OB-BP1/Siglec-6
A LEPTIN- AND SIALIC ACID-BINDING PROTEIN OF THE IMMUNOGLOBULIN SUPERFAMILY*

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We report the expression cloning of a novel leptin-binding protein of the immunoglobulin superfamily (OB-BP1) and a cross-hybridizing clone (OB-BP2) that is identical to a recently described sialic acid-binding I-type lectin called Siglec-5. Comparisons to other known Siglec family members (CD22, CD33, myelin-associated glycoprotein, and sialoadhesin) show that OB-BP1, OB-BP2/Siglec-5, and CD33/Siglec-3 constitute a unique related subgroup with a high level of overall amino acid identity: OB-BP1 versus Siglec-5 (59%), OB-BP1 versus CD33 (65%), and OB-BP2/Siglec-5 versus CD33 (56%). The cytoplasmic domains are not as highly conserved, but display novel motifs which are putative sites of tyrosine phosphorylation, including an immunoreceptor tyrosine kinase inhibitory motif and a motif found in SLAM and SLAM-like proteins. Human tissues showed high levels of OB-BP1 mRNA in placenta and moderate expression in spleen, peripheral blood leukocytes, and small intestine. OB-BP2/Siglec-5 mRNA was detected in peripheral blood leukocytes, lung, spleen, and placenta. A monoclonal antibody specific for OB-BP1 confirmed high expression in the cyto- and syncytiotrophoblasts of the placenta. Using this antibody on peripheral blood leukocytes showed an almost exclusive expression pattern on B cells. Recombinant forms of the extracellular domains of OB-BP1, OB-BP2/Siglec-5, and CD33/Siglec-3 were assayed for specific binding of leptin. While OB-BP1 exhibited tight binding (Kd 91 nM), the other two showed weak binding with Kd values in the 1–2 μM range. Studies with sialylated ligands indicated that OB-BP1 selectively bound Neu5Acα2–6GalNAcα (sialyl-Tn) allowing its formal designation as Siglec-6. The identification of OB-BP1/Siglec-6 as a Siglec family member, coupled with its restricted expression pattern, suggests that it may mediate cell-cell recognition events by interacting with sialylated glycoprotein ligands expressed on specific cell populations. We also propose a role for OB-BP1 in leptin physiology, as a molecular sink to regulate leptin serum levels.

Sialic acid-binding immunoglobulin superfamily member lectins (Siglecs),1 a recently designated family of cell surface molecules (1), are a subset of the I-type lectins (2), which in turn belong to the larger immunoglobulin superfamily. Siglecs share conserved cysteine residues which form two characteristic disulfide bonds: an intra-β-sheet bond within the NH2-terminal V-set immunoglobulin (Ig) domain, the other between the V-set domain and the proximal C2-set domain (3). Family members each have a single V-set NH2-terminal Ig domain followed by a variable number of C2-set Ig domains, as many as 16 for sialoadhesin (4), or as few as one for CD33 (5). While the family members are notable for structural similarities within their extracellular domains, overall primary amino acid sequence identities among them is relatively low (~30%). In contrast to the majority of immunoglobulin superfamily members which recognize protein ligands, Siglec family members have all been shown to bind to specific sialylated glycans (1–3).

Three Siglec family members appear to be tightly restricted in expression to specific populations within the hematopoietic lineage: sialoadhesin/Siglec-1 (Sn) to macrophages (6), CD33/Siglec-3 to cells of the myelomonocytic lineage (7), and CD22/Siglec-2 to B cells (8). Likewise myelin-associated glycoprotein (MAG)/Siglec-4 is only expressed on oligodendrocytes in the central nervous system and on Schwann cells in the peripheral nervous system (9, 10). For Sn and MAG, specific cell populations which bear the cognate “ligand” have been identified: Sn

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1 The abbreviations used are: Siglec, sialic acid-binding lectin of the immunoglobulin superfamily; Ig, immunoglobulin; SAP, SLAM-associated protein; SLAM, signaling lymphocyte activation molecule; Sn, sialoadhesin; MAG, myelin-associated glycoprotein; Ob-R, leptin receptor; rhOB-F, recombinant FLAG-tagged human leptin; rhOB, recombinant human leptin; rhGM-CSF, recombinant human granulocyte macrophage-colony stimulating factor; rhIL-10, recombinant human interleukin-10; PBL, peripheral blood leukocytes; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; IGFBP, insulin-like growth factor-binding protein; PBS, phosphate-buffered saline; SH2, Src homology domain 2; FACS, fluorescence-activated cell sorter.

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preferentially interacts with cells of the granulocytic lineage (11), and MAG with neuronal processes (12, 13). Additionally, for each Siglec family member, certain sialic acid ligand preferences have been determined (2, 3, 14). However, the identities of the individual glycoconjugates which carry the sialic acid determinants remain to be conclusively identified. By virtue of the restricted distribution of Sigidiscs, these lectins are thought to mediate highly specific cell-cell recognition events. Siglecs are also capable of transducing intracellular signals. For example, ligation of MAG results in activation of the Fyn tyrosine kinase (15) and contributes to the initiation and maintenance of myelination by the Schwann cells (16). Recent knockout studies of CD22 demonstrate that it is a dual modulator of signal transduction by B lymphocyte antigen receptors, acting as a negative regulator by activation of phosphatases and also a positive regulator by activation of kinases (17).

In the course of experiments intended to identify leptin-binding proteins, we cloned a novel Siglec family member, OB-BP1, from the TF-1 human erythroleukemic cell line. We initially undertook experiments to survey hematopoietic cell lines for leptin binding, based on structure prediction and fold recognition algorithms which revealed an unmistakable structural link between leptin and the diverse family of hematopoietic cytokines that have a unique four a-helical bundle fold (18, 19). We discovered OB-BP1 by FACS-based expression screening of a TF-1 cDNA library for leptin-binding cell surface molecules. Conventional cross-hybridization screening of the TF-1 cDNA library with an OB-BP1 probe led to the identification of a related molecule OB-BP2, which is identical to the recently reported Siglec-6 (20).

OB-BP1 and OB-BP2 display no similarity to the previously reported leptin receptor (Ob-R) (21, 22). The expression of each is restricted to a limited set of tissue and cell types. We further assessed putative interactions between these molecules and leptin by Biacore studies and found that only OB-BP1 bound with relatively high affinity. We also show a specific interaction of OB-BP1 with a sialic acid containing ligand, allowing its designation as Siglec-6.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**Unless otherwise indicated all cell lines were obtained from ATCC. HEL, HL60, Daudi, human EBV-transformed lymphoblastoid B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and HEL, HL60, Daudi, human EBV-transformed lymphoblastoid B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human揩eshi B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gift from Dr. Peter Parham, Stanford University), Jurkat, MOL-9, K562, ALL, and Jurkat, are human B cells (gi...
lymphoblastoid B cells, Jurkat, MOLT-4, THP-1, and U937) were analyzed for the expression of OB-BP1. Cells (1 × 10⁶) were incubated with 10F10 supernatant (1:1 in PBS, 1% bovine serum albumin) for 1 h at 4 °C, followed by incubation with 100 × diluted fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Pierce) for flow cytometry (Beckton-Dickinson FacScan). Peripheral blood mononuclear cells and neutrophils were isolated from human blood obtained from healthy volunteers using Mono-Poly Resolving Medium Ficoll-Hypaque (ICN) and analyzed for expression of OB-BP1. To analyze which subsets of peripheral blood mononuclear cells express OB-BP1, the staining with the 10F10 supernatant was followed by staining with the following subset-specific antibodies: tri-color-conjugated anti-human CD19 and CD14 (CalTag), cytochrome-conjugated anti-human CD4 and CD56, and phycoerythrin-conjugated CD8 and CD3 (Pharmingen). Cells were blocked with PBS, 1% bovine serum albumin, 5% mouse serum for 30 min before incubation with these antibodies. P3 × 63Ag8 (mouse-IgG₁, secreting myeloma; ATCC) supernatant was used as isotype control for the 10F10 supernatant.

**Analysis of the Sialic Acid Binding Properties of OB-BP1**—The extracellular domains of OB-BP1 were cloned into a FLAG-human Fc expression vector (pEIIc). COS-7 cells were transiently transfected at 60–70% confluence using LipofectAMINE Reagent (Life Technologies), in serum-free OptiMEM medium. After 5 h the medium was diluted 2 times with 10% fetal calf serum containing OptiMEM medium and the next day for the medium was changed for OptiMEM with 2% fetal calf serum. The COS-7 cell supernatants were collected 5–7 days after transfection. The fusion protein (OB-BP1/FLAG/human IgG-Fc) was purified on Protein A-Sepharose. Microwells (Nunc) were coated overnight at 4 °C with Protein A-Sepharose. Microwells (Nunc) were coated overnight at 4 °C with Protein A-Sepharose. The human erythroleukemic cell line TF-1 exhibited strong staining (Fig. 1A), whereas the mouse pro-B cell line Ba/F3 did not (Fig. 1B). The strong staining of TF-1 cells could be competed with non-FLAG-tagged rhOB-F, albeit with a significantly lower staining intensity than observed on OB-BP1-infected Ba/F3 cells (data not shown).

**Sequence Analysis**—The cDNA for OB-BP1 (1711 nucleotides) was sequenced and found to encode a type I membrane protein. An open reading frame of 441 was deduced for OB-BP1. We therefore constructed a TF-1 cDNA library in the nonredundant GenBank data base using the nucleotide sequences of either OB-BP1 or OB-BP2 revealed a striking similarity of both OB-BP1 and OB-BP2 to CD33 (5), a human leucocyte antigen of undetermined function which is expressed exclusively on cells of myelomonocytic lineage (7, 30). Following submission of our data to GenBank on 19 September 1996 (GenBank accession numbers U71382 and U71383), identical sequences appeared entitled CD33L identical to OB-BP1 (GenBank accession D86358) (27) and Siglec-5 (identical to OB-BP2) (20). The former was picked up by a group performing random cloning of cDNAs capable of protein expression in vitro (27) and the latter by a group using a commercial EST data base to specifically search for new Siglec family members (20). Data base searching with the amino acid sequences also detected similarities of the OB-BPs to other Siglec family members including MAG (31), Sn (4), and CD22 (32). At the primary sequence level, however, OB-BP1 and OB-BP2/Siglec-5 most closely resemble CD33 (overall sequence identity 63 and 56%, respectively) (see Fig. 2).

**OB-BP1 Is a Member of the SIGLEC Family**—The extracellular...
region of OB-BP1 is composed of Ig domains typical of Siglec family members (33): an NH₂-terminal V-set type followed by multiple C2-set Ig domains. OB-BP1 contains a total of 3 Ig domains while OB-BP2/Siglec-5 contains a total of 4 Ig domains. As with the other family members, the V-set domain of OB-BP1 and OB-BP2/Siglec-5 contains a pattern of conserved cysteines predicted to form two disulfide bridges, one as an intra-b-sheet disulfide bond and the other between the V-set domain and the proximal C-set Ig domain (33). Phylogenetic analysis of the entire Siglec family using Clustal W (34) and TreeView (35) revealed that OB-BP1, OB-BP2/Siglec-5, and human CD33 cluster together, with OB-BP1 being most closely related to CD33 (Fig. 3). Individual Ig domains of each molecule were compared with single Ig domains of Sn, MAG, CD33, and CD22. Domains displaying the highest degree of identity to the individual Ig domains of OB-BP1 or OB-BP2/Siglec-5 are shown in Table I. In the first two extracellular domains, OB-BP1 and OB-BP2 are generally more similar to CD33 than to each other, while in the third Ig domain they most closely resemble each other. The fourth Ig domain, present only in OB-BP2/Siglec-5, is most similar to MAG Ig domain 5.

Alignment of the intracellular domains of the Siglec family members with the OB-BPs revealed the highest degree of sequence identity and similarity to CD33, with notable conserva-
OB-BP1/Siglec-6

Fig. 4. Binding of sialylated ligands to the extracellular domain of OB-BP1. OB-BP1-Fc was immobilized via protein A on a microtiter plate at 500 ng/well. Biotinylated polyacrylamide conjugated to Neu5Acα2–6Galβ1–4Glc (6-SL and 3-SL, respectively), Neu5Acα2–6GalNAcα (sialyl-Tn), or GalNAcα (Tn) was added at the indicated concentrations and after washing binding was visualized with alkaline phosphatase-conjugated streptavidin and absorbance measured at 405 nm. Data show means ± S.D. of quadruplicates.

OB-BP1 Binds a Specific Sialylated Ligand, Allowing Its Designation as a Siglec Family Member—On the basis of sequence homology OB-BP1 meets one of the criteria to be a member of the Siglec family. Therefore we undertook further studies to examine if the key criterion, sialic acid binding, is met as well. The extracellular part of the protein was expressed as a fusion protein with the Fc part of human IgG. Solid-phase binding assays were performed with this recombinant protein to study binding to various biotinylated polyacrylamide conjugates: Neu5Acα2–6Galβ1–4Glc (sialyllactose), Neu5Acα2–6GalNAcα (sialyl-Tn), or GalNAcα (Tn). No binding was found to either sialyllactose or conjugates. In contrast, strong binding was found to the sialyl-Tn conjugate. No binding was found to the non-sialylated form, Tn, confirming the sialic acid dependence of the binding (Fig. 4). This allows the designation of OB-BP1 as a Siglec family member, Siglec-6.

The OB-binding Siglecs Are Differentially Expressed in Various Cell Types—Multiple tissue Northern blots were used to determine the size and tissue distribution of OB-BP1/Siglec-6 and OB-BP2/Siglec-5 mRNAs. Since the first three Ig domains of the two molecules contain nucleotide sequences with relatively long stretches of identity, we designed probes to minimize the likelihood of cross-reactivity under stringent hybridization and wash conditions (see “Experimental Procedures”). High expression of OB-BP1 mRNA was detected in placenta, with moderate expression in PBL (peripheral blood leukocytes), spleen, and small intestine (Fig. 5A). In PBL the largest transcript observed was approximately 1.8 kilobase pairs; therefore the 1711-base pair cDNA isolated from TF-1 cells is likely to represent a full-length species. Additional mRNAs are seen in other tissues. While the identity of the additional transcripts remains to be resolved, they are likely to be alternatively spliced mRNAs, particularly in light of the discovery of alternatively spliced forms of CD33 (42), CD22 (32), MAG (43–45), and Sn (4).

OB-BP2/Siglec-5 mRNA displays a somewhat different tissue distribution pattern than OB-BP1. The highest expression was detected in PBL, with moderate to low expression in spleen, lung, and placenta (Fig. 5B). The 2.2-kilobase pair mRNA in PBL is likely to represent the 2130-base pair full-length cDNA described above. As with OB-BP1, various other mRNA species are observed. The additional mRNAs for both molecules are more likely to represent alternatively spliced messages rather than different gene family members based on the stringency of the hybridization and wash conditions of the blots, since cross-hybridization was not observed between OB-BP1 and OB-BP2/Siglec-5 under these conditions.

A Monoclonal Antibody against OB-BP1 Detects Expression on B Cells and Placental Cells—Using OB-BP1ec as an immunogen, a specific mouse monoclonal antibody against OB-BP1 (10F10) was raised using conventional methodologies. Recombinant soluble forms of the extracellular domains of Siglec-5 and CD22 and Chinese hamster ovary cells stably expressing CD33 were used to show lack of cross-reactivity with these Siglecs (data not shown). The strong expression of OB-BP1 in placenta was confirmed using this monoclonal antibody, which indicated specific staining of the cyto- and synctiotrophoblastic cells (Fig. 6). Expression of OB-BP1 was found by flow cytometry on several cell lines of hematopoietic origin (Fig. 7A), but not on either of the T cell lines tested, Jurkat and MOLT-4. Expression in PBLs was studied by flow cytometry, indicating an almost exclusive expression pattern on B cells (Fig. 7C). On neutrophils a low level of expression of OB-BP1 was found and virtually no expression on T cells, monocytes or NK-cells (Fig. 7, B and C). Notably, this pattern of expression is distinct from that of OB-BP2/Siglec-5 which was recently reported by others to be predominantly expressed on neutrophils and monocytes (20).

OB-BP1ec Binds Leptin with a Higher Affinity than OB-BP2ec or CD33ec—Siglec family members typically bind sialic acid ligands. Since OB-BP1 was expression cloned using leptin as a target for binding, we used a second independent technique, surface plasmon resonance, to assess the ability of soluble forms of the OB-BPs to bind to leptin. When rhOB was injected over an OB-BP1ec surface in the flow cell of a sensor chip, strong binding was observed (Fig. 8A). As the ligand concentration was increased, both the association rate and the amount of protein bound increased. The association and dissociation rates, however, were relatively slow compared with the rates we had observed for leptin to the extracellular domain of Ob-R (kass = 1.55 × 106 M−1 s−1; kdis = 1.46 × 10−3 s−1 with a resulting Kd of 9.5 nM) (46) as well as rates observed for other cytokines to their receptors (47, 48). The average kass and kdis obtained for rhOB to OB-BP1ec were 3.2 × 109 M−1 s−1 and 2.9 × 10−4 s−1, respectively. The ratio of these constants yielded a Kd of 9.1 nM for the binding of leptin to OB-BP1ec. In contrast, no binding was observed when either rhGM-CSF (dotted line) or rHLL-10 (not shown) were injected over the OB-BP1ec surface at a concentration of 2 μM, suggesting that leptin binding was indeed specific. OB-BP1 was also expressed as an IgG fusion protein in mammalian cells (COS-7) and immobilized on the dextran matrix. This surface was also capable of recognizing long stretches of identity, we designed probes to mini-
and rhOB. Like OB-BP1<sub>ec</sub>, neither OB-BP2<sub>ec</sub> nor CD33<sub>ec</sub> displayed binding to rhGM-CSF (data not shown). When 2 µM rhOB was injected over the surface, both OB-BP2 and CD33 display low affinities for rhOB relative to OB-BP1 (Fig. 8B). For OB-BP2 an estimated $K_d$ of 880 nM was determined based on rate constants $k_{ass} = 205 \text{ M}^{-1} \text{s}^{-1}$ and $k_{diss} = 1.8 \times 10^{-4} \text{s}^{-1}$; binding to CD33 was weaker yet, with an apparent $K_d$ of 1.9 µM, derived from a slow $k_{ass}$ (74 M$^{-1}$ s$^{-1}$) and a $k_{diss}$ of $1.4 \times 10^{-4} \text{s}^{-1}$.

In summary, OB-BP1<sub>ec</sub> displayed specific binding only to leptin, but not to other helical cytokines, with an estimated $K_d$ value of ~50–90 nM. Both OB-BP2<sub>ec</sub> and CD33<sub>ec</sub> exhibited weak binding to leptin compared with OB-BP1 under similar conditions. In all experiments, we utilized recombinant leptin, rhOB, which was produced in E. coli and therefore not glycosylated. We conclude that the observed binding of OB-BP1 to leptin is specific for the structure of the protein and is not dependent on the glycosylation state of OB-BP1.

DISCUSSION

In the course of expression cloning of cell surface molecules which bind leptin, we have cloned two novel members of the Siglec gene family, OB-BP1 and OB-BP2. While this work was in progress, others reported the identical cDNAs as CD33L (27) and Siglec-5 (20). However, sialic acid binding was investigated only in the latter study. Our studies here demonstrate a sialic acid binding phenotype of OB-BP1, justifying its inclusion in the Siglec family as Siglec-6. The family now includes Sn/Siglec-1, CD22Sn/Siglec-2, CD33/Siglec-3, MAG/Siglec-4a, SMP/Siglec-4b, OB-BP2/Siglec-5, and OB-BP1/Siglec-6. These form a unique subset of the immunoglobulin superfamily by virtue of primary sequence and predicted structures (49). Like the other Siglec family members, OB-BP1 displays an NH$_2$-terminal V-set Ig domain with a characteristic pattern of conserved cysteines, followed by a variable number of C2-set type Ig domains, a single transmembrane domain, and a cytoplasmic tail of variable length. The extensive sequence similarity among OB-BP1, Siglec-5, and CD33 suggests the three represent a distinct subgroup within the Siglec family (49). This is supported by the close link of the genes for these three molecules on chromosome 19. Indeed, with the exception of Sn/Siglec-1, genes for all the other 5 mammalian Siglecs are known to be clustered in a region of human chromosome 19 and for CD22, CD33, and MAG in the syntenic region of mouse chromosome 7 suggesting that they arose by gene duplication, prior to the divergence of the mammalian orders.

To date, for two of the Siglec family members, MAG and CD22, the binding of the molecule to its sialic acid glycoprotein ligand is known to initiate intracellular signaling events. MAG
and CD22 transduce cell surface events to intracellular signaling pathways by modulating the activity of protein tyrosine kinases (15, 50) or phosphatases (51). In each instance, the tyrosine residues within the intracellular domain are phosphorylated upon activation of the protein, and the phosphotyrosine then serves as a recognition site for the kinases or phosphatases by a SH2 domain. Tyrosine residues within the cytoplasmic domain of the B-cell restricted CD22 molecule are phosphorylated upon surface Ig or CD22 ligation (52, 53). Interestingly, while some of the phosphotyrosines on CD22 serve as docking sites for kinases, others are embedded within sequences recognized by the SH2 domains of phosphatases. The physiological relevance of the negative and positive regulatory functions can be inferred from the analysis of CD22-deficient mice. In the absence of antigen, CD22 negatively regulates B cell antigen receptor signaling; in the presence of antigen, CD22 positively regulates signaling by the antigen receptor (17, 54, 55).

By inference from sequence analysis, other Siglec family members including OB-BP1/Siglec-6 and OB-BP2/Siglec-5 are also likely to be capable of signaling. The highly conserved consensus sequence surrounding the intracellular tyrosine residues of CD33, OB-BP1, and OB-BP2 (ELHYA(S/V)L–(12–18 residues)–TEYSE(I/V)(K/R)) suggests that the three are associated with common intracellular molecules that mediate signal transduction. The consensus does not match any previously identified phosphotyrosine binding motifs for either SH2 or phosphotyrosine-binding domains (56–58). However, we predict that as with Siglec family members MAG and CD22, the tyrosines in these molecules are likely to be phosphorylated upon engagement. Once the tyrosine is phosphorylated, the first motif is recognizable as an immunoreceptor tyrosine kinase (ITK).
Supernatants of OB-BP1ec, OB-BP2ec, or CD33ec were injected as in signifying kinetics with relatively slow on and off rates, which differ and CD33 bound with low affinities. The three exhibited binding OB-BP1 bound leptin with a moderate affinity, while OB-BP2 and CD33 were 91 nM, 880 nM, and 1.9 μM, respectively.

Fig. 8. Analysis of leptin binding to extracellular domains of OB-BP1, OB-BP2, or CD33. A. OB-BP1ec baculoviral supernatant was injected onto an M2 antibody surface. Various concentrations of rhOB, as indicated, or GM-CSF at 2 μM were then injected. B, baculoviral supernatants of OB-BP1ec, OB-BP2ec, or CD33ec, were injected as in A, followed by injection of 2 μM rhOB. Estimated Kd values for OB-BP1, OB-BP2, and CD33 were 91 nM, 880 nM, and 1.9 μM, respectively.

nase inhibitory motif (consensus: (L/I/V/S)XXX(L/V)), found in many members of the Ig superfamily, including CD22 (36–38). Interestingly, the second motif (TEYSE(I/V)) matches a sequence (TXXYY(I/V)) found in SLAM (signaling lymphocyte activating molecule) and several SLAM-like proteins, a family of immunoregulatory molecules also belonging to the Ig superfamily. This motif was recently identified in SLAM as the docking site for a new SH2-containing adaptor molecule SAP (SLAM-associated protein) (39–41). SAP was shown to act as an inhibitor by blocking recruitment of the SH2 domain-containing tyrosine phosphatase SHP-2 to its docking site in the SLAM cytoplasmic region. It is possible that a similar interplay exists between the recruitment of phosphatases to the immunoreceptor tyrosine kinase inhibitory motif in OB-BP1/Siglec-6, Siglec-5, and CD33 and the presence of SAP or SAP-like inhibitors in the SAP binding motif.

By definition Siglec family members should bind carbohydrate ligands terminating in unique sialic acid linkages. Measured affinities for CD22 were found to be in the low (5–30) micromolar range (59). Glycosylation of the receptor itself can regulate binding activity of CD22, CD33, or MAG but not Sn: in vitro CD22 requires glycosylation for activity (60) while CD33 binds only after deglycosylation (60, 61). The identification of the OB-BPs as Siglec family members with particularly close resemblance to CD33 suggests that these two molecules have the capacity to recognize and bind sialic acids. Indeed, the initial studies indicate that OB-BP-2/Siglec-5 can bind both α2-3- and α2-6-linked sialic acids (20), while OB-BP/Siglec-6 seems to selectively bind α2-6-linked sialic acid only when it is bound to GalNAc (the so-called sialyl-Tn motif).

In our surface plasmon resonance studies, we found that OB-BP1 bound leptin with a moderate affinity, while OB-BP2 and CD33 bound with low affinities. The three exhibited binding kinetics with relatively slow on and off rates, which differ significantly from typical receptor-cytokine kinetics in which both on and off rates are fast (47). The relative Kd values of 91 and 880 nM for OB-BP1 and OB-BP2(Siglec-5), respectively, were consistent with the results of our initial expression cloning: in the screen for leptin-binding proteins, we isolated only OB-BP1, which has the highest affinity for leptin. The lower affinity of OB-BP2 for leptin likely prevented its identification in this screen, despite its predicted abundance within the screened library. The affinity of OB-BP1 for leptin (91 nM) is significantly lower than the affinity we have observed for leptin to Ob-R (9.5 nM) in similar Biacore studies (46). The differential affinity leads us to suggest that OB-BP1 is unlikely to function as a second signaling receptor of leptin but rather as a binding protein.

The low but measurable binding of leptin by OB-BP2 and CD33 may result from the high degree of sequence similarity to OB-BP1. From the relatively high Kd values for OB-BP2 and CD33 (880 nM and 1.9 μM), we conclude that this binding is unlikely to be physiologically relevant. However, the binding of leptin by OB-BP1 will require further investigation, particularly in light of our observation that OB-BP1 does not promiscuously bind 4 α-helical bundle proteins such as GM-CSF or IL-10. Our results suggest that OB-BP1 has acquired the additional ability to recognize and bind a specific protein ligand. If so, OB-BP1 may have a second function, in addition to its likely role as a Siglec. If the affinity of OB-BP1 for leptin is physiologically relevant we suggest that other Siglec family members may similarly have protein ligands. Interestingly, the Kd of OB-BP1 for leptin is approximately 50–300 times higher than the published Kd values for the binding of Siglec members to their sialylated glycan cognates.

If leptin is an endogenous ligand of OB-BP1, we can speculate as to its role in leptin physiology. The reported Ob-R and its splice variants (21, 22) bind leptin with a high affinity (22, 46) and are expressed in tissues consistent with leptin functions deduced from the ob/ob phenotype. Based upon the Kd of 91 nM and the high expression of OB-BP1 in placenta and moderate expression on B cells we hypothesize that OB-BP1 regulates circulating levels of leptin or acts as a leptin carrier in blood via B cells. Binding proteins for other hormones and numerous cytokines have been identified. With only two exceptions (discussed below), the binding proteins are soluble forms of the authentic signaling receptors and are generated either by proteolytic cleavage of the receptor extracellular domain from the cell surface or by alternate splicing of the receptor mRNA. Soluble receptors which can act as binding proteins include those for tumor necrosis factor, interleukin-1, interleukin-4, and interleukin-7 (for a comprehensive review, see Ref. 62).

Binding proteins whose sequences are unrelated to those of the signaling receptors have been identified in two cases. In the first case, α2-macroglobulin acts as a promiscuous low affinity, high capacity binding protein for at least 7 growth factors or cytokines including platelet-derived growth factor and interleukin-1β (Ref. 63, for review see Ref. 64). In the second case, two signaling receptors and binding proteins of unrelated sequence have been reported for the insulin-like growth factors I and II. Each IGF preferentially binds its eponymous receptor: IGF-I to IGF-I receptor with a Kd of 0.23 nM and IGF-II to the IGF-I/mannose-6-phosphate receptor with reported Kd values which vary between 0.04 and 1.75 nM (65–68, 70). Of the 6 known insulin-like growth factor-binding proteins (IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6) Kd values have been reported only for IGFBP-3 binding of IGF-I (∼0.23 nM) and IGF-II (∼0.12 nM) (71). IGF-I binds to the binding protein IGFBP-3 with a Kd of approximately 1.5–9.4-fold higher than
that reported for binding to the IGF-I receptor. Similarly, for IGF-II given the wide range of reported $K_d$ values the $K_d$ of IGF-II for IGFBP-3 could be 3-fold greater or 15-fold lower than for the IGF-II receptor. Our observations of leptin binding to Ob-R and OB-1 (9.5 and 91 nM, respectively) are within the range of $K_d$ reported for the other IGFBPs and are consistent with the possibility that OB-1 acts as a leptin-binding protein.

It is also likely that OB-1 and OB-2 function as canonical Siglec family members and mediate cell-cell recognition events via the binding of a specific sialic acid determinant present on a discrete cell population. Other Siglec family members may serve as MAG and/or SN function as important molecular recognition proteins which mediate the interaction between specific cell populations. In addition, engagement of the receptor can signal the cell to initiate particular events. If the OB-1 has a sialic acid ligand, it may also confer specific recognition and signaling capacities upon the cells in which it is expressed, especially in the cyto- and syncytiohypoblasts of the placenta. The latter cells are not only the sites of high expression of OB-1, but produce leptin during pregnancy (72). Therefore, OB-1 may function as a leptin-binding protein in the placental barrier and play a role in embryonic development by modulating leptin levels (for review on leptin, see Ref. 73). In this respect it is of interest that Takei et al. (27) noted an alternately spliced form of OB-1 (CD33L2) that is predicted to encode a soluble form of the protein. Future investigations into the sialic acid binding capacities and the sialylated ligands of the OB-1s as well as the leptin binding properties of OB-1/Siglec-6 should help elucidate the physiological functions of these two molecules. Additional studies on the intracellular consensus motifs will also increase our understanding of the mechanism by which all of the members of this subfamily (OB-1, OB-2, and CD33) carry signaling functions.

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