The Enigmatic Role of Sulfatides: New Insights into Cellular Functions and Mechanisms of Protein Recognition

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Abstract

Sulfatides are sphingolipids commonly found at the surface of most of eukaryotic cells. Sulfatides are not just structural components of the plasma membrane but also participate in a wide range of cellular processes including protein trafficking, cell adhesion and aggregation, axon-myelin interactions, neural plasticity, and immune responses, among others. The intriguing question is how can sulfatides trigger such cellular processes? Their dynamic presence and specific localization at plasma membrane sites may explain their multitasking role. Crystal and NMR structural studies have provided the basis for understanding the mechanism of binding by sulfatide-interacting proteins. These proteins generally exhibit a hydrophobic cavity that is responsible for the interaction with the sulfatide acyl chain, whereas the hydrophilic, negatively charged moiety can be found either buried in the hydrophobic cavity of the protein or exposed for additional intermolecular associations. Since sulfatides vary in their acyl chain composition, which are tissue-dependent, more emphasis on understanding acyl chain specificity by sulfatide-binding proteins is warranted. Importantly, changes in cellular sulfatide levels as well as circulating sulfatides in serum directly impact cardiovascular and cancer disease development and progress. Therefore, sulfatides might prove useful as novel biomarkers. The scope of this review is to overview cell functions and mechanisms of sulfatide recognition to better understand the role of these lipids in health and disease.

Keywords

Sulfatides • Ceramide • Plasma membrane • Sulfatide-binding proteins • Platelet aggregation • Disabled-2 • Cluster of differentiation 1

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3.1 Introduction to Sulfatides

Sulfatides (also known as 3-*O*-sulfogalactosylceramides, sulfated galactocerebrosides, or SM4) are sphingolipids found at the extracellular leaflet of the plasma membrane of most eukaryotic cells. They were first isolated from human brain tissue by Thudichum in 1884 [105]. Sulfatides are not only membrane components but they are also involved in protein trafficking, cell adhesion and aggregation, axon-myelin interactions, modulation of sodium and potassium channels, learning and memory, and neural plasticity [9, 13, 16, 66, 109, 113]. Sulfatides are expressed in a variety of cells, predominantly in the myelin sheath of the nervous system, representing ~4 % of the total myelin lipids [47]. Also, these sphingolipids are largely found at the surface of blood cells such as erythrocytes [57], neutrophils [93], and platelets [85] and they are major component of lipoproteins in blood serum [100]. Sulfatides are esters of sulfuric acid with galactosylceramides at C3 of the galactose moiety, which is connected to the primary hydroxyl group of the *N*-acylated D-*erythro*-sphingosine base via a β-glycosidic bond (Fig. 3.1). The fatty acid chain length of sulfatides varies, with the majority being composed of C16 to C26, including 2-hydroxy fatty acids [47]. Sulfatides containing nervonic acid (C24:1) are the most abundant in myelin, whereas high levels of the lipid with stearic acid (C18:0) are present in the cortical grey matter [46]. Other structural variants of sulfatides (C22:0) are found in kidney tissue [47], with shorter acyl chains (C16:0) being predominant form in pancreas [23]. Sulfatides are also modified by hydroxylation at the α-2 carbon of the fatty acid by the fatty acid 2-hydroxylase [4] and both hydroxylated and nonhydroxylated forms of the lipid are found distinctly distributed in the cerebral cortex [119].

3.2 Sulfatide Synthesis and Degradation

Synthesis of sulfatides occurs in the endoplasmic reticulum and the Golgi apparatus. Initially, a galactose residue is transferred from UDP-

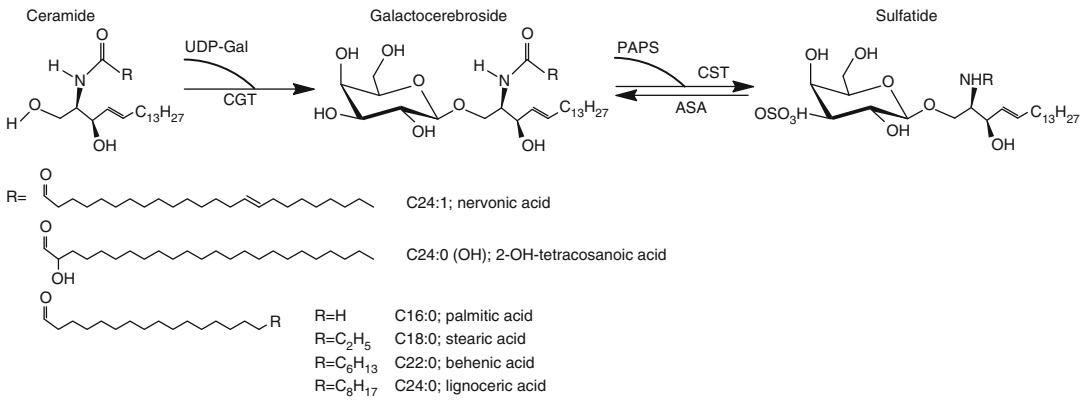

Fig. 3.1 The synthesis and degradation pathway of sulfatides. Ceramide is converted to galactocerebroside by addition of a galactose group from UDP-galactose, a reaction catalyzed by UDP-galactose:ceramide galactosyltransferase (CGT). Galactocerebroside is a substrate of 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST), which adds a sulfate group to the galactose moiety, using 3′phosphoadenosine-5′-phosphosulfate (PAPS), to generate sulfatide. Sulfatide turnover is mediated by arylsulfatase A (ASA), an enzyme that removes the sulfate group and generates galactocerebroside. ASA requires saposin B activity, a cysteine-rich protein that extracts sulfatides from membranes and allows ASA to catalyze the reaction on diffusible protein-lipid complexes. The chemical structure of ceramides is characterized by the presence of a sphingosine group and an additional fatty chain, which usually varies with different lengths and, therefore, depicted with an R group. Commonly found R-groups in ceramides and sulfatides are depicted at the *bottom*

galactose to 2-hydroxylated or nonhydroxylated ceramide at the luminal membrane leaflet of the endoplasmic reticulum, a reaction catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT; C 2.4.1.45) (Fig. 3.1). The product of this reaction, galactocerebroside, is delivered to the Golgi apparatus where it is modified by sulfation at position 3 of the galactose moiety through the action of a 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST; EC 2.8.2.11) [117]. Tissue-dependent expression of sulfatides correlates with the expression of both CGT and CST genes [35, 39, 123]. Recently, Aoyama and colleagues determined that the CST gene is transcriptionally stimulated by the activated peroxisome proliferator-activated receptor α and this effect directly enhances sulfatide levels in mice [75]. Mice lacking CST or CGT cannot produce sulfatides [38, 89]. Absence of the CST gene leads to disorganized paranodes and a lack of septate-like junctions, defects that promote a reduction of the nerve conduction velocity due to the lack of sulfatides [15, 19, 38]. Degradation of sulfatides is mediated by lysosomal arylsulfatase A (ASA; EC 3.1.6.8), which hydrolyzes the sulfate group from the galactose moiety leading to the formation of galactocerebroside. Sulfatide accumulation by the lack of ASA is associated with demyelination and metachromatic leukodystrophy (MLD), a lethal neurological disease [21, 84]. Overall, accumulated evidences indicate that alteration of sulfatide synthesis has a major impact on the generation of neuronal defects.

The reaction catalyzed by ASA depends upon the presence of saposin B, a sphingolipid activator protein that removes sulfatides from membranes and, thus, allows sulfatides to interact with ASA [56]. The crystal structure of saposin B shows a shell-like dimer of a helical bundle that encloses a hydrophobic cavity [2], a structural organization that is observed in many sulfatide-binding proteins [91]. Saposin B adopts a V-shaped conformation with five amphipathic α-helices, which associates to another saposin B molecule to build a large hydrophobic cavity in the dimer. The structure also reveals a region of elongated electron density that could be a potential lipid-binding site, an association that may require a conformational change of saposin B to expose its inner hydrophobic cavity to membranes [2].

Sulfatides can be intracellularly distributed by action of the glycolipid transfer protein (GLTP), a cytosolic peripheral protein that transfers glycolipids from the cytosolic leaflet of the plasma membrane or the endoplasmic reticulum and acts as a sensor of glycolipid levels [68]. GLTP employs a helical two-layer sandwich motif to transfer glycolipids and is able to recognize the sugar head group using hydrogen bonds and a hydrophobic pocket that associates with most of the nonpolar hydrocarbon chains of the ceramide region of the glycolipid [65]. There are two modes of glycosphingolipid binding by GLTPs [64]: (i) "*Sphingosine in*" mode, in which both the acyl and sphingosine chains are located in the same hydrophobic pocket of GLTP and (ii) "*Sphingosine out*" mode, in which the acyl chain of the sphingolipid remains in the hydrophobic pocket of GLTP, where the sphingosine backbone becomes exposed to the protein surface and allows interaction with another GLTP, forming a dimer. Recently, studies using the crystal structures of the wild-type human GLTP and a mutant ($Asp^{48}Val$; D48V) version of the protein in complex with sulfatides reveal that the D48V mutation favors the transfer selectivity to sulfatides by switching GLTP to the "*sphingosine in*" mode [91]. The D48V GLTP exhibits a cavity that allows the sulfate group to efficiently accommodate the sulfatide molecule in the protein, enhancing sulfatide binding over other neutral glycosphingolipids, such as galactoceramides. Consequently, sulfatides favor dimerization of GLTP, whose dimerization interface resembles the membrane-binding domains of the protein [54, 64].

3.3 Cellular Mechanisms Mediated by Sulfatides

3.3.1 Nervous System

Sulfatides are present in high levels in the myelin sheath, in both the central and peripheral nervous systems [108]. Myelin contains 70–75 % lipid,

4–7 % of which are sulfatides [77]. Sulfatides are also found in other glial cells, astrocytes, and neurons [11, 46, 81] and are myelin-associated inhibitors of central nervous system axon regeneration [114]. Increased cellular concentration of sulfatides is associated with MLD, in which patients exhibit accumulation of the lipid in lysosomes of oligodendrocytes, Schwann cells, macrophages, astrocytes, and neurons [79]; elevated levels of sulfatides are also associated with epileptic and audiogenic seizures [107]. Although unusual, deficiency in saposin B has also been observed in MLD [124]. Nonetheless, MLD leads to a progressive loss of myelin, in which the individual ultimately dies in a decerebrated state. Patients with Multiple Sclerosis or Parkinson's disease exhibit elevated levels of anti-sulfatide antibodies in serum and cerebrospinal fluid compared to healthy individuals [55]. Indeed, sulfatides act as autoantigens in Multiple Sclerosis patients [33]. Overall, these findings indicate that release of sulfatides from myelin is associated with the development of central nervous system diseases. Changes in sulfatide levels have been observed in other neuronal diseases, including epilepsy with mental retardation and Alzheimer's disease (for more details, see [20]).

3.3.2 Platelet Adhesion and Aggregation

Platelets represent an important linkage between thrombus formation and inflammatory processes. First, they prevent post-traumatic blood loss by forming fibrin-containing thrombi at the site of vascular injury, followed by the release of a battery of potent inflammatory and mitogenic molecules within the microenvironment that alters the chemotactic and adhesive properties of endothelial cells. These events facilitate the tethering and rolling of leukocytes over an inflamed vessel wall (activated endothelium [24, 25]), which then either firmly adhere and transmigrate into the arterial intima or simply detach [26, 27]. Among the various glycoproteins involved in these events, selectins are crucial for the initial contact between platelets and the vascular endothelium, and remarkably, mediate rosetting of platelets with monocytes and neutrophils to form platelet-leukocyte aggregates [60, 104]. Despite some contradictory results reviewed by Kyogashima [59], accumulated recent evidence suggests that sulfatides promote platelet adhesion and aggregation [18, 31, 70, 113].

One of the key cell surface receptors mediating leukocyte recruitment and exhibiting pro-aggregatory activity is P-selectin (for a review, see [12]). Most of the P-selectin ligands contain post-translational modifications needed for receptor binding and signal transduction in which sulfate moieties are frequently present [83, 88]. Sulfatides modulate P-selectin activity at the platelet surface [72] leading to further degranulation and increased surface P-selectin expression, which reinforces platelet aggregation [70, 113]. Moreover, P-selectin-sulfatide interaction leads to the formation of stable platelet aggregates and surface sulfatides enhance the formation of platelet-leukocyte aggregates [70]. Platelet P-selectin expression is decreased by fibrinogen deficiency [118].

Frequently, soluble platelet aggregation agonists bind to and induce conformational changes in the extracellular domains of the $\alpha_{IIb}\beta_3$ integrin receptor, triggering the inside-out integrin-signaling pathway [49]. Simultaneously, fibrinogen activates the outside-in signaling pathway by association with the $\alpha_{IIb}\beta_3$ integrin receptor via two Arg-Gly-Asp (RGD) motifs located in its α-chain [82]. In addition to fibrinogen, other integrin receptor agonists include the von Willebrand factor (vWF) and fibronectin and these associations stimulate platelet spreading and aggregation on vascular surfaces [49]. The adaptor protein Disabled-2 (Dab2) negatively regulates fibrinogen-$\alpha_{IIb}\beta_3$ integrin receptor association and, consequently, inhibits cell adhesion and cell signaling [18, 41]. The inhibitory function of the cytosolic pool of Dab2 is mediated by phosphorylation in its Ser24 residue, a post-translational modification that triggers the association of Dab2 to the cytoplasmic tail of the β3 subunit of the integrin receptor [41]. Binding of Dab2 to the integrin receptor is likely to be enhanced by phosphatidylinositol 4,5-bisphosphate-mediated membrane anchoring (Fig. 3.2). Consequently, Dab2 acts as

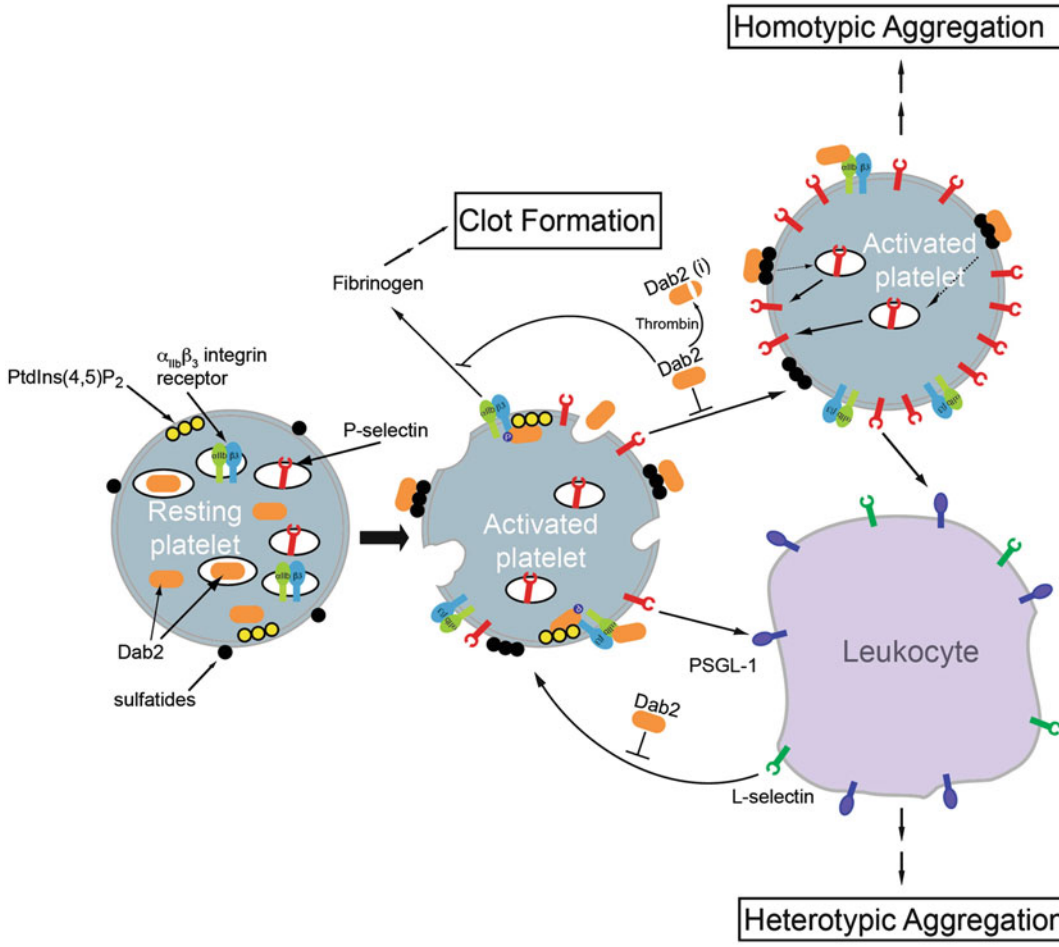

Fig. 3.2 An updated model of sulfatide- and Dab2-mediated modulation of platelet aggregation. Resting platelets are enriched in α-granules, which contain pro-coagulant (*i.e.*, P-selectin, $\alpha_{IIb}\beta_3$ integrin receptor) and anti-coagulant proteins (*i.e.*, Dab2). Another pool of platelet Dab2 is distributed cytosolically. Also, platelets contain signaling lipids including sulfatides (found at the outer leaflet of the plasma membrane) and PtdIns(4,5)P_2 (found at the inner leaflet of the plasma membrane). Upon activation, platelets change shape and release the α-granular content. Released Dab2 is partitioned in two pools: one associates with the α_{IIb} subunit of the integrin receptor through its RGD motif, and therefore, competes with fibrinogen for integrin receptor binding. Consequently, Dab2 negatively controls clot formation by modulating platelet aggregation. The second pool of Dab2 associates with cell surface sulfatides, whose levels are increased upon platelet activation. Upon platelet activation, cytosolic Dab2 is recruited to the plasma membrane in a phosphorylated state where interacts and inhibits the β3 subunit of the integrin receptor. Membrane recruitment of Dab2 is likely enhanced by its association to PtdIns(4,5)P_2. The fate of phosphorylated Dab2 after membrane recruitment is unknown. The function of extracellular Dab2 is modulated by the agonist thrombin, which cleaves Dab2 making it inactive (Dab2(i)). Both P-selectin and L-selectin bind to cell surface sulfatides mediating platelet-platelet platelet-leukocyte interactions, respectively. Furthermore, platelet-leukocyte interactions are enhanced by the association of P-selectin with PSGL-1. Both homotypic and heterotypic interactions are negatively modulated by Dab2. The presence of Dab2 at the cell surface is transient since it has been shown to be internalized back to α-granules (*dotted arrows*)

a negative regulator of integrin receptor inside-out signaling.

Dab2 is also localized in α-granules of both megakaryocytes [41] and resting platelets [18, 42]. Upon activation, Dab2 is secreted to the megakaryocyte and platelet surface via the α-granule secretory pathway where it binds to the $\alpha_{IIb}\beta_3$ integrin receptor, blocking fibrinogen-platelet

interactions [42]. Integrin-binding takes place because of the presence of an RDG motif in Dab2, an association that can be inhibited by the fibrinogen-derived Arg-Gly-Asp-Ser (RGDS) peptide [42]. Dab2 targets platelet surface membranes, as a result of platelet activation, via its N-terminal region containing the phosphotyrosine-binding (N-PTB) domain [18]. N-PTB is necessary and sufficient to inhibit platelet adhesion and aggregation by competing with fibrinogen for binding to the $\alpha_{IIb}\beta_3$ integrin receptor through its RGD motif [18]. In addition, Dab2 binds membrane sulfatides, an association that redistributes the protein at the platelet surface [18]. Dab2 recognizes sulfatides through the residues Lys25, Lys49, Lys51, and Lys53, which are located within the XBBXBX (B, basic residue; X, any residue) and BXBXBX motifs in its N-PTB region [18]. This class of basic clusters also mediates sulfatide binding of other cell adhesive proteins, including thrombospondins, laminins, and selectins [47]. The sulfatide-binding site of Dab2 overlaps with that of the phosphoinositide PtdIns(4,5)P_2 binding site [3], but competition likely does not occur in a physiological context since sulfatides are predominantly found at the plasma membrane surface, presumably in lipid rafts [96], whereas the phosphoinositide is predominantly found at the cytosolic leaflet of the plasma membrane [58]. Whereas sulfatides contribute to Dab2 membrane insertion, which is likely accompanied by a conformational change of the protein, phosphoinositide recognition occurs by electrostatic interactions associated with minor local structural changes in Dab2 [3]. Sulfatide recognition by Dab2 impairs cleavage by thrombin, a strong platelet agonist [18]. Consequently, a pool of Dab2 remains intact at the platelet surface upon activation, and is eventually internalized back to α-granules by an actin cytoskeleton-dependent mechanism [18]. Also, sulfatides modulate the availability of Dab2 for binding to the integrin receptor [18]. Taken together, Dab2 may be distributed in two pools at the platelet surface (Fig. 3.2). One pool of Dab2 competes with fibrinogen for binding to the integrin receptor, whereas a second pool binds sulfatides at the platelet surface. The second pool of Dab2 also exerts an additional layer of modulation of platelet aggregation since sulfatide binding by Dab2 blocks P-selectin-sulfatide interactions (Fig. 3.2) [113], which are required to sustain platelet aggregation [71]. Indeed, sulfatides promote surface expression of P-selectin in activated platelets [70, 113]. The N-PTB region of Dab2 not only blocks platelet-platelet interactions, but also controls the extent of heterotypic cell interactions, such as those with leukocytes *via* its recognition to cell surface sulfatides [113].

We have recently generated a Dab2-derived peptide that contains the two sulfatide-binding motifs (SBMs) of the protein [116]. The Dab2 SBM peptide adopts a helical and amphipathic structure when embedded in dodecylphosphocholine (DPC) micelles. The majority of the sulfatide-interacting residues map to the second sulfatide-binding motif with the basic residues Lys49, Lys51, and Lys53 as well as the nonpolar residues Ala52, Leu54 and Ile55 playing a major role in the interaction with the sphingolipid [116]. Using a combination of paramagnetic probes, we established that the peptide lies in a parallel orientation below the sulfatide-enriched DPC micellar surface but does not cross the hydrophobic core of the micelle. Using microfluidic devices that readily mimic vasculature, we showed that Dab2 SBM displays anti-aggregatory platelet activity, comparable to that described for the fibrinogen-derived peptide, Arg-Gly-Asp-Ser (RGDS) [116]. Thus, by binding to cell surface sulfatides, Dab2 SBM provides the basis for rational design, promising anti-aggregatory low-molecular mass molecules for therapeutic applications.

Sulfatides also interact with homeostatic cell adhesion proteins, such as vWF [86], chemokines [92], laminin [86], and thrombospondin [85]. Sulfatides inhibit vWF's platelet adhesion in flowing blood and under physiological shear stress conditions [9]. The sulfatide-binding site in vWF overlaps with that of the glycoprotein Ib and, consequently, the lipid can inhibit glycoprotein Ib-mediated platelet adhesion [9]. vWF binds sulfatides by a region comprising residues 1,391–1,409 within the A1 domain of the protein [5]. Further site-directed mutagenesis studies

demonstrated that the residues Arg1392, Arg1395, Arg1399, and Lys1423 are critical for sulfatide recognition as shown using ELISA-based plates coated with sulfatides [76]. The residues Arg1392 and Arg1395 within the A1 domain of vWF are also relevant for glycoprotein Ib binding [67], confirming that sulfatides and glycoprotein Ib compete with each other for vWF binding.

Chemokines are cytokines that bind to cell surface sulfated glycosaminoglycans, modulating the activity of chemokine receptors. In addition to glycosaminoglycan binding, chemokines bind sulfatides [92], although the role of sulfatide recognition by these proteins is not clear. Whereas chemokine production is reduced by sulfatides when tested in peripheral leukocytes and fat cells [10, 87], it is stimulated in brain immune cells [52].

Laminins contain a series of G-like modules of about 200 amino acids each that bind to sulfatides, an association that may facilitate the polymerization of the protein into networks [53]. Two XBBXBX and three BXBXBX sequences were initially suggested to be potential sulfatide-binding motifs for the protein [103]. Timpl and colleagues demonstrated that sulfatide binding is increased when laminin G-like modules are in tandem [102], indicating their cooperation in ligand recognition. Structural data indicate that residues K3027 and K3028 within the XBBXBX motif of laminin α2 G-like 4–5 domains are critical for sulfatide binding [37, 102]. Furthermore, residues K3088 and K3091 present in a basic cluster BXXBXXXB of the same protein contribute to sulfatide binding [37]. Likewise, the ^{2831}RAR and ^{2766}KGRTK residues of the related laminin α1 G-like 4–5 domains, which belong to potential BXBXBX motifs, are crucial for sulfatide binding [34]. However, other basic clusters involved in heparin recognition are dispensable for sulfatide binding [34], suggesting that the association of laminin to different ligands may trigger unique biological responses.

Thrombospondins are extracellular calcium-binding proteins that are involved in wound healing, angiogenesis, vessel wall biology, synaptogenesis, and connective tissue organization (for a review, see [1]). Thrombospondins are known to bind many partners [1]. Sulfatides and heparins show strong affinity to thrombospondin-derived peptides containing the WSXW (where X is any residue) sequence with no polybasic motif required for sulfatide binding [32]. Indeed, these peptides strongly inhibit sulfatide and heparin binding to the thrombospondin, blocking binding of this protein to melanoma cells [32].

3.3.3 Innate Immunity and Autoimmunity

T cells recognize antigens, such as foreign and self-lipids and peptides, leading to the production of cytokines and, therefore, contributing to immune responses [7]. T cells also use their cell surface receptor to recognize lipid antigen-bound cluster of differentiation 1 (CD1) molecules at the surface of professional antigen-presenting cells such as macrophages, dendritic cells, and a small group of B cells. There are three groups of CD1 surface proteins: (*i*) CD1a, CD1b, and CD1c (group 1), (*ii*) CD1d (group 2), and (*iii*) CD1e (group 3) [17]. CD1 proteins contain three extracellular domains (α1, α2, and α3), a transmembrane domain, and a cytoplasmic tail. The extracellular domains form a surface groove (named the lipid-binding groove) formed by two α-helices (α1 and α2) on top of a β-sheet [120, 122]. The lipid-binding groove, which is narrow and deep, contains hydrophobic residues that can interact with the acyl chains of the glycolipids [6], whereas the polar head group becomes exposed in the CD1-lipid complex, allowing recognition by T cell receptors [73]. In CD1a proteins, the lipid- binding groove contains two large hydrophobic regions termed A' and F' [120]. In the CD1a-sulfatide complex, the sulfatide adopts an S-shaped conformation in which the A' pocket contributes to the C_{18} sphingosine backbone recognition, and the acyl chain of the lipid emerges from the A' pocket and extends its association into the F' pocket [120]. The galactose moiety forms hydrogen bonds with residues Arg76 and Ser77, whereas the sulfate group forms hydrogen bonds with residues Arg76 and Glu154 and with water that is in complex between residues Arg73 and Glu154 [120]. Consequently,

the sulfated galactose residue becomes exposed at the surface of the complex for T cell receptor recognition. Sulfatides can be presented by all members of the CD1 group 1 and by CD1d [8, 94]. However, the sulfatide-binding affinity varies with each CD1 molecule, with the CD1a-sulfatide being the most stable complex [94].

Sulfatides have also been shown to be self-glycolipid antigens recognized by CD1d, assembling a complex that activates type II natural killer T (NKT) cells [50]. Sulfatides induce proliferation and expansion of memory, but not naïve, T cells [48]. The mechanism by which the T-cell receptor from type II NKT cells (XV19 hybridoma) interacts with the CD1d-sulfatide complex has been recently reported [78]. Whereas the type I NKT T-cell receptor exclusively contacts the F' pocket of CD1d, the type II NKT T-cell receptor binds orthogonally above the A' pocket of CD1d, emphasizing different CD1d points of contact. More importantly, T cells highly reactive to sulfatides are increased in number and CD1d is upregulated in the central nervous system of patients with experimental autoimmune encephalomyelitis [33, 50]. The presence of the sulfate group and the β-anomeric linkage are critical for CD1d activation-dependent T cells [94]. The dominant sulfatide species for CD1d-dependent immune responses is a C24:1 [121], which bears one unsaturation at the 8–9 position (Fig. 3.1). The crystal structure of the CD1d-C24:1 sulfatide complex shows the acyl chain in the A' pocket, whereas the sphingosine chain associates with the F' pocket, leaving the sulfated head group exposed at the protein surface [121].

3.3.4 Host-Pathogen Interactions

The action of protein toxins from pathogenic organisms requires specific sphingolipids at the cell surface to mediate protein endocytosis and to enhance the virulence of the pathogen. Sulfatide recognition by pathogen proteins includes the coli surface antigen 6, the heat-stable toxin b, and the 987P-fimbriae from *Escherichia coli* [14, 30, 51], and heat shock proteins from *Helicobacter pylori* [43, 44]. The only structural data reported for this class of toxins is that for the *Naja atra* Taiwanese Cobra cardiotoxin A3 (CTX -A3) in complex with sulfatides using hexaethylene glycol monodecyl ether detergent as a membrane mimetic [110]. CTX-A3 acts as a toxin by a sulfatide-dependent internalization mechanism that leads to pore formation in the host cell membrane [115]. The crystal structure of CTX-A3 reveals a dimer of two β-sheet proteins, an oligomerization state that is induced upon sulfatide binding. In the CTX-A3-sulfatide complex, the sulfatide head group is buried so that the sulfate group forms a hydrogen bond with the amino group of the residue Lys35, whereas the galactose sugar forms hydrogen bonds with the amino groups of the residues Lys12 and Lys18 and the carbonyl oxygen group on Arg36 and Cys38 [110]. The side chain of Lys44 interacts with the amide region of the ceramide backbone through a single hydrogen bond. The remaining lipid tail becomes exposed to the detergent-enriched solvent that facilitates the dimerization of CTX-A3. Membrane insertion and pore formation by CTX-A3 requires both protein and sulfatide conformational changes [106] and the presence of sulfatide-containing lipid domains [115].

Glycosphingolipids are also employed as receptors for virus infection. Both galactocerebrosides and sulfatides facilitate HIV type 1 virus binding to the $Cd4^{-}$ cell surface *via* the viral envelope gp120 protein [22]. Similarly, sulfatides are thought to be alternative cell surface receptors for the Influenza A virus [99] and the vaccinia virus [80]. In addition, sulfatides have been shown to enhance the formation and release of the progeny of infectious Influenza A viruses as well as translocation of newly synthetized viral nucleoprotein to the cytoplasm [101]. Indeed, sulfatide administration prevents cell viral infection [22, 99, 112] as it has been demonstrated for the bovine immunodeficiency virus, in which its internalization is inhibited by the glycosphingolipid during syncytium formation [112]. More recently, Kumar and colleagues demonstrated that sulfatide administration in mice inhibits HIV type 1 replication more efficiently than treatment with the nucleoside analog reverse transcriptase

inhibitor azidothymidine [98]. This is in agreement with the observation that antibodies that neutralize HIV-1 also recognize sulfatides [69]. Furthermore, the presence of sulfatides enhances mice hematopoiesis, which is usually lost during HIV-1 infection [98]. Overall, this evidence suggests that sulfatides represent novel tools to target viral infections.

3.4 Implications of Sulfatides in Disease Development and Progression

3.4.1 Cardiovascular Diseases

Sulfatides are known to play a critical role in the development of cardiovascular disease. Indeed, the measurement of serum sulfatide levels has been proposed to predict the incidence of cardiovascular disease in patients with end-stage renal disease (ESRD) [40]. The level of sulfatides in ESRD patients undergoing hemodialysis therapy and those with cardiovascular disease is consistently lower than in healthy individuals [40]. Patients with kidney transplantation show a significant increase of serum sulfatides in a time-dependent manner, which is correlated with an increment of platelet levels [111]. The recovery of sulfatide levels may be associated with the attenuation of the systemic oxidative stress triggered by the chronic kidney dysfunction in these patients [111]. Sulfatides are P-selectin ligands and as such mediate platelet-leukocyte interactions via P-selectin and CD11b/CD18 (Mac-1), an integrin receptor localized at the surface of monocytes, neutrophils, and T-cells [28]. Sulfatides increase Mac-1 surface expression in neutrophils, which may contribute to the development of intimal hyperplasia after endothelial injury [95]. Further studies demonstrate that sulfatides contribute to the progress of neointimal thickening after vascular injury, which can eventually trigger atherosclerosis [45]. In the same context, erythrocyte membrane sulfatides significantly increase in sickle erythrocytes and play a relevant role in sickle cell adhesion to endothelial cells [125].

3.4.2 Cancer Diseases

Increased levels of sulfatides have been observed in renal cell carcinoma [90], well-differentiated endometrial adenocarcinoma [97], some types of lung tumors [29], brain tumors [61], and colon [74], hepatocellular [36], and ovarian cancers [62, 63]. Sulfatides have been proposed as early predictors of ovarian cancer [63]. Recently, using a combination of mass spectrometry metabolite analysis and gene expression profiles, it has been established that sulfatide levels are elevated in ovarian cancer compared to normal ovarian tissue [62]. Consistent with this observation, higher levels of mRNA that codifies for the enzymes CGT and CST, required for sulfatide synthesis, are also detected in epithelial ovarian carcinoma cells, whereas the levels of ASA, saposin, and galactosylceramidase remain unchanged [62]. Taken together, measurements of sulfatide levels using mass spectrometry analysis of tumor tissues represent an excellent and sensitive tool to be used as serum biomarkers for early tumors.

3.5 Conclusions and Future Perspectives

As summarized in this review, the role of membrane sulfatides in the nervous system, innate and adaptive immunity, platelet adhesion and aggregation, and bacterial and viral infection is clearly emerging. However, there are several questions about how membrane sulfatides signal that need to be addressed. For example, a precise measurement of membrane sulfatide levels elicited by external cues is required to understand sulfatide-mediated signaling. Also, the levels of the enzymes that participate in the synthesis and degradation of sulfatides should play a key role in the modulation of the membrane levels of the sphingolipid.

The number of identified sulfatide-binding proteins has substantially increased over the past 15 years. The general sulfatide binding mechanism consists of the formation of hydrogen bonds between the acyl chains of the sphingolipid with

residues located in the hydrophobic cavity and accompanied by a few hydrogen bonds and electrostatic interactions between the side chain of basic residues of the protein and the negatively charged sulfate group of the galactose moiety. Perhaps, the key role of sulfatides center on the features of their acyl chains as they interact with protein hydrophobic cavities leaving, in some cases, the head group exposed at the surface of the protein. Thus, development of high-resolution methods for the discrimination of sulfatides with different fatty acid compositions is warranted. This is important as sulfatides with specific acyl chains lengths, unsaturation, or even hydroxylation modifications are tissue-dependent. Furthermore, predicting a sulfatide-binding site from the amino acid sequence of a protein is not an easy task. Whereas sulfatide-binding sites typically exhibit basic clusters of residues that follow the sequence BXBXBX or XBBXBX, some sulfatide-binding proteins exhibit unique sulfatide-binding basic motifs and some others do not employ basic residues at all.

With recent high-resolution structures of sulfatide-binding proteins we may also soon understand the role of sulfatides in protein membrane targeting as well as intra- and extracellular sulfatide-dependent protein dynamics. However, we still lack the information about sulfatide dynamics at membranes, its intracellular distribution of the glycosphingolipid, or its presence and relative concentration in lipid rafts. Moreover, the engagement of sulfatides in cardiovascular and cancer diseases makes this area of research clinically relevant. The identification of additional sulfatide-binding proteins and the appropriate measurement of sulfatide levels in serum and tumor tissues will certainly contribute to early prognosis.

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