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CERAMIDE-RICH PLATFORMS IN TRANSMEMBRANE SIGNALING

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Abstract

Recent evidence suggests that ceramide regulates stress signaling via reorganization of the plasma membrane. The focus of this review will be to discuss the mechanism by which acid sphingomyelinase (ASMase)-generated ceramide initiates transmembrane signaling in the plasma membrane exoplasmic leaflet. In particular, we review the unique biophysical properties of ceramide that render it proficient in formation of signaling domains termed ceramide-rich platforms (CRPs), and the role of CRPs in the pathophysiology of various diseases. The biomedical significance of CRPs makes these structures an attractive therapeutic target.

1. Introduction

Two decades ago, based on the proposal that sphingolipids may be bioeffector molecules [1], our laboratory proposed the existence of a signaling pathway initiated by hydrolysis of sphingomyelin (SM) to ceramide by the action of a sphingomyelinase (SMase) [2-4]. While initial studies conceived of a pathway similar in concept to that of the phosphoinositide pathway where ceramide served exclusively as a second messenger stoichiometrially activating protein targets to confer transmembrane signaling, experiments from a number of biophysical laboratories began to alter the perception of how this system might function. Specifically, studies in model membranes showed that ceramide generation changes membrane structure, leading to formation of macrodomains [5,6], concepts adapted for eukaryotes over the past decade [7]. This review details the capability of ceramide to reorganize membranes based on its unique biophysical properties, linking its biological and biomedical functions.

2. Ceramide: Biology and Biophysics

Ceramide is an evolutionarily conserved second messenger that plays a ubiquitous, conserved role in biologic processes as diverse as apoptosis, growth arrest, senescence and

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differentiation [8-12]. Ceramide is an N-acylsphingosine consisting of a fatty acid bound to the amino group of the sphingoid base, sphingosine. In nature, ceramides are usually found with monounsaturated or saturated fatty acids of various lengths from 2 to 28 carbon atoms, that sometimes may contain a hydroxyl group on either the C-2 position (α-hydroxyceramide) or on the terminal carbon atom (ω-hydroxyceramide) [13]. The fatty acid chain length significantly alters the physical properties of ceramide [14,15]. Ceramides with fatty acyl chains of 12 carbons or longer, i.e. long-chain ceramides, belong to the category of "non-swelling amphiphiles", as they cannot give rise to micelles or other aggregates in aqueous suspension, and hence cannot serve as detergents [16]. Alternatively, short-chain ceramides swell in water, a property that limits their utility in research. The most commonly-found mammalian ceramides in cellular membranes contain fatty acyl chains of 16-24 carbon atoms, and are among the least polar and most hydrophobic of membrane lipids. While free ceramides are found in abundance in skin stratum corneum, rendering impermeability to this barrier, these ceramides often contain free fatty acids of extraordinary length and unusual structure [13,17,18].

Depending on cell type and stimulus, ceramide can be generated either through sphingomyelinase (SMase)-dependent catabolism of sphingomyelin (SM), a *de novo* synthetic pathway, a salvage synthetic pathway, or at times through other cell-type specific mechanisms. SMase is a specialized form of phospholipase C, which cleaves the phosphodiester bond of SM, generating ceramide. Several SMase isoforms exist and are distinguishable by their pH optima, ion dependence and subcellular localization. SMases are classified in three main groups – acid SMase (ASMase), neutral SMase (NSMase), and alkaline SMase (Alk-SMase) [19]. While ASMase and NSMase were long believed to be the only two forms of SMase involved in signal transduction, new reports suggest potential signaling roles for Alk-SMase [20]. SMase-mediated ceramide generation is a rapid event localized primarily within the plasma membrane, however ceramide may also be generated in a more prolonged fashion via *de novo* synthesis, exclusively intracellularly within the endoplasmic reticulum (ER) or mitochondrial-associated membrane (MAM) [21-24]. *De novo* ceramide biosynthesis is regulated by ceramide synthase gene products which N-acylate sphinganine to form dihydroceramide, which is subsequently converted to ceramide by a desaturase [25]. In mammals, six isoforms of ceramide synthase have been identified, each displaying a high specificity toward acyl CoAs of different carbon backbone lengths, hence synthesizing ceramides of distinct chain lengths [26]. Findings in the recent years identified a novel mechanism of ceramide accumulation from the catabolism of complex sphingolipids that are eventually broken down into sphingosine, which is then reused through re-acylation to produce ceramide. This latter pathway has been referred to as either sphingolipid recycling or the salvage pathway. It involves a number of key enzymes that include ASMase, possibly glucocerebrosidase (acid-β-glucosidase), ceramidases, and (dihydro)ceramide synthases (for review of this topic see [27]). Finally, hydrolysis of gangliosides at the plasma membrane might also contribute to the generation of ceramide signaling in some systems [28].

As a second messenger, ceramide plays a ubiquitous role in diverse biologic processes [19,29]. While the exact mechanism by which ceramide performs its biologic functions is

not completely understood, findings in the last decade suggest that changes in local membrane structure induced by ceramide accumulation are essential for its biologic function. A better understanding of ceramide's effect on model and biologic membranes has been provided by a number of biophysical research groups, summarized below. Due to those properties ceramides mix poorly with phospholipid monolayers or bilayers, segregating into distinct, high temperature melting ceramide-enriched domains. In this review, we focus on four properties of ceramide that mediate its function: its ability to induce an increase in molecular ordering and domain formation, undergo lamellar phase destabilization and membrane fusion, confer membrane permeabilization, and facilitate transbilayer lipid movement.

2.1. Lipid Order and Domain Formation

Within lipid membranes, an increase in ceramide content results in increase in lipid order as determined by diphenylhexatriene (DPH) polarization and NMR spectroscopy. DPH, a fluorescence probe, routinely used to study lipid chain order, increases in fluorescence yield with increasing molecular order. Utilizing this method, Holopainen *et al.* showed that both natural ceramides and chemically-defined C_{16}-ceramide lead to dose-dependent increase in lipid acyl chain order of dimyristoylphosphatidylcholine and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) bilayers, respectively [30,31]. Moreover, *in situ* ceramide generation by SMase action upon SM/phosphatidylcholine (PC) bilayers leads to increased DPH polarization [31]. These findings were confirmed by Massey *et al.* who demonstrated that increasing ceramide concentration increased DPH polarization in dipalmitoylphosphatidylcholine (DPPC)/POPC/SM bilayers, ultimately increasing the gel-fluid phase transition temperature [32]. Similarly, H^2-NMR spectroscopy studies demonstrated that both bovine brain ceramide and synthetic C_{16}-ceramide lead to large increases in acyl chain order parameters of fully deuterated fluid state d_{62}-DPPC and d_{31}-POPC [33-35]. These studies also showed that ordering of ceramide chains was greater than that of phospholipids, such as POPC [33-35].

Ceramide-induced increase in lipid order in phospholipid bilayers ultimately leads to lateral phase separation and formation of ceramide-enriched domains, as initially demonstrated by H^2-NMR studies. These studies showed that ceramide addition induces lateral phase separation of fluid phospholipid bilayers into regions of gel and liquid phases, with ceramide partitioning into the gel phase [34,35]. Furthermore, these domains were detected by Holopainen *et al.* in PC membranes using a pyrene-labeled phospholipid (1-palmitoyl-2[(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine), a fluorescence probe sensitive to lateral mobility [31]. Similarly, differential scanning calorimetry and infrared spectroscopy studies demonstrated that in the lamellar phase, ceramides do not mix well with phospholipids, segregating into high-temperature melting ceramide-enriched domains [36]. In fact a small increases in ceramide levels can significantly affect membrane properties, as demonstrated by fluorescent spectroscopy and transmission electron microscopy (TEM) studies of C_{16}-ceramide/POPC bilayers, where at concentration as low as 2 mol% ceramide strongly affects POPC fluid matrix [37].

Ceramide-enriched domains in SM/ceramide vesicle bilayers were described by Sot *et al.* Giant unilamellar vesicles (GUVs) composed of SM and egg ceramide were doped with fluorescent DiI_{C18}, a probe that partitions preferentially into more fluid membrane regions. Fluorescence microscopy analysis indicated that while pure SM vesicles appear uniformly stained, those containing egg ceramide displayed dark areas corresponding to rigid, ceramide-rich domains at as low as 5 mol% ceramide. Increasing ceramide concentration further increased the dark areas, and caused a shape change from circular to elongated eventually leading to coalesce of these domains into a single continuous domain [38].

Since in biologic systems and in particular during apoptotic signaling, membrane ceramide is generated via SMase-catalyzed cleavage of the phosphodiester bond of SM, a number of biophysical studies of domain formation were performed in ceramide/SM mixtures to show that ceramide generated *in situ* by SMase segregates laterally in the same way as natural or synthetic ceramide added exogenously. Holopainen *et al.* first observed that addition of bacterial SMase to SM-containing vesicles changes lateral membrane organization, forming detectable ceramide-rich domains by fluorescence spectroscopy [31]. Subsequently, Nurminen *at el.* showed that contacting SMase-tethered amino-derivatized acrylate microspheres to GUVs containing fluorescent Bodipy(Bdp)-labeled SM leads to formation of ceramide-rich domains. As observed by fluorescence microscopy, ceramide generation reorganized GUV membrane, with Bdp-labeled ceramide molecules accumulating at one pole of the GUV, whereas prior to ceramide generation Bdp-labeled SM was homogeneously distributed across the membrane (Figure 1) [39].

In situ ceramide generation by SMase action in model membranes of biologic relevance was further examined by combining fluorescence and atomic force microscopy (AFM). These studies showed that *in situ* ceramide formation leads to changes in height and shape of lipid domains in equimolar DOPC/SM/cholesterol supported bilayers, as well as in bilayers containing protruding SM/cholesterol-rich domains that mimic lipid rafts [40-42]. In fact, studies by Ira and Johnson provided direct visualization of ceramide-induced clustering of small nanometer-sized domains into large ordered domains with diameters as large as 1 μm. Many of these domains were raised 2 nm above the surface of the membrane, likely caused by formation of ceramide in the upper membrane leaflet. Alternately**,** high ceramide concentration in the lower leaflet resulted in negative membrane curvature, discussed in the following sections [41-43].

2.2. Non-lamellar Phase Transition, Membrane Fusion and Fission

Ceramide facilitates transition from a lamellar to non-lamellar membrane phase, more specifically to an inverted hexagonal phase, in model phospholipid membranes as demonstrated by ^{31}NMR spectroscopy, differential scanning calorimetry and X-ray scattering [15,36,44]. The ability of ceramide to stabilize inverted phases has been attributed to its geometry, as ceramides, unlike most other lipids, have a small headgroup, easily accommodated into non-lamellar phases [45]. This property has been subsequently linked to ceramide's ability to facilitate membrane curvature leading to fusion and fission [45,46]. Currently, the most widely-accepted mechanism for membrane fusion involves transient participation of a non-lamellar structure, the "stalk", a semitoroidal structure that allows

merger of apposed membrane leaflets. Due to its ability to promote non-lamellar phase stabilization, ceramide can similarly facilitate stalk formation [6,47].

Effects of ceramide on membrane fusion were first demonstrated by Ruiz-Arguello in large unilamellar vesicles (LUVs) containing SM, phosphatidylethanolamine (PE) and cholesterol. SMase treatment of these vesicles led to ceramide generation in seconds accompanied by vesicle aggregation, indicated by cryo-transition or TEM. Further, addition of ceramide at 5-10 mol% in PC-containing vesicles reduced the time required for fusion of smaller vesicles in response to addition of phospholipase C [44,48]. Similar to fusion, ceramide-induced budding of vesicles, i.e. fission was demonstrated following addition of SMase by microinjection or coupled to amino-derivitized acrylate microspheres to PC/SM and SM GUVs, respectively. SMase-induced SM cleavage and ceramide generation resulted in shedding of small membrane vesicles into the GUV interior, visualized by fluorescence microscopy [39,49]. The authors attributed this phenomenon to the tendency of ceramide to separate into domains, induce negative spontaneous curvature and the augmented bending rigidity of the ceramide-enriched domains, leading to bilayer invagination and vesiculation.

Biologic significance of the biophysical findings confirming fusagenic properties of ceramide was demonstrated in ATP-depleted macrophages and fibroblasts. Although ATP hydrolysis has been regarded as a general requirement for internalization processes in mammalian cells, treatment of ATP-depleted macrophages and fibroblasts with exogenous SMase rapidly induced formation of numerous vesicles, approximately 400 nm in diameter, not enriched in clathrin or caveolin, that pinched off from plasma membrane into cytoplasm [50]. The authors hypothesized that the reason that SMase activity and ceramide formation override the requirement for ATP hydrolysis in the process of vesicular formation lies in the ability of ceramide to cause negative curvature, as demonstrated by Helfritch *et al.* [45]. High ceramide concentrations would in turn lead to vesicle fission by the mirror image of their facilitation of fusion [36]. Ceramide-induced vesicle fusion was also demonstrated in a cellular system, sea urchin cortical vesicles treated with SMase [51]. Finally, ability of ceramide to induce membrane curvature, leading to fusion and fission, may have an important function in pathogenic infections, which will be described in more detail in subsequent sections of this review. ASMase has been shown to be obligatory for internalization of a number of pathogens, as well as a subsequent intracellular immune response, early stages of which are mediated by fusion of intracellular phagosome with lysosome leading to pathogen eradication [52].

2.3. Membrane Permeability

Ceramide-induced membrane permeability was initially demonstrated in model membranes by Ruiz-Arguello *et al.* The authors used LUVs composed of SM, PE and cholesterol that contained an entrapped fluorescent dye solution [44]. SMase treatment induced ceramide formation and leakage of intravesicular aqueous content. This effect, independent of the specific lipid composition of the LUV or the type of the dye entrapped, was also observed in fluorescent dye loaded and resealed erythrocyte ghosts after SMase addition. These findings were confirmed by Montes *et al.* in LUVs loaded with fluorescin-derivatised dextrans,

where addition of *Bacillus* SMase or long-chain ceramides released dextrans of molecular mass of ~20 kDa [53].

Physiologic relevance of ceramide-induced membrane permeability was suggested by studies of Siskind and Colombini. Using electrophysiologic detection methods, the authors provided evidence that ceramide forms channels in isolated mitochondria leading to increased permeability of mitochondrial outer membranes to cytochrome c and other small proteins [54,55]. An additional aspect of these studies, strenghtening the argument that ceramide-induced increase in membrane permeability mediates apoptosis induction following certain stimuli, is the demonstration that dihydroceramide, which is inactive in promoting apoptosis at the cellular level, has minimal permeabilizing activity in planar lipid bilayers, and in fact inhibits mitochondrial permeabilization by ceramides [55]. Further, recent evidence by Siskind *et al*. reveals the relationship between ceramide channels in mitochondria and Bcl-2 family members, key regulators of mitochondria-mediated apoptosis. These studies showed that anti-apoptotic proteins, Bcl-xL and Bcl-2, disassemble ceramide channels in the mitochondrial outer membranes of isolated mitochondria from rat liver and yeast, suggesting ceramide channels as one mechanism for releasing pro-apoptotic proteins from mitochondria during the induction phase of apoptosis [56].

While a mechanism for ceramide-induced increase in permeability has not yet been elucitated, this phenomenon was attributed to the ceramide's molecular structure, its preference for domain formation and transmembrane movement [6]. Colombini and co-workers postulated a model of a ceramide pore consisting of columns of four to six ceramide molecules linked by hydrogen bonds via amide groups and arranged as parallel or anti-parallel staves [57]. Since addition or generation of ceramide *in situ* is required to induce leakage, it has been suggested that the process of generation of microdomains rich in ceramide within the disordered sea of lipids induces membrane leakage at the interfaces between ceramide-rich and poor domains [58]. Additionally, the ability of ceramide to undergo "flip-flop" diffusion (described below), leading to transbilayer lipid movement could lead to discontinuities in the membrane perneability barrier [6].

2.4. "Flip-flop" Transbilayer Diffusion

Spontaneous transbilayer movement of ceramide has been demonstrated in lipid vesicles and cell membranes. Kinetic studies of "flip-flop" movement of a fluorescent analog of ceramide in POPC vesicles by Bai and Pagano reported that ceramide has a considerably faster flip-flop rate than phospholipids [59]. Lopez-Montero *et al.* subsequently showed that incorporation of a small percentage of ceramide into the external leaflet of egg PC GUVs leads to a reversible shape change from prolate to pear-shaped. The reversibility of the shape change implied transmembrane movement of lipids, more specifically ceramide, which was confirmed with spin-labeled ceramide analog incorporated into LUVs [60]. Additional studies demonstrated ceramide "flip-flop" in model and cellular (i.e. LUVs and erythrocyte ghost, respectively) membranes following ceramide generation *in situ* [61]. When GM3 was limited to the outer leaflet of neuramidase-containing SM/PE/cholesterol LUVs, Contreras *et al.* demonstrated that external addition of SMase caused GM3 hydrolysis. The most parsimonious explanation of these data is that ceramide generation in the outer leaflet

promoted lipid exchange with the inner leaflet, thus permitting GM3 access to the entrapped neuramidase [61]. In a parallel study, a preparation of SM-containing liposomes or erythrocyte ghosts was labeled with a fluorescent energy donor NBD-PE in the inner leaflet. Addition of SMase to hydrolyze SM to ceramide was accompanied by fluorescence energy transfer to an impermeable acceptor in the outer aqueous medium, suggesting the NBD-PE flipped to the outer monolayer [61]. Similar effects were observed when egg ceramide, but not dihydroceramide, was added externally to pre-formed vesicles [62].

The suggested reasons for the ceramide-induced transbilayer lipid movements have been two fold [6]. The first is ceramide's property to facilitate lamellar to non-lamellar phase transition, mentioned above. The idea is that formation of ceramide on one side of the membrane would induce the transient formation of non-lamellar structural intermediates, leading to loss of membrane bilayer asymmetry, mixing of surrounding lipids, and as the structure collapses transbilayer lipid exchange. The second reason was attributed to general membrane properties, such as a tendency for mass conservation in each membrane leaflet. When one membrane layer becomes enriched with ceramides, they diffuse towards the other leaflet, leading to movement of non-ceramide lipids in the opposite direction so that the net mass transfer between monolayers is avoided. The observation of the flip-flop movement of ceramide between membranes would appear particularly relevant to its biologic effects in mammalian cells, especially in the SMase signaling pathway. SMase catalyzed SM hydrolysis and ceramide formation occur on the outer plasma membrane because most mammalian SM is found on the exoplasmic leaflet of the plasma membrane. Spontaneous transbilayer movement of ceramide is hence necessary for its interaction with intracellular targets. The following section attempts to describe the biologic effects of ceramide in mammalian membranes in terms of the biophysical parameters described above.

3. Ceramide-rich Platforms

ASMase-mediated ceramide generation on the exoplasmic leaflet of the plasma membrane alters membrane structure with substantive consequence for transmembrane signaling. The unique biophysical properties of ceramide, described in the previous section, such as its preferential self-association and ability to act as a fusagen, mediate formation of ceramide-rich platforms (CRPs) with diameters of 200 nm up to several microns. These macrodomains can be visualized by a variety of techniques including scanning electron microscopy, and conventional and confocal fluorescence microscopy, as depicted in Figure 2 [63]. CRPs appear to be sites of protein oligomerization for the purpose of transmembrane signaling, reviewed below. While recent evidence indicates that NSMase, and perhaps even Alk-SMase may be involved in transmembrane signaling, far less is known about signaling via these two enzymes than via ASMase. Hence, the remainder of this review will focus on ASMase-initiated signaling at the plasma membrane.

3.1. Biology of ASMase

While ASMase was originally considered strictly lysosomal because of its pH optimum at 4.5-5.0, Liu and Anderson observed ASMase localization within secretory vesicles at the plasma membrane [64]. Subsequent studies determined that the enzyme exists in two forms, termed lysosomal SMase (L-ASMase) and secretory SMase (S-ASMase), differing in

glycosylation pattern and NH_2-terminal processing, and consequently in subcellular targeting. Contrary to original belief, Schissel *et al.* determined that because only the on and off rate of the substrate, rather than the catalytic activity of the enzyme, is regulated by pH, ASMase can also hydrolyze SM at the neutral pH found at the cell surface, albeit with lower efficiency [24].

L-ASMase and S-ASMase are derived from the same gene and 698 amino acid protein precursor, as first demonstrated by Sandhoff and colleagues [65]. Using pulse-chase metabolic labeling, they suggested a stepwise and compartment-specific processing of a common primary translation product of 75 kDa (preproform), converted into 72 kDa precursor (proform) in the ER/Golgi complex. Soon after entry into the ER, the N-terminal signal sequence (amino acids 1-46) is removed by a signal peptidase. Several additional residues following the signal sequence are also removed, as recombinant human mature ASMase was reported to contain 570 amino acids, pointing to a loss of the first 59 amino acids in total. In fact, the N-terminal amino acid sequence analysis reported by Tabas and colleagues revealed that ASMase isolated from the lysosomal fraction of CHO cells began with Gly_{66}, whereas S-ASMase purified from conditioned media of these cells contained an additional 6 amino acids and began with His_{60} [66]. Sandhoff and colleagues reported additional N-terminal processing of L-ASMase that was isolated from human placenta. N-terminal sequencing of this particular enzyme determined that Gly_{83} was the first amino acid [67]. These data collectively explain the more rapid migration of L-ASMase than S-ASMase on SDS-PAGE, which is due both to additional N-terminal proteolytic processing of L-ASMase and differences in the oligosaccharide profile between the two enzyme forms. Glycosylation of ASMase is thought to promote proper folding and trafficking, as well as serve a protective role, preventing destruction in the harsh environment of the lysosome. There are six predicted N-glycosylation sites on the ASMase polypeptide chain, of which five have been confirmed. The differences in glycosylation pattern between L-ASMase and S-ASMase are such that while L-ASMase possesses a high mannose N-glycan composition, and hence is sensitive to Endo H cleavage (Endo H cleaves Asn-linked mannose rich oligosaccharides, but not highly processed complex oligosaccharides), S-ASMase exhibits a complex type pattern [66].

Following glycosylation, the mannosylated polypeptide precursor enters either the lysosomal or secretory pathway. In the lysosomal pathway, ASMase undergoes modification and trafficking which is typical for lysosomal proteins – acquisition of mannose-6 phosphate residues by the sequential actions of N-acetyl glucosamine-1-phosphotransferase and N-acetyl glucosamine phosphodiesterase on mannose residues of the precursor. Vesicles containing mannose-phosphate receptors then shuttle the modified enzyme to the early endosome or late endosome/prelysosome. In contrast, S-ASMase escapes the mannose-6 phosphate shuttle and is directed into the secretory pathway [66].

Both forms of ASMase are metalloenzymes containing several highly conserved Zn^{2+} binding motifs and are activated by Zn^{2+}. Unlike S-ASMase, L-ASMase is exposed to Zn^{2+} during trafficking to or within lysosomes (and/or during cellular homogenization), and does not require addition of exogenous Zn^{2+} for *in vitro* activity. Studies by Tabas and colleagues confirmed the prediction that ASMase is exposed to and binds intracellular Zn^{2+} during

trafficking to lysosome by following endocytosis of highly purified secreted FLAG-tagged S-ASMase (which is Zn^{2+}- dependent) in ASMase null fibroblasts [66]. Following 16 hours incubation, catalytically active ASMase was detected in the lysosomes, where its *in vitro* activity was not dependent on exogenously-added Zn^{2+}. In contrast, activity of ASMase that was not endocytosed was almost entirely Zn^{2+}-dependent [66]. An exception to the Zn^{2+} requirement for *in vitro* activity of S-ASMase was observed in endothelial cells, which are a particularly rich source of this form of ASMase. S-ASMase from endothelial cells is not entirely dependent on the exogenous addition of Zn^{2+}, indicating that subcellular localization of Zn^{2+} and the exposure of ASMase to its intracellular stores might be subjected to cell type variation or regulation [68].

Activation of ASMase by various stimuli has been extensively studied in the past decade. ASMase is activated via engagement of the TNF receptor superfamily members - Fas [69-71], CD40 [72], DR5 [73] and TNFα [74,75]. Further, a number of groups demonstrated activation of ASMase by various stress stimuli, such as LPS [76,77], disruption of integrin signaling [78], engagement of the PAF-receptor [79], UV-light (UV-A [80] and UV-C [81-83]), heat [84,85], oxidative stress [86], chemotherapeutic agents (cisplatin [87], gemcitabine [88], doxorubicin [89], etoposide (Jacobi and Haimovitz-Friedman, unpublished)), ionizing radiation (IR) [90,91] and accumulation of Cu^{2+} [92]. Further, *Neisseriae gonorrhoea* [93], *Pseudomonas aeruginosa* [94], *Staphylococcus aureus* [95], rhinovirus [96] and Sindbis virus [97] lead to ASMase activation, which is instrumental in pathogen internalization and host cell response. Finally, receptors with various functions that mediate differentiation, phagocytosis, regulation of cell adhesion or induce inflammation, namely CD20 [98], Interleukin-1 [99], CD5 [100], CD28 [101], LFA-1 [102] and FcγRII [103] have also been shown to activate ASMase.

Initial studies that demonstrated ASMase activation by cellular stress were performed by Gulbins *et al.*, who demonstrated that Fas stimulation increases the V_{max} of ASMase, accompanied by enzyme translocation onto the outer leaflet of plasma membrane, bringing it into the proximity of its substrate, SM [69,71,104,105]. While it has not been formally shown which form of ASMase (or perhaps both) is the form involved in transmembrane signaling, by virtue of its location and cell-type preferential expression, we believe that S-ASMase in most instances represents the stress signaling form.

The mechanisms mediating surface translocation and activation of ASMase are not well understood. Fas triggers this process by recruitment of a small amount of the adaptor protein FADD and initiator caspase 8 (see below for details). In this regard, mutation of the Fas death domain, which mediates the interaction with the adaptor protein FADD and with caspase 8, or genetic and pharmacologic inhibition of caspase 8, inhibit ASMase activation [106,107]. Alternately, Rotolo *et al*. demonstrated that while Fas-induced ASMase activation and ceramide generation is inhibited in caspase inhibitor zVAD-treated or in caspase 8- or FADD-deficient cells, UV-C triggers these events independently of caspase activity [82]. Furthermore, ASMase activation via the TNF-R55 does not seem to be mediated by caspase 8, but rather by another unknown initiator caspase [108]. These data suggest that several pathways exist to initiate surface translocation and ASMase activation, even in response to stimuli that lead to the same outcome.

Additional *in vitro* studies by Qui *et al.* demonstrated that oxidation of cysteine 629 of ASMase leads to constitutive enzyme activity [109]. Although not yet proven *in vivo*, it is an attractive proposal since many stress stimuli known to activate ASMase induce formation of oxygen radicals. In fact, Charruyer *et al.* showed that UV-C irradiation results in redox-dependent activation and translocation of ASMase to the outer leaflet of the plasma membrane, ceramide generation and apoptosis [81]. Similarly, Dumitru *et al.* demonstrated that TRAIL (TNF-related apoptosis-inducing ligand) induces rapid release of ROS prior to ASMase activation [73]. In this regard, the antioxidants Tiron or N-acetycysteine blocked TRAIL-induced ASMase activation, ceramide generation and apoptosis in BJAB cells or splenocytes [73]. Finally, studies in a radiosensitive human head and neck squamous carcinoma cell line (SCC61) showed that IR induces ASMase translocation and activation, abolished by ROS scavengers [110].

3.2. Structure of Ceramide-rich Platforms

CRP formation was first described following stimulation of the Fas receptor in Jurkat T and JY B lymphocytes [69,71]. The authors demonstrated that Fas ligand (FasL) activates ASMase and signals its translocation onto the extracellular leaflet of the plasma membrane. Electron microscopy studies using nanogold-coupled anti-ASMase antibody suggested that the enzyme localizes within intracellular vesicles, mobilized upon Fas stimulation. These vesicles subsequently fuse with plasma membrane exposing ASMase on the outer leaflet bringing it into contact with its substrate SM [69,104]. ASMase activation and translocation was detected within minutes of Fas stimulation, leading to ceramide generation and CRP formation within the same time frame. The mechanism of Fas-induced CRP formation was thereafter shown operative in many other cell types including SKW 6.4, JY B cell lymphoma, H9 T cell lymphoma, human peripheral blood lymphocytes, epithelial and mouse granulose cells, lung epithelial cells, primary murine splenocytes and hepatocytes, to list a few [111].

Because of the membrane localization of SM, CRPs are assumed to be derived from rafts, liquid ordered microdomains within plasma membrane, however definitive experimental proof for this hypothesis has yet to be provided. SM is the main sphingolipid component of plasma membrane and as much as 70% of SM is found in the outer leaflet of plasma membrane. Sphingolipids have significantly higher melting temperature than other phospholipids and interact with each other via hydrophilic interactions between their head groups and hydrophobic interactions between their side chains. Their molecules pack tightly in membranes due to their saturated hydrocarbon chains, contrasting with unsaturated fatty acyl chains of other phospholipids in the membrane. Interactions between sphingolipid molecules are additionally stabilized by cholesterol, which fills void spaces by interacting with SM via hydrogen bonds and hydrophobic van der Waals interactions of the sterol ring system and SM's ceramide moiety [112-114]. Tight packing of sphingolipids, hydrophilic and hydrophobic interactions between SM molecules, stabilized by cholesterol, lead to lateral separation of these lipids from bulk phospholipids, resulting in spontaneous formation of sphingolipid and cholesterol-enriched liquid ordered microdomains, termed rafts [114]. Treatment with drugs that sequester or extract cholesterol from membrane, such

as β-cyclodextrin, filipin or nystatin, leads to disruption of these ordered domains, confirming the stabilizing function of cholesterol [115].

Translocation of ASMase appears targeted to rafts, as fluorescence microcopy studies in Jurkat T lymphocytes demonstrated that surface ASMase co-localizes with the raft marker GM1 [69,71,104]. Further, studies in model membranes showing that cholesterol can modify the activity of ASMase provide an additional hint regarding the location of its plasma membrane activity [24,116]. Contreras *et al.* showed that while ASMase is almost inactive on LUVs without cholesterol, the addition of cholesterol results in remarkable enhancement of enzyme activity [116].

3.3. Function of Ceramide-rich Platforms

Functionally, CRPs provide a platform to re-organize (and re-compartmentalize) receptor and signaling molecules at the cell membrane to facilitate amplification of signaling processes. In particular, CRP formation sorts proteins on the cell surface, providing a mechanism for spatial re-organization of receptors into clusters upon cellular stimulation. Ultimately, high receptor density within this small area of cell membrane, which seems prerequisite for signal transmission, facilitates downstream activation of molecules that associate with the receptor. For example, in Fas signaling, engagement of pre-trimerized Fas receptor activates within seconds a small percentage (1-2% of maximum) of procaspase 8 and the adaptor protein FADD, sufficient for ASMase translocation and CRP formation. CRPs in turn provide a platform for Fas oligomerization, which facilitates formation of the death-induced signaling complex (DISC), an event essential for the other 98% of Fas-induced FADD and Caspase 8 activation, and eventually apoptosis of these cells [106]. This amplification function of CRPs in Fas signaling was delineated in studies in B lymphocytes lacking functional ASMase [69,71,104]. In contrast to wild type cells, Fas stimulation in ASMase null lymphocytes is unable to generate ceramide, and in turn CRP formation is not observed. Subsequently, deficiencies in DISC formation were detected. Weak recruitment of FADD was accompanied by markedly attenuated activation of caspase 8, reaching only 1% of the level achieved after maximal Fas activation in wild-type cells, and abrogation of apoptosis. Addition of nanomolar quantities of C_{16}-ceramide directly initiated CRP formation, in turn restoring Fas clustering and Fas-initiated signaling of apoptosis in ASMase-deficient cells. These studies showed that following Fas stimulation, CRPs serve as signaling platforms that cluster activated receptor molecules, providing a feed forward mechanism that ultimately results in significant amplification of a weak primary signal leading to a signal transduction cascade [69,71,104].

Furthermore, CRPs could function as domains that recruit molecules transmitting the signal initiated by the receptor, as with Fas, while at the same time exclude molecules that negatively interfere with the signal that the receptor transmits. The mechanism, however, that mediates preferential partitioning or exclusion of proteins into CRPs is still not understood. Studies by Bock *et al.* provided initial insights into the molecular mechanism of receptor clustering in CRPs following CD40 stimulation. The authors replaced the membrane-spanning region of CD40, known to localize within CRPs following receptor stimulation, with the membrane-spanning region of CD45, which localizes outside of rafts,

at least in some cells [117]. The studies revealed that while CD40 clustered in CRPs following stimulation with CD40 ligand (CD40L), CD40/CD45 chimeras were unable to cluster within these structures, and in fact were excluded from CRPs. The mutant also failed to initiate signaling upon cellular stimulation, such as p38MAPK phosphorylation, typically observed in 3A9 T cell hybridoma after stimulation via CD40. Nonetheless, forced crosslinking of the CD40/CD45 mutant was sufficient to restore p38MAPK phosphorylation, confirming the notion that CD40 clustering in membrane platforms leads to signal amplification. Further, these studies demonstrated that, at least for CD40, the membrane-spanning region determines preferential partitioning of proteins into CRPs [117].

Finally, by limiting lateral diffusion of proteins and lipids within CRPs, these structures might serve as stabilizing platforms for receptor-ligand interaction, inducing a change in receptor conformation and increasing receptor affinity for the ligand. The phenomenon that receptor and ligand are immobilized within CRPs has been shown for Fas/FasL and CD40/CD40L pairs [69,118,119]

Besides providing a signaling platform by clustering receptors and amplifying a signal generated on the plasma membrane, CRPs could also directly regulate protein function by interacting with proteins on the cytoplasmic leaflet of the membrane. This function of CRPs is enabled by a unique property of ceramide to spontaneously flip to the cytoplasmic leaflet. Ceramide has been shown to interact and activate a number of proteins, namely phospholipase A2, kinase suppressor of Ras (KSR), atypical protein kinase C isoforms, c-Raf-1, Cathepsin D and ceramide-activating protein phosphatases (CAPP) [120-124]. Whereas the exact mechanism of ceramide-mediated regulation of most of these proteins remains to be explored, recent evidence suggests that in at least some instances interaction with ceramide targets proteins to CRPs [125]. As demonstrated by Yin *et al.*, EGF stimulation induces translocation of KSR1 and its subsequent activation. Specifically, KSR binds to and selectively traffics its substrate c-Raf-1 into CRPs, the sites of c-Raf-1 activation. Mutations within the ceramide-binding domain of KSR-1, the atypical C1 domain, attenuate EGF-induced KSR-1 membrane translocation, KSR-1 kinase activation and c-Raf-1 mediated cell proliferation [125].

Additionally, rafts, caveolae and CRPs are employed by a wide range of pathogens including bacteria, viruses and parasites to infect mammalian cells. These structures have been implicated in many aspects of infection, such as pathogen internalization, intracellular maturation of phagosomes, lysis and fusion of phagosomes, virus budding, immune receptor signaling and induction of cell death upon infection and release of cytokines [126,127]. Specifically, the involvement of ASMase and ceramide has been demonstrated in many of these infectious states, including infections with *N. gonorrhoea*, *P. aeruginosa*, *S. aureus*, Rhinovirus and Sindbis virus. CRP formation, however, has only been shown in the cases of *P. aeruginosa* and Rhinovirus [127]. The involvement of CRPs in at least some aspects of infection with pathogens, such as pathogen internalization, virus budding and phagosome maturation, lysis and fusion, is most likely mediated by ceramide's ability to induce membrane curvature, leading to membrane fusion/fission, as described above.

Finally, CRPs can function by regulating formation of membrane channels. As demonstrated following infection with *P. aeruginosa*, ASMase activation generates CRPs, which serve as sites into which cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels insert, oligomerize and become functional [94]. Further, the ability of ceramide to form pores in membranes of intracellular organelles, opens up a possibility that CRP formation is not restricted to the plasma membrane. As previously noted, Colombini and colleagues demonstrated that ceramide forms channels in isolated mitochondria, leading to increased permeability of mitochondrial outer membrane to cytochrome c, a crucial commitment step in the intrinsic apoptotic pathway signaling cascade [54-56]. Whether CRPs are involved in this process, however, remains to be explored.

The properties of CRPs indicate that they possess a general function in signal transduction for a variety of stimuli. This theory is also supported by the fact that CRPs are formed in response to a variety of somewhat unrelated cellular stimuli (see Table 1). However, it is not clear whether CRPs form in all cell types, nor whether every stimulus that activates ASMase and/or induces ceramide elevation leads to CRP formation. As CRP formation has not been studied in each instance where ASMase is activated, further investigation is necessary to better understand the link between the two processes.

The outcome of CRP formation in cells depends on the stimulus that leads to their generation. For example, TNF-receptor superfamily members CD40 and Fas both lead to formation of CRPs in JY B lymphocytes [104,118]. These receptors localize within CRPs, leading to downstream activation of molecules that associate with the receptor and the cognate signal transduction cascade, however signaling is very different in the two instances. While Fas-induced CRP formation leads to DISC complex generation and ultimately apoptosis [104], CD40 engagement results in a pro-survival cascade mediated by engagement of the MAPK signaling pathway and IL-12 release, ultimately leading to B-cell activation. Both of these processes are CRP-dependent and impaired in ASMase null cells. These phenomena further suggest that CRPs play a general function in transmembrane signal transduction [118].

The explanation why ASMase activation and CRP formation occur in response to certain stimuli in some cell types, but not in others, remains unknown. For example, CRP formation has been demonstrated in endothelial cells and hepatocytes stimulated by IR and FasL. While endothelial cells form CRPs in response to both of these stimuli, hepatocytes form CRPs in response to Fas engagement but not IR. This finding is confirmed by the cellular outcomes in these two cell types. While endothelial cells treated with IR and FasL undergo ceramide-dependent apoptosis, apoptosis is only observed in hepatocytes stimulated by FasL, as hepatocytes appear radioresistant. The reason for these observations has not been elucidated, however it is clear that additional, cell specific signals are needed for CRP formation, pre-requisite for cellular responses to specific stimuli.

4. Manipulation of CRPs in Disease Treatment

At present no drugs exist that specifically target CRPs to either prevent or amplify their function. Nevertheless, data generated from a number of groups suggests that CRPs mediate

diverse disease pathologies and are engaged in disease treatment, summarized in Table 2. While, CRP generation has not been directly demonstrated in each of these disease states, primarily due to technical constraints of visualizing CRPs *in vivo*, ASMase and in some instances ceramide synthase have been shown to directly mediate aspects of disease processes, pointing to likely involvement of CRPs. Due to space constraints, however, we will focus only on diseases where involvement of CRPs has been experimentally shown or strongly suggested, namely vascular disorders, metabolic disorders and infectious diseases. We hence propose that development of drugs that prevent or amplify the function of CRPs might lead to novel therapies for these diseases.

Vascular dysfunction mediated by ASMase-generated ceramide has been characterized for a number of stimuli, including platelet-activating factor (PAF) and IR. Goggel *et al.* showed that PAF-induced pulmonary edema is regulated, in part, by activating ASMase in pulmonary microvessels, leading to elevated ceramide levels, which in turn induce vascular leakage and pulmonary edema in mice [79]. PAF-induced pulmonary edema was reduced in *asmase*$^{-/-}$ mice and in wild type littermates treated with the non-specific ASMase inhibitors xanthogenate D609 or imipramine, as well as with a monoclonal anti-ceramide IgM antibody [79]. Although ASMase-generated CRPs were not documented in this model, attenuation of PAF-induced edema by exogenously-added antibody suggests that ceramide is indeed generated on the outer plasma membrane. These findings are clinically relevant, as several studies indicate that ceramide derivatives are markedly elevated in bronchoalveolar lavage fluid in patients with acute respiratory distress syndrome, also known as ARDS. Further, plasma ceramide is increased in sepsis patients, the principle inducer of ARDS, correlating with mortality [128]. Collectively, these studies provide a basis for a new therapeutic approach identifying ASMase, ceramide, and presumably CRPs as potential targets in treatment of acute lung injury.

Ceramide-mediated vascular dysfunction induced by IR and the therapeutic potential of engaging this phenomenon were demonstrated in solid tumors and the gastrointestinal (GI) tract. Garcia-Barros *et al.* showed that tumor stem cell clonogen (SCC) lethality after exposure to single high-dose radiotherapy, an emerging therapeutic modality, is conditionally linked to an early and massive wave of ASMase-mediated apoptosis in the microvascular endothelium of exposed tissue [129]. These studies further showed that early-phase microvascular endothelial apoptotic injury is mandatory for tumor cure, as MCA/129 fibrosarcomas grown in *asmase*$^{-/-}$ mice, which provide apoptosis resistant vasculature, were completely resistant to the curative effects of up to 15 Gy IR, a dose that normally induces 50% cure of tumor implanted into wild-type littermates [129]. Although direct proof for CRP involvement in the tumor response to IR in this system is still lacking, due to technical problems, preliminary studies from our laboratory demonstrate CRP formation in an isolated population of tumor endothelium irradiated *ex vivo* (Stancevic and Kolesnick, unpublished). These data highlight the importance of CRPs in microvascular cellular responses to IR, in particular in endothelial cell apoptotic death. Along these lines, restoring or amplifying CRP formation in the endothelium may radiosensitize tumors. In fact, recent *in vivo* data by Smith *et al.* demonstrated that local generation of ceramide in tumors, achieved through administration of recombinant ASMase (rhASM), acts synergistically with IR, leading to a

three-fold decrease in tumor size [130]. The authors provide evidence that the endothelial compartment mediates the tumor response to IR enhanced by rhASM administration, as a significant decrease in the number of endothelial vessels per microscopic field of treated tumors was documented [130]. Taken together these studies suggest that amplifying CRP generation in tumor microvasculature via increasing ASMase-mediated ceramide generation represents a promising therapeutic strategy requiring further development.

As with tumor tissue, the endothelial-stem cell linkage mechanism was shown to mediate normal tissue damage after single high-dose IR exposure in the GI tract. The lethal GI syndrome not only limits the efficacy of IR in cancer therapy, but also presents a potential threat as a result of whole body irradiation in other clinical contexts, such as a nuclear accident. In the murine small intestines, IR-induced GI toxicity is mediated by a wave of microvascular endothelial apoptosis within the lamina propria, which regulates survival of IR-injured crypt SCCs. As in tumor, endothelial apoptotic injury in the GI tract is mediated by ASMase, as genetic or pharmacologic inactivation of ASMase conferred resistance to IR-induced microvascular apoptosis and, in turn, SCC lethality, preserving the GI tract and leading to animal survival [90]. These studies suggest a potential approach to protect the GI tract from IR lethality by decreasing ceramide levels, leading to an increase in the therapeutic index during cancer treatment of organs in the abdomen and prevention of a lethal GI syndrome resulting from total body irradiation even in cases as extreme as a radiological terrorist attack.

In addition to the GI tract, reduction of ceramide levels may protect other tissues from the side effects of cancer therapies, namely oocytes and tissues affected during acute Graft-versus-host disease (GVHD). Published data indicate that chemotherapy- and IR-induced apoptosis of oocytes may be mediated by ASMase and its second messenger ceramide. Specifically, Tilly and colleagues demonstrated that oocytes lacking ASMase are resistant to doxorubicin-induced apoptosis *in vitro*. Further, administration of sphingosine-1-phosphate (S1P), a ceramide metabolite, which counteracts ceramide's pro-apoptotic effects, inhibits apoptosis induced by anti-cancer therapy *in vivo*. In these studies, IR-induced oocyte loss in adult wild-type female mice, the event that drives premature ovarian failure and infertility in female cancer patients, was completely prevented by injection of S1P into the ovary just prior to IR [89]. Additionally, mating trials of wild-type mice subjected to IR treatment showed that preservation of oocytes by S1P pretreatment leads to an increase in pregnancy rates from 12.5% to 75% [131]. More importantly, this preservation of fertility was accomplished without propagating genetic damage in the offspring of S1P-treated irradiated females. These data collectively demonstrate that preservation of ovarian function and fertility post-irradiation can be safely and effectively achieved *in vivo* using S1P, providing a basis for the development of S1P-based therapies for the treatment of infertility.

GVHD is a common complication of allogeneic bone marrow transplantation mediated by cytotoxic T lymphocyte (CTL)-induced apoptosis leading to host organ damage. Rotolo *et al.* recently showed that ASMase is involved in development and progression of acute GVHD. Using clinically-relevant mouse models of acute GVHD in which allogeneic bone marrow and T cells were transplanted into wild-type and *asmase*$^{-/-}$ hosts, the authors identified host ASMase as critical for full blown GVHD. More specifically, the mechanism

by which ASMase mediates CTL-induced apoptosis of target cells (hepatocytes, small and large intestinal epithelium and endothelium, skin) appeared to require CRP formation on target cell membranes [132]. The lack of host ASMase, and in turn abrogation of CRP formation (defined using an *ex vivo* 2 cell model of GVHD in which *in vivo*-activated T cell effector cells were coincubated with *asmase*$^{+/+}$ or *asmase*$^{-/-}$ hepatocyte target cells), attenuated organ injury and consequent cytokine storm, decreasing morbidity and mortality. These findings, delineating a requirement for target cell ASMase in evolution of GVHD, provide potential new targets for disease management through inhibition of CRPs and/or ASMase activity.

Wilson's disease is a metabolic disorder in which impaired hepatic secretion of Cu^{2+} results in its inappropriate accumulation within the liver parenchyma, and ultimately cirrhosis and hemolytic anemia. Recent studies by Lang *et al.* showed that Cu^{2+}-triggered hepatocyte apoptosis is mediated by ASMase activation, ceramide release and subsequent CRP formation [92]. Genetic deficiency or pharmacologic inhibition of ASMase by amitriptyline, and presumably abrogation of CRP formation, prevented Cu^{2+}-induced hepatocyte apoptosis, protecting rats genetically prone to develop Wilson's disease from acute hepatocyte injury, liver failure and early death. Furthermore, these studies indicated a novel mechanism for Cu^{2+}-mediated anemia in which Cu^{2+} triggered secretion of ASMase from leukocytes, resulting in ceramide formation in erythrocytes and subsequent exposure of phosphatidylserine at the cell surface. *In vivo*, phosphatidylserine exposure led to immediate clearance of affected erythrocytes from blood, an event absent in *asmase*$^{-/-}$ animals. Specifically, erythrocytes in *asmase*$^{-/-}$ mice were resistant to Cu^{2+} treatment, failing to elevate ceramide and expose phosphatidylserine, and hence avoided *in vivo* elimination. The relevance of this model was shown in human subjects, as patients with Wilson' disease exhibited elevated plasma levels of ASMase and displayed constitutively increased ceramide- and phosphatidylserine-positive erythrocytes [92]. Hence, these data indicate the significance of ceramide and CRPs for pathogenesis of Wilson's disease, and provide a basis for treatment by pharmacologic inhibition of ASMase and/or CRP formation.

Inhibition of infectious diseases by manipulation of ceramide metabolism presents another area with exciting therapeutic potential. As noted previously, CRPs appear involved in many aspects of infection with various pathogens – internalization of pathogen, activation of intracellular signaling pathways, induction of host cell death, and cytokine release, to name a few. As demonstrated by Grassme and colleagues, CRPs are critical for internalization of *P. aeruginosa* into epithelial cells and fibroblasts, induction of apoptosis in the infected cell and regulation of host cell cytokine release (the innate immune response) [94]. CRP formation seemed required for infection, as pharmacologic (sphingolipid raft disrupting agents - filipin, nystatin or β-cyclodextrin) or genetic (ASMase-null) inhibition of CRP formation in lung epithelial cells correlated with a failure to internalize bacteria and lack of apoptosis of infected cells *in vitro* and *in vivo*. Additionally, CRP formation was shown to mediate the host defense against the acute *P. aeruginosa* infection. Specifically, inhibition of CRP formation in ASMase null mice led to an eight-fold increase in IL-1β release and IL-1-mediated septic death of these mice. Addition of C_{16}-ceramide led to restoration of CRP formation, bacterial internalization and epithelial cell apoptosis, as well as normalization of

IL-1β release in cultured lung epithelial cells. These data indicate ASMase-generated ceramide and CRPs may play a central role in both *P. aeruginosa* infection, as well as the host defense against the pathogen, making ASMase a potential, but complicated therapeutic target.

In fact, recent findings by Gulbins and colleagues suggest that careful titration of ASMase activity to normalize chronically-elevated ceramide levels in affected lungs of cystic fibrosis patients may provide a therapeutic advantage in treatment of chronic *P. aeruginosa* infection [133]. The concept here would be to titrate ceramide back to physiologic levels but not further to avoid the scenario described above regarding the uncontrolled innate immune response. Cystic fibrosis is caused by mutations in the transmembrane conductance regulator (CFTR) and is characterized by chronic lung inflammation and frequent chronic lung infections with *P. aeruginosa*, *Burkholderia cepacia* and *Haemophilus influenzae*. The molecular mechanism responsible for pulmonary inflammation and high susceptibility to infection has been recently revealed by Teichgraber *et al.* In their studies, CFTR deficiency resulted in alkalization of intracellular vesicles in epithelial cells that contain ASMase and acid ceramidase. While an increase in the pH to 6.0 completely inactivates acid ceramidase, it decreases the activity of ASMase only by 30-40%, resulting in the imbalance between ASMase cleavage of SM to ceramide and acid ceramidase consumption of ceramide [133]. Subsequent ceramide accumulation was detected in respiratory epithelial cells, and submucosal glands in CFTR-deficient mice, corroborated, in lung specimens and nasal epithelial cells from patients with cystic fibrosis. As demonstrated by Teichgraber *et al.*, ceramide accumulation leads to chronic respiratory epithelial cell death by apoptosis and release of DNA into bronchi, where it serves as an adhesin for *P. aeruginosa* resulting in high susceptibility to persistent infection. Pharmacologic treatment of CFTR-deficient mice with amitriptyline or partial genetic deficiency of ASMase (haploinsufficiency) normalized pulmonary ceramide concentrations and prevented all pathologic effects, including the susceptibility to infection. These findings suggest that inhibition of ASMase and normalization of ceramide levels might be a new important treatment strategy to prevent and treat bacterial infections in cystic fibrosis patients, a concept currently being tested in Phase II trials in Germany [134].

5. Conclusion

In summary, accumulating evidence indicates that the unique biophysical properties of ceramide dictate its biological function through membrane reorganization and formation of CRPs. CRPs form in response to diverse stimuli and represent novel sites of signal transduction initiation and transmission across the plasma membrane. Crucial involvement of CRPs in a number of disease initiation states, as well as disease treatments, makes them an attractive therapeutic target. Specifically, strategies for increasing CRP formation in tumor vasculature may potentially sensitize tumors to curative effects of IR and chemotherapy. Alternately, attenuation of CRP formation could potentially inhibit lethal side effects of cancer therapies, such as GI syndrome and GVHD, increasing the therapeutic index. Additionally, the central role of CRPs in infection of mammalian cells with at least some pathogenic bacteria, suggests their manipulation as a strategy to alter infection, particularly in highly susceptible individuals, such as cystic fibrosis patients. Overall,

identified less than a decade ago, CRPs still represent novel signaling structures, the biomedical importance of which is just starting to be revealed. As such, further research in this largely untapped field will undoubtedly identify involvement of CRPs in many more processes, and more clearly elucidate how these enigmatic structures are capable of transferring specific information regarding diverse stresses.

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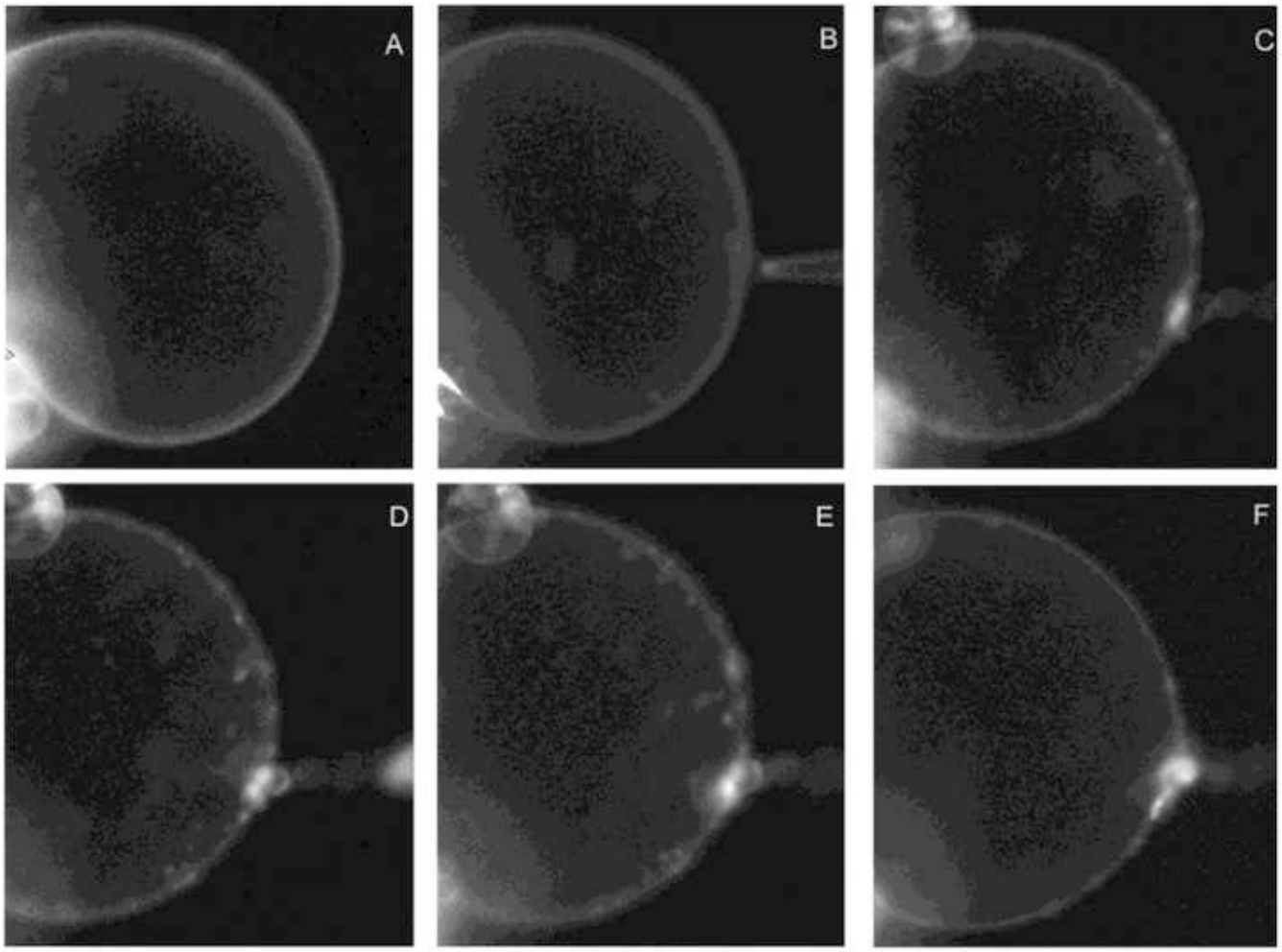

Figure 1. Ceramide-rich platform formation in a model membrane

Still fluorescent microscopy images depicting membrane reorganization of SOPC:C16-0 SM:Bdp-SM (0:75:0.2:0.05) GUV (A) following ceramide formation by SMase coupled onto amino-derivatized acrylate (B-F). Contacting membrane surface with immobilized SMase (B) resulted in generation of ceramide patches in one pole of the GUV depicted at 45 min (C), 50 min (D), 55 min (E) and 90 min (F). Adapted from Nurminen *et al.* JACS, 2002 [39]

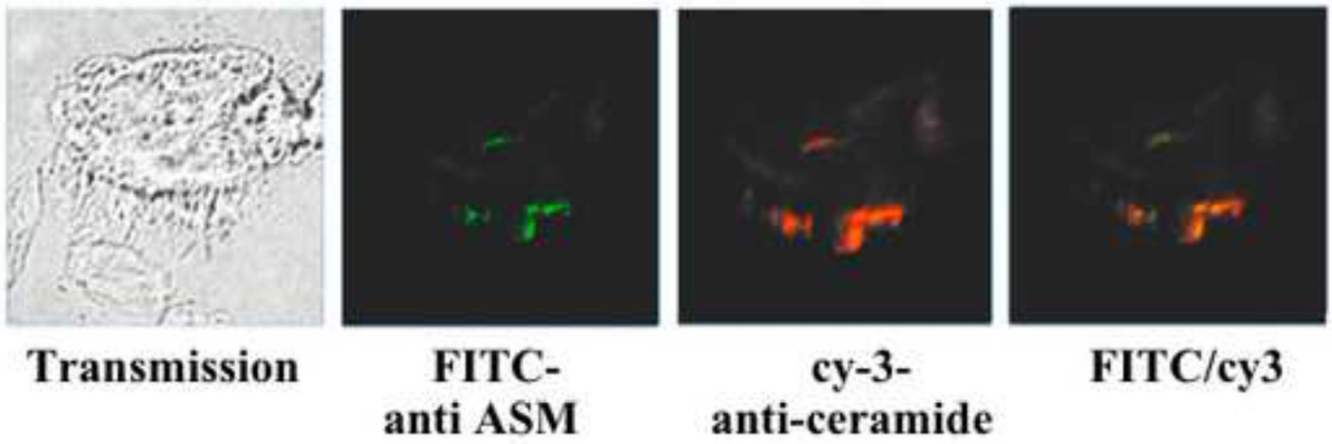

Figure 2. Ceramide-rich platforms in murine tracheal epithelial cells following *in vivo* infection with *Pseudomonas aeruginosa*

Fluorescence microscopy images demonstrate colocalization of surface ceramide with *Pseudomonas aeruginosa* and ASMase on the cilia of *in vivo*-infected murine tracheal epithelial cells at 20 minutes post infection. Adapted from Grassme *et al.* Nature Medicine, 2003 [94]

Table 1
Ceramide-rich platform formation in biological systems

Stimulus	Cell type	Cellular outcome	Reference
FasL, CH 11	Jurkat T lymphocytes JY B Cell Lymphocytes H9 (Human T Cells) SKW 6.4 Cells (Human B Lymphocytes) K50 Cells (Burkitt Lymphoma Cells) WI 38 Cells (Human Lung Fibroblasts) H9 T Cell Lymphoma Human PBL* Murine Granulosa Cells* Lung Epithelial Cells Murine and Human Lymphocytes* Murine Splenocytes and Hepatocytes* Coronary Artery Endothelial Cells*	Apoptosis	[69,71,106,111,135 138]
TNFα	Coronary Artery Endothelial Cells*	Apoptosis	[136]
Endostatin	Coronary Artery Endothelial Cells*	Apoptosis	[136,139]
CD40L	JY B Lymphocytes Human Aortic Endothelial Cells*	Activation	[72,118] [140]
Rituximab (CD20)	Daudi Cells (Burkitt Lymphoma) RL Cells (Follicular lymphoma) Chronic Lymphocyte Leukemia (CLL) Cells*	Growth inhibition	[98]
TRAIL	Murine T Splenocytes* BJAB Cells (Burkitt Lymphoma) A549 (Carcinomic Human Alveolar Basal Epithelial Cells) L929 Cells (Murine Aneuploid Fibrosarcoma)	Apoptosis	[73]
UV-C	U937 Cells (Human Myeloblastoma) Jurkat T Lymphocytes	Apoptosis	[81,82]
IR	Jurkat T Lymphocytes Bovine Aortic Endothelial Cells* SCC61 Cells (Head and Neck Squamous Carcinoma)	Apoptosis	(Zhang and Kolesnick, unpublished) (Stancevic and Kolesnick, unpublished) [110]
P. aureginosa	Human Nasal Epithelial Cells* Chang Conjuctive Epithelial Cells Murine Tracheal Epithelial Cells (*in*	Apoptosis Internalization IL-12 release	[94,141]

Stimulus	Cell type	Cellular outcome	Reference
	vivo) Murine Lung Fibroblasts* WI-38 Cells (Human Lung Fibroblasts) Alveolar Macrophages*		
Rhinovirus	Chang Epithelial Cells Murine Nasal Cells*	Apoptosis Internalization	[96,142]
Cisplatin	HT29 Cells (Colon Carcinoma)	Apoptosis	[87]
Etoposide	Bovine Aortic Endothelial Cells* Human Coronary Artery Endothelial Cells*	Apoptosis	(Jacobi and Haimovitz-Friedman, unpublished)
Cytolytic T-cells	Murine Hepatocytes and Splenocytes*	Apoptosis	[132]
ROS	Peripheral Blood Neutrophils*	Apoptosis	[135]
Anti- FCγRII antibody	U937 (Monolytic Cells)	FCγRII phosphorylation	[103]
Anti-tumor ether lipid (ET-18-OCH_3)	Jurkat T Lymphocytes HL-60 Cells	Apoptosis	[143]
Cu^{2+} treatment	Murine Hepatocytes*	Apoptosis	[92]
Ceramide (CD14 engagement)	Monocytes*	Innate immune	[77]
Endotoxin (LPS)	THP-1 Cells (Human Acute Promonocytic Leukemia)	TNFα production	[144]
Oxotremorine (Muscarinic type 1 receptor agonist)	Bovine Coronary Arterial Myocytes (CAMs)*	Production of cADPR and coronary artery constriction	[145]

* primary cells

Table 2
Role of ASMase and ceramide-rich platforms in pathologic conditions

	Disease	Reference
Vascular Disorders	PAF, TNF-induced pulmonary edema (ALI)	[79]
	IR-induced tumor vascular dysfunction	[129]
	GI Syndrome	[90]
	Ischemic stroke	[146]
	Atherosclerosis	[147]
	Chronic heart failure	[148]
Metabolic Disorders	Wilson's disease	[92]
	Diabetes*	[149-151]
Cancer	Cancer chemotherapy (daunorubicin, cisplatin, gemcitabine)	[87,88,152,153]
	IR- and chemotherapy-induced side effects (GVHD, infertility)	[89,131,132]
Infections	*Pseudomonas aureginosa*	[94]
	Rhinovirus	[96]
	Sindbis virus	[97]
	Neisseriae gonorrhoea	[93]
	Staphylococcus aureus	[154]
	Sepsis	[155]
Lung Diseases	Cystic fibrosis	[133]
	Emphysema*	[156]
Liver disease	Autoimmune hepatitis	[157]
Central Nervous System	Alzheimer's disease	[158]

* Ceramide synthase has also been shown to be involved in generation of ceramide that mediates these pathologies