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Visions & Reflections

How sphingolipids bind and shape proteins: molecular basis of lipid-protein interactions in lipid shells, rafts and related biomembrane domains

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Abstract. Understanding the molecular mechanisms controlling the association of proteins with lipid rafts is a central issue in cell biology and medicine. A structurally conserved motif (the 'sphingolipid binding domain') has been characterized in unrelated cellular and microbial proteins targeted to lipid rafts. I propose that the structuration of a sphingolipid shell around the sphingolipid binding domain not only extracts the protein from the liquid-disordered phase of the plasma membrane, and ensures its delivery to lipid rafts, but also influences its conformation. The chaperone activity of sphingolipids in shells and rafts may play an important role in infectious and conformational diseases (human immunodeficiency virus-1, prions, Alzheimer).

Key words: Plasma membrane; microdomain; infection; AIDS; Alzheimer; prion; chaperone; HIV-1; virus fusion; lipid rafts; caveolae.

Membrane lipids do not form a homogenous phase consisting of glycerophospholipids (e.g. phosphatidylcholine) and cholesterol, but a mosaic of domains with specific biochemical composition and physicochemical properties [1]. Among these domains, those containing sphingolipids and cholesterol, referred to as lipid rafts, have received much attention in the last few years [1–5]. Because they are excluded from the liquid-disordered (Ld) phase of glycerophospholipids, sphingolipids segregate into specific, cholesterol-enriched microdomains. These microdomains, which include caveolae [1], are in a liquid-ordered (Lo) phase floating in the more liquid glycerophospholipid-rich/cholesterol-poor bulk (Ld phase) of the plasma membrane. Lipid rafts, caveolae and related domains are dynamic entities that stir, fuse and continuously modify their shape, so that they might be compared to a myriad of mercury sheets perpetually moving on the surface of a mirror.

The characteristic partitioning of raft-associated lipids into ordered lipid phases renders them relatively insoluble in certain detergents such as Triton X-100 at 4 °C [6] or Brij 98 at 37 °C [7]. Accordingly, rafts can be readily purified as detergent-resistant membranes (DRMs) by ultracentrifugation on sucrose density gradients. Under these conditions, DRMs are recovered as complexes from the buoyant fractions. Biochemical analysis of DRMs demonstrates a specific enrichment in sphingolipids [glycosphingolipids (GSLs) and sphingomyelin] and cholesterol. Lipid rafts and caveolae serve as platforms dragging a variety of integral and peripheral proteins, including glycosyl-phosphatidyl inositol (GPI)-anchored proteins [6, 8]. Because they can diffuse laterally in the plasma membrane, lipid rafts behave as floating shuttles able to bring together activated receptors and transducer molecules, thereby coordinating the spatiotemporal organization of signal transduction pathways within selected

areas of the plasma membrane [9]. Finally, lipid rafts are preferential sites for host-pathogen/toxin interactions [10] and are involved in the generation of pathological forms of proteins associated with Alzheimer's and prion diseases [4].

Although the biochemical structure of sphingolipids and cholesterol may give a rational explanation for the self-organization of these lipids into specific domains [11], the factors governing the association of proteins with lipid rafts are mostly unknown. According to a recent theory developped by Anderson and Jacobson, proteins that are targeted to lipid rafts are first encased in a shell of cholesterol and sphingolipids that confer to the complex a light buoyant density [12]. The concept of a lipid shell surrounding membrane proteins is not new: for instance, Marsh used a similar metaphor in 1993 [13], underscoring that 'each integral protein is surrounded by a shell of lipids which mediate the coupling between the hydrophobic intramembranous surface residues and the fluid bilayer'. In particular, lipid head groups were thought to interact with the protein residues located at the polar-apolar interface of the membrane. In the lipid shell model postulated by Anderson and Jacobson [12], proteins targeted to rafts interact preferentially with sphingolipids and/or cholesterol (rather than glycerophospholipids), and those lipids form a specific type of shells (or condensed complexes) characterized by (i) an increased mobility in the plane of the membrane and (ii) a molecular compatibility with the lipids of the Lo phase, so that lipid shells have an affinity for preexisting lipid rafts. Accordingly, lipid shells target the protein they encase to these microdomains. The lipid shell model, although speculative, is particularly attractive, especially because it may lead to a thorough reevaluation of the role of lipid-protein interactions in the organization of membrane domains [14].

A consequence of the model is that both peripheral and integral membrane proteins should be capable of forming lipid shells, suggesting that raft-associated proteins might use multiple protein-lipid interactions to stabilize their association with condensed complexes. In this respect, the characterization of structural motifs involved in the interaction of proteins with sphingolipids and cholesterol is of primary importance. Curiously, one of the first steps towards the identification of such motifs came from studies of human immunodeficieny virus-1 (HIV-1) and amyloid proteins that undergo significant conformational transitions following binding to sphingolipids [4, 15]. Starting from the observation that a disulfide-linked domain (the V3 loop) of the HIV-1 surface envelope glycoprotein gp120 mediated its binding to the GSL galactosylceramide (GalCer) [16], we looked for a similar domain in the prion protein (PrP), another GalCer-binding protein [17]. Structure similarity searches using the combinatorial extension method [18] revealed the presence of a V3-like domain not only in PrP, but also in the Alzheimer β-amyloid peptide [15]. The motif, which will be referred to as the sphingolipid-binding domain (SBD), is a hairpin structure (e.g. α-helix-turn-α-helix or β-strand-turn-β-strand) with a turn containing at least one aromatic residue. Two typical examples of SBDs, the V3 loop of HIV-1 gp120, and the receptor binding site of the bacterial Shiga-like toxin, are shown in figure 1. The aromatic rings of the Phe^{20} residue of gp120 and the Phe^{30} and Trp^{34} residues of the toxin are critical for the interaction with the sugar moiety of surface GSLs (GalCer, Gb3) [4, 15, 19]. Indeed, protein-carbohydrate interactions involve such aromatic residues that are exposed to the solvent and stack against sugar rings [19–21]. This 'stacking' is typical for many protein-carbohydrate interactions and especially for the β-anomer of galactose residues (this is due to the particular stereochemistry of this sugar). From a chemical point of view, the interaction is driven by the proximity of the aliphatic protons of the sugar ring, which carry a net positive partial charge, and the π-electron cloud of the aromatic ring [22, 23]. This particular type of molecular association, which may be considered as an unusual case of H bond, is referred to as CH-π interaction. The respective orientation of both partners (the sugar and the aromatic rings) is essential for the interaction, since the sugar ring has to be parallel to the plane of the aromatic ring [23]. Because of this particular geometry, this arrangement is frequently described as stacking. CH-π interactions also occur between unrelated bacterial toxins and their GSL receptors [19, 21, 24]. The presence of aromatic residues in the V3 loop of gp120 and

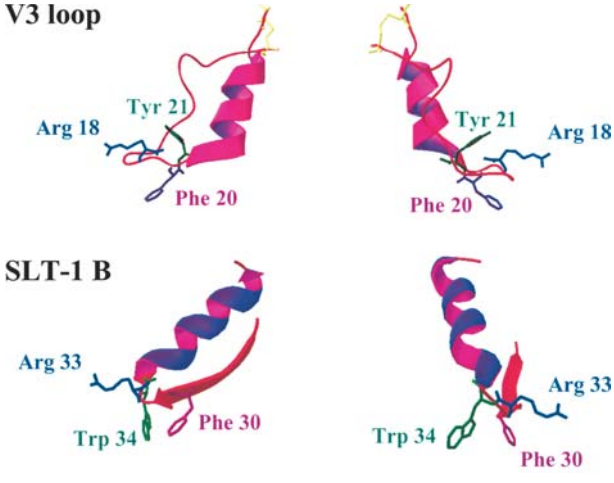

Figure 1. Structural homology between the V3 loop of HIV-1 gp120 and the B subunit of Shiga-like toxin (SLT-1 B). In each case, two symmetrical orientations of the SBD are shown. The structural alignment between the V3 loop (PDB entry: 1CE4) and SLT-1 B (PDB entry: 1CQF) has been obtained by the Combinatorial Extension (CE) program [16] and slightly refined with Deep View (Swiss-PDB viewer) software [53] to superimpose the aromatic residues Phe^{20} and Phe^{30}, and the basic residues Arg^{18} and Arg^{33}, respectively, in the V3 loop and SLT1-B. The disulfide bridge of the V3 loop is shown in yellow.

in the SBD of PrP is consistent with the establishment of CH-π interactions with the sugar head of raft GSLs [15]. When the aromatic ring faces the pyranose ring, ordered water molecules are being released from both rings, resulting in the establishment of entropy-driven interactions. In this respect, CH-π interactions can be compared with the well-known π-stacking interactions involving aromatic residues of the hydrophobic core of proteins [25]. The reorganization of water molecules during the binding reaction is highly significant, since the affinity between two ligands depends on the number of structured water molecules that are released to bulk solution as a result of the interaction [26].

On the basis of molecular modelling and physicochemical studies [4], I have proposed a model in which raft GSLs lock up the cellular prion protein PrP in its non-pathological conformation enriched in α-helix (i.e. the protease-sensitive, cellular form of the prion protein PrP^{c}). The SBD of PrP is a disulfide-linked loop that includes the α2 and α3 helix of PrP [15]. Interestingly, this region (especially the α2 helix) has a strong propensity to form a β sheet [27], indicating that a stabilizing factor might be necessary to maintain thermodynamically unfavoured α helices in PrP^{c}. In my opinion, raft GSLs might constitutively inhibit the formation of β structures in PrP by embedding the aromatic residues in the Lo phase of the raft environment. Interestingly, in a recent study focused on the role of rafts in the conversion of PrP, Baron et al. [28] concluded that the association of PrP^{c} with rafts might sterically hinder its binding to exogenous PrP^{sc} (i.e. the protease-resistant, infectious 'scrapie' form of the prion protein). Previous studies on the sites of interaction between PrP^{c} and PrP^{sc} indicated that binding occurs via sites on PrP^{c} that are close in space to the C terminus where the GPI anchor is attached [29]. Overall, these data strongly support the involvement of SBD-raft interactions in the locking of raft-bound PrP^{c} in a form unable to interact with PrP^{sc}, the binding site for PrP^{sc} being masked by 'protective' sphingolipids such as GalCer. Changing the physicochemical properties of the raft or its biochemical composition (for instance through PEG-induced membrane fusion [28] or upon fumonsin B1 or sphingomyelinase treatment [30]) could induce the dissociation of PrP^{c} from these sphingolipids, allowing the formation of a PrP^{c}/PrP^{sc} heterodimer. In particular, the availability of aromatic side chains in the SBD of PrP^{c} would allow a tight packing (through $\pi-\pi$ interactions) between adjacent helices of PrP^{c}, using the accessible aromatic side chains of PrP^{sc} as templates [31]. This would lead to the formation of a PrP^{sc}/PrP^{sc} homodimer, a key step in the generation of amyloid fibrils [32], which is likely to proceed through an ordered polymerization of parallel β-helix structures. The β helix is a particular fold found in some β-sheet-rich proteins [33] and, most important, in the only available structure of PrP^{sc} [34]. It is an unusually stable structure generally found in proteins subjected to harsh, denaturating environments (e.g. antifreeze proteins, bacterial or viral virulence factors) [33]. The conversion from PrP^{c} to PrP^{sc} (classically an α-helix $\rightarrow$ β-sheet transition) can thus be understood as stabilization of a proto-β-helical motif by a neighbouring PrP^{sc} molecule and subsequent extension to form the complete β helix [34]. The formation of a β helix involves the stacking of nonpolar residues (either aliphatic or aromatic). Moreover, parallel β helices provide flat sheets for lateral assembly into filamentous oligomers. From this discussion, I would like to underscore (i) the key role of aromatic residues in the self-assembly of amyloid fibrils and (ii) the potential protective effect of raft sphingolipids which could stabilize thermodynamically unfavoured α-helix structures in amyloid fibril-forming proteins [4, 15].

The characterization of a common SBD in unrelated proteins shed some light on the molecular mechanisms involved in the interaction of membrane proteins with condensed lipid complexes (lipid shells). The first step of the binding reaction is the superposition of the sugar ring of the GSL and of the aromatic ring of the SBD, which may proceed through an induced-fit mechanism to allow an optimized stacking (CH-π) interaction (fig. 2). The GSL schematized in figure 2 is GalCer with an α-hydroxylated fatty acid, which exhibits a high affinity for HIV-1 gp120 [35] and PrP [15]. Nevertheless, the model of interaction can be extrapolated to other GSL receptors such as LacCer, Gb3, Gb4 and gangliosides. Crystallographic studies have shown that due to intramolecular hydrogen bonds involving the α-OH group, the rigid galactose ring of GalCer is parallel to the plane of the membrane, so that the molecule adopts a typical L shape (or 'shovel') conformation [36, 37]. The sugar ring is then only free to rotate about the β-glycosidic bond (i.e. C1 of galacose-O1 of ceramide) as shown in figure 2. Thus, the sugar head inclination can be modulated according to the orientation of the aromatic ring of the SBD. The potential accessibility of the hydrophobic face of the sugar residue (often a galactose unit) on the one hand, and the conformational adjustments of both ligands (SBD and GSL) through an induced fit mechanism on the other, explain why so many pathogens and toxins select α-hydroxylated vs. nonhydroxylated galactose-containing GSLs [17, 35, 38, 39]. Secondary interactions, especially those involving basic amino acid residues of the SBD, would then further stabilize the complex through adequate electrostatic interactions with the negatively charged phosphate group of sphingomyelin and/or sialic acids of gangliosides (fig. 2). In this respect, there is a striking analogy between the SBD residues that are critical for the binding to sphingolipids, and the residues of transmembrane proteins most commonly found in the membrane-water interfacial regions, i.e. aromatic and basic residues in both cases

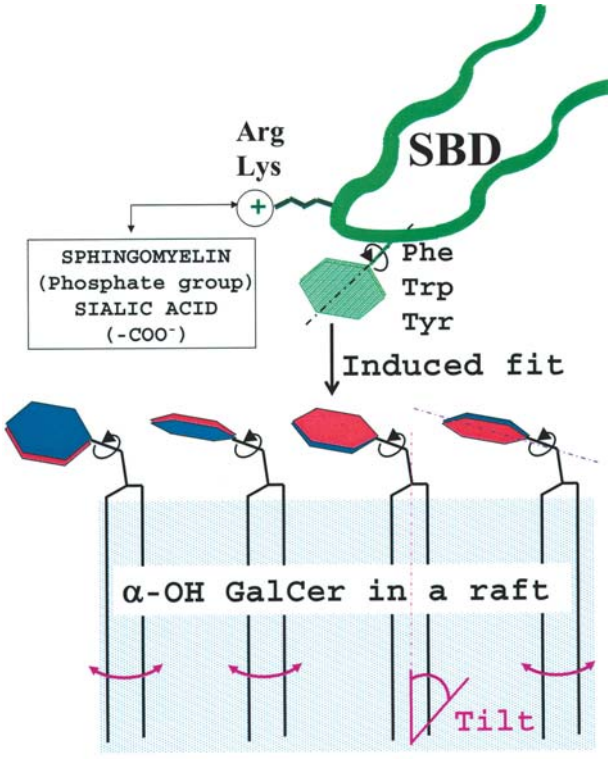

Figure 2. Potential interaction between the SBD and a GSL in condensed complexes of the plasma membrane. The model is based on crystallographic data obtained with natural α-OH GalCer [36, 37]. In particular, note that the conformation of the GSL allows a favourable orientation of the galactose ring and of the polar parts of the ceramide moiety. This may explain why many pathogens and toxins preferentially bind to sphingolipid receptors with α-OH fatty acids [38]. The free rotation of the aromatic ring (around the Cα–Cβ bond) at the crown of the SBD fold is also important for adjusting the orientation of the SBD over the hydrophobic face of the galactose ring in the early steps of the binding process (CH-π interaction). Accordingly, the binding reaction is envisionned as an induced-fit process implying conformational rearrangements in both partners (the protein and the glycolipid). In the crystal, the hydrocarbon chains of GalCer adopt a tilt of 40–45°, thereby establishing lateral close-packing contact. In the liquid-ordered phase of the plasma membrane, it is likely that the ceramide part could adopt several orientations by modifying the tilt, and this process might be controlled by hydrophobic associations with intercalated cholesterol molecules. This cholesterol effect would also contribute to adjust the orientation of the sugar head for an optimal interaction [51].

[40]. Obviously, the aromatic residues of the SBD interact with the sugar unit of a glycolipid, whereas the aromatic residues of transmembrane segments interact at the polar/apolar interface near the lipid carbonyl region. Therefore, both the type of interaction and the location of the interaction are different. However, this may not be the case for basic residues, which might play a similar role in the SBD and in transmembrane segments. Lys and Arg have a relatively long aliphatic side chain with a positive charge at the end. Accordingly, the aliphatic part interacts with the hydrophobic core of the membrane bilayer, whereas the positively charged end is located in the polar part where it can interact with negatively charged phosphate groups of phosphatidylcholine (PC) ('snorkeling' phenomenon). Since PC and sphingomyelin (SM) share the same polar head group (i.e. phosphorylcholine), Lys and Arg residues of transmembrane domains of integral proteins may alternatively interact with each type of phospholipid (PC and SM), resulting in a reversible exchange between the bulk membrane (PC rich, Ld phase) and lipid shells (SM rich, Lo phase) [12]. Consistent with this hypothesis, Yamabhai and Anderson [41] have been able to localize the principal raft targeting information in the epidermal growth factor (EGF) receptor to a 60-amino-acid-long (residues 581–641) juxtamembrane region in the extracellular domain of the protein. Secondary structure predictions suggest that this segment may form two adjacent SBD-like folds, each containing both aromatic and basic residues (Trp^{608}, Tyr^{610}, Tyr^{626}, Lys^{609}, Lys^{642}) [J. Fantini, unpublished results]. Similarly, a SBD fold could be predicted in the extracellular domains of several raft-associated transmembrane proteins such as the platelet-derived growth factor (PDGF) receptor, prominin, plasmolipin or the glucose transporter GLUT-1 [J. Fantini, unpublished results]. Edidin also reported the presence of a motif structurally related to the SBD in class I major histocompatibility complex (MHC) protein [14]. In the case of GPI-anchored proteins, it has been recently suggested that in addition to the anchor insertion into the bilayer, the protein itself could interact with the membrane surface [42]. The characterization of an SBD in cellular PrP, a GPI-anchored protein, strongly supports this hypothesis [15]. The motif has also been found in several GPI-anchored proteins, including Thy-1 and intestinal alkaline phosphatase [J. Fantini, unpublished results]. The presence of an SBD near the GPI anchor may certainly strenghten the association of such proteins with the membrane, thereby limiting their spontaneous release.

As a working hypothesis, I propose that the association with a lipid shell might significantly affect the conformation of the protein. The 'pitching' of the sugar ring over the surface of the plasma membrane, and of the hydrocarbon chains inside the membrane (fig. 2), is expected to facilitate the establishment of a wide variety of lipid-protein interactions during the transport of the protein to lipid microdomains [12]. The coordinated setting of these interactions may affect the conformation of the protein. Slight conformational changes may arise from minor adjustements of the orientation of lipid-interacting residues in the SBD. In addition, I postulate that the lipid shell may stabilize a thermodynamically unstable conformation at a minimal energetic cost, just like a small wedge can efficiently stop a car on a sloping street. In my model, the shaping of the protein is a progressive and multistep process, beginning in the lipid shell and ending up in a lipid raft. The influence of sphingolipids on protein con-

formation has been demonstrated for several proteins, including HIV-1 gp120 [43], PrP [44], the Alzheimer β-amyloid peptide [45] and proinsulin [46]. Overall, these data suggest the interesting possibility that specific sphingolipids in lipid shells, lipid rafts and related domains of biomembranes act as molecular chaperones [4], as previously postulated for some glycerophospholipids [47]. Indeed, although molecular chaperones have been initially described as a class of proteins that bind transiently to hydrophobic surfaces of proteins, thereby prevented self-aggregation or misfolding [48], nucleic acids, lipids or even carboxyl functions bound to synthetic surfaces do also have a chaperone activity [49, 50]. As a matter of fact, the stacking CH-π interaction between pyranose rings of GSLs and aromatic residues of the SBD may prevent the spontaneous aggregation of proteins due to $\pi-\pi$ interactions between solvent-accessible aromatic side chains. Finally, the protein may affect the structural organization of the lipid shell, as recently demonstrated for the GPI-anchored Thy-1 protein incorporated in lipid vesicles [42]. During this process, cholesterol may adjust the orientation of sphingolipids in the interface region of the membrane, allowing an optimized presentation of sugar rings to the SBD. In agreement with this concept, we observed that cholesterol is required for high-affinity binding of the V3 loop of gp120 to the GSL Gb3 [51]. It is interesting to note that this 'tuning' effect of cholesterol is functionally similar to the conformational effect of the α-OH group (in the acyl chain of the ceramide moiety), which optimizes the interaction of glycolipid receptors suchs as GalCer with various pathogen ligands, including PrP [17], HIV-1 gp120 [35] and *Helicobacter pylori* [39] (fig. 2).

In conclusion, I propose that lipid shells may not only extract specific proteins from the liquid-disordered phase of the plasma membrane, and subsequently sort them to lipid rafts, but also have a critical effect on their conformation. The chaperone activity of sphingolipid shells may prepare the protein to interact with and function within lipid rafts. I also hypothesize that in some instances, lipid shells may lock up unstable α-helix structures in proteins that would otherwise adopt β conformations known to favour the formation of amyloid fibrils [27]. Pathological forms of the prion protein and of the Alzheimer β-amyloid peptides are generated in lipid rafts when the association of their SBD with specific sphingolipids (GalCer, sphingomyelin) has been weakened by an external factor [4, 15, 30]. Several bacterial/viral toxins and adhesins also use an SBD to interact with various sphingolipid receptors in lipid rafts. Synthetic analogues of sphingolipids that bind SBDs with high affinity [51, 52] will be of great interest for future antiinfectious, antiadhesive and/or antiamyloid therapies. Finally, I hope that this article will stimulate constructive discussions among researchers involved in the study of protein-lipid interactions and that the role of these interactions in the sorting and shaping of raft-associated proteins will receive a definitive experimental confirmation in the near future.

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