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Biochimica et Biophysica Acta 1746 (2005) 284 – 294
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Ceramide-enriched membrane domains

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Received 14 April 2005; received in revised form 5 September 2005; accepted 6 September 2005
Available online 26 September 2005

Abstract

Cellular activation involves the re-organization of receptor molecules and the intracellular signalosom in the cell membrane. Recent studies indicate that specialized domains of the cell membrane, termed rafts, are central for the spatial organization of receptors and signaling molecules. Rafts are converted into larger membrane platforms by activity of the acid sphingomyelinase, which hydrolyses raft-sphingomyelin to ceramide. Ceramide molecules spontaneously associate to form ceramide-enriched microdomains, which fuse to large ceramide-enriched membrane platforms. The acid sphingomyelinase is activated by multiple stimuli including CD95, CD40, DR5/TRAIL, CD20, FcγRII, CD5, LFA-1, CD28, TNF, the Interleukin-1 receptor, the PAF-receptor, CD14, infection with *P. aeruginosa*, *S. aureus*, *N. gonorrhoeae*, Sindbis-Virus, Rhinovirus, treatment with γ-irradiation, UV-light, doxorubicin, cisplatin, disruption of integrin-signaling and under some conditions of developmental death. Ceramide-enriched membrane platforms serve the clustering of receptors, the recruitment of intracellular signaling molecules and the exclusion of inhibitory signaling factors and, thus, facilitate signal transduction initiated by the specific stimulus.

Keywords: Ceramide; Acid sphingomyelinase; Membrane domain; Cellular infection

1. Membrane rafts

Singer and Nicolson suggested in 1972 the classical fluid mosaic model of the cell membrane, which was based on the finding that most physiological phospholipids exhibit low melting temperatures and, therefore, most likely exist in a liquid disordered phase [1]. However, many studies in the last years modified this model and suggested that mammalian membranes contain very small domains that are in a liquid-ordered phase [2–6]. Biological membranes of eukaryotic cells contain large amounts of sphingolipids and cholesterol in addition to glycerophospholipids. Sphingolipids and cholesterol predominantly localize to the outer leaflet of the cell membrane. Sphingomyelin is the most prevalent membrane sphingolipid and is composed of a hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup. Ceramide also forms the backbone of other complex sphingolipids, e.g., cerebrosides and gangliosides. Ceramide is composed of D-*erythro*-sphingosine and a fatty acid containing 2–28 carbon atoms in the acyl chain. Most physiological ceramides display C_{16}- through C_{26}-chains. The sphingosine base is connected with the acyl chain via an amide ester bond. Ceramide is predominantly synthesized in the endoplasmatic reticulum as is cholesterol, while the biosynthesis of sphingomyelin occurs in the Golgi apparatus and to a lower rate also in the plasma membrane [7–9]. The two pools of sphingomyelin are connected by vesicular transport.

Sphingolipids, which have a much higher melting temperature than other phospholipids in the cell membrane, interact with each other via hydrophilic interactions between the sphingolipid headgroups [3–5]. These interactions are stabilized by cholesterol that functions as a spacer between the bulky sphingolipids and interacts with sphingolipids via hydrogen bonds and hydrophobic van der Waal interactions of the sterol ring system and the ceramide moiety of sphingolipids. Cholesterol seems to fill the void spaces between bulky sphingolipids [3–5]. The tight interaction between sphingolipids and cholesterol results in a separation of sphingolipid- and cholesterol-enriched membrane domains from other phospholipids in the cell membrane. Interference with cholesterol metabolism or extraction of cholesterol from rafts destroys these membrane domains indicating the necessity of cholesterol for the integrity of these structures. The formation of distinct

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0167-4889/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2005.09.001

sphingolipid- and cholesterol-enriched membrane domains results in a lateral organization of the cell membrane. Since these distinct domains seem to float in the membrane they were named rafts [3]. Support for the existence of these domains was deducted from the observation that the cell membrane contains domains that are resistant to solubilization by non-ionic detergents such as Triton X-100 at low temperatures [10]. It is thought that the highly ordered spacial structure and the composition of rafts result in a relative resistance of rafts to detergents. These membrane domains were therefore also termed detergent-insoluble glycolipid-enriched membranes (DIGs) or detergent-resistant membranes (DRM). Atomic force microscopy was employed to determine the size of rafts and suggested an approximate diameter of rafts of 50 nm [11]. Many studies made use of labeled cholera toxin that binds to GM1-gangliosides enriched in membrane rafts to indirectly visualize rafts [12].

Sphingomyelin is predominantly, maybe even exclusively, present in the outer leaflet of the cell membrane. Accordingly, rafts may exist in the outer leaflet of the cell membrane, while it is unknown whether the inner leaflet of the cell membrane also contains such distinct domains. It might be possible that the higher organization of lipids in the outer leaflet and the long side chains of ceramide alter the structure/composition of the inner leaflet of the cell membrane and, thus translate the suborganization of the outer membrane leaflet into rafts also to the inner leaflet. It is also possible that unknown principles of lipid organization in the inner leaflet of the cell membrane exist.

Finally, we would like to indicate that the existence of rafts is still controversial [13], although many studies support the idea of membrane rafts.

2. Ceramide-enriched membrane platforms

Many studies in the last years indicated that rafts are merged into large membrane domains upon hydrolysis of sphingomyelin and generation of ceramide within rafts [14–29]. Initial studies indicated that CD95 increases the catalytic activity V_{max} of the acid sphingomyelinase [30–32], which belongs to a class of enzymes that hydrolyzes sphingomyelin and generates ceramide. In addition to a stimulation of the acid sphingomyelinase, CD95 also triggers a translocation of the enzyme onto the extracellular leaflet of the cell membrane bringing the acid sphingomyelinase into close vicinity to its substrate sphingomyelin [19,21] (Fig. 1). The translocation of the acid sphingomyelinase seems to be directed into rafts, since surface acid sphingomyelinase co-localizes with fluorescent-labeled cholera toxin [19], which is used as a marker for rafts. Activation and translocation of the acid sphingomyelinase occurred very rapidly after cellular stimulation and was observed within 10–30 s after stimulation via CD95 [19–21,25]. Ceramide molecules dramatically change the biophysical properties of rafts: Ceramide molecules spontaneously self-associate and have the tendency to form small ceramide-enriched membrane microdomains [33,34]. These microdomains are able to spontaneously fuse to one or a few large ceramide-enriched macrodomains, so called platforms [18,33,34] (Fig. 2). In addition, the generation of ceramide within rafts permits very tight packing of the lipids [34] and strongly stabilizes rafts [35]. Further, ceramide may alter the composition of pre-formed rafts by the exclusion of cholesterol from rafts, which was shown in biophysical studies on model membranes [36]. The formation of ceramide-enriched membrane platforms was demonstrated in fluorescence microscopy studies employing two different antibodies against ceramide [21–29,37]. Furthermore, the local treatment of a phosphatidylcholine/sphingomyelin-composed unilamellar vesicle with sphingomyelinase that was immobilized onto a microbead demonstrated rapid formation of large ceramide-enriched domains in the membrane [18]. This observation indicates that the formation of ceramide-enriched membrane domains does not require cytoskeletal elements, however, the study certainly does not exclude that ceramide-

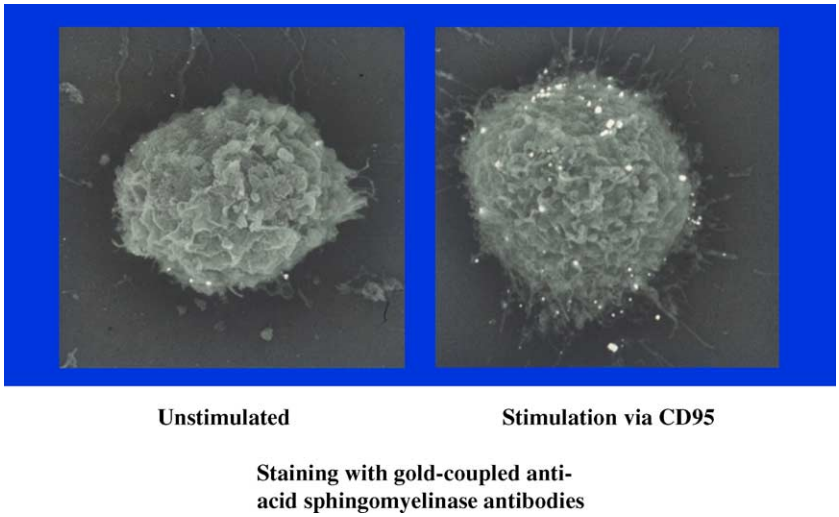

Fig. 1. CD95 induces surface exposure of the acid sphingomyelinase. Jurkat T-lymphocytes were stimulated for 2 min via CD95, fixed and stained with gold-coupled anti-acid sphingomyelinase antibodies. The cells were then analyzed by scanning electron microscopy. The gold particles appear in the analysis as small white dots. The studies demonstrate that CD95-mediated stimulation of the cells results in a marked accumulation of the acid sphingomyelinase on the cell surface. (Printed with permission of the JBC.)

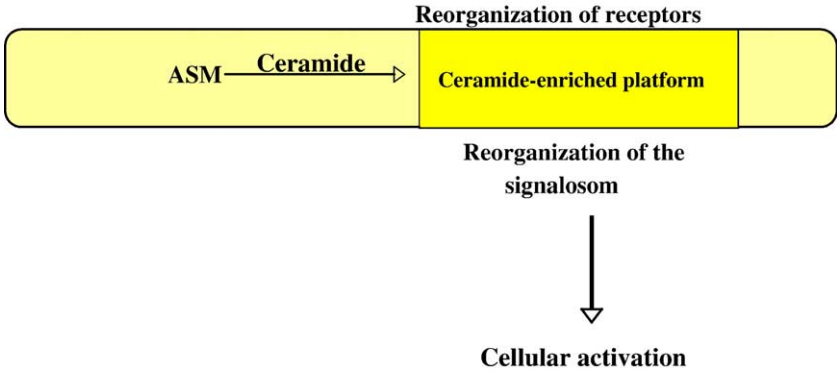

Fig. 2. Ceramide-enriched membrane platforms facilitate signal transduction. Activation and surface-translocation of the acid sphingomyelinase result in the generation of ceramide in the outer leaflet of the cell membrane. Surface ceramide forms large ceramide-enriched membrane platforms that serve the re-organization and clustering of receptor molecules and/or the intracellular signalosom, an event that is required for cellular activation.

enriched membrane domains interact with the cytoskeleton in vivo. Further magnetic resonance spectroscopy studies revealed that incorporation of ceramide into model membranes results in the formation of stable, distinct domains that correspond with a transition of fluid phospholipid bilayers into a gel-like phase [14]. This is consistent with the observation that small amounts of ceramide significantly increase the gel-to-fluid transition temperature of sphingomyelin and, thus, stabilize the liquid ordered state [36]. Further experiments on model membranes demonstrate spontaneous formation of ceramide-enriched domains with as little as 5 mol% ceramide in the membrane [15]. Finally, atomic force microscopy studies on artificial membranes employing C_{16}-ceramide, one of the most prevalent natural ceramides, confirmed the laminar phase separation of long chain ceramides in glycerophospholipid/cholesterol bilayers [16].

In the recent years, a variety of studies supported this model and demonstrated that many receptors or stimuli induce an activation of the acid sphingomyelinase and/or a release of ceramide and/or the formation of ceramide-enriched membrane domains and/or require expression of the acid sphingomyelinase for transmission of the specific biological effect. Those receptors and stimuli include CD95 [19–21,30,31,38–40], CD40 [23], DR5/TRAIL [41], CD20 [27], FcγRII [28], CD5 [42], LFA-1 [43], CD28 [44], TNF [45–47], the Interleukin-1 receptor [48], the PAF-receptor [49], CD14 [50], infection with *P. aeruginosa* [24] (Fig. 3), *S. aureus* [51], *N. gonorrhoeae* [52,53], Sindbis-Virus [54], Rhinovirus [55], γ-irradiation [56,57], UV-light [29,58,59], doxorubicin [60], cisplatin [26], gemcitabine [61], disruption of integrin-signaling [62] and some conditions of developmental death [59,63]. Although the formation of ceramide-enriched membrane domains has not been formally shown for all of those events (in fact for CD95, CD40, DR5, CD20, FcγRII, *P. aeruginosa*, rhinoviruses, UV-light, cisplatin and developmental death of neutrophils), these data may suggest that ceramide-enriched membrane platforms may function as a general motif in signaling transduction.

2.1. Function of ceramide-enriched membrane domains

In general, ceramide-enriched membrane platforms appear to function as a tool that re-organizes receptor and signaling molecules in and at the cell membrane to facilitate and amplify signaling processes via a specific receptor. In this regard, ceramide-enriched membrane domains do not seem to be part of the specific signaling cascade of the activated receptor molecule, allowing these membrane domains to have a general function in many signaling pathways. The reorganization of receptor molecules into ceramide-enriched membrane domains may result in clustering of these receptor molecules and a very high receptor density, which seems to be prerequisite for an effective transmission of many signals into cells. A high

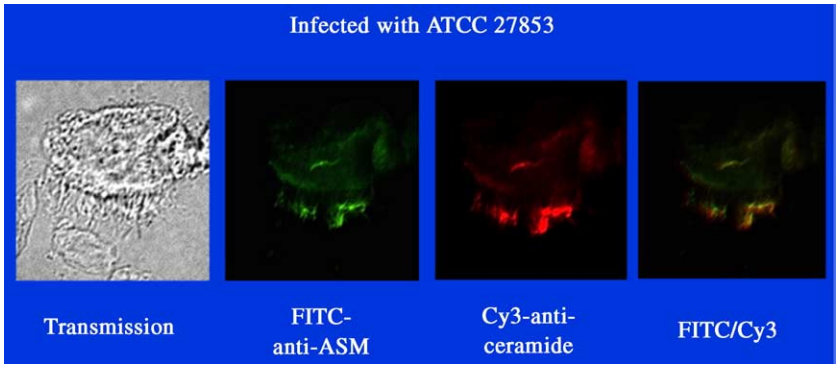

Fig. 3. *P. aeruginosa* induces the formation of ceramide-enriched membrane platforms. Tracheal epithelial cells were infected in vivo with *P. aeruginosa*, the cells were fixed, isolated and stained with FITC-coupled anti-acid sphingomyelinase and Cy3-labeled anti-ceramide antibodies. The results demonstrate the formation of a ceramide-enriched membrane platform that contains the acid sphingomyelinase at the bacterial infection site. (Printed with permission of Nat. Med.)

density of receptor molecules might facilitate downstream activation of molecules that associate with the receptor. Furthermore, ceramide-enriched membrane domains may serve to recruit intracellular signaling molecules that transmit the signal generated by receptor activation into the cell and/or exclude molecules that negatively interfere with the signal transduction pathway initiated by the receptor. The recruitment of signaling molecules to the clustered receptor then enables the receptor to successfully transmit the activation signal. However, at present, it is unknown how ceramide-enriched membrane domains in the outer leaflet of the cell membrane are translated into changes of the protein composition of the inner plasma membrane leaflet. Finally, clustering of a receptor into rafts may stabilize the interaction with the receptor ligand presented on a stimulatory cell, for instance in the immunologic synapse. An increased ligand affinity might be also achieved by a ceramide-mediated alteration of receptor conformation.

All of those events have been described for the CD95 receptor [25]: CD95 clusters in ceramide-enriched membrane domains, which facilitates recruitment of the adapter protein FADD, caspase 8 as well as caspase 3 to the aggregated CD95 receptor and transactivation of associated caspase 8 molecules [25,64].

CD95 clustering, which may serve as a paradigm, is not restricted to a single cell type and was recently demonstrated by six independent groups to occur within seconds in Jurkat cells, SKW 6.4 and JY B cell lymphoma, H9 T cell lymphoma, epithelial and mouse granulosa cells, and in primary murine spenocytes and hepatocytes [65]. The significance of CD95 clustering for the biological effects of this receptor was shown in studies employing acid sphingomyelinase-deficient cells or animals, respectively [19,20,25,39,40]. Acid sphingomyelinase-deficient cells failed to release ceramide upon CD95 stimulation and resisted CD95-induced apoptosis [19,20]. Reconstitution of natural C_{16}-ceramide to acid sphingomyelinase-deficient cells restored the potential of CD95 to trigger apoptosis, supporting the central role of ceramide for CD95-triggered apoptosis. In vivo studies on acid sphingomyelinase-deficient mice confirmed this notion and revealed that acid sphingomyelinase-deficient hepatocytes were more than 10-fold more resistant to induction of apoptosis after intravenous injection of stimulatory anti-CD95 antibodies [39,40]. Very similar data were obtained for TNF-receptor stimulation and acid sphingomyelinase-deficient animals, which were protected from TNF-α induced apoptosis of hepatocytes, hepatic failure and death [46].

Consistent with the notion of an important role of membrane domains for signaling of CD95 are experiments that revealed an inhibition of CD95-induced apoptosis upon extraction of cholesterol or neutralization of ceramide in ceramide-enriched membrane domains using anti-ceramide antibodies [19,37,66,67]. This finding was challenged by a study from Algeciras-Schimnich et al. who failed to show a blockade of apoptosis upon treatment with methyl-β-cyclodextrin [68]. However, it should be mentioned that efficient extraction of cholesterol and destruction of rafts was not controlled and it might be possible that a certain threshold of cholesterol-extraction must be reached to destroy rafts and prevent CD95-induced apoptosis.

Although the above-described studies indicate a central role of the acid sphingomyelinase for CD95-induced apoptosis, it should be noted that some studies came to a different conclusion for the function of the acid sphingomyelinase in CD95- [69–71] or γ-irradiation- [71] triggered apoptosis. A study by Lin et al. [38] demonstrated that acid sphingomyelinase-deficient hepatocytes and mice, respectively, are protected from CD95-induced apoptosis or death, while acid sphingomyelinase-deficient and wildtype thymocytes or Concanavalin- or lipopolysaccharide-pre-stimulated lymphocytes were equally sensitive to CD95-induced apoptosis.

How are ceramide-enriched membrane domains formed if they are required for initiation of signaling for instance via CD95? This hen-and-egg problem is best illustrated by experiments on the ordering of signaling events initiated by CD95 [25]. Ligand binding and cross-linking of CD95 trimers results in a very weak recruitment of FADD and stimulation of caspase 8, which reaches approximately 1% of the levels that are observed for maximal activation of caspase 8. This weak activation of caspase 8 is even observed in acid sphingomyelinase-deficient cells, but it is not sufficient to trigger apoptosis. However, the low activity of caspase 8 is sufficient to trigger the translocation and activation of the acid sphingomyelinase and the formation of ceramide-enriched membrane platforms. In a feed-forward mechanism, these domains then cluster dispersed CD95 molecules and permit efficient recruitment and full activation of caspase 8 to execute death. These studies indicated that clustering of CD95 in ceramide-enriched membrane domains mediates an approximately 100-fold amplification of the signal intensity of the receptor. Very similar findings were recently observed for the DR5/TRAIL system [41].

Many studies demonstrated that a function of ceramide-enriched membrane platforms is not restricted to receptor-mediated signaling. It was shown that γ-irradiation [56,57], UV-A and UV-C light [29,58,59], some chemotherapeutics [26,27,60] and bacterial as well as viral infections [51–54] require the acid sphingomyelinase to trigger apoptosis. The significance of the acid sphingomyelinase and ceramide system for the biological effects of stress was best investigated for γ-irradiation, which may serve as a paradigm for the function of the acid sphingomyelinase and ceramide in the cellular stress response. Acid sphingomyelinase-deficient T- and B-lymphocytes [56] and murine embryonic fibroblasts [72] failed to release ceramide upon γ-irradiation. Moreover, these cells as well as acid sphingomyelinase-deficient oocytes [60,73] and endothelial cells [57] were resistant to γ-irradiation-induced cell death. Likewise, acid sphingomyelinase-deficient mice were protected from radiation-induced ceramide formation and damage in the lung [56], small intestine [57] or brain [74], while radiation-induced cell death was readily observed in normal mice. γ-irradiation of the gastrointestinal tract and the central nervous system primarily targeted endothelial cells and killed those cells via an activation of the acid sphingomyelinase [51,74], while endothelial cells lacking the acid sphingomyeli-

nase were protected from radiation-induced cell death. Endothelial cell apoptosis leads to vascular dysfunction and the gastrointestinal (GI)-syndrome characterized by loss of the water barrier resulting in diarrhoea and dehydration, and loss of the barrier for enterobacteria predisposing to sepsis. Accordingly, acid sphingomyelinase-deficient mice were protected from the development of a GI-syndrome upon radiation, while wildtype mice developed a severe GI-syndrome and died.

A very similar phenomenon was observed for the irradiation of tumors: Tumors that were implanted into acid sphingomyelinase-deficient mice were resistant to γ-irradiation, although the same tumors implanted into wildtype mice were radiosensitive [75]. These studies by R. Kolesnick and Z. Fuks revealed that γ-irradiation triggered apoptosis of wildtype endothelial cells, while acid sphingomyelinase-deficient endothelial cells of the tumor vessels were resistant to irradiation-induced apoptosis resulting in resistance of the tumor to radiation [75]. These data prove a central role of the acid sphingomyelinase in γ-irradiation-induced cell death. However, formally it remains to be determined whether ceramide-enriched membrane platforms mediate the effects of γ-irradiation.

3. Mechanisms of acid sphingomyelinase activation and surface translocation

At present, the molecular mechanisms mediating surface translocation and activation of the acid sphingomyelinase are not well understood. CD95 has been shown to trigger translocation and activation of the acid sphingomyelinase via a recruitment of an adaptor protein, FADD, and activation of a protease, caspase 8 [25,76]. The association of CD95 with FADD and caspase 8 is mediated by a short intracellular domain, the death domain [77,78]. Accordingly, mutation of the death domain and genetic or pharmacological inhibition, respectively, of caspase 8 prevented activation of the acid sphingomyelinase [76,79]. Vice versa, overexpression of FADD or caspase 8, or transfection of a constitutively active caspase 8 mutant resulted in increased ceramide release [80]. In contrast, activation of the acid sphingomyelinase by TNF-R does not seem to be mediated by caspase 8 and an unknown initiator caspase was suggested to function between the receptor and the acid sphingomyelinase [81]. However, since caspases are cytosolic enzymes and the acid sphingomyelinase localizes to abluminal compartments, i.e., vesicles or the outer leaflet of the cell membrane, it seems to be unlikely that caspases directly activate the acid sphingomyelinase, e.g. through limited proteolysis. A recent study employing cells treated with z-VAD or deficient for caspase 8 or FADD, respectively, also demonstrated that activation of capases is required for acid sphingomyelinase-mediated release of ceramide and the formation of ceramide-enriched membrane platforms upon stimulation via CD95, while UV-C light induced these events independent of caspase-activity [59]. These data suggest that several pathways exist to induce an activation of the acid sphingomyelinase and the formation of ceramide-enriched membrane domains.

Further studies indicated an association of the acid sphingomyelinase with phosphatidylinositol-3-kinase (PI-3-K) upon nerve growth factor stimulation of PC12 cells via TrkA [82]. These authors suggested a direct interaction of the acid sphingomyelinase with the regulatory p85 subunit of PI-3-K within rafts. Nerve growth factor-mediated activation of PI-3-K resulted in an approximately 50% inhibition of the activity of the acid sphingomyelinase suggesting that phosphatidyl-inositol-phosphates negatively regulate the acid sphingomyelinase. However, the interaction of the two proteins is difficult to reconcile on the background of the abluminal localization of the acid sphingomyelinase and a cytoplasmic presence of PI-3-K. It might be possible that the acid sphingomyelinase folds into the cell membrane and, thus, might be able to interact with intracellular proteins or lipids, although the acid sphingomyelinase is not known to contain an obvious transmembranous domain.

Several recent studies also demonstrated an inhibition of the acid sphingomyelinase by inositol-phosphates [83,84]. However, the physiological significance of these findings remains to be determined since inhibition of the acid sphingomyelinase required very high phosphate concentrations.

Finally, a study by Qui et al. [85] demonstrated that in vitro oxidation of the cysteine residue 629 of the acid sphingomyelinase molecule results in constitutive activation of the enzyme. Likewise, a copper-promoted dimerization of the acid sphingomyelinase via this cysteine residue stimulated the enzyme's activity. Since many stress stimuli induce the formation of oxygen radicals, an oxidation of the acid sphingomyelinase might also activate the enzyme in vitro, although this mechanism has to be proven in vivo.

The enzyme activity of the acid sphingomyelinase reaches an optimum at an acidic pH suggesting that the enzyme should be only active in the lysosome. However, only the substrate affinity is affected by neutral pH values, which increase the K_m value, but do not alter the activity [V_{max}] of the enzyme [86]. Since many factors including membrane lipids such as cholesterol or LDL have been shown to modify the activity of the acid sphingomyelinase, it is very likely that full activity of the enzyme on the cell surface is achieved even at neutral pH values [87]. For instance a *Bacillus cereus* sphingomyelinase is almost inactive on large unilamellar vesicles without cholesterol [88]. Small quantities of cholesterol slightly increased the enzyme's activity, while an increase of the cholesterol concentration above 25 mol% resulted in a remarkable enhancement of the activity of the enzyme. This finding might also explain why interference of nystatin, filipin or β-methylcyclodextrin with cellular cholesterol prevents the hydrolysis of sphingomyelin by the acid sphingomyelinase.

4. Mechanisms of receptor clustering in ceramide-enriched membrane domains

Although receptor clustering is a general and fundamental mechanism of signal transduction into cells, the molecular and structural mechanisms of the preferred partitioning of a receptor in membrane domains are poorly characterized.

Molecules that are constitutively present in rafts, for instance GPI-linked proteins, might cluster simply by the fusion of small ceramide-enriched microdomains to a large ceramide-enriched membrane domain.

The situation is much more complex for receptors that primarily reside outside of rafts. Studies from our laboratory indicate a central function of the transmembranous domain of CD40 for trapping and localization of CD40 in ceramide-enriched membrane domains [22]. These studies employed chimeric proteins that consisted of the extra- and intracellular domain of CD40 and the transmembranous domain of CD45. CD45 is known to localize outside of rafts, at least under most circumstances. These studies revealed that wildtype CD40 translocates and becomes trapped in ceramide-enriched membrane domains after stimulation, while a chimeric protein consisting of the transmembranous domain of CD45 did not localize into ceramide-enriched membrane domains. This correlated with a failure of the mutant protein to cluster and to signal into the cell upon stimulation under physiological conditions. However, forced, artificial clustering of the CD40/CD45 chimeric protein resulted in activation of JNK, which is a known target of CD40 [89,90], indicating that the exchange of the transmembranous domain did not inactivate the mutant protein. It further emphasizes the importance of receptor clustering for cellular activation under physiological conditions. At present, it is unknown how the transmembranous domain initiates trapping of receptor molecules in ceramide-enriched membrane domains. Binding of the ligand might change the conformation of the transmembranous domain of the cognate receptor and might promote direct interaction of the transmembranous domain with ceramide. However, it also seems to be possible that the formation of ceramide-enriched membrane domains alters the diameter of the cell membrane in this domain. Therefore, the number of hydrophobic amino acids in the transmembranous domain of a receptor may determine whether trapping of a transmembranous protein in ceramide-enriched membrane domains occurs or whether the presence of the protein outside of these domains is energetically more favorable.

5. Regulation of protein function by ceramide-enriched membrane domains

Ceramide generated by the acid sphingomyelinase in the outer leaflet of the cell membrane or within intracellular vesicles may also flip to the cytoplasmic leaflet, although the mechanisms of flipase-like translocation of ceramide from one membrane to another membrane leaflet are unknown. Intracellular ceramide has been described to interact and activate phospholipase A_2 [91], kinase suppressor of Ras (KSR; identical to ceramide-activated protein kinase) [92], ceramide-activated protein serine-threonine phosphatases [93], protein kinase C isoforms [94] and c-Raf-1 [95]. Further, intravesicular ceramide was demonstrated to bind cathepsin D [96]. Ceramide may bind directly to PLA_2 [91] and cathepsin D [96], while the exact mechanism of ceramide-mediated regulation of most of the above proteins is still unknown. The interaction of ceramide with cathepsin D has been matched to a short domain in cathepsin D, which triggers the autocatalytic cleavage of cathepsin D to its active form and seems to promote the translocation of the enzyme from endosomes into the cytoplasm [96,97]. Whether these proteins interact with individual ceramide molecules or with the hydrophobic platform of a ceramide-enriched membrane domain remains to be investigated.

In addition, ceramide-enriched membrane platforms are involved in the regulation of ion channels, in particular the Kv1.3 channel and calcium release activated calcium (CRAC) channels [98–101]. Kv1.3 channels are central for the activation, differentiation, proliferation and regulation of apoptosis in lymphocytes [102,103]. Recent studies indicated that the potassium channel Kv1.3 is constitutively present in membrane rafts and localizes to ceramide-enriched membrane domains after stimulation via CD95 [22]. Translocation of the channel protein into ceramide-enriched membrane domains correlates with an inactivation of the Kv1.3 channel [22]. Likewise, disruption of rafts by cholesterol-extracting drugs inhibits Kv1.3. Ceramide-enriched membrane domains may serve to recruit intracellular signaling molecules, in particular Src-like tyrosine kinases, that inhibit the activity of Kv1.3 [104]. Vice versa, rafts may separate the channel from Src-like tyrosine kinases and, thus, enable the channel to remain in its active status. Therefore, the sorting function of ceramide-enriched membrane platforms may bring the channel into close vicinity to its regulators and permits, for instance, inhibition of Kv1.3 by tyrosine phosphorylation of the protein within ceramide-enriched membrane domains. However, the alteration of the lipid composition in ceramide-enriched membrane domains may also have a direct effect on the activity of the channel. This concept is supported by data from D. Oliver et al. who demonstrated a regulation of A-type potassium channels by membrane lipids, in particular phosphoinositides and arachidonic acid [105]. It could be envisioned that ceramide-enriched membrane domains alter the conformation of Kv1.3 resulting in an inactivation of the channel, while intact rafts promote a conformation that permits channel activity. Alteration of rafts by sphingomyelin hydrolysis and ceramide generation or destruction of rafts by cholesterol-extraction may then inhibit the channel.

Similar effects were described for CRAC channels. CRAC channels are central in the regulation of cellular Ca^{2+} concentrations and, thus, involved in multiple cellular pathways [106]. Stimulation of cells via CD95 [100,107] or the TNF-receptor [101] results in inhibition of CRAC channels, which is dependent on expression of the acid sphingomyelinase. Furthermore, synthetic ceramides, C_2-, C_6- and C_{16}-ceramides, inhibit the activity of CRAC channels. Endogenous ceramide and long chain ceramides may inhibit CRAC channels by the transformation of rafts into ceramide-enriched membrane domains as discussed for Kv1.3, while incorporation of short chain ceramides may interfere with the integrity of rafts.

6. Mitochondrial ceramide-enriched membrane platforms

Several recent studies indicate that ceramide is also present in mitochondria [108–111]. Mitochondrial ceramide could be generated via the de novo synthesis pathway, a reverse activity of the ceramidase [112] and activity of the acid sphingomyelinase, which has been shown to reside in the space between the inner and outer mitochondrial membrane [113]. At present, only the de novo pathway and the acid sphingomyelinase have been implied in mitochondrial ceramide generation. Mitochondrial ceramide has been suggested to be involved in apoptosis [109], however, the mechanisms how mitochondrial ceramide mediates apoptosis remain to be defined. It is possible that mitochondrial ceramide also forms ceramide-enriched membrane domains that serve the re-organization of proteins involved in apoptosis. Furthermore, it has been described that C_{16}-ceramide is able to form large channels [114], which may permit the exit of cytochrome *c* from mitochondria upon induction of apoptosis.

7. Infections via ceramide-enriched membrane platforms

Rafts have been shown to serve many pathogens for infection of cells including several bacteria such as *Escherichia coli*, *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Clostridium difficile*, *Clostridium tetani*, *Salmonella typhi* and *typhimurium*, *Shigella flexneri*, some viruses such as *Inflenza virus*, *HIV*, *Measles virus*, *Respiratory syncytial virus*, *Ebolavirus, Papillomaviridae*, *EBV*, *Echovirus*, *Sindbis virus*, various parasites such as *Plasmodium falciparum*, *Trypanosoma*, *Leishmania* and *Toxoplasma gondii* and even *Prions* (for recent reviews see [115,116]). However, at present it is unknown whether all these pathogens employ membrane platforms for infection of the cells or whether unmodified small sphingolipid- and cholesterol-enriched membrane rafts are sufficient to transmit the infection. Our studies indicate that at least some bacteria and viruses modify rafts into ceramide-enriched membrane platforms. We demonstrated that *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Neisseriae gonorrhoeae* (*N. gonorrhoeae*) and Rhinovirus use the acid sphingomyelinase for the generation of ceramide and ceramide-enriched membrane platforms that serve the infection of the mammalian target cells [24,51–53,55].

These pathogens activated the acid sphingomyelinase and induced a translocation of the enzyme onto the cell surface within minutes after tethering and adhering to the cell, which resulted in the release of ceramide and the formation of large ceramide-enriched membrane platforms in epithelial cells, fibroblasts and endothelial cells. Genetic deficiency or pharmacological inhibition of the acid sphingomyelinase prevented the release of ceramide and subsequent internalization of *P. aeruginosa*, *S. aureus*, *N. gonorrhoeae* as well as induction of apoptosis of the host cells by *P. aeruginosa* or *S. aureus* [24,51–53]. Furthermore, infection of epithelial cells with *P. aeruginosa* resulted in a release of pro-inflammatory mediators, which was significantly increased in cells lacking the acid sphingomyelinase.

In vivo studies with *P. aeruginosa* confirmed a central role in vivo of the acid sphingomyelinase for pulmonary *P. aeruginosa* infections [24]. While normal mice were able to clear a pulmonary *P. aeruginosa* infection, acid sphingomyelinase-deficient mice were unable to eliminate the bacteria, bronchial epithelial cells failed to undergo apoptosis and to internalize the bacteria and the mice responded with a massive release of cytokines in the lung. The mice finally died by sepsis.

P. aeruginosa induces the clustering of CD95 and the cystic fibrosis transmembrane conductance regulator (CFTR) in ceramide-enriched membrane domains [24]. We and others demonstrated that *P. aeruginosa*-induced apoptosis is mediated by CD95 activation [117–119] and, thus, clustering of CD95 in ceramide-enriched domains is very likely the initial event for triggering apoptosis of the host cell. Mechanisms that mediate the internalization of *P. aeruginosa* are much less characterized. It was suggested that CFTR functions as a receptor for *P. aeruginosa* and mediates internalization of the bacteria [120,121]. However, even *P. aeruginosa* strains that do not interact with CFTR are internalized and, thus, additional receptors may exist that mediate internalization [121]. Thus, clustering of CFTR might initiate *P. aeruginosa* internalization in some cases, but additional mechanisms that are also dependent on ceramide-enriched membrane domains seem to be operative in parallel. Finally, at present, it is unknown how ceramide-enriched membrane domains regulate the release of pro-inflammatory mediators such as Interleukin-1 upon infection with pathogens.

In summary, although some receptors that cluster in ceramide-enriched membrane domains upon infection of mammalian cells have been identified [24], the mechanism how ceramide-enriched membrane domains mediate the infection of mammalian cells require further definition.

Further studies indicated that the acid sphingomyelinase and ceramide-enriched membrane platforms are also involved in viral infections of mammalian cells. Data from our group [55] reveal that rhinoviruses very rapidly activated the acid sphingomyelinase, triggered a release of ceramide and induced the formation of large ceramide-enriched membrane platforms. Genetic or pharmacological inhibition of the acid sphingomyelinase prevented rhinoviral infection of human epithelial cells. Furthermore, it was demonstrated that *Sindbis virus* activated the acid sphingomyelinase and mediated the release of ceramide, which was required for the induction of apoptosis in infected cells by this virus [54].

Recent studies discovered a novel function of the acid sphingomyelinase and ceramide in the signaling of platelet activating factor (PAF) and pulmonary failure caused by PAF [49,122]. PAF, one of the most important mediators of septic shock and multi-organ failure, very rapidly activated the acid sphingomyelinase and triggered a release of ceramide in the lung. Intravenous injection of PAF induced pulmonary edema, which was significantly reduced in acid sphingomyelinase-deficient mice. Combined inhibition of the acid sphingomye-

linase and the cycloxygenase, which is also activated by PAF, completely prevented pulmonary edema. PAF was also demonstrated to induce externalization of phosphatidylserine on the surface of erythrocytes, which resulted in a pre-mature deletion of erythrocytes from the blood [122]. Inhibition of the acid sphingomyelinase prevented these events elicited by PAF. Therefore, it seems likely that further systemic events in patients with sepsis are mediated by the acid sphingomyelinase and ceramide.

Finally, recent studies on the maturation of phagosomes suggest that sphingolipids and ceramide are involved in the intracellular processing of vesicles. Internalization of bacteria into mammalian cells results in the formation of vesicles, so called endosomes. Endosomes are transported in the cell, mature and fuse with lysosomes to form a phagolysosome, which are central for the degradation of the bacteria. The fusion of the two vesicles requires the coordinated assembly of actin filaments on phagosomes. Studies by Anes et al. [123] demonstrated that this fusion process is regulated by sphingolipids including ceramide that seem to serve as a binding and nucleation site for the interaction of actin with phagosomes.

7.1. Perspectives

The comprehensive concept of ceramide-enriched membrane domains and the ceramide-mediated transformation of very small “inactive” membrane rafts into large, “active” signaling platforms explains the function of ceramide for cellular activation by many and diverse receptors, stress stimuli and even developmental processes. Ceramide does not seem to play a specific role in many of these signaling pathways, but reorganizes receptor molecules and the signalosom in the cell and is a pre-requisite for signal transduction by the above-discussed pathways. The important role of ceramide and ceramide-enriched membrane platforms, respectively, in cellular signal transduction, suggest that a pharmacological manipulation of these membrane domains might be useful to treat at least some forms of cancer, infectious diseases or chronic degenerative disorders.

Acknowledgement

The authors would like to thank S. Moyrer for excellent support.

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