THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 27, Issue of July 5, pp. 24466–24474, 2002

Printed in U.S.A.

Cloning and Characterization of Human Siglec-11

A RECENTLY EVOLVED SIGNALING MOLECULE THAT CAN INTERACT WITH SHP-1 AND SHP-2 AND IS EXPRESSED BY TISSUE MACROPHAGES, INCLUDING BRAIN MICROGLIA*[S]

Received for publication, March 25, 2002, and in revised form, April 26, 2002
Published, JBC Papers in Press, May 1, 2002, DOI 10.1074/jbc.M202833200

Takashi Angata‡§, Sheena C. Kerr§¶‖, David R. Greaves‡‡, Nissi M. Varki‡, Paul R. Crocker¶, and Ajit Varki‡§§**

*From the ‡Glycobiology Research and Training Center, Departments of Medicine and Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California 92093-0687, the ¶Wellcome Trust Biocentre, Division of Molecular Cell Biology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, United Kingdom, and the **Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom*

Siglecs are sialic acid-recognizing animal lectins of the immunoglobulin superfamily. We have cloned and characterized a novel human molecule, Siglec-11, that belongs to the subgroup of CD33/Siglec-3-related Siglecs. As with others in this subgroup, the cytosolic domain of Siglec-11 is phosphorylated at tyrosine residue(s) upon pervanadate treatment of cells and then recruits the protein-tyrosine phosphatases SHP-1 and SHP-2. However, Siglec-11 has several novel features relative to the other CD33/Siglec-3-related Siglecs. First, it binds specifically to α2–8-linked sialic acids. Second, unlike other CD33/Siglec-3-related Siglecs, Siglec-11 was not found on peripheral blood leukocytes. Instead, we observed its expression on macrophages in various tissues, such as liver Kupffer cells. Third, it was also expressed on brain microglia, thus becoming the second Siglec to be found in the nervous system. Fourth, whereas the Siglec-11 gene is on human chromosome 19, it lies outside the previously described CD33/Siglec-3-related Siglec cluster on this chromosome. Fifth, analyses of genome data bases indicate that Siglec-11 has no mouse ortholog and that it is likely to be the last canonical human Siglec to be reported. Finally, although Siglec-11 shows marked sequence similarity to human Siglec-10 in its extracellular domain, the cytosolic tail appears only distantly related. Analysis of genomic regions surrounding the Siglec-11 gene suggests that it is actually a chimeric molecule that arose from relatively recent gene duplication and recombination events, involving the extracellular domain of a closely related ancestral Siglec gene (which subsequently became a pseudogene) and a transmembrane and cytosolic tail derived from another ancestral Siglec.

Siglecs (sialic acid-binding immunoglobulin superfamily lectins)[1] are a family of cell surface lectins defined by certain shared structural motifs in the first two Ig-like domains and by their ability to recognize sialic acids via the first Ig V set domain (1–4). In humans, ten canonical Siglecs (3–5) and one Siglec-like molecule (6, 7) have been reported so far. Each Siglec shows distinct expression patterns in different cell types (3–6), suggesting that these molecules play unique roles in the cells expressing them. Some Siglecs have strict ligand requirements, whereas others show more relaxed specificity. Thus, CD22/Siglec-2 is highly specific for α2–6-linked sialic acids (8–10); myelin-associated glycoprotein/Siglec-4 shows α2–3 linkage specificity, which is strongly affected by the adjacent glycan structure (11, 12); and Siglec-6/OB-BP1 only recognizes sialyl-Tn (13). In contrast, sialoadhesin/Siglec-1 (14, 15), CD33/Siglec-3 (15, 16), Siglec-5 (17), Siglec-7 (18), Siglec-8 (19), Siglec-9 (20, 21), and Siglec-10 (5) all recognize both α2–3- and α2–6-linked sialic acids to differing degrees. Some Siglecs, especially Siglec-7, also bind α2–8-linked sialic acids (15, 22, 23).

Most Siglecs have tyrosine-based putative signaling motifs in the cytosolic tails, suggesting their involvement in intracellular signal transduction (3, 4). Notably, most CD33/Siglec-3-related Siglecs (3, 4), a subgroup of the Siglec family defined by their sequence similarity, have immunoreceptor tyrosine-based inhibitory motifs. In keeping with this, several of these Siglecs have been shown to interact with protein-tyrosine phosphatases SHP-1 and/or SHP-2 (Src homology domain 2-containing phosphatases 1 and/or 2) upon tyrosine phosphorylation (7, 24–30), and some studies have shown that cross-linking of these Siglecs induces inhibitory cellular signals (27, 31).

In this paper, we report the molecular cloning and characterization of a novel human Siglec, Siglec-11. Some of its unique properties are presented, such as a preference for α2–8-linked sialic acids and expression in tissue macrophages, including microglia in brain. Our analysis of genomic DNA data bases suggests that Siglec-11 is the last canonical Siglec to be reported and that it evolved via recent gene duplication and recombination events that occurred after the split of the primate and rodent lineages.

* This work was supported by United States Public Health Service Grant PO1 HL57345–05 (to A. V.) and a Senior Fellowship from the Wellcome Trust (to P. R. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at *http://www.jbc.org*) contains the sequences of Siglec-P14 and Siglec-P16.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF337818.

§ These authors contributed equally to this work.

‖ Supported by a studentship from the Biotechnology and Biological Sciences Research Council.

‡‡ British Heart Foundation Basic Science Lecturer.

§§ To whom correspondence should be addressed: UCSD School of Medicine 0687, La Jolla, CA 92093-0687. E-mail: avarki@ucsd.edu.

[1] Part of the data on mouse genomic sequences discussed here was generated through use of the Celera Discovery System and Celera's associated data bases. As per the agreement with the University of California, the relevant sequences have been submitted by Celera Genomics to the GenBank™ with accession number AY115107.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, all of the reagents were purchased from Fisher or Sigma. Isotype control mouse monoclonal antibody was from BD Biosciences Pharmingen, and fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody was from DAKO. The antibodies to SHP-1 (rabbit polyclonal, C-19), SHP-2 (rabbit polyclonal, N-16), and horseradish peroxidase-conjugated anti-rabbit IgG antibody were from Santa Cruz Biotechnology, Inc. The 4G10 anti-phosphotyrosine hybridoma was a kind gift from Lars Nitschke (University of Würzburg, Germany). The horseradish peroxidase-conjugated horse anti-mouse IgG antibody was from Vector Laboratories.

Cloning of Siglec-11 cDNA—We identified a Siglec-like putative gene by a homology search of human genome sequences in the nonredundant and high throughput genome sequencing divisions of the GenBank™ data base, using known human Siglec cDNA sequences as templates. Primers for cDNA cloning of this putative gene, denoted Siglec-11, were designed based on the genomic DNA sequence. The full-length coding region of Siglec-11 cDNA was cloned with Expand High Fidelity PCR System (Roche Molecular Biochemicals) following the manufacturer's protocols, using human fetal liver Marathon-Ready cDNA library (BD Biosciences CLONTECH) as template and SL-11 5′-UTR[2] (5′-GGGACAGGCCCAGCCCCAGAGCCC-3′) and SL-11 3′-UTR2 (5′-GAGTCCAGTTCTGGCCGTCACACC-3′) as primers. PCR products were cloned into pCRII-TOPO TA cloning vector (Invitrogen), and the sequences were analyzed. The 3′-end cDNA sequence of Siglec-11 was obtained by 3′-rapid amplification of cDNA ends (RACE) from the human fetal liver Marathon-Ready cDNA library using the Expand High Fidelity PCR System, as follows. Gene-specific cDNA fragments were amplified by PCR using primers SL-11 3′-RACE-1 (5′-GGTGATCAGAGACGCGCAGAGGG-3′) and AP1 (BD Biosciences CLONTECH, provided with the cDNA library), followed by a nested PCR using primers SL-11 3′-RACE-3 (5′-CCCTAGAAGAACCAGACCAAGCAC-3′) and AP2 (BD Biosciences CLONTECH, provided with the cDNA library). The 5′-end cDNA sequence was obtained in the same manner by 5′-RACE, using primers SL-11 5′-RACE-1 (5′-GCCATAAGCAGCAGTAGACTCGTCC-3′) and AP1 in the first round PCR, followed by a nested PCR using primers SL-11 5′-RACE-2 (5′-TAGGAGAGGTTGCAAGACACGATGACAC-3′) and AP2. PCR products were cloned into pCRII-TOPO TA cloning vector, and the sequences were analyzed.

RT-PCR Analysis of mRNA Expression in Human Tissues—Human multiple tissues cDNA panel 1 and Titanium *Taq*DNA polymerase were purchased from BD Biosciences CLONTECH. PCR reactions to amplify a 400-bp segment of Siglec-11 cDNA were carried out according to the manufacturer's instructions, using the primers 11RTUTR (5′-TGCTGGTGTCCTGTTGCCATGGAGACCTC-3′) and 11RTd5F (5′-TCGCTGCCCTGCTCGCTTTCTGTTCTT-3′).

Preparation of a Siglec-11 Expression Construct—The full-length coding region of Siglec-11 cDNA was amplified by PCR from a sequence-verified cDNA clone described above to introduce Kozak sequence, using *Pwo* polymerase (Roche Molecular Biochemicals) and the primers SL-11 Chi 5′ (5′-CCCTCTAGAGCCACCATGCTGCTGCTGCCCCTGCTGCTGCCC-3′; Kozak sequence underlined, preceded by an *Xba*I site) and SL-11 3′-UTR-Hd (5′-GCGCAAGCTTCCGTCACACCAGTGCGACTCCC-3′; *Hin*dIII site underlined). PCR products were digested with *Xba*I and *Hin*dIII and subcloned into *Xba*I-*Hin*dIII sites of pcDNA3.1(−) (Invitrogen), and the sequences were verified.

Preparation of Siglec-11-Fc Chimera Protein—The Siglec-11 cDNA fragment encoding the first three Ig-like domains was amplified from the sequence-verified cDNA clone by PCR using *Pwo* polymerase with primers SL-11 Chi 5′ and SL-11 Chi 3′(3D) (5′-ATCCATCACTCTCAGGTTCTCTGGAGGA-3′), digested with *Xba*I, and cloned to *Xba*I-*Eco*RV sites of EK-Fc/pEDdC vector (6, 13, 21, 32). Upon mammalian cell transfection, the resulting construct (Siglec-11-EK-Fc/pEDdC) expressed a recombinant soluble Siglec-11-Fc protein (a fusion protein of the first three Ig-like domains of Siglec-11 and human IgG Fc fragment, with an enterokinase cleavage site/FLAG tag (DYKDDDDK) in between). Because of the poor production of the fusion protein from this construct, the DNA fragment coding the fusion protein was subcloned into pcDNA3.1(−).

The fusion protein was prepared by transient transfection of Chinese hamster ovary TAg cells (32) with Siglec-11-EK-Fc/pcDNA3.1(−) using LipofectAMINE reagent (Invitrogen). The culture supernatant was changed 24 h after transfection to 2.5% low IgG fetal bovine serum (HyClone, Logan, UT) in Opti-MEM (Invitrogen). Siglec-11-Fc was purified from culture supernatant by adsorption to protein A-Sepharose (Amersham Biosciences), as described previously (13, 21, 32).

Expression constructs for Siglec-11-Fc point mutants (R120K and R120A) were prepared by introducing point mutations into Siglec-11-EK-Fc/pcDNA3.1(−) using QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions, using primer pair SL-11 R120K sense (5′-GGCATGGTACTTCTTTAAAGTGGAGAGAGGAAGCC-3′; Lys codon underlined) and SL-11 R120K antisense (complementary to SL-11 R120K sense) or primer pair SL-11 R120A sense (5′-GGCATGGTACTTCTTTGCCGTGGAGAGAGGAAGCC-3′, Ala codon underlined) and SL-11 R120A antisense (complementary to SL-11 R120A sense), respectively. Recombinant proteins were prepared as described above.

Ligand Binding Specificity of Siglec-11—Sialic acid linkage specificity was analyzed by a standard enzyme-linked immunosorbent assay as described previously (13, 21, 32), using biotinylated polyacrylamide probes (PAA-Bio) multiply substituted with sialylated oligosaccharides (Glycotech, Rockville, MD). Mild periodate treatment of $(Neu5Ac)_2$-PAA-Bio probe to specifically truncate the glycerol-like side chain of sialic acids was performed as described previously (13, 32).

Preparation of Mouse Monoclonal Antibodies against Siglec-11—Balb/c mice were immunized subcutaneously with Siglec-11-Fc (15 μg) in Freund's complete adjuvant followed by Siglec-11-Fc (15 μg) in Freund's incomplete adjuvant 28 days later and were immunized further with Siglec-11 extracellular domain (10 μg; Fc part removed from the fusion protein by enterokinase digestion and protein A-agarose adsorption) in Freund's incomplete adjuvant after an additional 28 days. The hybridomas were generated by fusing immune spleen cells with the Sp-2 myeloma following standard methods (33). A positive well reacting specifically with Siglec-11-Fc was identified by enzyme-linked immunosorbent assays as described previously (20). The hybridoma was cloned four times by limiting dilution, and the monoclonal antibody was designated 4C4 (subclass IgG2a). 4C4 was used both as a purified IgG and a tissue culture supernatant. Potential cross-reactivity of 4C4 against other human Siglecs (CD33/Siglec-3, Siglec-5, Siglec-7, Siglec-8, Siglec-9, and Siglec-10) was analyzed by flow cytometry using Chinese hamster ovary cells stably expressing each of these Siglecs and confirmed negative for all of the Siglecs tested (data not shown).

Flow Cytometry Analysis of Peripheral Blood Leukocytes—The leukocytes were prepared from peripheral blood of healthy volunteers as described previously (19). Single labeling of cells for flow cytometry was performed following standard protocols (34) and fixed in 2% formaldehyde in phosphate-buffered saline following staining. Following staining, all of the samples were analyzed on a FACSort (BD Biosciences Immunocytometry Systems).

Immunohistochemical Analysis of Siglec-11 Expression—Paraffin-embedded formalin-fixed tissue sections mounted on glass slides (prepared in-house or purchased from DAKO) were deparaffinized, treated to inactivate endogenous peroxidase, and then subjected to antigen retrieval (35, 36). The slides were blocked with bovine serum albumin and streptavidin/biotin to prevent spurious staining and incubated overnight at 4 °C with the following: anti-Siglec-11 monoclonal antibody 4C4 (culture supernatant, diluted at 1:10 with 1% bovine serum albumin/phosphate-buffered saline), anti-CD68 antibody (clone KP1), anti-CD23 antibody (clone MHM6), or anti-CD79a antibody (clone JCB117; these three antibodies were supplied prediluted from DAKO). The slides were then washed with 0.1% Tween 20 in Tris-buffered saline. The antibody binding signals were detected using a catalyzed signal amplification system (DAKO) following the manufacturer's protocol, except that NovaRed substrate (Vector Laboratories) was used for color development. The slides were counter-stained with Meyer's hematoxylin solution and viewed under a Zeiss microscope. The digitized images were captured using a DKC-5000 digital photo camera (Sony), NIH Image, and Adobe Photoshop.

Analysis of Tyrosine Phosphorylation of Siglec-11 and Interaction with SHP-1 and SHP-2—Full-length Siglec-11 cDNA was subcloned into pcDNA3CD68 (37) and transfected into RAW 264.7 cells by electroporation. Stable cell lines were established by selection with 1 mg/ml G418 and two rounds of limiting dilution. The control cells were prepared likewise, except that empty vector was used for transfection. The cells were either untreated or treated with 0.04 mM pervanadate for 30 min at 37 °C and lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, and protease inhibitors). The lysates were precleared with protein G-Sepharose and immunoprecipitated overnight at 4 °C with 4C4 and protein G-Sepharose. For immunoblot analysis, the proteins were resolved by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide gels) and transferred to

[2] The abbreviations used are: UTR, untranslated region; PAA-Bio, biotinylated polyacrylamide; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; RT, reverse transcription.

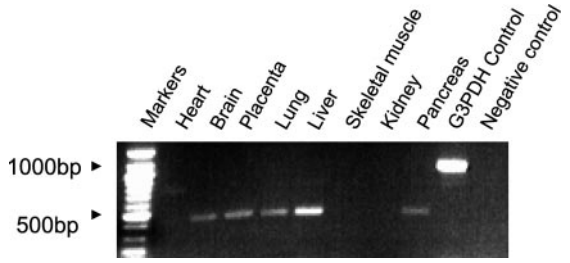

FIG. 1. **RT-PCR analysis of Siglec-11 transcripts in human tissues.** The CLONTECH human multiple tissue cDNA panel was subjected to 40 cycles of PCR using Siglec-11-specific primers, and the DNA products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The expected 400-bp band was prominent in cDNA from brain, placenta, lung, liver, and pancreas but undetectable in heart, skeletal muscle, and kidney. The positive control (glycerol-phosphate dehydrogenase) and negative control (water) are also shown.

nitrocellulose membrane for blotting. The membranes were blocked with either 3% bovine serum albumin in 0.1% Tween 20 in Tris-buffered saline (for 4G10 anti-phosphotyrosine antibody) or 5% nonfat dry milk in 0.1% Tween 20 in Tris-buffered saline (for all other antibodies) and incubated with the indicated primary antibody. Antibody binding was detected with the appropriate horseradish peroxidase-conjugated secondary antibody and visualized with ECL (Amersham Biosciences) according to the manufacturer's instructions.

RESULTS

Molecular Cloning of Siglec-11—In our search for novel Siglec candidates in human genomic DNA sequences, we identified a Siglec-like putative gene on the bacterial artificial chromosome clone CTC-326K19 (GenBank™ accession number AC011452). Proof for the active transcription of this putative gene, tentatively denoted Siglec-11, was provided by two EST clones (clones EST32579 from whole embryo and EST46736 from fetal kidney; GenBank™ accession numbers AA328836 and AA341128, respectively) and by RT-PCR analysis of several human tissues using Siglec-11-specific primers (Fig. 1). Nucleotide sequence analysis of the clone EST46736 revealed that it is an incomplete splicing product, and evaluation of the clone EST32579 indicated that this clone has been displaced by an adjacent clone (EST32578). Because both EST clones are of embryonic origin and the mRNA is most abundant in liver among adult tissues tested (Fig. 1), we isolated the full-length coding region of the cDNA by PCR from a human fetal liver cDNA library and obtained the sequences of untranslated regions by 3′- and 5′-RACE.

The complete cDNA encodes a Siglec-like molecule (686 amino acids) with five extracellular Ig-like domains, a single-pass transmembrane domain, and cytosolic tail (Fig. 2*A*). The amino acid sequence of the translated product contains almost all of the defining structural features of Siglecs. These include conserved amino acids (an arginine residue and an aromatic amino acid near the N terminus) whose side chains were shown to interact with sialic acid in Siglec-1/sialoadhesin (38), as well as three conserved cysteine residues in the first and second Ig-like domains (Fig. 2*A*). Notably, an aromatic amino acid on the β-strand G, conserved among all other Siglecs, was replaced by a histidine (His^{129}) in this molecule. This new molecule shows high sequence similarity with CD33/Siglec-3-related Siglecs (3, 4), especially with Siglec-10 (5, 39), but only in its extracellular domain (Fig. 2*B*). The cytosolic tail contains three tyrosine residues, two of which conform to the two tyrosine-based putative signaling motifs typically found in the cytosolic tails of CD33/Siglec-3-related Siglecs (3, 4). In contrast to the extracellular domain, the cytosolic tail was more closely related to that of Siglec-5 (Fig. 2*B*).

Ligand Binding Specificity of Siglec-11—Unlike the case with most other Siglecs, COS-7 cells transiently transfected with a full-length Siglec-11 expression construct failed to show sialic acid-dependent binding of human erythrocytes (data not shown). This was not due to lack of expression at the cell surface because staining of transfected COS cells with anti-Siglec-11 mAb 4C4 (see below) revealed bright specific surface labeling (data not shown). This could mean either that this molecule does not recognize sialic acids or that the preferred ligand structure is not present on human erythrocytes. A recombinant fusion protein Siglec-11-Fc was then prepared and used in an enzyme-linked immunosorbent assay, with various biotinylated polyacrylamide probes carrying multiple copies of sialylated oligosaccharides (6, 13, 15, 21, 32). As shown in Fig. 3*A*, Siglec-11 binds weakly but specifically to α2–8-linked sialic acids, with the preference toward shorter oligomers. The maximal binding signal observed was 3–40-fold weaker compared with those observed using other Siglecs and their optimal ligands in parallel analyses (data not shown). This apparent strict linkage specificity of Siglec-11 toward α2–8-linked sialic acid is unusual, although some other Siglecs recognize α2–8-linked sialic acid, as well as α2–3- and/or α2–6-linked sialic acids (15, 22, 23). Mild periodate treatment of $(Neu5Ac)_2$-PAA-Bio probe did not negatively affect the binding but rather slightly increased the binding to Siglec-11 (by ~30%; data not shown). This is also an unusual property among Siglecs, which in all other cases reported so far except for Siglec-6 (*i.e.* Siglecs-1–5, -7, and -9) actually require the glycerol-like side chain of sialic acid for optimal recognition (11, 15, 21, 32, 40).

All of the functional Siglecs studied to date have a conserved arginine residue on the β-strand F in the V set domain that is required for optimal recognition of sialic acids. Site-directed mutagenesis of this residue to alanine was previously shown to abolish or greatly reduce the ability to recognize sialic acids by mouse sialoadhesin/Siglec-1 (41), mouse CD22/Siglec-2 (42), human CD33/Siglec-3 (24), mouse myelin-associated glycoprotein/Siglec-4 (43), and mouse Siglec-F (44). Mutation of this residue to lysine in mouse sialoadhesin/Siglec-1 (41), mouse CD22/Siglec-2 (42), human Siglec-7 (32), and human Siglec-9 (21) also greatly reduced or abolished binding. Furthermore, a naturally occurring mutation of this residue to cysteine in human Siglec-L1 also greatly diminished binding (6). Unexpectedly, point mutations of the corresponding arginine residue in Siglec-11 (R120K and R120A) only reduced but did not abolish this binding, suggesting that this arginine residue is not absolutely essential for sialic acid recognition by this molecule (Fig. 3*B*). This difference from other Siglecs could be related to the linkage specificity of Siglec-11 toward α2–8-linked sialic acids.

Expression Pattern of Siglec-11—RT-PCR analysis using Siglec-11-specific primers with a panel of human cDNAs showed that Siglec-11 transcripts were readily detectable in brain, placenta, lung, liver, and pancreas but could not be detected in heart, skeletal muscle, or kidney (Fig. 1). A monoclonal antibody 4C4 against Siglec-11 was prepared as described under "Experimental Procedures" and was used for the analysis of Siglec-11 protein expression in human tissues. Flow cytometry analysis of peripheral blood leukocytes failed to reveal any cell population positive for Siglec-11 (data not shown). This is in contrast to most other CD33/Siglec-3-related Siglecs, which are predominantly expressed by leukocyte subsets (3, 4).

Immunohistochemical analysis of human tissue sections revealed low but distinct expression of Siglec-11 in Kupffer cells in liver, intestinal lamina propria macrophages, brain microglia, and perifollicular cells in spleen (Fig. 4, *A–D*). The staining pattern observed with 4C4 in these tissues was generally similar to that with anti-CD68 antibody (a marker for tissue macrophages; Fig. 4, *F–I*). However, in spleen the 4C4 antibody

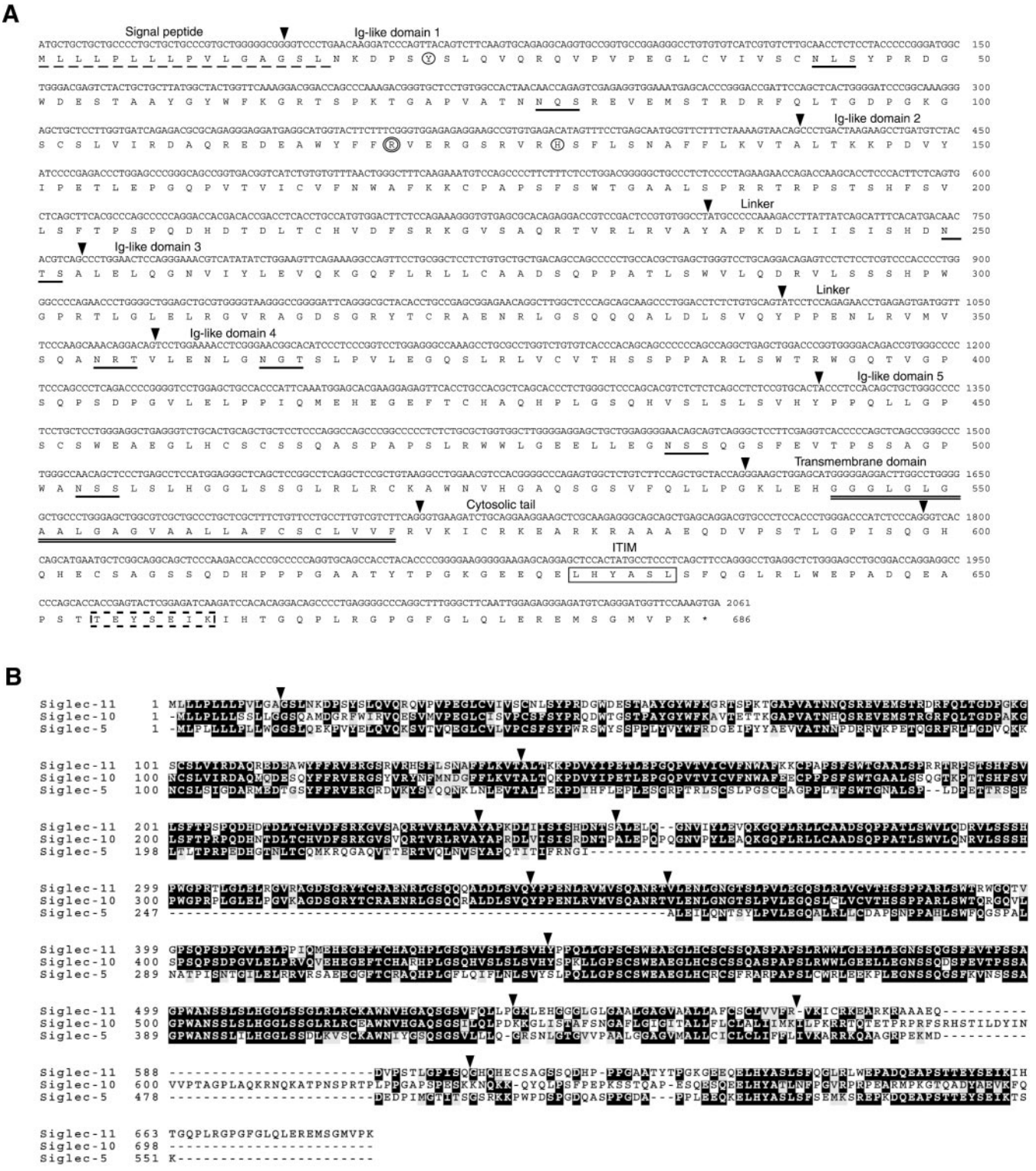

FIG. 2. **Primary sequence of Siglec-11.** *A*, cDNA and amino acid sequences of Siglec-11. *Double circle*, "essential" arginine in typical Siglec V set domains; *circle*, aromatic amino acid residues typical of Siglec V set domains; underlined with hatched and double lines, signal peptide and transmembrane domain, respectively; arrowheads, exon junctions; underlined, potential *N*-glycosylation sites; boxes with solid and hatched lines, putative immunoreceptor tyrosine-based inhibitory motif and another tyrosine-based motif conserved among Siglecs, respectively. *B*, sequence alignment of Siglec-11 with Siglecs-5 and -10. Domain borders (exon junctions) are shown with *arrowheads*.

stained cells closer to germinal center (cells in corona and marginal zone; Fig. 4*D*), whereas anti-CD68 antibody marked cells outside the follicles and in the red pulp (Fig. 4*I*). This observation suggests that whereas Siglec-11 is mainly found on tissue macrophages, its expression does not completely overlap with that of CD68. In addition to this, some infiltrating mononuclear leukocytes in chronically inflamed tissues were intensely stained with 4C4 antibody (Fig. 4*E*), suggesting that Siglec-11 expression is up-regulated in these cells in chronic inflammatory conditions. This staining also did not overlap with that seen with anti-CD68 (Fig. 4*J*). The precise nature of these infiltrating cells is yet to be determined.

To further analyze the nature of the cells expressing Siglec-11 in human tissues, sections of tonsil (Fig. 5, *A–D*) and

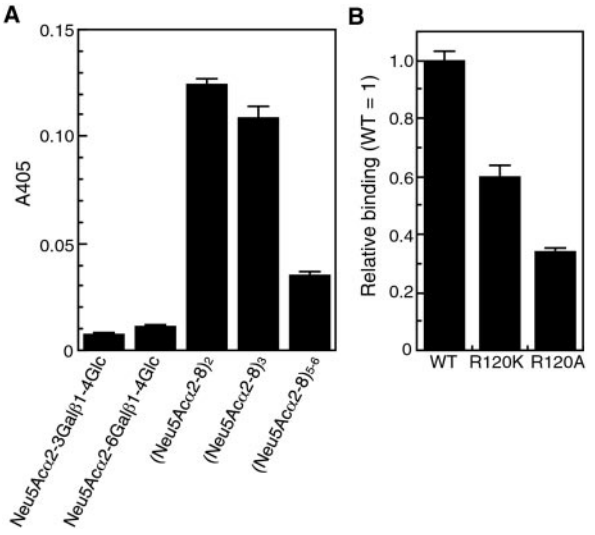

FIG. 3. **Ligand binding of Siglec-11.** *A*, sialic acid linkage specificity of Siglec-11. The Siglec-11-Fc recombinant fusion protein was immobilized on the wells of 96-well plate and binding of PAA-Bio probes (biotinylated polyacrylamide array carrying multiple sialylated oligosaccharides) were analyzed as described under "Experimental Procedures." The mean read outs at 405 nm (*A405*) of triplicate wells for each ligand were plotted. The *error bars* indicate the standard deviations. Note that the α2–8-linked Neu5Ac oligomers were conjugated to polyacrylamide backbone via Neu5Ac aminoalditol as a spacer, which is excluded from the number of Neu5Ac residues listed. *B*, effect of point mutation of the conserved arginine, Arg^{120}. Siglec-11-Fc (wild type (*WT*), R120K, or R120A) was immobilized on the wells of a 96-well plate and probed with $(Neu5Ac)_2$-PAA-Bio as above.

appendix (Fig. 5, *E–H*) were stained with antibodies against Siglec-11, CD68, CD23 (mature B-cells, mantle zone B-cells, and follicular dendritic cells), and CD79a (immature and mature B-cells). In the tonsil, Siglec-11 antibody stained a subset of the cells in the mantle zone and in between the follicles (Fig. 5*A*). In contrast, CD68 was expressed within tingible body macrophages in the follicles and in macrophages outside the follicles (Fig. 5*B*), and CD23 was expressed on a subset of mantle zone B-cells and follicular dendritic cells (Fig. 5*C*). The expression patterns of the CD45RO and CD8 were also different from that of Siglec-11 (data not shown). In the appendix, the Siglec-11 antibody stained lamina propria cells and some cells in the lymphoid nodule (Fig. 5*E*), whereas CD68 and CD23 were expressed on lamina propria macrophages (Fig. 5*F*) and the B-cells in the lymphoid nodule (Fig. 5*G*), respectively. The expression pattern of CD8 was again different from that of Siglec-11 (data not shown). In summary, the expression of Siglec-11 did not completely overlap with any of the single markers used in the analysis but rather resembled those of CD68 (in many tissues) and CD23 (in lymphoid tissues) combined.

Tyrosine Phosphorylation of Siglec-11 and Interaction with SHP-1/SHP-2—Others have shown that some CD33/Siglec-3-related Siglecs can be tyrosine-phosphorylated following treatment with pervanadate and then associate with the protein-tyrosine phosphatases SHP-1 and SHP-2 (7, 24–30), suggesting their roles in intracellular signaling. To analyze whether Siglec-11 shares this property, we studied tyrosine phosphorylation and association with SHP-1 and SHP-2 in stably transfected RAW 264.7 mouse macrophage-like cells. Siglec-11 could be immunoprecipitated as a ~100-kDa band under reducing conditions (Fig. 6) and at ~180-kDa under nonreducing conditions (not shown), suggesting that it exists naturally in the membrane as a disulfide-linked dimer. With untreated RAW cells, no Siglec-11-associated tyrosine phosphorylation could be detected. However, following treatment with sodium pervanadate, a potent inhibitor of tyrosine phosphatases, tyrosine phosphorylation was clearly evident, and this was accompanied by co-immunoprecipitation of both SHP-1 and SHP-2 (Fig. 6). Interestingly, low levels of SHP-2 could also be seen in immunoprecipitates from non-pervanadate-treated cells, under conditions where phosphorylation of Siglec-11 was undetectable (Fig. 6). No specific bands were observed in RAW cells transfected with empty vector and treated identically.

Gene Structure of Siglec-11—As mentioned above, the Siglec-11 gene was identified on the bacterial artificial chromosome clone CTC-326K19, which originated from human chromosome 19. This bacterial artificial chromosome clone was localized on cytological band 19q13.3–13.4 (45), but it does not fall within the CD33/Siglec-3-related gene cluster on the cytological band 19q13.4 (44). Rather, it is located about 1 Mb upstream of the cluster (according to the bacterial artificial chromosome tile mapping by the Lawrence Livermore National Laboratory; greengenes.llnl.gov/genome-bin/loadmap?region=mq). The gene structure of Siglec-11 was determined by sequence alignment of the cDNA and genomic DNA sequences, following conventional rules for splice site prediction (Fig. 7).

Interestingly, the human genome contains a 3.1-kb DNA segment that shows ~97% overall identity (~96% in the exon coding regions and ~98% in the noncoding regions) to a part of the Siglec-11 gene located immediately (~8 kb) upstream of the Siglec-11 gene (Fig. 7). This segment contains potential coding sequences for the first eight exons, encoding the extracellular domain (signal peptide, five Ig-like domains, and linker peptides) and the introns in between, as well as the 5′-UTR (~0.3 kb). Even if this DNA segment is transcribed into RNA and properly processed, it most probably represents a pseudogene, because the second exon (encoding the first Ig-like domain) contains a four-nucleotide deletion, causing a reading frameshift. Notably, the Siglec-10 gene locus also contains a segment which shows 89% identity to this Siglec-11/Siglec-11-like pseudogene segment over 2.8 kb. As with Siglec-11, this segment encompasses the first eight exons encoding the extracellular domain (signal peptide, five Ig-like domains, and linker peptides) as well as the corresponding introns of Siglec-10 gene. This observation strongly suggests that the DNA segment encoding extracellular domains of Siglec-10, Siglec-11, and the Siglec-11-like pseudogene (named Siglec-P16; the theoretical cDNA for this pseudogene is provided in the Supplementary Material) are derived from a common ancestor by a series of segmental duplication (46) events.

Immediately downstream of the highly conserved segment of Siglec-P16, there are three exon-like DNA segments that show distinct similarity to some other Siglec-like pseudogenes, such as Siglec-P3 and Siglec-P14 (GenBank™ accession number AF150143 and Supplementary Materials, respectively; see also Ref. 6 and its supplement for the nomenclature and partial sequences of Siglec-like pseudogenes). Of particular interest is Siglec-P14, which is a processed (intron-less) pseudogene on chromosome 1. Siglec-P14 shows high overall sequence identity with Siglec-P16 including the putative exon (fossils) for transmembrane and cytosolic domains, although it lacks the segment corresponding to exons 5–8 (encoding the third, fourth, and fifth Ig-like domains and the linker peptide between the third and fourth Ig-like domains). The presence of this processed pseudogene, which is likely to have derived from an mRNA of ancestral Siglec-P16 via reverse-transcription and chromosomal integration (47), indicates that the ancestral Siglec-P16 was once actively transcribed. It also suggests that it was transcribed in germ-line cells (egg and/or sperm precursor), because a reverse-transcription of an mRNA and chromo-

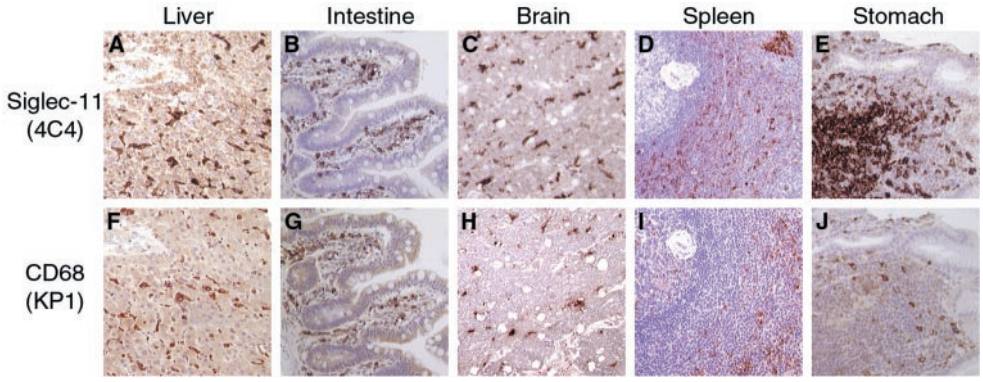

FIG. 4. **Immunohistochemical analysis of Siglec-11 expression in human tissues.** Staining of human tissue sections with 4C4 anti-Siglec-11 monoclonal antibody was performed as described under "Experimental Procedures." Examples of positive staining (*brown*) with 4C4 antibody in normal tissues are shown, including Kupffer cells in liver (*A*), lamina propria macrophages in intestine (*B*), microglia cells in brain (*C*), and perifollicular cells in spleen (*D*). In inflammatory tissues, the infiltrating cells were stained intensely (*E*, stomach). Adjacent sections stained with anti-CD68 antibody are shown for comparison (*F–I*). The controls with secondary reagents alone showed no brown staining (data not shown). All of the pictures are at 200× magnification.

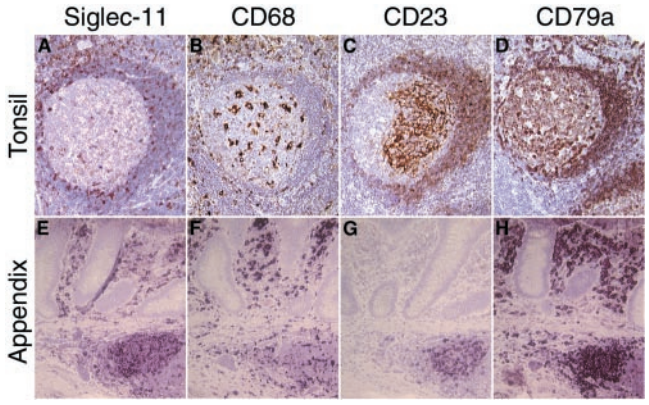

FIG. 5. **Immunohistochemical analysis of Siglec-11 expression in tonsil and appendix.** Serial sections of tonsil (*A–D*) and appendix (*E–H*) were probed with antibodies against Siglec-11 (*A* and *E*), CD68 (*B* and *F*), CD23 (*C* and *G*), or CD79a (*D* and *H*). The *tonsil panel* demonstrates a tonsillar follicle, and the *appendix panel* shows mucosal epithelium with goblet cells and lamina propria with a lymphoid nodule (*bottom right corner*). *Brown* indicates positive staining with the antibody. All of the pictures are at 200× magnification.

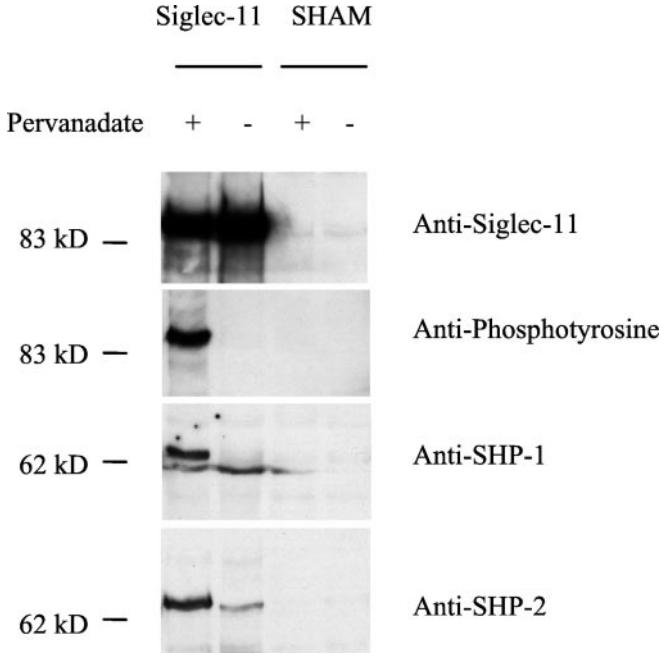

FIG. 6. **Tyrosine phosphorylation and association of Siglec-11 with SHP-1 and SHP-2.** Siglec-11- or sham-transfected RAW cells were left untreated or treated with pervanadate, and the cell lysates were immunoprecipitated with anti-Siglec-11 mAb. Western blots of reduced immunoprecipitates were probed with the indicated antibodies. Siglec-11 is tyrosine-phosphorylated following pervanadate treatment, and this leads to recruitment of SHP-1 and SHP-2. A small amount of SHP-2 is constitutively associated with Siglec-11 in the absence of phosphorylation. The *lower band* in the anti-SHP-1 tracks is nonspecific, because the degree to which this band appeared varied in different experiments.

somal integration would not leave a trace (processed pseudogene) unless it happened in a germ-line cell.

We also compared the human genomic DNA region containing the Siglec-11 gene with the syntenic region in mouse genome (Fig. 7). In the human genome, Siglec-11 and Siglec-P16 are flanked by the genes for vaccinia-related kinase 3 and activating transcription factor 5. On the other hand, the mouse syntenic region (on chromosome 7) flanked by vaccinia-related kinase 3 and activating transcription factor 5 lacks a Siglec-11-like gene and only contains a relic of a Siglec-P16-like pseudogene. This fact strongly suggests that the ancestral Siglec-P16 was present in the common ancestor of primates and rodents.

DISCUSSION

Here we have described the molecular cloning and characterization of a novel human Siglec, Siglec-11. Our analysis of human genome data bases suggests that this may be the last canonical human Siglec to be reported. Siglec-11 shows several unique properties in terms of its ligand binding. First, it shows binding specificity toward α2–8-linked sialic acids. Second, the arginine residue conserved among all Siglecs is not absolutely essential for ligand recognition by Siglec-11. Third, it does not require the glycerol-like side chain of sialic acids for efficient recognition. All of these facts point to the possibility that Siglec-11 recognizes different aspects of ligand(s) than other Siglecs. One possibility is that it has two binding sites for each sialic acid in the α2–8-linked sialic acid dimer. However, introduction of point mutations at two potential candidates of such "second contact site," Arg^{65} and His^{129}, did not alter ligand binding specificity appreciably.[3] Identification of the amino acid residue(s) responsible for the unique binding specificity of Siglec-11 may require block swapping experiments with Siglec-10, as was recently demonstrated between Siglec-7 and -9 (23).

Despite repeated attempts to demonstrate interaction be-

[3] T. Angata and A. Varki, unpublished results.

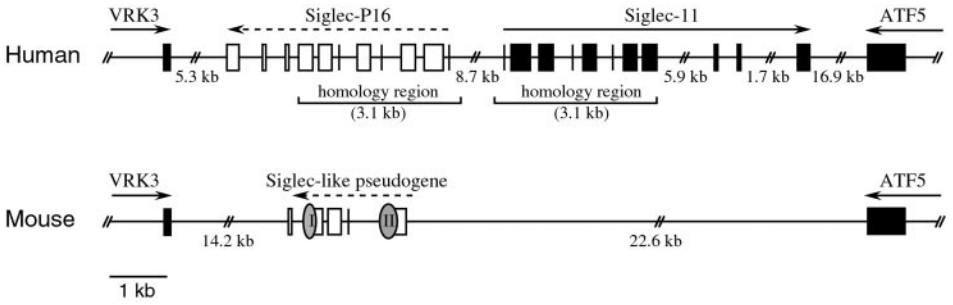

FIG. 7. **Gene structure of Siglec-11 and comparison with the mouse syntenic region.** Exons of functional genes (human Siglec-11, human and mouse VRK3 (vaccinia-related kinase 3), and human and mouse ATF5 (activating transcription factor 5)) and pseudogenes (human Siglec-P16 and mouse counterpart) are represented by *filled* and *open boxes*, respectively. The untranslated regions were not included in the box representation. Only the exons containing termination codons are shown for VRK3 and ATF5 genes. *Gray circles* on the mouse genome represent repetitive elements interrupting exon fossils.

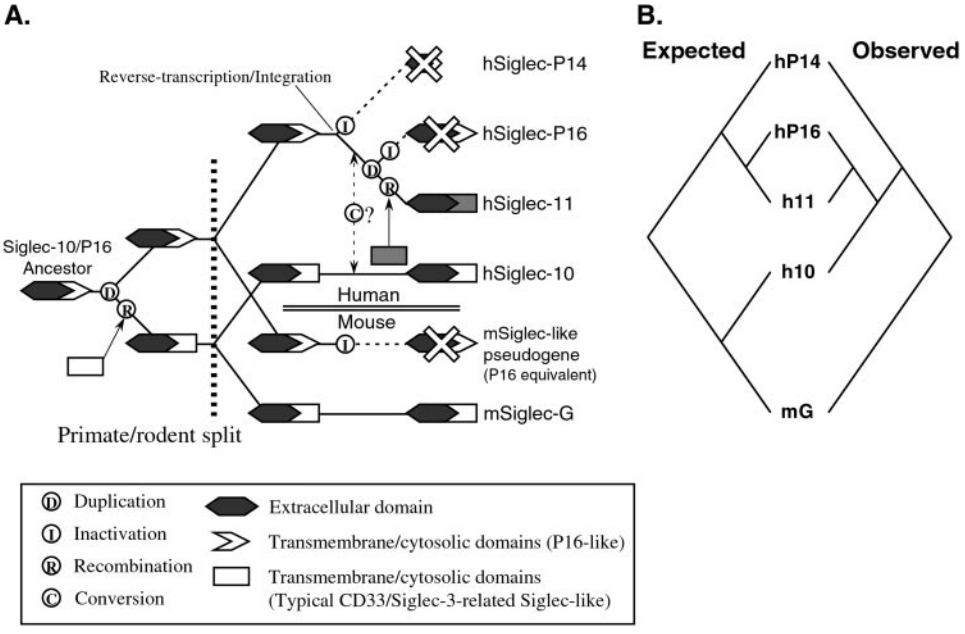

FIG. 8. **Proposed evolutionary relationships of genes and pseudogenes related to Siglec-11.** See text for discussion. *A*, proposed scenario for the evolution of Siglecs closely related to Siglec-11 involving duplications, inactivations, recombinations among modular units represented by groups of exons, as well as a possible gene conversion. Processed (pseudo)genes such as hSiglec-P14 are usually inactive at the moment of generation ("dead on arrival"), because the process of reverse transcription is error-prone and/or because of the lack of appropriate transcriptional regulatory elements. *B*, comparison of phylogenetic trees (cladograms) of the extracellular domain-coding sequences, as predicted from the scenario in *A* or as constructed using sequences of the first three exons (or equivalent) of human Siglec-10, -11, -P14, and -P16 and mouse Siglec-G (putative Siglec-10 ortholog). The mouse equivalent of human Siglec-P16 was omitted from this analysis, because of the absence of the first three exons (see Fig. 7). The "observed" tree was constructed from a DNA distance matrix using the neighbor-joining method (63). Human Siglec-5 was used as an outgroup to define the root.

tween Siglec-11 and gangliosides carrying α2–8-linked sialic acids, we could not observe clear binding signals with the α2–8-linked sialic acid-bearing gangliosides tested (GD3, GD1b, and GT1b).[3] This may be because these gangliosides are not preferred ligands for Siglec-11. Alternative candidates for the natural ligands of Siglec-11 are oligo/polysialic acids found on glycoproteins. Recent studies by Kitajima and co-workers (48–50) have revealed that glycoproteins modified with α2–8-linked sialic acid oligomers (oligosialic acids) are widely distributed in mammalian tissues and not just limited to the neural cell adhesion molecule. Such oligosialylated glycoproteins include adipo Q (49), integrin α_5 subunit (50), and numerous unidentified glycoproteins in brain (48).

Although it is beyond the scope of this paper, the native functions of Siglec-11 and the roles of sialic acid interaction in such functions are obviously of great interest. The unique expression pattern of Siglec-11 on tissue macrophages and infiltrating mononuclear leukocytes in inflammatory tissues, and its ability to interact with both SHP-1 and SHP-2 protein-tyrosine phosphatases, suggest that this molecule might be involved in the regulation of innate immune responses against foreign antigens through signal transduction. Because the lack of mouse ortholog prevents us from analyzing natural functions of Siglec-11 *in vivo* by gene disruption approach, we need a model system employing human cells to explore this hypothesis.

By analogy to other Siglecs, such as CD33/Siglec-3 (24–26), Siglec-L1/S2V (7), Siglec-10 (28), and mouse Siglec-E/MIS (29, 30), the Siglec-11 interaction with SHP-1 and SHP-2 is likely to be predominantly mediated by the phosphotyrosine (Tyr^{632}) in the membrane-proximal tyrosine-based motif that conforms to the canonical immunoreceptor tyrosine-based inhibitory motif. If this is the case, what is the ligand for the second (membrane-distal) tyrosine-based motif, which is also well conserved among CD33/Siglec-3-related Siglecs? This question in fact applies to all CD33/Siglec-3-related Siglecs and awaits an answer.

Our analysis of the human genomic region containing Siglec-11 gene and comparison with the syntenic region in mouse

genome revealed interesting clues regarding the evolutionary history of Siglec-11 (Fig. 8*A*). First, the pseudogene Siglec-P16, which shows extremely high nucleotide sequence identity with Siglec-11, appears to have a pseudogene equivalent in mouse genome, as defined by its syntenic location and the orientation (Fig. 7). On the other hand, there is apparently no equivalent of Siglec-11 itself in mouse genome. Thus, Siglec-P16 is very likely to be ancestral to Siglec-11. Siglec-P16 was also apparently actively transcribed in the past, judging from the presence of a processed pseudogene (Siglec-P14) elsewhere in the human genome that appears to have arisen from it.

Second, the sequence identity between Siglec-P16 and Siglec-11 ceases abruptly between the fifth Ig-like domain and the transmembrane domain. The remaining transmembrane-cytosolic domains of Siglec-11 show higher similarity to those of Siglec-5. This suggests that Siglec-11 is a chimeric molecule that arose from relatively recent gene duplication and recombination events, whereby the extracellular domain duplicated from the functional ancestor of Siglec-P16 became recombined with the transmembrane-cytosolic domain-coding region of another ancestral Siglec (Fig. 8*A*). Because the nucleotide sequence identity between the transmembrane-cytosolic domains of Siglec-11 and Siglec-5 is not very high (62%), this segment is not likely to have originated directly from Siglec-5 but rather from another ancestral Siglec. Nevertheless, Siglec-11 serves as a clear example indicating that Siglec genes are modular in origin, appearing to have undergone partial and/or full gene duplications and uneven genetic recombinations or deletions to reach their current organizational status.

Third, judging from the extensive sequence identity between Siglec-11 and Siglec-P16, this partial gene duplication probably happened relatively recently. Our preliminary analysis using PCR[3] suggests that the similar gene configuration (head-to-head repeat of Siglec-11 and the Siglec-11-like (pseudo)gene P16) is conserved in the genomes of all other great apes (chimpanzee, bonobo, gorilla, and orangutan). If this is confirmed by further work, this gene duplication must have happened more than 13 million years ago, when the orangutan lineage diverged from the African great ape lineage (which lead to the rest of the great apes, including humans) (51, 52). On the other hand, the ~97% identity of the P16 pseudogene to the Siglec-11 extracellular domain is in the general range of genomic DNA sequence differences typically found between humans and orangutans (53–55). Thus, the appearance of Siglec-11 might not have occurred much earlier than in the orangutan–human common ancestor. Also intriguing is the fact that the coding regions of Siglec-11 seemed to have diverged more rapidly (~96% identity) than that of the noncoding regions (~98% identity). This suggests that this gene has been under positive selection pressure during hominid evolution (56). Determination of the precise timing of this event would of course require genomic DNA analysis of other primates and possibly that of other mammalian groups. It is also formally possible that the duplication of Siglec-11 and -P16 is more ancient but that these two copies were maintained so unusually similar to each other by concerted evolution, as has been suggested for α-globin genes (57). This possibility must also be examined by careful genomic DNA sequence analysis of primate Siglec-11/Siglec-P16 loci.

From the topology of the Siglec phylogenetic tree (see Fig. 8 of Ref. 44), it is likely that Siglec-10/Siglec-11 clade (including Siglec-P16) diverged earlier from the rest of CD33/Siglec-3-related group of Siglecs. In other words, it is likely that a Siglec-10/Siglec-11-like gene was ancestral to all other CD33/Siglec-3-related Siglecs. In this regard, it is interesting to ask whether the Siglec-P16 is ancestral to Siglec-10 (and ultimately to all CD33/Siglec-3-related Siglecs in the gene cluster) or whether it is derived from a Siglec-10 ancestor. The Siglec-P16 pseudogene in human genome is very compact and appears to be devoid of repetitive elements in the coding region, which would limit the likelihood of DNA recombination between the coding regions for the extracellular domain (highly conserved among Siglec-10, -11, and -P16) and the transmembrane-intracellular domain (not conserved). Thus, it is tempting to suggest that Siglec-P16 is also ancestral to Siglec-10. If the scenario presented above (Fig. 8*A*) is correct, the phylogenetic tree of the extracellular domain-coding sequences of human Siglec-10, -11, -P14, and -P16 and mouse Siglec-G (putative mouse ortholog of human Siglec-10) is expected to appear as shown in Fig. 8*B*. However, the phylogenetic tree constructed from the first three exons (or equivalent) of these genes and pseudogenes (Fig. 8*B*) is not consistent with this scenario. This incongruity is likely to be due to a gene conversion between Siglec-10 and Siglec-P16 ancestors, which has presumably taken place sometime after the generation of Siglec-P14 but before the generation of Siglec-11. To answer this question, we would have to wait until genomic DNA sequences of the CD33/Siglec-3-related gene cluster and Siglec-P16/Siglec-11 segment from other primates and other mammals become available.

Siglec-11 and Siglec-P16 stand as clear evidence that the CD33/Siglec-3-related Siglec genes are rapidly evolving and fit Ohno's model of evolution by gene duplication and the "birth and death of genes" (58, 59). However, unlike other rapidly evolving gene families like the olfactory receptors genes (60, 61) and the killer cell inhibitory receptor genes (62), the CD33/Siglec-3-related genes are not all confined to a single cell type. Thus, the evolution of these genes must have also involved the recruitment of novel regulatory sequences to each new gene after it emerged. Another fascinating question relates to what might have been the selection pressures favoring the rapid evolution of these Siglecs. This question is of course intimately tied to understanding the primary functions of the CD33/Siglec-3-related Siglecs in the various cell types that they are expressed in.

Acknowledgment—Part of the mouse genomic data used for this paper was generated through the use of the Celera Discovery System under a Client agreement with the University of California System.

REFERENCES

1. Crocker, P. R., Clark, E. A., Filbin, M., Gordon, S., Jones, Y., Kehrl, J. H., Kelm, S., Le Douarin, N., Powell, L., Roder, J., Schnaar, R. L., Sgroi, D. C., Stamenkovic, K., Schauer, R., Schachner, M., Van den Berg, T. K., Van der Merwe, P. A., Watt, S. M., and Varki, A. (1998) *Glycobiology* **8**, v
2. Kelm, S., and Schauer, R. (1997) *Int. Rev. Cytol.* **175,** 137–240
3. Crocker, P. R., and Varki, A. (2001) *Trends Immunol.* **22,** 337–342
4. Crocker, P. R., and Varki, A. (2001) *Immunology* **103,** 137–145
5. Munday, J., Kerr, S., Ni, J., Cornish, A. L., Zhang, J. Q., Nicoll, G., Floyd, H., Mattei, M. G., Moore, P., Liu, D., and Crocker, P. R. (2001) *Biochem. J.* **355,** 489–497
6. Angata, T., Varki, N. M., and Varki, A. (2001) *J. Biol. Chem.* **276,** 40282–40287
7. Yu, Z., Lai, C. M., Maoui, M., Banville, D., and Shen, S. H. (2001) *J. Biol. Chem.* **276,** 23816–23824
8. Powell, L. D., Sgroi, D., Sjoberg, E. R., Stamenkovic, I., and Varki, A. (1993) *J. Biol. Chem.* **268,** 7019–7027
9. Powell, L. D., and Varki, A. (1994) *J. Biol. Chem.* **269,** 10628–10636
10. Powell, L. D., Jain, R. K., Matta, K. L., Sabesan, S., and Varki, A. (1995) *J. Biol. Chem.* **270,** 7523–7532
11. Collins, B. E., Kiso, M., Hasegawa, A., Tropak, M. B., Roder, J. C., Crocker, P. R., and Schnaar, R. L. (1997) *J. Biol. Chem.* **272,** 16889–16895
12. Schnaar, R. L., Collins, B. E., Wright, L. P., Kiso, M., Tropak, M. B., Roder, J. C., and Crocker, P. R. (1998) *Ann. N. Y. Acad. Sci.* **845,** 92–105
13. Patel, N., Brinkman-Van der Linden, E. C. M., Altmann, S. W., Gish, K., Balasubramanian, S., Timans, J. C., Peterson, D., Bell, M. P., Bazan, J. F., Varki, A., and Kastelein, R. A. (1999) *J. Biol. Chem.* **274,** 22729–22738
14. Crocker, P. R., Kelm, S., Dubois, C., Martin, B., McWilliam, A. S., Shotton, D. M., Paulson, J. C., and Gordon, S. (1991) *EMBO J.* **10,** 1661–1669
15. Brinkman-Van der Linden, E. C. M., and Varki, A. (2000) *J. Biol. Chem.* **275,** 8625–8632
16. Freeman, S. D., Kelm, S., Barber, E. K., and Crocker, P. R. (1995) *Blood* **85,** 2005–2012
17. Cornish, A. L., Freeman, S., Forbes, G., Ni, J., Zhang, M., Cepeda, M., Gentz,

R., Augustus, M., Carter, K. C., and Crocker, P. R. (1998) *Blood* **92,** 2123–2132
18. Nicoll, G., Ni, J., Liu, D., Klenerman, P., Munday, J., Dubock, S., Mattei, M. G., and Crocker, P. R. (1999) *J. Biol. Chem.* **274,** 34089–34095
19. Floyd, H., Ni, J., Cornish, A. L., Zeng, Z. Z., Liu, D., Carter, K. C., Steel, J., and Crocker, P. R. (2000) *J. Biol. Chem.* **275,** 861–866
20. Zhang, J. Q., Nicoll, G., Jones, C., and Crocker, P. R. (2000) *J. Biol. Chem.* **275,** 22121–22126
21. Angata, T., and Varki, A. (2000) *J. Biol. Chem.* **275,** 22127–22135
22. Ito, A., Handa, K., Withers, D. A., Satoh, M., and Hakomori, S. (2001) *FEBS Lett.* **498,** 116–120
23. Yamaji, T., Teranishi, T., Alphey, M. S., Crocker, P. R., and Hashimoto, Y. (2002) *J. Biol. Chem.* **277,** 6324–6332
24. Taylor, V. C., Buckley, C. D., Douglas, M., Cody, A. J., Simmons, D. L., and Freeman, S. D. (1999) *J. Biol. Chem.* **274,** 11505–11512
25. Ulyanova, T., Blasioli, J., Woodford-Thomas, T. A., and Thomas, M. L. (1999) *Eur. J. Immunol.* **29,** 3440–3449
26. Paul, S. P., Taylor, L. S., Stansbury, E. K., and McVicar, D. W. (2000) *Blood* **96,** 483–490
27. Falco, M., Biassoni, R., Bottino, C., Vitale, M., Sivori, S., Augugliaro, R., Moretta, L., and Moretta, A. (1999) *J. Exp. Med.* **190,** 793–801
28. Whitney, G., Wang, S. L., Chang, H., Cheng, K. Y., Lu, P., Zhou, X. D., Yang, W. P., McKinnon, M., and Longphre, M. (2001) *Eur. J. Biochem.* **268,** 6083–6096
29. Ulyanova, T., Shah, D. D., and Thomas, M. L. (2001) *J. Biol. Chem.* **276,** 14451–14458
30. Yu, Z. B., Maoui, M., Wu, L. T., Banville, D., and Shen, S. H. (2001) *Biochem. J.* **353,** 483–492
31. Vitale, C., Romagnani, C., Falco, M., Ponte, M., Vitale, M., Moretta, A., Bacigalupo, A., Moretta, L., and Mingari, M. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 15091–15096
32. Angata, T., and Varki, A. (2000) *Glycobiology* **10,** 431–438
33. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 139–243, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Kantor, A. B., and Roederer, M. (1997) in *Weir's Handbook of Experimental Immunology* (Herzenberg, L. A., Weir, D. M., Herzenberg, L. A., and Blackwell, eds) Vol. II, pp. 49.1–49.13, Blackwell Science, Inc., Malden, MA
35. Gown, A. M., Wever, N., and Battifora, H. (1993) *Appl. Immunohistochem.* **1,** 256–266
36. Taylor, C. R., Shi, S.-R., Chaiwun, B., Young, L., Imam, S. A., and Cote, R. J. (1994) *Hum. Pathol.* **25,** 263–270
37. Gough, P. J., Gordon, S., and Greaves, D. R. (2001) *Immunology* **103,** 351–361
38. May, A. P., Robinson, R. C., Vinson, M., Crocker, P. R., and Jones, E. Y. (1998) *Mol. Cell* **1,** 719–728
39. Li, N., Zhang, W. P., Wan, T., Zhang, J., Chen, T. Y., Yu, Y. Z., Wang, J. L., and Cao, X. T. (2001) *J. Biol. Chem.* **276,** 28106–28112
40. Collins, B. E., Yang, L. J. S., Mukhopadhyay, G., Filbin, M. T., Kiso, M., Hasegawa, A., and Schnaar, R. L. (1997) *J. Biol. Chem.* **272,** 1248–1255
41. Vinson, M., Van der Merwe, P. A., Kelm, S., May, A., Jones, E. Y., and Crocker, P. R. (1996) *J. Biol. Chem.* **271,** 9267–9272
42. Van der Merwe, P. A., Crocker, P. R., Vinson, M., Barclay, A. N., Schauer, R., and Kelm, S. (1996) *J. Biol. Chem.* **271,** 9273–9280
43. Tang, S., Shen, Y. J., DeBellard, M. E., Mukhopadhyay, G., Salzer, J. L., Crocker, P. R., and Filbin, M. T. (1997) *J. Cell Biol.* **138,** 1355–1366
44. Angata, T., Hingorani, R., Varki, N. M., and Varki, A. (2001) *J. Biol. Chem.* **276,** 45128–45136
45. Dehal, P., Predki, P., Olsen, A. S., Kobayashi, A., Folta, P., Lucas, S., Land, M., Terry, A., Zhou, C. L. E., Rash, S., Zhang, Q., Gordon, L., Kim, J., Elkin, C., Pollard, M. J., Richardson, P., Rokhsar, D., Uberbacher, E., Hawkins, T., Branscomb, E., and Stubbs, L. (2001) *Science* **293,** 104–111
46. Lander, E. S., *et al.* (2001) *Nature* **409,** 860–921
47. Esnault, C., Maestre, J., and Heidmann, T. (2000) *Nat. Genet.* **24,** 363–367
48. Sato, C., Fukuoka, H., Ohta, K., Matsuda, T., Koshino, R., Kobayashi, K., Troy, F. A., II, and Kitajima, K. (2000) *J. Biol. Chem.* **275**, 15422–15431
49. Sato, C., Yasukawa, Z., Honda, N., Matsuda, T., and Kitajima, K. (2001) *J. Biol. Chem.* **276,** 28849–28856
50. Nadanaka, S., Sato, C., Kitajima, K., Katagiri, K., Irie, S., and Yamagata, T. (2001) *J. Biol. Chem.* **276,** 33657–33664
51. Stewart, C. B., and Disotell, T. R. (1998) *Curr. Biol.* **8,** R582–R588
52. Goodman, M., Porter, C. A., Czelusniak, J., Page, S. L., Schneider, H., Shoshani, J., Gunnell, G., and Groves, C. P. (1998) *Mol. Phylogenet. Evol.* **9,** 585–598
53. Sibley, C. G., Comstock, J. A., and Ahlquist, J. E. (1990) *J. Mol. Evol.* **30,** 202–236
54. Koop, B. F., Goodman, M., Xu, P., Chan, K., and Slightom, J. L. (1986) *Nature* **319,** 234–238
55. Chen, F. C., and Li, W. H. (2001) *Am. J. Hum. Genet.* **68,** 444–456
56. Johnson, M. E., Viggiano, L., Bailey, J. A., Abdul-Rauf, M., Goodwin, G., Rocchi, M., and Eichler, E. E. (2001) *Nature* **413,** 514–599
57. Liebhaber, S. A., Goossens, M., Kan, Y. W. (1981) *Nature* **290,** 26–29
58. Ohno, S. (1970) *Evolution by Gene Duplication*, Springer-Verlag, New York
59. Nei, M., Gu, X., and Sitnikova, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7799–7806
60. Brand-Arpon, V., Rouquier, S., Massa, H., de Jong, P. J., Ferraz, C., Ioannou, P. A., Demaille, J. G., Trask, B. J., and Giorgi, D. (1999) *Genomics* **56,** 98–110
61. Sharon, D., Glusman, G., Pilpel, Y., Khen, M., Gruetzner, F., Haaf, T., and Lancet, D. (1999) *Genomics* **61,** 24–36
62. Canavez, F., Young, N. T., Guethlein, L. A., Rajalingam, R., Khakoo, S. I., Shum, B. P., and Parham, P. (2001) *J. Immunol.* **167,** 5786–5794
63. Saitou, N., and Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425