Apoptosis (2007) 12:941–949
DOI 10.1007/s10495-007-0757-1

Dynamics of lipid raft components during lymphocyte apoptosis: The paradigmatic role of GD3

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Published online: 9 February 2007

Abstract Several investigations have been carried out since many years in order to precisely address the function of lipid rafts in cell life and death. On the basis of the biochemical nature of lipid rafts, composed by sphingolipids, including gangliosides, sphingomyelin, cholesterol and signaling proteins, a plethora of possible interactions with various subcellular structures has been suggested. Their structural and functional role at the plasma membrane as well as in cell organelles such as endoplasmic reticulum and Golgi apparatus has been analyzed in detail in several studies. In particular, a specific activity of lipid rafts has been hypothesized to contribute to cell death by apoptosis. Although detected in various cell types, the role of lipid rafts in apoptosis has however been mostly studied in lymphocytes where the physiological apoptotic program occurs after CD95/Fas triggering. In this review, the possible contribution of lipid rafts to the cascade of events leading to T cell apoptosis after CD95/Fas ligation are summarized. Particular attention has been given to the mitochondrial raft-like microdomains, which may represent preferential sites where some key reactions can take place and can be catalyzed, leading to either survival or death of T cells.

Keywords Lipid rafts · Apoptosis · Mitochondria · Gangliosides · GD3

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What are the lipid rafts

Lipid rafts are small and highly dynamic evolutionarily conserved structures which can play a role in signal transduction by concentrating molecules involved in signaling pathways, by allowing their molecular interaction, and/or by modulating signaling functions [1, 2]. They are envisaged as lateral assemblies of specific lipids and proteins in cellular membranes proposed to function in processes such as membrane transport, signal transduction, and cell adhesion [2, 3].

Biochemically, they are specifically enriched in certain lipids (sphingolipids, including gangliosides, sphingomyelin, and cholesterol), whereas other lipids (e.g., glycerophospholipids) are selectively depleted [4]. In particular, GM1 [5] or GM3 [6] have been proposed as markers for lipid rafts. However, the key role of these structures in signal transduction is strictly depending on their (glyco)protein composition. Indeed, a large variety of proteins has been detected in these microdomains isolated from different cell types, including tyrosine kinase receptors (EGF-R) [7], mono- (Ras, Rap) [8] or heterotrimeric G proteins [9], Src-like tyrosine kinases (lck, lyn, fyn) [10], PKC isozymes [11] and GPI-anchored proteins [12, 13]. Furthermore, multiple classes of cell adhesion proteins are GPI-anchored, e.g., F3, LAMP, NCAM120, TAG1, and BIG-1 [14] and they are localized in lipid rafts [15]. Thus, these microdomains have been suggested to participate directly in the mechanisms of cell adhesion. In particular, lipid rafts play a key role in regulating integrin function [16, 17]. At the end, these structures may play a role in the mechanisms of protein and lipid sorting at the trans-Golgi network level as well as for apical delivery in polarized cells [18]. In this regard, redistribution of rafts during cell migration is a pivotal step in achieving polarity [19]. In addition, partitioning of molecules into rafts may contribute to localize proteins at the front or the rear of moving

cells [19]. Thus, two different raft subtypes, distinguished by their peculiar ganglioside composition, segregate to each cell pole, with leading-edge rafts (L-rafts), enriched in GM3 and uropod rafts (U-rafts) enriched in GM1 [20, 21]. Analysis of lipid raft dynamics during chemotaxis confirmed segregation of distinct raft subtypes during cell migration [19].

Finally, a general function of lipid rafts in signal transduction may be to allow the lateral segregation of proteins within the plasma membrane, providing a mechanism for the compartmentalization of signaling components, concentrating certain components in lipid rafts, including those of importance in apoptosis (see below) and excluding others [1, 22, 23]. This selective confinement has suggested that rafts could function as platforms for the formation of multicomponent transduction complexes. Thus, they represent a sort of "chamber", where they can concentrate receptors for interaction with ligands and effectors on both sides of the membrane, thus speeding up binding during signaling and preventing inappropriate crosstalk between pathways [1], although the different signaling pathways may depend on the cell type.

How they are studied

The study of lipid rafts takes advantage of different methodological approaches. The use of non-ionic detergent extraction to generate low-density detergent-resistant membranes (DRMs) has had a major role in implicating rafts in cellular functions [24]. Although this treatment disrupts most lipid-lipid interactions, a minor fraction of cell membranes is preserved and can be isolated as DRMs. Since detergent extraction also disrupts several lipid-protein interactions, only few proteins, strongly interacting with highly ordered domains, retain their association with lipids and are recovered in DRMs. Thus, isolation of DRMs represents a valuable tool for the analysis of lipid rafts and an useful starting point for defining membrane subdomains. Applying a variety of detergents may reveal subtle differences in lipid-protein interactions [25]. Morphological analyses, including scanning confocal microscopy and electron microscopy (EM) (Fig. 1) with consequent quantitative statistical analyses prompted to analyze raft distribution on whole cells [26]. Specific protein-lipid interactions within rafts have been studied by coimmmunoprecipitation experiments [6, 27] or by fluorescence resonance energy transfer (FRET) [28]. In addition, many new approaches for detecting heterogeneity in cell membranes have emerged [28, 29], that rely on the distinct diffusion characteristics or enhanced proximity between raft components. Single-particle tracking (SPT) [30, 31] have enabled to measure the diffusion characteristics of GPI-anchored proteins. Recently, spectroscopic measurements, e.g. by Fluorescence Correlation Spectroscopy technique, that combine different evaluations of biophysical properties of the plasma membrane, e.g. ordered state, fluidity and deformability, can contribute to better analyze the dynamics of raft components in living cells [32]. Cellular lipid assemblies in their *a priori* state are likely to be small, indicating an intrinsic diversity of composition. Functional rafts (that is, larger platforms) are then induced as required in specific cellular contexts of sorting or signaling. The understanding of the mechanisms that govern the aggregation of rafts and their role in determining cell fate may represent an emerging field of investigation in the coming years.

The role of lipid rafts in lymphocytes

Many lines of evidence reveal the existence of lipid rafts in human lymphocytes [33, 34]. In these cells, lipid rafts may play a general role in signaling via immunoreceptors, such as TCRs, B-cell receptors and FcRs [22, 23]. Indeed, the phosphorylated immunoreceptors may associate with other protein-tyrosine-kinases (Syk family), inducing the activation of downstream members of signaling cascades such as PLCγ, phosphatidyl-inositol 4,5 diphosphate, and proteins regulating the activity of the small G-protein Ras [8].

The role of lipid rafts in T cell activation has been emphasized by the recruitment of TCR to lipid rafts upon receptor stimulation [35, 36]. The recruitment of cross-linked TCR into lipid rafts is independent of signaling events, but may be a consequence of raft clustering at the plasma membrane. There is evidence that the composition of raft-associated proteins changes after T cell stimulation. Several acylated proteins involved in the early phases of TCR signaling, such as Lck and Fyn [37], the adapter protein LAT [38], the Csk-activating protein [39] and Lck interacting molecule (LIME) [40] reside constitutively in lipid rafts. Upon TCR triggering, many signaling proteins become concentrated in lipid rafts, including the Syk family kinase Zap-70, which binds PLCγ and strictly interact with GM3 [6], the exchange factor Vav [8], the protein kinase Cθ (PKCθ) [41] and the protein kinase B (PKB) [42]. In addition, Lck changes its interaction with lipid rafts dependently on TCR activation. Indeed, it is present in rafts in an inactive form, but, upon T cell activation, the active form is accumulated within these microdomains [22, 23]. These findings, together with the observation that lipid raft disruption abolishes TCR-mediated signaling events [8], prompt to conclude that lipid rafts are indeed the platforms for TCR signaling. After T cell activation, these platforms are constitutively assembled as membrane patches that may translocate to immune synapses. It has been suggested that these large platforms could play a very important role in signal transduction pathways from the plasma membrane through the cell cytoplasm [43]. Targeting of lipid rafts to immune synapses was found to be

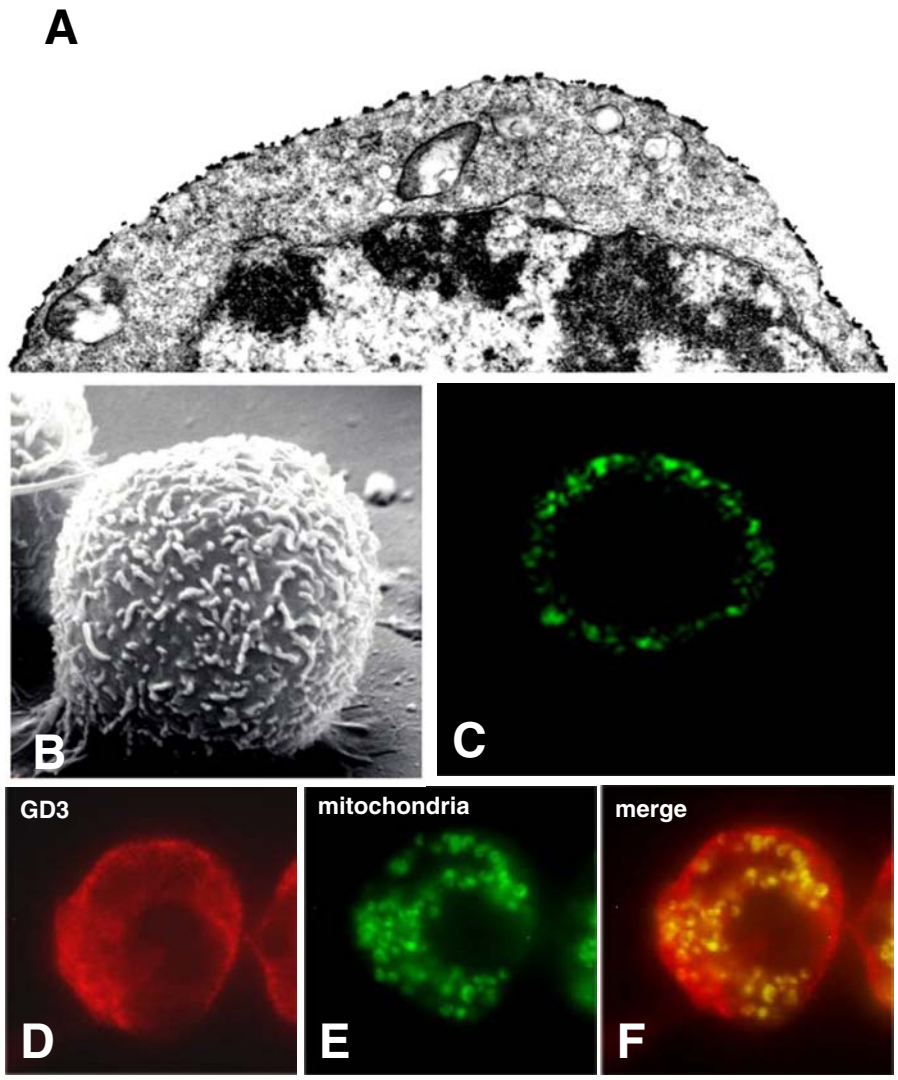

Fig. 1 Morphological analyses carried out by immunogold transmission electron microscopy show the typical clustered distribution of gold particles, corresponding to ganglioside-enriched microdomains, on cell plasma membrane of a T cell (A). Panel (B) shows the scanning electron microscopical features of a lymphoblastoid cell and panel (C) the confocal microscopical features of the same cell type after GD3 immunolabeling. Note the typical microvillous structures in (B) and the localization of GD3 glycosphingolipid at the T cell plasma membrane in (C). After CD95/Fas triggering, GD3 is visible as localized in mitochondria of T cells (D–E and merge picture F)

actin-dependent and requires PI3K activity and myosin motor proteins [43].

Specific rafts assemble into large-scale domains to create plasma membrane asymmetries at specific cell locations coordinating temporally and spatially cell signaling in these processes: the general function of lipid rafts in lymphocytic cells may thus be to vehicle targeting molecules to functional active portions of cell plasma membrane. This scenario is of relevance, considering the key role played by activation in T cells: resting lymphocytes are in fact resistant to apoptosis induction, whereas activated T cells undergo apoptosis following CD95/Fas ligation (activation-induced cell death, AICD).

The role of lipid rafts in apoptosis

Based on the lines of evidence indicating that gangliosides have to be considered as constitutive components of the plasma membrane [44] several studies have been carried out in the recent years as regards the possible implications of lipid rafts in cell physiology and in the maintenance of cell homeostasis. In particular, the possibility that lipid rafts could be involved in the complex framework instructing the apoptotic cascade has been investigated in a series of works carried out with diverse cell systems [27, 45–47]. As a general rule, in consideration of the various mechanisms involved, two different pathways have been established to occur in the apoptotic cascade: one takes into account the role of specific death receptors on the plasma membrane (type I cells, see below), while the alternative pathway (mitochondrial pathway) encompasses the series of events occurring downstream to mitochondria (type II cells). The prototypic molecule related to the occurrence of the receptor-mediated cell death program is represented by CD95/Fas. This belongs to the TNF receptor family and has been extensively and deeply investigated in a series of works describing the death signaling cascade and the involvement of the apical

caspases that are involved in the early events of this type of apoptosis. The second one, the mitochondrial pathway, is instead due to a direct activity of various molecules, mainly mitochondrially targeted drugs, that modify the organelle integrity and function, e.g. the mitochondrial membrane potential (MMP) and lead to the opening of the so-called megapore with release of apoptogenic factors, formation of the apoptosome and activation of executioner caspases. Anyway, these two pathways are profoundly intertwined to form a complex framework of events, e.g. via Bid molecular signaling.

In this scenario, the possible involvement of lipid rafts has been suggested since many years. First, on the basis of literature data indicating the presence of acidic glycosphingolipids at the plasma membrane [44], several efforts have been made to shepherd through the possible role of these peculiar structures on receptor-mediated cell death process. This has been investigated in various cell types. However, lymphocytes are the most widely investigated cell model. In human lymphocytic cells monosialoganglioside GM3 is the main constituent [48] and disialoganglioside GD3 is also well expressed. These molecules are mainly concentrated in lipid rafts (Fig. 1), where they are complexed with several proteins implied in signal transduction including, after T cell activation, the Syk family kinase Zap-70 [6, 35].

As regards apoptosis, a role for gangliosides as structural components of the multimolecular signaling complex involved in CD95/Fas receptor-mediated apoptotic pathway as well as in other receptor mediated apoptosis pathways was reported in different cell types [49, 50]. In type I cells, i.e. in cells susceptible to receptor-mediated apoptosis via initiator caspase-8, it was suggested that ceramide can play an essential role in CD95/Fas-mediated clustering in lipid rafts controlling both the fate and the activation of T cells [45, 51]. Accordingly, it was also shown that lipid rafts mediate CD95/Fas induced aggregation [27, 52, 53] and that the death-inducing signaling complex (DISC) associates with these domains upon CD95/Fas engagement [54, 27]. In particular, although not all cell types show the raft-dependency of CD95/Fas clustering [49, 55], in lymphocytic cells the GEM-dependency of the initiation of receptor clustering and signaling, including caspase 8 activation, was demonstrated by using cholesterol depleting agents, such as methyl-β-cyclodextrin (MβCD) [27]. In this context, cholesterol and sphingolipids have also been investigated in order to assess their impact on MHC function and immunological synapse formation. They have been considered as supervisors of the plasma membrane of immune cells [56]. For instance, in type I cells, CD59 and CD55 surface receptors appear as localized into different lipid submicrodomains with respect to CD28 [57]. The same authors found that CD55 or CD59 are negative regulators, whereas CD28 recruitment amplifies the Fas signaling pathway. They argue that, at the T cell surface, different types of microdomains with distinct functional properties may play a role in determining cell fate. In the same vein, Fas ligand (FasL), that has a well-conserved intracellular portion, is constitutively localized in lipid rafts. Interestingly, increased amounts of FasL recruited in rafts following FasL/Fas receptor interaction probably influence cell death induction [58]. Furthermore, CD95/Fas redistribution into membrane lipid rafts can also be induced by drugs, e.g. cisplatin, thus sensitizing cells to apoptosis [59]. Even in enucleated cells, i.e. in erythrocytes, a sort of apoptosis has been described that implicates caspase activity [60] and CD95/Fas-mediated triggering. Strikingly, translocation of CD95/Fas into rafts could trigger caspase activation in these cells too [61]. A series of works on raft function has also been carried out by investigating the effects of filipin, MβCD or cholesterol synthesis blockers, such as mevastatin, as raft disruptors and analyzed the resulting alterations of the apoptotic machinery. For example, studies on the role of insulin-like growth factors in regulating the apoptotic response to different ligands of TNF superfamily have proposed that segregation of IGF receptor in and out of the rafts may regulate the IGF receptor-mediated pro-apoptotic effects of TNF family members by influencing the recruitment to the lipid rafts of regulatory molecules, e.g. Akt/PKB [62]. The effects of raft disruption on pro-apoptotic signaling have also been demonstrated in B cells, where lipid rafts play a crucial role in CD20-induced caspase activation [63] or in leukemic cells where lipid rafts disruption prevents cladribine induced apoptosis [64]. Finally, by using MβCD and filipin, it was also demonstrated a role for lipid rafts in cell sensitization to cathepsin B-dependent apoptosis via lipopolysaccaride [65]. This argues in favor of an essential role of lipid raft-dependent signaling also when the apoptotic cell death occurs via a caspase-independent pathway.

The paradigmatic role of GD3

Different sphingolipids have been analyzed in order to assess their possible involvement in specific subcellular activities. For instance, it was hypothesized that a portion of intracellular GD3, a glycosphingolipid with two sialic-acid residues, can play a role in apoptotic machinery, since it is able to propagate CD95/Fas-mediated apoptotic signals [66, 67], Activation of death receptors (CD95/Fas, TNFα receptor) induces an intracellular flow of GD3, probably carried entirely by raft-containing vesicular transport [68]. Moreover, physical interaction and accumulation of GD3 in mitochondria were demonstrated in different cells following C2 ceramide [69] or TNFα (70) administration. In particular, in hepatocytes, GD3 synthesis increased and the ganglioside concentration decreased from the cell surface. Indeed, GD3 colocalizes with Rab-5 and Rab-7 in early and late endosomes via coordinated secretory /endocytic vesicular trafficking [70]. In

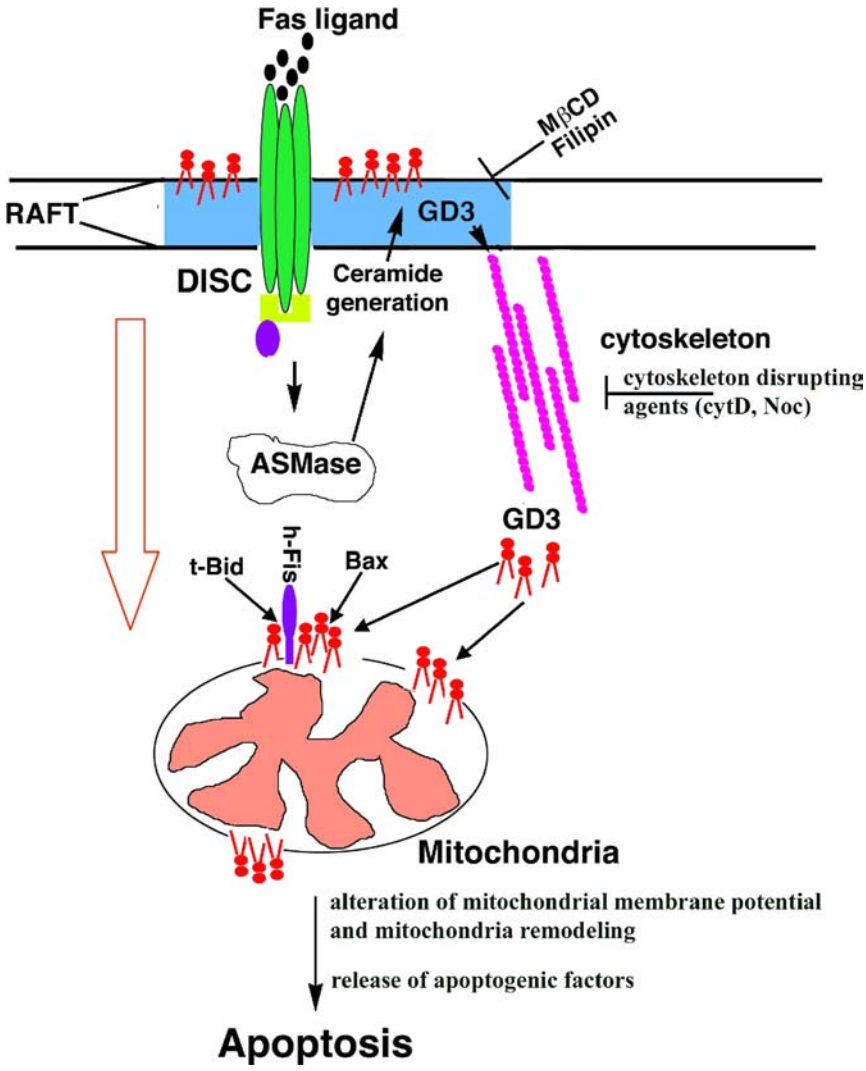

Fig. 2 Schematic drawing depicting the possible role of lipid rafts in apoptotic cascade. MβCD: methyl beta cyclodextrin; cytD: cytochalasin D; Noc: nocodazole

line with these findings, it has been hypothesized that an association with cytoskeleton can play a role in cytoplasmic movements of GD3 molecule finally targeted to mitochondria [71]. However, the molecular mechanisms are still under investigation.

The role of cytoskeleton

It has been shown that human T cells, that are susceptible to CD95/Fas-mediated apoptosis, undergo polarized morphology, forming uropods, and CD95/Fas polarization [72]. This cell remodeling is instructed by cell cytoskeletal components. In fact, it was hypothesized that actin microfilament system plays a key role. In particular, the so-called FERM family proteins (4.1 ezrin radixin and moesin) have been discovered to contribute to immunological synapse formation [73] and to regulate, via association with the actin cytoskeleton, CD95/Fas redistribution at the cell membrane, a pre-requisite for cell susceptibility to CD95/Fas-mediated apoptotic signal [72]. Accordingly, low concentrations of microfilament perturbating agents, e.g. cytochalasins, impaired CD95/Fas-mediated apoptosis cascade. Furthermore, other studies also suggested that disruption of lipid rafts and interference with actin cytoskeleton prevented CD95/Fas clustering and apoptosis [27, 74]. This indicates that membrane raft microdomains mediate lateral assemblies involved in CD95/Fas-mediated apoptosis. In other terms, an association between gangliosides, e.g. GD3 and cytoskeletal elements, such as ezrin molecule, is mandatory in order to induce CD95/Fas-mediated apoptosis [71]. In fact, actin-linking proteins ezrin, moesin, RhoA small GTPase, and RhoGDI were found to be conveyed into Fas-enriched rafts after anti-Fas MoAbs administration and actin cytoskeleton appeared to be involved in the formation of CD95/Fas clusters in lipid rafts [74]. In this context, cytoskeletal elements could act as

concentrators of death receptors in a sort of chamber where apoptotic cell death could easier be triggered. Interestingly, the release of ezrin from lipid rafts (and of lipid rafts from actin cytoskeleton) can exert a regulatory function also in B cell antigen receptor signaling [75]. This could also be of relevance in the modulation of B cell apoptosis.

Raft-like microdomains on mitochondria

Since receptor-mediated apoptosis in type I cells leads to apical caspase activation, *t*-bid-mediated mitochondrial alterations and subsequently to apoptosis execution via release from mitochondria of apoptogenic factors and activation of executioner caspases, this organelle is the first and essential executioner of apoptosis in type II cells or after the induction of apoptosis by mitochondriotropic drugs. Hence, the analyses of the possible implications of mitochondria remodeling and lipid rafts redistribution during apoptosis recently emerged from literature. In fact, although lipid rafts are considered as ubiquitous constituents of plasma membrane, recent lines of evidence also indicated that they are associated to intracellular organelles, including the Golgi apparatus and a subcompartment of the endoplasmic reticulum (ER) [76, 77]. More recently, it has been observed that ER, although contains relatively low levels of cholesterol and sphingolipids compared with other organelles, shows the presence of lipid raft-like domains characterized by the presence of specific ER lipid raft associated proteins (erlins) [78]. Furthermore, gangliosides are key modulators of intracellular calcium flux. It has been hypothesized that ganglioside accumulation influences the endoplasmic reticulum calcium homeostasis thus contributing to the occurrence of apoptosis [79]. This could be of relevance in consideration of the recently assumed role of ER (ER stress) [80] in the apoptotic process. In this regard, the role of a close association of the ER with mitochondria in apoptosis regulation has recently been suggested [81, 82]. In addition, the possible role of gangliosides in organelle scrambling processes has also to be taken into account [79].

Glycosphingolipids, synthesized from ceramide in the Golgi, are subsequently distributed to different compartments, most predominantly to the plasma membrane. However, a trafficking of ganglioside GD3 to mitochondria has been recently reported [83], thus revealing a novel function of this lipid as a death effector. The presence of a metabolic pathway of sphingolipids, including several enzyme activities of sphingolipid metabolism, has in fact been described in mitochondria [84]. The dynamic mitochondriotropic redistribution of GD3 has been investigated in some recent works. This ganglioside seems to act as an intracellular lipid messenger inducing apoptosis by directly targeting mitochondria [70]. Accordingly, it was demonstrated that proapoptotic activity of GD3 was counteracted by acetylated GD3 (9-O-acetyl GD3) [85] thus indicating the key role played by ganglioside molecule in inducing apoptosis. For instance, raft-like domains, enriched in gangliosides (GD3,GM3), but with a relatively low content of cholesterol, are present on mitochondrial membrane, where Bcl-family proteins (truncated Bid and Bax) are recruited. It was suggested that these domains could bolster mitochondrial subcompartmentalization hijacking human T cells towards a CD95/Fas apoptotic prone phenotype [83].

As regards the loss of mitochondrial membrane potential, a well known alteration of this organelle associated with apoptosis execution via release of apoptogenic factors, e.g. AIF and cytochrome *c*, some studies have been performed [83, 86, 87]. In this regard, GD3 specifically induces gradual depolarization of the inner mitochondrial membrane that is suppressed by cyclosporin A, a mitochondrial pore opening inhibitor [86]. Furthermore, it has been shown that the mitochondrial effects of GD3 ganglioside are selective, since they cannot be mimicked by either GD1a or GM3 gangliosides and lead to the opening of the permeability transition pore [87].

Finally, taking into account the above mentioned implication of cytoskeletal components in ganglioside trafficking through the cell cytoplasm, some insights deriving from different laboratories seem to indicate that GD3 mitochondrial targeting could depend on cytoskeleton function. For example, following TNF-alpha exposure, GD3 undergoes a rapid redistribution, that seems to depend from actin cytoskeleton function [70]. In particular, it has been suggested that cytoplasmic movements of GD3 molecule towards mitochondria are of importance in receptor-mediated apoptosis, e.g. after TNF alpha treatment or CD95 ligation, and could depend on its association with cytoskeleton [70, 71]. Conversely, staurosporin, a drug capable of inducing apoptosis by directly acting on mitochondrial membrane transition, induced neither GD3 redistribution nor GD3-cytoskeleton association [71].

The role of mitochondrial fission and fusion processes has recently been taken into consideration in the apoptotic cell death process [88, 89]. It has been hypothesized that the fusion process could be associated with cell senescence and survival whilst the mitochondrial fission process may occur in cells undergoing apoptosis. Fusion and fission processes are instructed by a series of molecules [89]. Members of the sphingomyelin pathway seem to have a profound influence on these mitochondrial changes occurring in the apoptotic cascade [90]. For example, DRP1 molecule (dynein related protein 1) and *h*-Fis, the human homologue of yeast molecule, known to play a role in mitochondrial fission, seem to play a key role in the apoptotic cell death pathway [88, 91]. In fact, the latter has been described as an integral protein of the outer mitochondrial membrane participating to

the membrane scission events. We have recently suggested a role for GD3 (and possibly GM3) as a mitochondrial structural component involved in the opening of the mitochondrial permeability transition pore and forming a multimolecular complex that includes VDAC-1, Bcl-2 family and fission proteins, e.g. *h*-Fis [83]. The role of gangliosides in this multimolecular system could be to facilitate the transient and local formation of inverted hexagonal structures that undergo the fission process. Mitochondrial lipid microdomains may thus instruct a sort of mitochondrial "chamber" where specific reactions can take place and can be catalyzed, leading to either survival or death of T cells (Fig. 2). Further studies are required to elucidate the role of raft-like microdomains on signal transduction pathways involving mitochondria.

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