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Role of very long fatty acid-containing glycosphingolipids in membrane organization and cell signaling: the model of lactosylceramide in neutrophils

Sandro Sonnino · Alessandro Prinetti · Hitoshi Nakayama · Mitsuaki Yangida · Hideoki Ogawa · Kazuhisa Iwabuchi

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Abstract Glycosphingolipids are highly enriched in specialized membrane microdomains ("lipid rafts", caveolar domains and glycosynapses), and they participate to the process of transduction of information across the membrane. Lactosylceramide (LacCer) is specifically coupled with the Src family kinase Lyn in plasma membrane microdomains of human neutrophils. Ligand binding to LacCer activates Lyn, resulting in neutrophil functions, such as superoxide generation and migration. The β-Gal-(1–4)-β-Glc disaccharide structure of LacCer is necessary, but it is not sufficient for LacCer-mediated Lyn activation. For this function, the presence of a LacCer molecular species with ceramide containing a very long fatty acid chain is also required. In this manuscript, we discuss the importance of interdigitation within the membrane, promoted by the presence of glycosphingolipid species with very long fatty acyl chains as determinants for membrane organization, instrumental to the signaling process.

Keywords Membrane organization · Interdigitation · Lactosylceramide · Very long fatty acids · Neutrophil

S. Sonnino (✉) · A. Prinetti
Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Università degli Studi di Milano,
Via Fratelli Cervi 93,
20090 Segrate, Italy
e-mail: sandro.sonnino@unimi.it

A. Prinetti
e-mail: Alessandro.prinetti@unimi.it

H. Nakayama · M. Yangida · H. Ogawa · K. Iwabuchi (✉)
Institute for Environmental and Gender-specific Medicine,
Juntendo University Graduate School of Medicine,
Tomioka 2-1-1 Urayasu,
Chiba 279-0021, Japan
e-mail: iwabuchi@med.juntendo.ac.jp

Introduction

Glycosphingolipids (GSL) are amphiphilic membrane components consisting in a hydrophobic moiety, ceramide, and a hydrophilic oligosaccharide headgroup [1]. Ceramide is responsible for their insertion into the outer layer of plasma membranes, with the oligosaccharide chain protruding in the extracellular environment. More than 400 GSL oligosaccharide chains have been identified, but the number of existing GSL molecular species is at least ten times higher, due to the heterogeneity in their ceramide fatty acyl chains [1, 2].

GSL participate to the process of transduction of information across the membrane [3] determining the lateral organization of cellular membranes and modulating the function of several classes of membrane proteins [4]. Their functions rely at least in part on their ability to form clusters with sphingomyelin, ceramide, cholesterol and signal transducing proteins such as GPI-anchored, and acylated proteins (including Src family-kinases), to form specialized membrane domains usually known with the generic term of lipid rafts [2].

Specific features of the sphingolipid structure can be invoked to explain the role of glycosphingolipids in the formation and stabilization of membrane lipid domains. On the other hand, the molecular mechanisms of GSL-mediated cell functions are still largely to be elucidated. In this manuscript, we will present a new vista in this insight, based on the following considerations. that we believe of particular relevance.

1. The presence of GSL in cellular membranes reduces the fluidity and dynamics of membrane-associated proteins through (a) the creation of a network of hydrogen bonds promoted by the amide linkage at the water–lipid

interface, (b) the large surface area required by the GSL monomer inserted into the surface and the oligosaccharide-oligosaccharide interactions, direct or mediated by water, thermodynamically in favor of segregation (c) the high melting temperature of the hydrocarbonyl chains and (d) the partial exclusion of water from the membrane, due to interaction with cholesterol. All these factors favor stable intermolecular interactions and the formation of complexes within proteins.

2. GSL modulate the protein conformation by specific interactions. This affects the protein function (*e.g.*, its enzyme activity) and/or allows new interactions among different proteins.
3. GSL containing very long fatty acids might participate in reducing the membrane thickness through interdigitation of the two membrane leaflets. This allows contacts between proteins of the external and cytoplasmic leaflets as well as between glycosphingolipids on the cell surface and components of the cytoplasmic leaflet.

Membrane organization and the complex lipid ceramide: role of very long fatty acids

There is a wide information on the ability of GSL to interact with proteins, in some cases in a highly specific manner, modulating their properties [1, 3]. This has been considered the starting step in the process of transferring the information across the membrane. But for some sphingolipid-protein interactions, there is no information on how the process continues after the initial interaction. GSL show a characteristic and sometimes heterogeneous hydrophobic moiety, the ceramide. Some evidence suggests that ceramide, the portion of the molecule that is inserted into the outer layer and makes the GSL to be component of the membrane, could be involved in the intermediate steps of signalling process.

There is a general consensus on the role played by the sphingolipid ceramide moiety in promoting the formation of membrane lipid domains and on their stabilization [2, 4]. The amide group of ceramide is a rigid group, with a perpendicular orientation towards the two hydrocarbonyl chains, which parallel orientation is stabilized by the presence of an unsaturation at positions 4–5 of sphingosine. The hydroxyl group at position 2 of sphingosine, the amidic proton and the carbonyl oxygen, enable the ceramide moiety to form hydrogen bonds, acting as hydrogen bond donors and acceptors at the same time. This cannot be realized by glycerophospholipids, that can act only as acceptors of hydrogen bonds. On the other hand, cholesterol as well has a very limited capacity to form hydrogen bonds. The orientation of the hydrogen bond donor and acceptor groups of sphingolipids is optimal to form lateral interactions and to considerably increase the stability in lipid–lipid association. This, together with a reduced GSL dynamics due to GSL–protein interactions, are very good requisites to promote the formation of rigid zones within the membrane. In these areas, a network of hydrogen bonds segregate GSL and cholesterol within the surrounding glycerophospholipid-rich environment.

The ceramide moiety contains a long chain base linked to a fatty acid chain. Sphingosine [(2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene] is largely the main long chain base in mammals. However, the structure with 20 carbons is relatively abundant in neurons, where its content progressively increases along aging [5]. This introduced the hypothesis of an involvement of C20-sphingosine containing GSL in functional processes. On the other hand, GSL ceramide is heterogeneous concerning its fatty acid content.

Glycerophospholipids (GP) are the main components of cellular membranes, where they can be compared to the bricks in a wall. In the membranes, GP comprise for many species differing in the fatty acid hydrocarbonyl structure (number of carbons and unsaturations). The structure of GP fatty acids is someway specifically associated to cell and tissue type. In general, the necessity to have a certain degree of membrane fluidity requires the presence of fatty acids with low transition temperatures, *i.e.*, characterized by a high degree of unsaturation. Detergent-Resistant Membrane fractions (DRM) likely corresponding to isolated membrane lipid domains are enriched in sphingolipids and cholesterol. On the other hand, GP remain their major components. Among GP, dipalmitoyl phosphatidyl choline is the main GP species, and therefore the main component of DRM. In addition, palmitic acid is the main fatty acid released from the complex lipid mixture from DRM. Palmitic and stearic acid are abundant in glycosphingolipids, mainly in those from neuronal membranes, and over 60% of total membrane glycosphingolipids belong to DRM [6, 7]. Thus, the DRM fraction contains membrane domains highly enriched in components with high transition temperature. Lipids with high transition temperature reduce the membrane fluidity and realize a super-stable structure. In addition to this, a further consideration of the fatty acid content of DRM complex lipids deserves attention.

The thickness of a cellular membrane is related to the length of the hydrocarbonyl chains present in the membrane lipids. The maximum length of ceramide is determined by the length of sphingosine and of the fatty acid hydrocarbonyl portions. The first five carbons of sphingosine belong to the water–lipid interface, so that, with the exception of less abundant ceramides containing very short chain fatty acids, it is the fatty acid structure that defines the length [8] of the whole ceramide portion inserted into the outer membrane

layer (Fig. 1). Thus, DRM that are highly enriched in lipids that contain C16 and C18 fatty acids, display an outer layer thicker than the non DRM outer portion with fatty acid C16–C22. Nevertheless, DRM sphingolipids, mainly those from extranervous system membranes maintain some heterogeneity of the fatty acid moiety. Thus, it is necessary to understand how geometrically a lipid with very long chain fatty acid (C24 or more), can stay inserted into the outer layer of a bilayer ordered membrane. On the basis of neutron diffraction experiments on artificial membranes, it has been proposed that a partial interdigitation between the two leaflets can occur, resulting from the tendency of very long fatty acids to fit into the membrane [9]. Specific lipid organization has been also proposed in the past to explain the role of very long hydrocarbonyl chain containing lipids in mammalian skin [10]. Interdigitation can participate to stabilize the bilayer, due to side-by-side interaction between the two membrane layers. On the other hand, cholesterol, which is indeed abundant in DRM, has been proposed to be main component of the cytoplasmic layer [11, 12], but at the same time it displays a very high flip-flop capability, thus moving between the two layers according to the necessities. However, at the same time the free space can be filled by reducing the membrane thickness (Fig. 2). This could be a way to have new protein contacts linking the two layers of a membrane, determining the initiation of a signal across the membrane itself.

Laccer and neutrophil

Neutrophils after infection immediately migrate toward the infecting pathogen and phagocytose it via pattern recognition

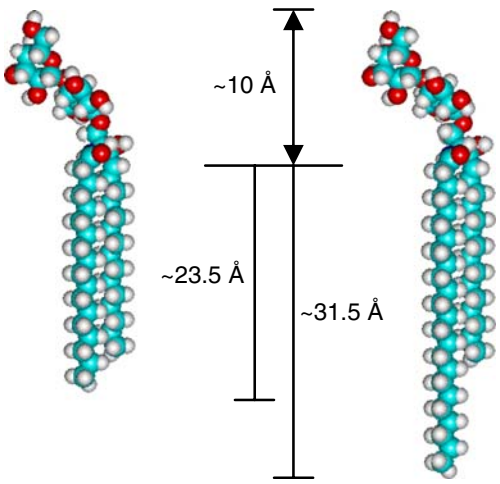

Fig. 1 Representation of the LacCer containing C16:0 and C24:0 fatty acid chain. The figure shows one of the possible conformers defined by different couples of glycosidic torsional angles. Both the Gal-Glc and Glc-Cer linkages have been shown to be dynamics with high motional

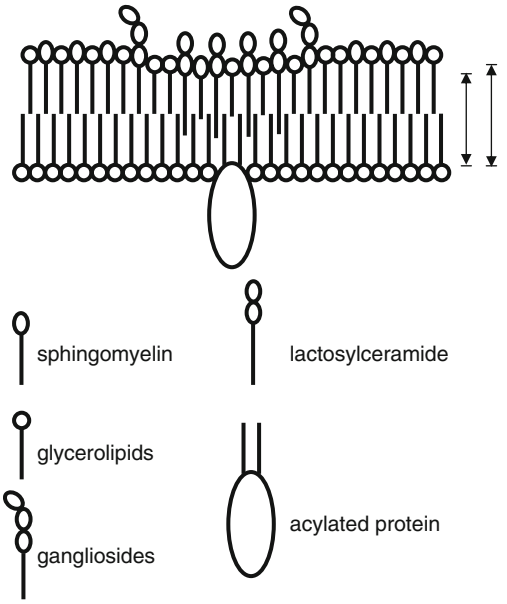

Fig. 2 Schematic representation of the membrane with partially interdigitated layers. The presence of components with very long fatty acid, here represented by LacCer, requires a partial interdigitation to allow the insertion of the long hydrocarbonyl chain; reducing the membrane thickness

receptors (PRRs) such as beta(2) integrin Mac-1(CD11b/CD18, CR3), CD14 and Dectin-1. In the second step, neutrophils kill the microorganisms with microbicidal molecules and through superoxide generation. LacCer accounts for about 70% of the GSL in human neutrophils and it has been shown to bind specifically to several types of pathogenic microorganism. In addition, chemotactic and superoxide-generating activities are induced by addition of anti-LacCer monoclonal antibodies to cells. On neutrophil plasma membranes, LacCer forms lipid microdomains with the Src family kinase Lyn, and serve as PRR responsible for chemotaxis, phagocytosis and superoxide generation of neutrophils [13–15] (Fig. 3). Anti-LacCer monoclonal antibody activates Lyn, which becomes phosphorylated, and it immunoprecipitates Lyn-associated domains from a detergent resistant membrane fraction prepared from cell membranes.

Dimethyl sulfoxide-treated neutrophilic differentiated human promyelocytic leukemia HL-60 cells (DMSO-HL-60 cells) possess chemotactic and superoxide-generating activities induced by formyl peptide fMLP. Moreover, they have a content of LacCer in the detergent resistant membrane fraction similar to that of neutrophils. Nevertheless, anti-LacCer antibodies do not induce chemotactic and superoxide generating activities.

In both DMSO-HL-60 cells and neutrophils LacCer contains the base (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadec-4-ene. Instead, in DMSO-HL-60 cells LacCer contains 50% of C16:0 and 50% of the hydroxyl derivative, while in

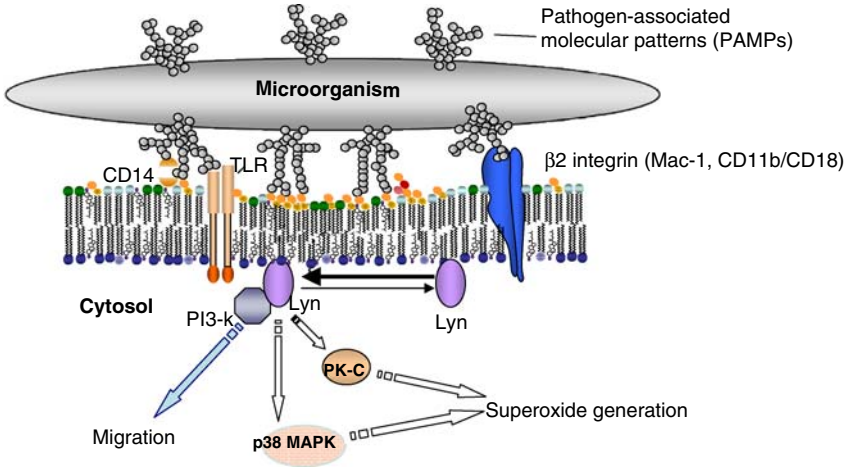

Fig. 3 Role of neutrophils in innate immunity. The binding of microorganisms to LacCer induces clustering of LacCer-enriched lipid domains, leading to superoxide generation and migration through Lyn-, PI3-k-, p38 MAPK-, and protein kinase C-dependent signal transduction pathways

neutrophils LacCer contains 60% of C24:0 and C24:1, 10% of C22:0 and 40% of C16:0 (Fig. 4). This information suggested that the LacCer species containing very long fatty acids are necessary to link LacCer with Lyn and to induce cell signalling in neutrophils. In support of this hypothesis, incorporation of very long fatty acid containing LacCer into the plasma membranes of DMSO-HL-60 cells by exogenous administration, showed LacCer-mediated neutrophil superoxide generation, Lyn activation by LacCer crosslinking and Lyn-coimmunoprecipitation with anti-LacCer antibody. These observations suggest that C24 fatty acid chains of LacCer play an important role in the formation of LacCer enriched lipid rafts coupled with Lyn as functional lipid domains responsible for chemotaxis and superoxide generation. LacCer forms about 44 nm clusters, and about 25% of LacCer clusters is colocalised with Lyn in neutrophils and C24:1-LacCer-loaded, but not untreated or C16:0-LacCer-loaded, HL-60 cells (Fig. 5). In addition, silencing of Lyn prevents migration of C24-LacCer loaded D-HL-60 cells. Neutrophils phagocytosed non-opsonized zymosans (NOZs), the cell wall of *Saccharomyces cerevisiae*. This phagocytosis was significantly diminished not only by anti-CD11b, but also anti-LacCer antibodies [14]. Mac-1 and LacCer are accumulated and colocalized in the actin-enriched phagocytic cup regions during neutrophil phagocytosis of non-opsonized zymosans. In contrast, D-HL-60 cells hardly phagocytosed NOZs. However, loading of DMSO-HL-60 cells with C24:0-LacCer or C24:1-LacCer resulted in reconstruction of functional Lyn-associated LacCer-enriched lipid rafts, and restored DMSO-HL-60 cell phagocytosis of NOZs. This latter event was inhibited not only by anti-LacCer but also by anti-CD11b mAbs. These results indicate that C24 fatty acid chain-containing LacCer-enriched lipid rafts-1-dependent neutrophil phagocytosis of NOZs.

Mac-1 (CD11b/CD18 integrin) plays a central role in neutrophil activation at sites of inflammation. Mac-1

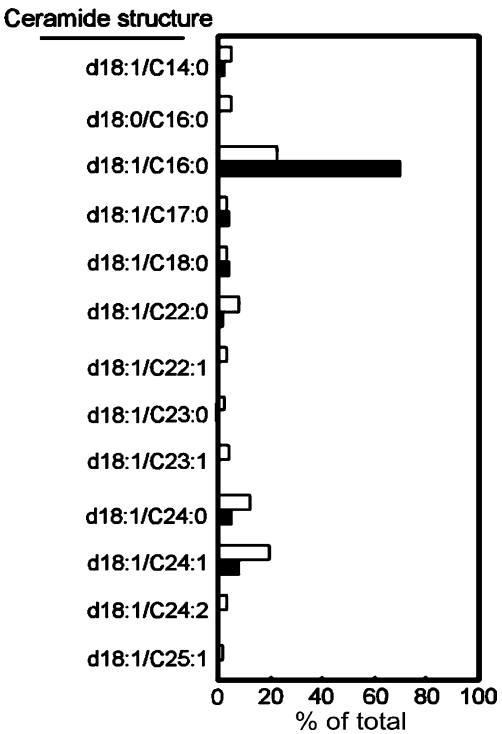

Fig. 4 Distribution of molecular species of LacCer derived from the plasma membrane DRMs of neutrophils and D-HL-60 cells. Neutrophils and D-HL-60 cells were disrupted by nitrogen cavitation, and the plasma membrane and granular DRMs were isolated from the cavitates. The lipids, which were extracted with chloroform/methanol 2:1 (*v*/*v*) from the plasma membrane and granular fractions of D-HL-60 cells, and the plasma membrane fractions of neutrophils, were separated by high-performance thin-layer chromatography with chloroform/methanol/water 65:25:4 (*v*/*v*/*v*). The migrated lipids were visualized by primulin spray. LacCer bands isolated by HPTLC were recovered by scraping, LacCer was extracted with chloroform/methanol (2:1 *v*/*v*), followed by LC-ESI-MS^n. The abundance of LacCer species was calculated from the peak area ratios on the LC chart of LC-ESI-MS analysis. Each bar shows the percentage (mean of 3 independent experiments) of each LacCer molecular species in total LacCer. *Open bar* Neutrophils. *Closed bar* DMSO-HL-60 cells

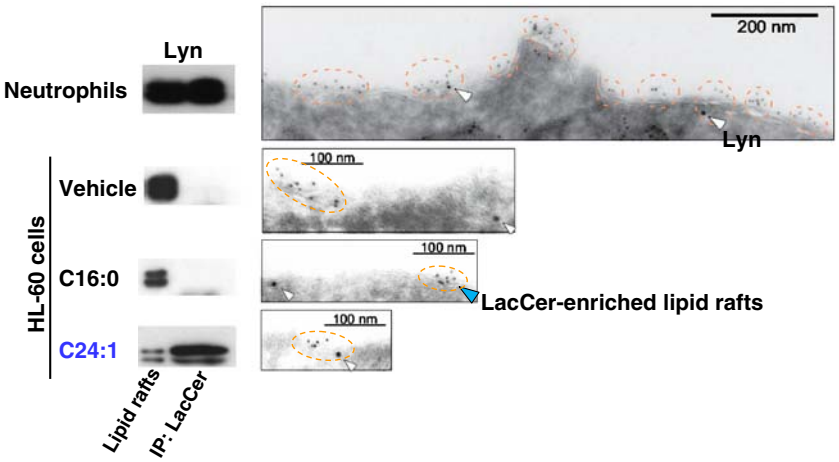

Fig. 5 Association of LacCer with Lyn in C24-LacCer loaded D-HL-60 cells. *Left panels* Co-immunoprecipitation of Lyn with anti-LacCer antibody in C24:1-LacCer-loaded but not C16:0-loaded DMSO-HL-60 cells. Plasma membrane DRM were isolated from neutrophils and DMSO-HL-60 cells loaded with different LacCer species or 0.1% DMSO (vehicle), and immunoprecipitated with anti-LacCer IgM Huly-m13. The immunoprecipitates were analyzed by SDS-PAGE/immunoblotting using rabbit anti-Lyn IgG. *Right panels* Electron micrograph showing the results of double-immunolabeling cytochemistry using anti-LacCer and anti-Lyn antibodies. Neutrophils, 0.1% DMSO-treated (vehicle), C16:0-, or C24:1-LacCer-loaded DMSO-HL-60 cells were fixed, and ultra-thin sections of these cells were incubated with anti-LacCer IgM Huly-m13 and with rabbit polyclonal IgG to Lyn. As a secondary antibody, we used 5-nm gold-conjugated anti-mouse IgM and 10-nm gold-conjugated anti-rabbit IgG (Fab')2. *Hatched circles* indicate LacCer clusters on the plasma membrane. *White arrow* Lyn

regulates several neutrophil functions, including adhesion, migration, chemotaxis, phagocytosis, respiratory burst, and degranulation [16]. Normally, Mac-1 binds poorly to ligands unless the cells are exposed to inflammatory stimuli, such as chemokines, bacterial products, and cytokines [17–20]. These stimuli cause an increase in avidity of CD11b and CD18 through a process called "inside-out" signaling [21]. Inside-out signaling *via* specific cytokine or chemoattractant receptors, such as IL-8, initiates conversion of CD11b/CD18 from the non-adhesive

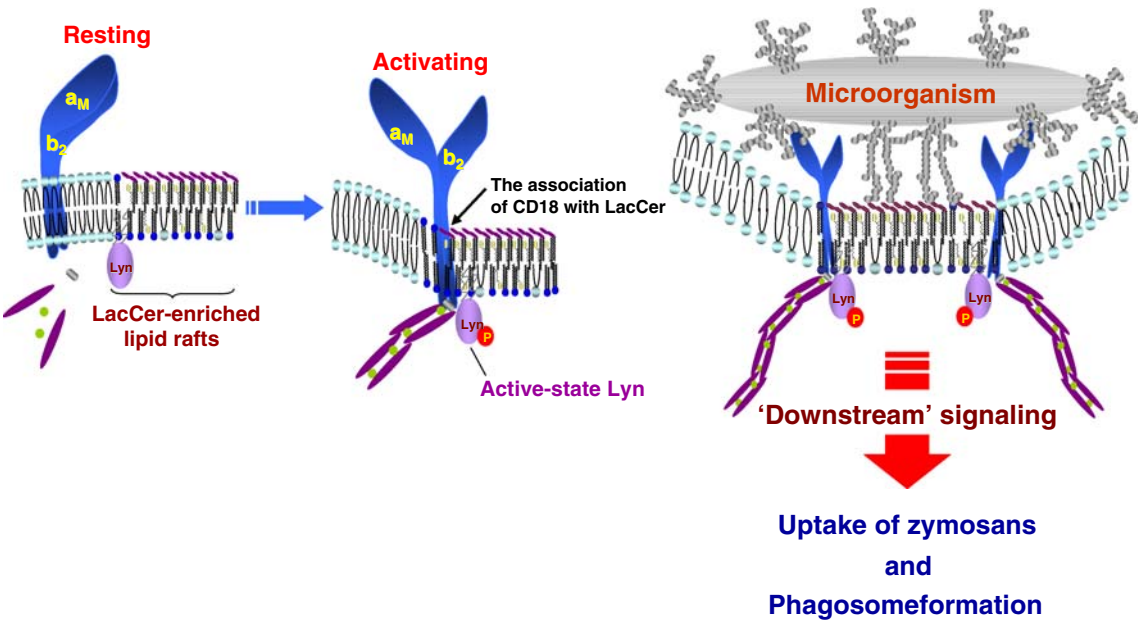

Fig. 6 Beta(2) integrin Mac-1-dependent neutrophil phagocytosis is mediated by LacCer-enriched lipid rafts. When neutrophils phagocytose non-opsonized microorganisms, Mac-1 tightly associated with LacCer-enriched lipid rafts in activated state, resulting in phagocytosis of microorganisms. Activation of Mac-1 induces the rearrangement of cytoskeletal proteins, resulting in translocation of Mac-1 into LacCer-enriched lipid rafts, and allows Mac-1 to transmit the stimulatory signals to Lyn through LacCer-enriched lipid rafts. The association of Mac-1 with LacCer in activating states could be mediated by epitope localized in residues 345–612 in β subunit CD18 of Mac-1

into the adhesive state [22]. Ligand binding to CD11b/CD18 delivers Src family kinase-dependent outside-in signals, resulting in neutrophil activation [22]. Binding of ligands to CD11b can directly induce conformational changes and activation of CD11b, leading to killing of iC3b-target cells [23]. Although the CD11b/CD18-induced outside-in signaling is highly dependent on Src family kinases [22, 24], CD11b/CD18 has short cytoplasmic domains and is devoid of catalytic activities responsible for signaling inside the cells [25, 26]. Stimulation with CD11b-activating antibody induces colocalization of Mac-1 and LacCer on plasma membranes of C24:1-LacCer—but not C16:0-LacCer-loaded DMSO-HL-60 cells, and phosphorylation of LacCer-associated Lyn. Therefore, it can be speculated that activation of CD11b/CD18 induces the rearrangement of cytoskeletal proteins, resulting in translocation of CD11b/CD18 into LacCer-enriched lipid rafts. This would allow CD11b/CD18 to transmit the stimulatory signals to Lyn through LacCer-enriched lipid rafts (Fig. 6). C24 fatty acid chains are crucial for the association of the Lyn molecule with LacCer, which is responsible for LacCer-mediated outside-in signal transduction. This association is indispensable for LacCer-enriched lipid raft-mediated neutrophil functions.

Conclusions

Glycosphingolipids are components of the membranes as a heterogeneous species differing in both the oligosaccharide and ceramide moieties. Some data suggest that the oligosaccharide chains specifically participate to interaction processes with membrane components. Unclear is the role of ceramide species. Hypothesis on a possible correlation between ceramide hydrophobicity and ceramide hydroxylation with the aging process have been presented [5, 27]. Recent papers reported on a specific enrichment of very long fatty acid containing lactosylceramide in the neutrophil lipid rafts and on its involvement in signal transduction processes. Now we propose, on the basis of a series of data, the specific role of the very long fatty acid containing lactosylceramide in modulating membrane interdigitation through the very long acyl chain. Then, interdigitation is proposed to be the switch for the transduction of information throughout the membrane perhaps by allowing contact and interaction between proteins belonging to the two layers.

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