIRM1 [36]. mSiglec-E was isolated in a yeast two-hybrid screen using the protein tyrosine phosphatase Src homology 2-domain-containing protein tyrosine phosphatase-1 (SHP-1) as bait [37]. Finally, hSiglec-8, -9, -10, -L1 and mSiglec-E, -F and -H were identified by the combined approaches of PCR and low-stringency cDNA/genomic library screening (J. Zhang and P.R. Crocker, unpublished work).

The human siglec-like sequence hSiglec-L1, which is discussed above, was identified in public genomic and EST databases [8,38]. The same gene was isolated in a yeast two-hybrid screen using SHP-1 as a bait and was, surprisingly, later shown to bind to red blood cells in a sialic-acid-dependent manner when expressed in COS cells [20]. However, this result is controversial, especially in light of the findings that the chimpanzee orthologue of hSiglec-L1 has retained the critical arginine in the N-terminal V-set domain and mediates robust sialic-acid-dependent binding, in stark contrast with hSiglec-L1 [8]. Further genomic analyses in other species are required to shed light on the evolutionary pathways taken by CD33-related siglecs.

**Comparisons of human and mouse CD33-related siglecs**

Recent access to the almost complete human and murine (Celera Genomics) genomic databases has allowed the precise cataloguing and mapping of all human and mouse CD33-related genes [7,39]. While the human genome contains eight CD33-related siglecs, one siglec-like gene and approx. 16 pseudogenes, the mouse genome contains only five CD33-related genes and two pseudogenes [7,33]. All eight of the human siglecs have two conserved immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails, which are implicated in inhibitory signalling functions (see below). In contrast, only three of the five murine CD33-related genes encode ITIM-containing siglecs and those that lack ITIMs do not have obvious orthologues in humans (Figure 1 and Table 1).

Consistent with the earlier *in situ* hybridization work carried out on hSiglec-5, -6, -7, -8 and -10, these genes are clustered within a 506-kb region on human chromosome 19q13.3–13.4. hSiglec-11 is located approx. 1 Mb upstream
from the cluster, but still within the cytological band 19q13.3–13.4 [33]. Apart from mSiglec-H, all murine CD33-related siglecs lie in a syntenic region on chromosome 7p. Chromosome 19 in humans is the most gene-rich chromosome; it contains large families of Ig-related receptors, including the carcinoembryonic antigen family, killer inhibitory receptors and Ig-like transcripts, in addition to the CD33-related siglecs (reviewed in [40]). The dramatic expansion of Ig-like families on chromosome 19q may be due, at least in part, to the presence of chromosome 19-specific minisatellites that facilitated gene duplication by acting as nucleation sites for unequal cross-overs during meiotic recombination [16,41]. Such exchanges can also introduce nucleotide changes in the non-coding regulatory regions, which, in the case of siglecs, could be important for their acquiring cell-type-specific expression patterns in the immune system.

The sequence similarity shared between all of the CD33-related siglecs in humans and mice (approx. 50–80%) falls generally within the range for orthologous pairs of immune system receptors. This makes assignments of orthologues potentially problematic. Indeed, in sequence alignments and phylogenetic analysis of all known human and mouse siglecs, only siglec-1, -2, -4 and -10 can be seen to have clear orthologues in both species. Where studied, the corresponding proteins in humans and mice exhibit similar sialic-acid-binding properties and cellular expression patterns [3,42–44] (P.R. Crocker and J. Zhang, unpublished work). Based on gene structure, sequence alignments, relative chromosomal localization and, where available, functional properties, it has been suggested that murine CD33, mSiglec-E and mSiglec-F are orthologues of human CD33, hSiglec-9 and hSiglec-5 respectively [7]. However, murine CD33 has a completely different cytoplasmic tail from human CD33 and it lacks ITIM-like motifs [5,45]. Unlike mSiglec-G and hSiglec-10, which are approx. 60% identical, mSiglec-E and -F are only approx. 50% identical with hSiglec-9 and -5 respectively (Table 1) and show significant differences in sialic-acid-binding specificity and cellular expression patterns [7] (P.R. Crocker and J. Zhang, unpublished work). Therefore, based on currently available data, it is possible

<table>
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<tr>
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Table 1 Percentage sequence identities derived from alignments of the indicated human and mouse siglecs. Alignments were made using the full-length protein sequences, except for those made using human CD33, for which only the Ig domains were used.
that siglec-1, -2, -4 and -10 were the principal siglecs present in the common ancestor of humans and mice. The remaining CD33-related siglecs could have evolved independently in different species, involving a process of exon deletion and gene duplication, starting from a siglec-10-like precursor. This would have been particularly extensive in the primate lineages, leading to the generation of eight functional CD33-related siglecs and one siglec-like molecule.

**Sialic-acid-binding specificity**

Sialic acid is a generic term for a large family of 9-carbon sugars that are all derivatives of neuraminic acid or oxo-deoxynonulosonic acid. They are typically found at the exposed, non-reducing ends of oligosaccharide chains attached to a wide variety of proteins and lipids. At least 18 sialyltransferases exist in mammals and these catalyse the transfer of different types of sialic acid to various acceptors, to generate α2,3-, α2,6- or α2,8-glycosidic linkages. Despite the high degree of sequence similarity, the CD33-related siglecs exhibit clear differences in their sialic-acid-binding preferences, both at the level of apparent affinity and preference for glycosidic linkage. hSiglec-7, for example, shows a striking preference for α2,8-linked disialic acids in contrast with the highly related hSiglec-9 which strongly prefers α2,3-linkages [19]. The biological significance of these sugar-binding preferences is unclear at present and it is likely to require an understanding of the precise functions of each molecule, about which very little is known at present.

With the exception of hSiglec-6, which shows restricted specificity for the sialyl Tn antigen [35], all of the known siglecs recognize forms and linkages of sialic acid that are commonly found at cell surfaces and in the extracellular environment [7,16,21–23,25,30,31,33,43]. One consequence is that the siglec-binding sites can be masked by *cis* interactions with sialic acids on the same cell, thereby preventing them from mediating cell–cell interactions. Since all CD33-related siglecs on circulating leucocytes appear to be masked [46], one possibility is that the binding specificity of each siglec is tailored towards the sialylation pattern of the host cell, thereby facilitating *cis* interactions with relevant ligands that play a role in modulation of cellular functions. The importance of such sialic-acid-dependent *cis* interactions has been demonstrated recently for CD22/siglec-2, which associates with the B-cell receptor and inhibits B-cell activation [47]. CD22 specifically binds α2,6-linked sialic acids on N-glycans that are generated by the ST6GalI sialyltransferase, an enzyme that is expressed abundantly by β1,4-galactoside α2,6 sialytransferase [48]. Addition of a novel CD22-specific sugar inhibitor to B-cells was found to enhance B-cell activation, thereby showing that sialic-acid-dependent *cis* interactions are important for the signalling functions of CD22 [49].

**Expression pattern of CD33-related siglecs**

CD33 was originally identified as a marker of myeloid progenitor cells that is absent from multipotential stem cells in the bone marrow [50,51]. This...
restricted specificity has been extremely useful in providing a means of distinguishing subpopulations of haemopoietic progenitors in samples of bone marrow and umbilical cord blood. Furthermore, its expression on the great majority of acute myeloid leukaemic blasts has been exploited in the development of a humanized antibody conjugated to the toxin calicheamicin (Mylotarg®), to eliminate residual leukaemia cells after relapse (reviewed in [52]). Recent studies have shown that, similar to CD33 and other siglecs, the novel CD33-related siglecs are expressed in a very cell-type-specific manner, largely within the haemopoietic system. While much remains to be learned, some intriguing observations have emerged from studies of normal human circulating blood leucocytes and selected tissues using panels of monoclonal antibodies. Some siglecs are expressed quite broadly, e.g. hSiglec-9 is found on neutrophils, monocytes and on a substantial fraction of NK cells and B-cells [30]. Others are much more restricted in their expression, notably hSiglec-8, which is found only on circulating eosinophils and at very low levels on basophils [24,53]. On the other hand, several siglecs can be present on the same cell type; for example, monocytes express hCD33, hSiglec-5, -7, -9 and -10, and circulating B-cells express hSiglec-5, -6, -9 and -10, suggesting some degree of functional redundancy at the cellular level [21,22,25,30,35,54]. Siglec-11 is the only CD33-related siglec characterized thus far that is undetectable on blood leucocytes [33]. Interestingly, siglec-11 is expressed by certain tissue macrophages, including brain microglia and liver Kupffer cells and certain other leucocytes, especially in inflammation [33]. There is only limited information available at present on the expression of the other CD33-related siglecs on cells outside of the haemopoietic system, although hSiglec-6 is expressed at high levels by cytotrophoblasts and syncytiotrophoblasts of the placenta, in addition to B-cells [35]. Interestingly, using a chicken antibody, siglec-L1 was observed at high levels on the apical edge of epithelial cells in various tissues [8]. However, other studies have shown a pattern of siglec-L1 mRNA expression that is more consistent with haemopoietic expression, especially on macrophages [20,38] (J. Zhang and P.R. Crocker, unpublished work).

**CD33-related siglecs as inhibitory receptors**

It is now well established that balancing positive and negative signals is crucial for setting activation thresholds for cells of the haemopoietic and immune systems. The paradigm that has emerged from studies of classical inhibitory receptors such as the FcγRIIB and killer inhibitory receptors is that when cellular activation is triggered by receptors with immunoreceptor tyrosine-based activation motifs (ITAMs), counteracting inhibitory signals are delivered through receptors bearing ITIMs (reviewed in [55]). Tyrosine-phosphorylated ITIMs recruit and activate cytosolic phosphatases, either the protein tyrosine phosphatases Src homology 2-domain-containing protein tyrosine phosphatase (SHP)-1 or SHP-2, or the inositol polyphosphate 5′ phosphatase SHIP (Src homology 2-containing inositol-polyphosphate 5′ phosphatase). These phosphatases inhibit signalling pathways by distinct mechanisms, which results in raised activation thresholds (reviewed in [55]).
The presence of two conserved ITIM-like motifs in the cytoplasmic regions of all hCD33-related siglecs (Figure 2B) and the differential expression of these proteins on different types of leucocyte, suggest a generic role in regulating cellular activation. hSiglec-8, which was originally thought to lack ITIM-like motifs, has now been found to exist in alternatively spliced forms containing either a short cytoplasmic tail without ITIMs or a long tail with ITIMs [23–25,56,57]. While the membrane proximal motif of CD33-related siglecs fits the consensus ITIM sequence (Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-(Leu/Val), the membrane distal motif does not (Figure 2B). Detailed studies of hCD33, hSiglec-10, hSiglec-L1 and mouse inhibitory siglec (MIS)/mSiglec-E have shown that both SHP-1 and SHP-2 can be recruited following pervanadate treatment of cells to inhibit tyrosine phosphatases. Mutagenesis studies have shown that for all of the above siglecs the distal ITIM-like motif binds more weakly to SHP-1 and SHP-2, and is dispensable for phosphatase binding, presumably reflecting its departure from the consensus ITIM sequence. However, the distal motif is highly conserved (Figure 2B), which suggests that it is important for interacting with other regulatory molecules. Interestingly, this motif is similar to a tyrosine-based motif [Thr-Xaa-Tyr-Xaa-Xaa-(Val/Ile)] found in SLAM (signalling lymphocyte activation molecule) and SLAM-related proteins that binds SAP (SLAM-associated protein), a single-SH2-domain protein that inhibits SHP-2 recruitment and activates additional signalling pathways (reviewed in [58]). This motif has recently been designated ‘immunoreceptor tyrosine-based switch motif’ (ITSM), which reflects its apparent role in switching between different signalling pathways [59].

Functional evidence indicating that siglecs can mediate inhibitory signals has been obtained using antibodies to co-cross-link hCD33 or MIS/mSiglec-E with an activating human receptor, FcγR1 [34,60,61]. This resulted in reduced Ca2+ influx compared with cross-linking FcγRI alone. Likewise, siglec-7 was identified as an inhibitory NK cell receptor in a redirected killing assay in which anti-siglec-7 antibodies were used to cluster siglec-7 at the NK cell–target-cell interface [54]. In other functional studies, the addition of intact anti-hCD33 or anti-hSiglec-7 monoclonal antibodies to haemopoietic cell cultures resulted in reduced cell growth and prevention of dendritic cell development [36,62]. The physiological relevance of these interesting findings remains unclear, since antibodies rather than natural ligands were used to cluster the siglecs. Clearly, additional experimental approaches are needed to understand the functions of CD33-related siglecs and the importance of sialic acid recognition in mediating these functions.

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