REVIEW ARTICLE

Ceramide: second messenger or modulator of membrane structure and dynamics?

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The physiological role of ceramide formation in response to cell stimulation remains controversial. Here, we emphasize that ceramide is not *a priori* an apoptotic signalling molecule. Recent work points out that the conversion of sphingomyelin into ceramide can play a membrane structural (physical) role, with consequences for membrane microdomain function, membrane vesiculation, fusion/fission and vesicular trafficking. These processes contribute to cellular signalling. At the Golgi, ceramide takes part in a metabolic flux towards sphingomyelin, diacylglycerol and glycosphingolipids, which drives lipid raft formation and vesicular transport towards the plasma membrane. At the cell surface, receptor clustering in lipid rafts and the formation of endosomes can be facilitated by transient ceramide formation. Also, signalling towards mitochondria may involve glycosphingolipid-containing vesicles. Ceramide may affect the permeability of the mitochondrial outer membrane and the release of cytochrome *c*. In the effector phase of apoptosis, the breakdown of plasma membrane sphingomyelin to ceramide is a consequence of lipid scrambling, and may regulate apoptotic body formation. Thus ceramide formation serves many different functions at distinct locations in the cell. Given the limited capacity for spontaneous intracellular diffusion or membrane flip-flop of natural ceramide species, the topology and membrane sidedness of ceramide generation are crucial determinants of its impact on cell biology.

Key words: apoptosis, diacylglycerol, lipid raft, sphingolipid, vesicular trafficking.

INTRODUCTION

The sphingolipid ceramide (Cer) is almost universally generated during cellular stress and apoptosis, either by *de novo* synthesis or via the action of acid sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase) [1–4]. This fact, together with the knowledge that synthetic, short-chain Cer species can induce cell cycle arrest or apoptosis, has led to the widely held belief that endogenous Cer plays a role in apoptotic signalling. By analogy with diacylglycerol (DAG), Cer has been suggested to fulfil a second-messenger function by binding to specific intracellular protein targets. This idea, however, has remained very controversial [5–9].

In the present review, we highlight the physicochemical properties of natural Cer (which differ dramatically from those of short-chain Cer) in membranes, and emphasize that endogenous Cer formation should be considered in its topological context. In this perspective, direct evidence for a role of Cer as a classic second messenger is scarce, while evidence is emerging for its indirect impact on cellular signalling, resulting from alterations in membrane structure and intracellular vesicle transport following the conversion of sphingomyelin (SM) into Cer or *de novo* Cer formation. Newly identified functions of Cer in domain coalescence, receptor clustering and vesicle formation and fusion also conform to its important role as a membrane structural component.

PHYSICAL PROPERTIES OF CER IN MEMBRANES: A NEW PERSPECTIVE ON SIGNALLING

Natural Cer remains restricted to membranes, self-aggregates and modulates signalling from microdomains

Although DAG and Cer have a similar structure, their occurrence, location and structural behaviour in membranes are quite different [10,11]. Through intermolecular hydrogen bonding and its two long, saturated hydrophobic chains, Cer fits better than DAG in a lipid bilayer structure. It has only recently become clear that, in contrast with DAG, Cer does not fluidize membranes, but increases the order of the acyl chains in the bilayer (tighter packing; rigidization) [12,13]. This can be measured most easily by fluorescence polarization of the membrane probe diphenylhexatriene [14]. Unlike DAG, Cer has a tendency to self-aggregate in-plane, even more so in the presence of SM. As a result, Cer segregates laterally into membrane microdomains (rafts/caveolae), packed tightly in homodimers/multimers in association with the other sphingolipids and cholesterol [7,11,13,15,16]. In fact, substantial amounts of Cer are encountered in isolated rafts/caveolae, even from unstimulated cells [16–18]. It promotes and stabilizes raft/caveolae formation. For the same physico-chemical reason, natural Cer does not transfer spontaneously between lipid bilayers, and does not flip-flop as readily as DAG. Fluorescently labelled Cer has an estimated spontaneous flip-flop rate ($t_{1/2}$) of approx. 22 min [19].

Abbreviations used: aSMase, acid sphingomyelinase; Cer, ceramide; C_2-Cer, *N*-acetylsphingosine; C_6-Cer, *N*-hexanoylsphingosine; DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; GCS, glucosylceramide synthase; JNK, c-Jun N-terminal kinase; KSR, kinase suppressor of Ras; MAPK, mitogen-activated protein kinase; nSMase, neutral sphingomyelinase; PC, phosphatidylcholine; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; SAPK, stress-activated protein kinase; SM, sphingomyelin; TGN, *trans*-Golgi network; TNF, tumour necrosis factor.

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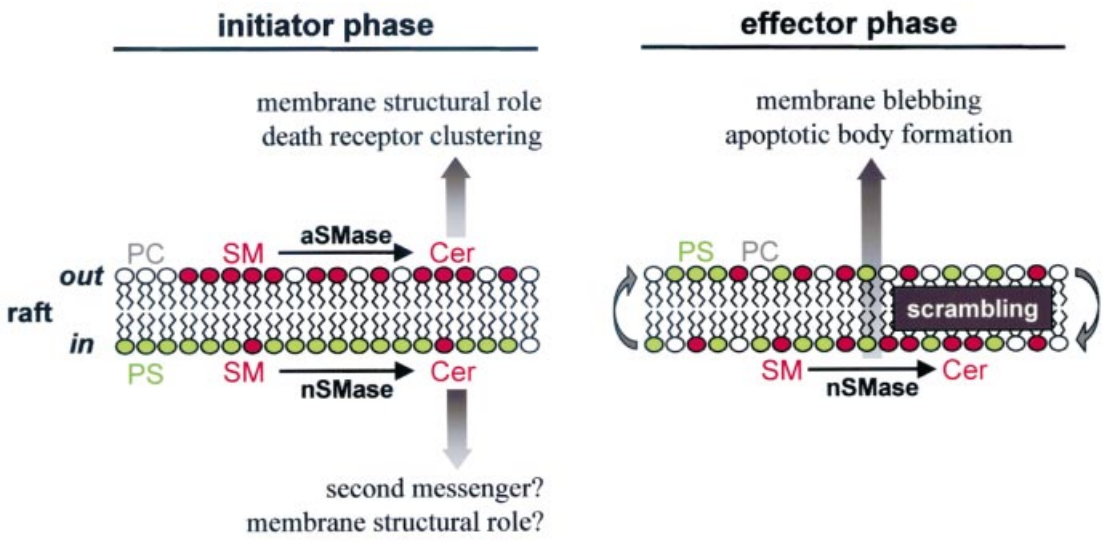

Figure 1 Plasma-membrane sidedness of Cer formation, and function of SM hydrolysis during the initiation and effector phases of apoptosis

aSMase acts in the initiator phase of apoptosis (left panel) and delivers Cer to the exoplasmic side of the plasma membrane, where it facilitates death receptor clustering. nSMase acts at the inner leaflet of the plasma membrane. In the initiator phase, nSMase uses the minor SM pool (red) located in the inner leaflet. In the effector phase (right panel), nSMase hydrolyses bulk SM (red) that moves first from the outer to the inner leaflet due to phospholipid scrambling, i.e. loss of lipid asymmetry in the plasma membrane. Phosphatidylserine (PS; green) thereby becomes exposed at the cell surface, a recognition signal for professional macrophages. Large shaded arrows indicate distinct effects of SM breakdown: rapidly formed Cer may have membrane structural and possibly second-messenger functions. Reduced SM levels and concomitant loss of cholesterol allows membrane blebbing and shedding of vesicles in the effector phase of apoptosis [71]. It may possibly also be instrumental in the formation of apoptotic bodies.

This rate is much lower for natural Cer, so that Cer might be restricted, for quite some time, to the side of the bilayer where it is generated [7,20].

The limited transbilayer movement of Cer predicts that, if generated from the major pool of SM in the exoplasmic side of membrane rafts, Cer is unlikely to act as a rapidly generated second messenger on a cytoplasmic target protein at the opposite side of the membrane. Rather, it could modify subtle in-plane intermolecular interactions within and among lipid rafts, with consequences for raft coalescence, membrane curvature and (apoptotic) signalling emanating from these domains. Indeed, Gulbins and Kolesnick and their co-workers [18,21,22] recently provided compelling evidence for this view. They showed that natural Cer generated or inserted exogenously in the outer leaflet of the raft bilayer can facilitate clustering of the CD95/Fas death receptor, residing in rafts [23], and the subsequent induction of apoptosis under sub-optimal concentrations of cross-linking ligand or antibodies.

An important new concept emerging from these studies is that Cer can act as a structural component and functional modulator of the membrane raft outer leaflet, as opposed to a possible second messenger in the inner leaflet (Figure 1, left panel).

Short-chain Cer differs dramatically from natural Cer in physical properties and its behaviour in membranes and cells

Since natural, long-chain Cer is not water-soluble and is difficult to insert from outside into a phospholipid bilayer, synthetic short-chain Cers, particularly *N*-acetylsphingosine (C_2-Cer) and *N*-hexanoylsphingosine (C_6-Cer), which are water soluble (form micelles) and membrane-permeable, are used widely as experimental tools [7,11]. However, use of these exogenous short-chain Cer analogues as mimics of endogenous Cer is, in general, not justified. In fact, much of the current confusion in the literature on the role of Cer in cellular signalling stems from the fact that effects of short-chain Cer cannot be extrapolated to natural Cer species.

The main reason why C_2-Cer does not mimic endogenous Cer is its different partitioning and behaviour in biomembranes. C_2-Cer, similar to DAG, tends to form and to promote a hexagonal, non-bilayer, inverted micellar structure. It disorders (fluidizes) the membrane and causes lipid-packing defects in the bilayer [24]. This contrasts with natural long-chain Cer, which has an ordering/packing (rigidizing) effect on the phospholipids in the membrane and stabilizes the gel phase [13,25]. Solely for this reason, C_2-Cer is suspected to have a different lateral distribution in the membrane, and is also likely to differ with regard to its distribution/partitioning into microdomains such as rafts and caveolae. Also important is the fact that natural Cer stays relatively tightly bound to the membrane where it is generated [7,20], and any possible interaction with a target protein must occur in this membrane or in a membrane vesicle derived directly (by fission) from this membrane. C_2-Cer, on the other hand, due to its amphiphilic nature, can leave the (plasma) membrane and translocate, via the cytosol, to other membranes inside the cell. A clear example of this different intracellular behaviour of C_2-Cer has been reported by Tepper et al. [26]: exogenous C_2-Cer (taken up via the plasma membrane) is converted into GlcCer in the Golgi, while endogenous Cer generated in the plasma membrane after CD95/Fas ligation or by exogenous (bacterial) SMase does not reach the Golgi and thus is not glycosylated.

The pro-apoptotic effect of C_2-Cer may be related to its facile intracellular diffusion. For example, exogenously applied C_2-Cer can reach the endoplasmic reticulum, where it inhibits the CDP pathways for the biosynthesis of phosphatidylcholine (PC) and phosphatidylethanolamine [27–30]. In the case of PC, this inhibition occurs directly at the rate-determining enzyme, CTP: phosphocholine cytidylyltransferase, and was found to be

inversely related to the length of the N-linked carbon chain of Cer (hydrophobicity), as was the ability of these Cer derivatives to trigger apoptosis [30]. The blockade of phospholipid synthesis/turnover by C_2-Cer may severely impede intracellular vesicular trafficking [31] (see below) and is a direct cause of apoptosis in at least some cell types, since supplementation of, for example, PC by other means (such as acylation of lyso-PC) rescues the cells from apoptosis induction [29,32]. Other (pro-apoptotic) effects of short-chain Cer that may depend on its easy intracellular diffusion are seen in mitochondria (see below), on the translocation and activation of protein kinase Cζ (PKCζ) [33] (see below), and on the stimulation of formation of endogenous, long-chain Cer. The latter occurs either by rapid (minutes) activation of a nSMase [34] or via relatively slow (hours) deacylation/reacylation at the sphingosine backbone by a CoA-dependent Cer synthase, probably located at the Golgi [35].

C_2-dihydroceramide, which lacks the 4–5 double bond in the sphingoid base, is often used as a control for specific effects of C_2-Cer. Dihydroceramide is the direct metabolic precursor of Cer, and its chemical structure differs by only one double bond. Ideal as it may seem, therefore, in reality it is not an appropriate control, since it behaves as a different molecule in physical terms [24,36]. This difference may be due to the more hydrophilic allyllic hydroxy group in C_2-Cer as compared with (saturated) C_2-dihydroceramide, which, for example, makes the latter compound 5-fold less soluble in ethanol [24]. Stereochemically, C_2-Cer has a different, more rigid, conformation, owing to the 4–5 double bond [24].

Cer promotes membrane permeability and channel formation

One striking property of Cers that may be physiologically relevant is their ability to restructure the permeability barrier of model and cell membranes. The early observations of Cer-induced release of aqueous contents from liposomes and erythrocyte ghosts [37] were extended by studies in platelets [24] and mitochondria [36,38]. In the latter organelle, short-chain as well as long-chain (natural) Cers were found to induce cytochrome *c* release. However, in contrast with C_2-Cer, long-chain Cer was unable to dissipate the mitochondrial inner membrane potential, which is in line with its restricted inter-membrane movement mentioned above. Montes et al. [39] have recently shown that *in situ*-generated Cer (through enzymic cleavage of SM) from lipid bilayers can give rise to the release of fluorescein-labelled dextrans of approx. 20 kDa, i.e. larger than cytochrome *c*. In cells, such permeability effects are presumably occurring locally, in small membrane regions (such as rafts and caveolae), or at certain local sites in mitochondria. Two properties of Cer, namely its capacity to induce negative monolayer curvature and its tendency to segregate into Cer-rich domains, appear to be important in the membrane restructuring process.

Siskind and Colombini [40] first reported that C_2-Cer and C_{16}-Cer form large channels in artificial planar membranes. Recently, they extended these studies to isolated mitochondria [41]. They showed that Cer does not trigger a particular mechanism for the secretion or release of cytochrome *c*, but simply increases the permeability of the mitochondrial outer membrane (via Cer channel formation) for a number of small proteins, including cytochrome *c*. *In vivo*, the release of cytochrome *c* from mitochondria is known to depend on additional factors, such as Bcl-2 family members (Bax, Bad, tBid) and possibly glycosphingolipids [the Cer-derived glycosphingolipid (ganglioside) G_{D3}] that might catalyse the restructuring of lipids to induce pore formation (see below).

Cer facilitates membrane fusion and fission, budding and vesicle formation

Membrane fusion and fission, inherent in the processes of endo/exocytosis and vesicular trafficking in cells, require at some stage a phospholipid transition from the lamellar to the non-lamellar (hexagonal) state. Phosphatidylethanolamine, with its relatively small polar headgroup and space-occupying poly-unsaturated acyl chain(s), may transiently and locally adopt such an inverted position in the membrane, which is facilitated by Cer [25]. Since Cer has a small hydroxy headgroup, it is cone-shaped and relatively poorly hydrated, which has consequences for the structure and curvature of the membrane microdomain where it is generated. Enzymic generation of Cer in SM-containing large unilamellar vesicles leads to extensive vesicle aggregation [37,42]. This has been attributed to the localized, asymmetrical generation of Cer-rich patches on the vesicle surface. The relative dehydration and exposure of hydrophobic surfaces at these patches would mediate aggregation, driven mainly by hydrophobic forces. Its relative dehydration also causes Cer to induce tight acyl packing in the plane of the membrane, even more so in association with SM [15]. When Cer is generated asymmetrically in the membrane, by a local action of SMase, this may have consequences for the vectorial budding of vesicles, as demonstrated elegantly by Holopainen et al. [43]. These authors used SM-containing giant liposomes to show that application of SMase to one side of the liposome caused vesicle formation on the other side. SMase treatment also resulted in endocytic vesicle formation in macrophages and fibroblasts [44]. Formation of these vesicles was different from other forms of endocytosis, because it was ATP-independent and the vesicles lacked any obvious coat [44]. The authors speculated that cells may use endogenous SMase to alter the local lipid composition and to promote membrane budding and fusion/fission. Alternatively, induction of endocytic vesicles and enlarged endosomes could also be achieved, in fibroblasts, by exogenous addition of C_6-Cer [45], which first intercalates in the outer leaflet of the plasma membrane. This phenomenon was reversed when the lipid was removed from the culture medium.

This induction of vectorial membrane budding may well be physiologically relevant. For example, infection by *Neisseria gonorrhoeae* requires entry of the bacterium into mucosal epithelial cells. This phagocytic process was triggered by, and is absolutely dependent on, aSMase activity at the cell surface [46]. More generally, inward vesiculation and subsequent endocytic vesicular traffic towards Golgi or mitochondria might be promoted by an aSMase producing Cer at cell surface rafts/caveolae. Also, the endocytosis and further downstream signalling of raft-localized activated receptors, such as CD95/Fas [47], might be facilitated by this physical mechanism.

To conclude, Cer formation facilitates the transient and local formation of inverted hexagonal structures in membranes that undergo a process of fusion and fission. In addition, Cer tends to self-aggregate, and its asymmetrical formation in the membrane (e.g. in lipid rafts) may induce negative membrane curvature, which precedes budding and vesiculation.

TOPOLOGY OF CER FORMATION DETERMINES ITS FUNCTION

Cer can be generated at several subcellular locations [9,48]. Classically, *de novo* synthesis of Cer occurs in the endoplasmic reticulum. Cer then translocates to the Golgi, where it has a major function as a metabolic precursor of glycosphingolipids, SM and DAG, each of which have important cell biological functions themselves (see below). Through vesicular traffic, SM and the glycosphingolipids become highly enriched in micro-

domains (lipid rafts and caveolae) on the cell surface. Only some 10–20 % of the SM in the plasma membrane resides in the inner leaflet. SM at both sides of the membrane is subject to hydrolysis back to Cer, and each type of hydrolysis, occurring on different time scales after cell stimulation, has a different function.

Sidedness of Cer formation at the plasma membrane

At the plasma membrane, Cer can be generated from SM by aSMase and/or nSMase as a consequence of cell stimulation by a variety of cytokines, death receptor ligands, differentiation agents, anti-cancer drugs/treatments or other stress agents (for an excellent overview, see [2]). It was thought for some time that, in stimulated cells, SM hydrolysis would only represent the small proportion present in the inner leaflet. Relatively rapid SM hydrolysis at the cytosolic side of the plasma membrane following stimulation of cells with, e.g., tumour necrosis factor α (TNFα), CD40 ligand or vitamin D_3 is believed to be caused by an as yet unidentified nSMase [49–52], that is probably controlled positively by cytosolic phospholipase A_2 [53] and (in an indirect way) negatively by glutathione levels in the cell [54,55]. Activation of nSMase by ligation of the CD40 and TNF death receptors was shown to depend on the adapter protein FAN that associates physically with these receptors [51,52]. The function of the relatively rapid formation of Cer (within 15–60 min) at the cytosolic side of the plasma membrane is unknown. It has been suggested to serve as a second messenger for an enzymic target protein in (apoptotic) signal transduction (to be discussed below). Zhang et al. [56] stably transfected Molt-4 leukaemia cells with bacterial nSMase under the control of an inducible promoter, and showed that the induced nSMase activity generated Cer from an intracellular pool of SM (at the inner leaflet of the plasma membrane?), which resulted in apoptosis, while Cer generated in the outer leaflet by an exogenous SMase did not cause apoptosis. It would be interesting to know whether or not the small SM pool in the plasma-membrane inner leaflet corresponds topologically to rafts/caveolae [17].

The conversion of SM into Cer has also been observed in the outer leaflet of the plasma membrane. van Helvoort et al. [57] described a reverse action of a SM synthase on the basolateral surface of MDCK cells, producing Cer concomitantly with PC. This is a phosphocholine transferase activity, distinct from SM hydrolysis by a SMase that would liberate the phosphocholine headgroup. This activity requires the input of DAG, generated in or inserted into the plasma membrane. The identity of the enzyme involved and the physiological relevance of its activity, as well as its possible regulation, remain unknown. Recent evidence suggests that Cer at the plasma-membrane outer surface can also be produced by aSMase. This enzymic activity was detected in lipid rafts as a rapid response to stimulation of TNF receptor family members, and will be described in the next section. The enzyme aSMase is well characterized and has been cloned, and is traditionally thought to be active only in endosomal/lysosomal compartments [2]. Its relevance for Cer formation as a necessary step in apoptosis induction has been the subject of a long-standing debate [2,5,6,8,58–62]. The distinction of rapid and slow (sustained) phases of Cer formation during apoptosis induction has allowed this controversy to be partially resolved (see below).

Rapid Cer formation at the cell surface promotes death receptor clustering in lipid rafts

As alluded to above, Gulbins and Kolesnick and their co-workers [18,21,22,63] found rapid Cer formation (within 1 min) on the surface of T and B cells following activation of the TNF receptor family members CD95/Fas and CD40 respectively. This Cer was generated in lipid rafts by aSMase, and facilitated the ligand-induced clustering of these receptors, located in lipid rafts [23,64]. Receptor clustering and activation were associated with recruitment to these rafts of downstream signalling proteins: FADD (Fas-associated death domain) and caspase-8 in the case of CD95/Fas [23], and TRAF (TNF receptor-associated factor) in the case of CD40 [64]. Further CD40 signalling towards extracellular-signal-regulated kinase (ERK) activation and cytokine production, in dendritic cells, is initiated within lipid rafts by activation of Src family kinases, such as Lyn [64].

It was shown that receptor activation resulted in translocation of aSMase from intracellular stores to the extracellular leaflet of the plasma membrane, where it co-localized with sphingolipids in rafts [18,63]. Since aSMase is a glycoprotein [8] and has indeed also been detected in isolated rafts [16,65], this makes sense, but with its low pH optimum the enzyme faces unfavourable activation conditions there at neutral pH. An attractive idea, therefore, is that aSMase becomes transiently activated in recycling endosomes, at low pH, where it generates Cer from SM at the luminal side. Upon endosomal fusion with the plasma membrane, Cer will then appear in the outer leaflet of the plasma membrane, as actually shown [18,63]. There, it facilitates raft clustering and the patching and capping of the (death) receptors [18,21,22,63]. Support for an amplifying role of Cer in CD95/Fas-mediated apoptosis came from Burek et al. [66], who used primary cells from a patient with Farber disease. These cells accumulate Cer as the result of an inherited deficiency of acid ceramidase.

The involvement of aSMase in death receptor clustering was demonstrated by using aSMase-deficient cells, in which addition of natural Cer could overcome the decreased sensitivity to CD95/Fas ligation. The raft-dependency of the initiation of receptor clustering and signalling was demonstrated by using raft-disrupting agents, such as cholesterol-depleting methyl-β-cyclodextrin [23]. However, not all cell types show this raft-dependency of CD95/Fas clustering [47]. Also, decreased sensitivity to CD95/Fas stimulation in the absence of aSMase holds only for certain cell types, such as hepatocytes [22,61]. It is a subtle phenomenon that is only observed at sub-optimal concentrations of agonistic anti-CD95 antibody [21,61]. Further, CD95/Fas-induced aSMase-mediated Cer production is a rapid (1 min) event and occurs locally (in rafts). It is conceivable that, if basal Cer levels in these rafts are too high (which may easily be the case [16,17]), stimulated acute Cer production may escape detection and may also no longer be effective in facilitating CD95/Fas clustering and apoptosis induction.

As a cautionary note, aSMase$^{-/-}$ mice, used to demonstrate the involvement of aSMase-mediated Cer formation in apoptosis induction, develop a Niemann–Pick-type disease and their cells have many abnormalities [8,67]. These include altered T cell receptor responses, a severely altered composition of sphingolipids and cholesterol in their membranes, and a defect in the formation of functional lipid rafts, which can no longer be isolated by standard procedures based on detergent insolubility [67]. One can imagine that general alterations in lipid composition, rather than defective Cer formation, may determine apoptosis sensitivity in such cells. We and others [58,59] have found that, in aSMase-deficient B lymphocytes from a Niemann–Pick patient, restoration or overexpression of aSMase did not affect apoptosis induction by CD95/Fas or by radiation and anti-cancer drugs. Clearly, aSMase activity is not in general essential for the induction of apoptosis, but seems to facilitate this process in some cases.

Slow and sustained Cer formation during apoptosis may serve apoptotic body formation

While both aSMase and nSMase activities may rapidly produce small transient elevations in Cer (less than a doubling of control levels) at either side of the plasma membrane (Figure 1, left panel), a slow, sustained and substantial elevation of Cer levels (at least 5-fold over control) is found in the effector phase of apoptosis, induced by death receptor stimulation, anti-cancer drugs, ionizing radiation or serum deprivation [68–70]. This Cer production occurs by an entirely different mechanism (Figure 1, right panel). While aSMase deficiency, as occurs in Niemann–Pick-disease cells, may prevent rapid Cer production in some cells (see above), it does not affect the slow Cer formation [59]. From data obtained in Jurkat T cells stimulated via CD95/Fas or by γ-radiation or etoposide, we concluded that this Cer pool is produced from plasma-membrane SM by an undefined nSMase, downstream of inducer caspase activation [70,71]. Furthermore, we found that, during the apoptotic effector phase, scrambling of membrane phospholipids (loss of bilayer asymmetry) and phosphatidylserine exposure at the cell surface leads to concomitant transbilayer movement of SM. This translocation of SM to the cytoplasmic side of the plasma membrane [71] appeared to be sufficient for immediate hydrolysis by a nSMase, without any apparent activation step. The identity of this enzyme remains unknown. The nSMase isoenzymes that have been cloned [72–74] have an as yet unknown function in the cell. They localize in the endoplasmic reticulum and/or nucleus, and we and others found that their (over)expression in cells does not affect Cer formation during apoptosis, nor apoptosis itself ([8,75] and references cited therein).

What is the function of the slow Cer formation in apoptosis? Phospholipid scrambling and loss of SM have profound effects on the physico-chemical properties (e.g. cholesterol content and fluidity [14,71]) of the plasma membrane (microdomains). Together with cytoskeletal changes [76], this may facilitate membrane blebbing, vesicle shedding and apoptotic body formation. Replenishment of SM in the apoptotic cell prevented these surface changes. Thus the breakdown of SM, rather than the production of Cer as such, seems to be important for these morphological changes in the apoptotic cell [71], and may serve to facilitate apoptotic body formation (Figure 1, right panel).

Cer formation at the Golgi apparatus: a metabolic precursor of SM and DAG in vesicular trafficking

Cer is a precursor of more complex sphingolipids, such as SM and the glycosphingolipids synthesized in the Golgi apparatus [48]. The enzyme SM synthase transfers a phosphocholine group from PC on to Cer, thereby liberating DAG. Like other lipid-metabolizing enzymes, such as phosphoinositide 3-kinase (PI 3-kinase) and phospholipase D [77], SM synthase has recently been shown to play an important role in secretory vesicular transport at the *trans*-Golgi network (TGN) [78,79]. In this process, DAG formation appears to be essential in recruiting protein kinase D and consequent vesicle biogenesis at the TGN (Figure 2). Compromising SM synthase-mediated DAG production by blocking *de novo* Cer synthesis (fumonisin B1 treatment) blocked vesiculation and generated morphological abnormalities (tubules) at the TGN [80]. An essential role for DAG in vesicular trafficking has also been demonstrated in yeast, where the PC transfer protein Sec14p negatively regulates the metabolic flux of PC synthesis and the consumption of DAG [31,81,82]. When Sec14p is defective, PC is overproduced at the expense of DAG, and vesicular transport from the TGN is consequently blocked. Mammalian cells express a Sec14p orthologue, PITPβ (phosphatidylinositol transfer protein β), a PC/SM transfer protein at the Golgi, that might have a similar function as yeast Sec14p in controlling DAG homoeostasis and vesicle biogenesis [78].

Cells with relatively high metabolic activity, such as simian virus 40-transformed fibroblasts, displayed much higher SM synthase activity and higher DAG/Cer ratios than metabolically inactive normal (wild-type) fibroblasts [83,84]. Since DAG, unlike Cer, is often associated with activation of PKC and/or RasGRP (guanine nucleotide releasing protein) in cell proliferation, SM synthase was proposed to be a 'biostat' in the regulation of cell viability and cell death [83,84]. However, in addition to DAG/Cer steady-state levels, SM synthase activity may also determine the metabolic flux from Cer to DAG, and the associated activity of vesicular traffic, which conceivably is higher in transformed/proliferating cells.

While SM synthase is classically located in the Golgi, its activity has also been found at other subcellular locations, depending on cell type [85]: in a compartment close to, or at, the cytosolic side of the plasma membrane [86], and even at the cell surface [57], but not in endosomes [87] (although this remains a controversial issue [88]). Interestingly, SM synthase activity and the consequent production of DAG can be inhibited by the putative PC-specific phospholipase C inhibitor D609 [83,89], suggesting that this activity may account for many of the results obtained with D609 that have been attributed to inhibition of PC-specific phospholipase C [89]. Notably, by inhibiting SM synthase, D609 may thus block membrane vesicular trafficking.

As a cautionary note, while *de novo* synthesized (endogenous) Cer is necessary for vesicle biogenesis at the Golgi [80], exogenous C_2-Cer, in contrast, prevented coated vesicle formation and exocytosis from the Golgi, by preventing the association of ADP-ribosylation factor with the Golgi membrane [90]. This, again, illustrates the differences in cell biological effects of short-chain and natural Cer, probably related to their different physical properties in membranes.

Glucosylceramide synthase (GCS) at the Golgi does not convert Cer derived from SM at the plasma membrane

Cer is a metabolic precursor of complex glycosphingolipids. GCS utilizes Cer at the cytosolic side of the Golgi membrane and may thus potentially lower cellular Cer levels [1]. Cabot and co-workers [91,92] indeed found that overexpression of GCS attenuated the Cer levels accumulating in MCF-7 breast cancer cells in response to various chemotherapeutic drugs or to TNFα. Moreover, it protected these cells against these cytotoxic compounds. In Jurkat cells, however, GCS overexpression had no effect on apoptosis induction or on Cer levels accumulating during apoptosis in response to CD95/Fas stimulation or exposure to etoposide or γ-radiation [26]. Cer generated in this way or by bacterial SMase was not glycosylated by GCS [26]. Similar results were obtained in glucosylceramide-deficient GM95 melanoma cells that were stably transfected with functional GCS and exposed to various drugs (R. J. Veldman and T. Levade, unpublished work). Thus GCS located in the Golgi is topologically segregated from Cer produced from SM in the plasma membrane.

In contrast, *de novo* synthesized Cer, as well as exogenous cell-permeable C_2-Cer (which induces apoptosis), have access to GCS and are efficiently glycosylated [26]. In MCF-7 and other breast cancer cell lines, unlike the Jurkat cell system, drug-induced Cer appeared to be produced via the *de novo* pathway [93,94]. This

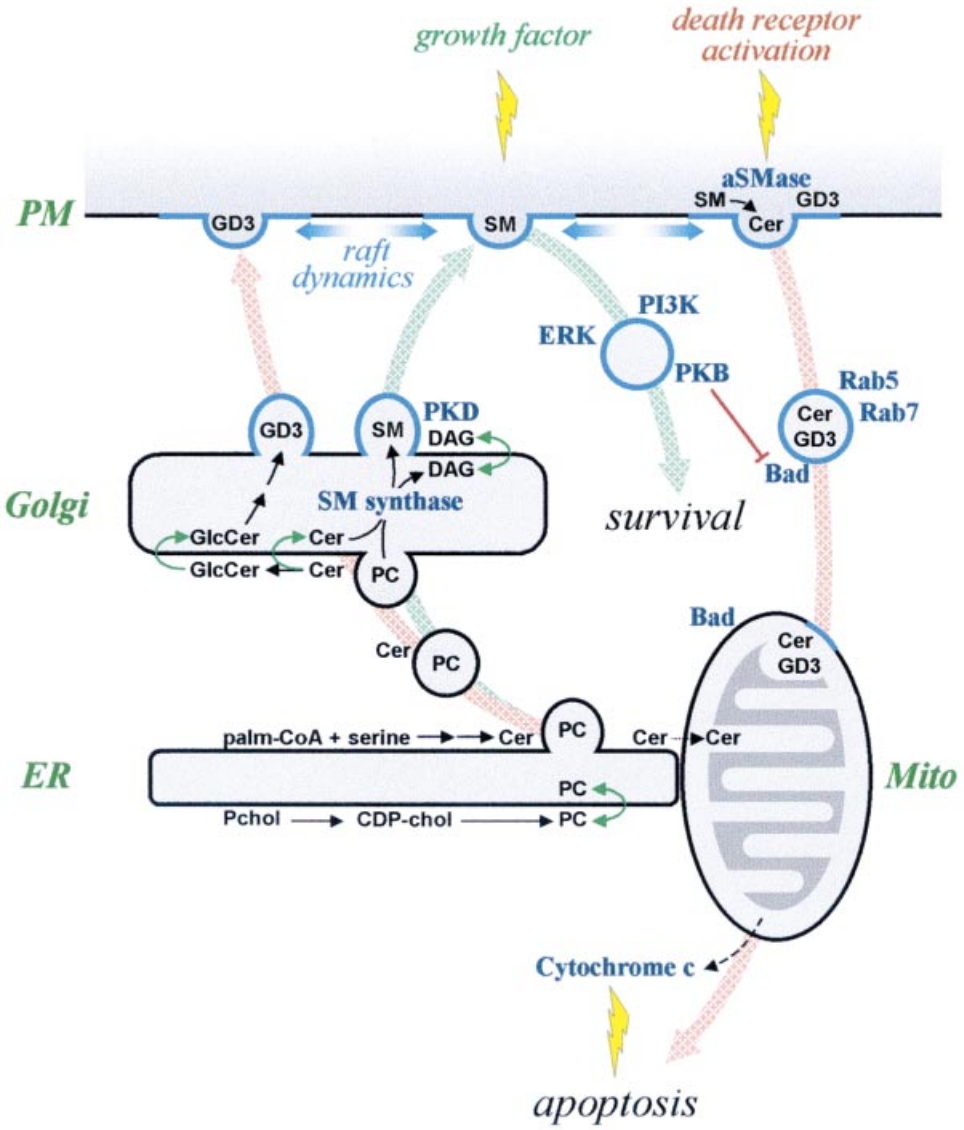

Figure 2 Pathways of lipid trafficking involved in survival and death signalling

The *de novo* synthesis of Cer and PC occurs in the endoplasmic reticulum (ER). Under the control of growth factor or death receptors, key decisions of complex sphingolipid synthesis and anterograde vesicular routing are taking place at the Golgi apparatus (green and red routes respectively). SM synthase may serve as a 'biostat', regulating the metabolic flux through and levels of Cer and DAG, while synthesis and routing of the ganglioside G_{D3} may be involved in death signalling. Lipid rafts (blue) containing sphingolipids (SM, G_{D3}) are important signalling platforms [16,174] and are actively involved in vesicular trafficking [114,175–177] and sphingolipid recycling [178]. Retrograde vesicular transport occurs after receptor-controlled raft dynamics at the plasma membrane (PM) and endocytosis. Proliferative and survival routes towards activation of ERK and/or PKB (green) are distinguished from the death route (red) directed towards mitochondria (Mito). Cross-talk between these pathways exists, e.g. through phosphorylation of Bad by PKB, which sequesters Bad from the mitochondria. Cer may act at multiple sites: it may enter mitochondria at sites of close contact with the ER, or through retrograde vesicular transport, in which process it may play a facilitating role (SM cycle). In the Golgi, Cer is an essential component of the SM synthase 'biostat' and a precursor of the complex (glyco)sphingolipids in rafts. PKD, protein kinase D; palm-CoA, palmitoyl-CoA; Pchol, phosphocholine; CDP-chol, CDP-choline.

explains why GCS expression attenuates Cer levels in MCF-7, but not in Jurkat, cells. It remains unclear why GCS expression would protect MCF-7 cells against apoptosis induction. Is it the diminution of (deleterious?) Cer as such, or an indirect consequence, such as an alteration in vesicular trafficking? We note that the negative correlation between GCS expression/activity and apoptosis does not hold for other cell types [26,95]. Since effects on lipid levels and viability in the MCF-7 system were seen only after a very long period (days) of exposure to the cytotoxic drug or to TNFα [91,92], they might be secondary to general alterations in cellular lipid homoeostasis and vesicular trafficking.

Role of Cer in mitochondria

Mitochondria play a key role in the orchestration of death signals [70,96]. These organelles have recently been appreciated to contain small amounts of a variety of sphingolipids [97] and sterols [98], the function of which is unknown. Mitochondria also contain Cer [97], which may be received directly from the endoplasmic reticulum via intimate membrane contacts [98–100], or derived from SM during apoptosis. There are a number of indications that Cer could be a mediator of apoptosis at the mitochondrial level: Cer levels are elevated in (isolated) mito-

chondria during CD95/Fas-, TNF- and radiation-induced apoptosis [101,102]. A mitochondrial ceramidase with reciprocal (Cer synthase) activity has been identified [103,104]. Birbes et al. [105] found that selective hydrolysis of a mitochondrial pool of SM induced apoptosis. They transfected MCF-7 cells with a bacterial SMase targeted to various subcellular organelles, and observed cytochrome *c* release and apoptosis induction only when the enzyme was targeted to the mitochondria. As mentioned above, Cer can form membrane channels in isolated mitochondria, large enough to release cytochrome *c* and other small proteins [41]. *In vivo*, the release of cytochrome *c* from mitochondria is not that simple. We know that proteins such as Bax, Bad and tBid are involved in permeabilizing the mitochondrial outer membrane to cytochrome *c* [96]. It is conceivable that these proteins catalyse the restructuring of lipids to induce pore formation [106]. For example, pro-apoptotic tBid translocates from the cytosol to mitochondria, where it binds to the specific mitochondrial lipid cardiolipin [107], thus promoting negative curvature and, as a result, destabilization of the lipid bilayer [108].

Another lipid implicated in mitochondrion-dependent apoptosis is the Cer-derived glycosphingolipid (ganglioside) G_{D3} [109–113]. G_{D3} is synthesized in the Golgi and becomes enriched on the cell surface in lipid rafts/caveolar microdomains. Activation of death receptors (CD95/Fas, TNF receptor) induces an intracellular flow of G_{D3}, probably carried entirely by raft-containing vesicular transport [114] (Figure 2): G_{D3} synthesis increased and the lipid disappeared from the cell surface via raft-dependent endocytosis, and moved via vesicular transport towards the mitochondria [115]. Overexpression of G_{D3} synthase induced apoptosis in HuT78 T cells [109], while suppression of G_{D3} synthase expression, by antisense oligodeoxynucleotides, inhibited apoptosis induced by low K^+ or by staurosporine in neurons [111]. In contrast with natural Cer, G_{D3} appears to act on the mitochondrial permeability transition pore, secondary to the generation of reactive oxygen species, leading eventually to cytochrome *c* release [116,117]. The function of Cer here is twofold: first, it is a metabolic precursor of G_{D3} (in the Golgi); second, Cer (as a product of aSMase) may facilitate endocytosis and loss of G_{D3} from the plasma membrane and its subsequent appearance in the mitochondrion [113,115] (Figure 2). In between these locations, the apolar G_{D3} must remain membrane (vesicle)-bound, and was indeed found to be co-localized with the early and late endosomal markers Rab5 and Rab7 respectively [115]. The induction of raft coalescence, local negative membrane curvature and vesicle budding by Cer formation may facilitate this vesicular transport of G_{D3}. Perhaps G_{D3}, alone or together with some pro-apoptotic Bcl-2 family member (such as tBid, dephosphorylated Bad [118] or Bax [119]), may serve to specifically guide/direct this vesicular apoptotic signal towards the mitochondrial target. Interestingly in this regard, Bad is attached to lipid rafts in interleukin-4-stimulated T cells, but dissociates from rafts and associates with mitochondria in interleukin-4-deprived cells, which then die by apoptosis [120]. It may therefore be possible that the apoptotic signal from the cell surface towards the mitochondrion involves the vesicular transport of lipid-raft-bound G_{D3} and Bad or another pro-apoptotic Bcl-2 family member, which is facilitated by Cer formation (Figure 2).

THE SECOND-MESSENGER CONCEPT

Cer is commonly stated to be a second messenger in apoptotic signalling. However, the scientific basis for this notion is not as strong as for other lipid second messengers, such as DAG and the phosphoinositides. We will reconsider the experimental data supporting this second messenger concept in the light of the recent membrane structural perspectives.

Lipid second messengers in signal transduction are usually produced rapidly and transiently, i.e. within seconds or minutes, after stimulation of cells. Thus a rapid induction of Cer formation, if genuine and detectable, could act in apoptotic signalling. However, rapid Cer formation is hard to detect, and reported increases are moderate (less than a doubling) [68]. In fact, quite a number of investigators have been unable to measure

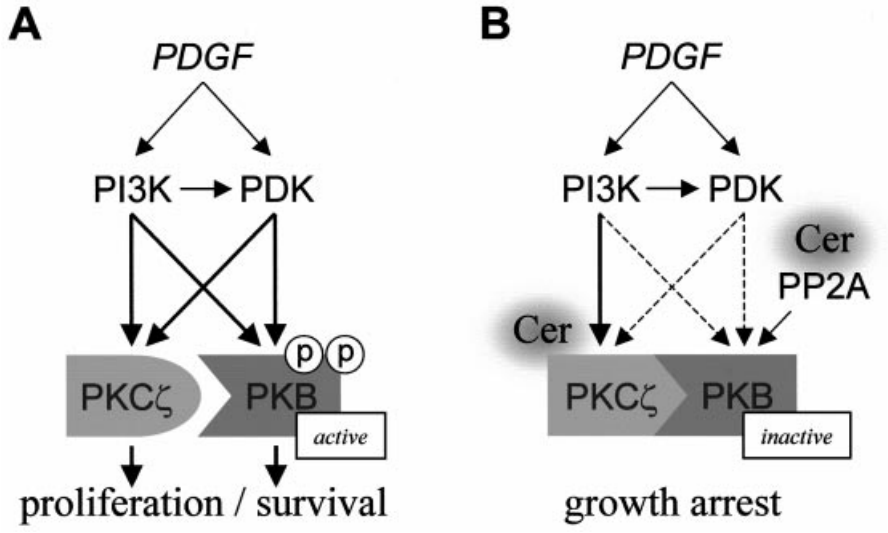

Figure 3 Ceramide-activated PKCζ and protein phosphatase PP2A negatively regulate the Akt/PKB survival pathway

Growth factors such as platelet-derived growth factor (PDGF) stimulate cell proliferative and/or survival pathways (**A**), via activation of PI 3-kinase (PI3K), 3-phosphoinositide-dependent protein kinase (PDK), PKCζ and Akt/PKB at the membrane level. Cer activates PKCζ, directly or indirectly (see the text), such that PKCζ binds to PKB. This, together with a Cer-induced, PP2A-mediated dephosphorylation at PKB residues Thr-308 and Ser-473, inactivates this kinase, which leads to the arrest of cell growth (**B**). Solid arrows represent active routes, and broken arrows represent inhibition of these routes.

these minor increases in Cer levels, for reasons that are not entirely clear. It is conceivable that Cer is generated only locally or, once generated, is metabolized very rapidly (e.g. to SM), so that we are in fact dealing with a rapid Cer flux (difficult to measure) rather than a transient Cer accumulation.

Bona fide lipid second messengers have well defined physiological target proteins. Several possible intracellular target enzymes for Cer have been proposed, such as the protein kinases PKCζ [121,122], kinase suppressor of Ras (KSR) [123] and Raf-1 [124]. However, particularly for the last two kinases, this notion has not been verified consistently by follow-up studies [125,126]. Better characterized potential Cer targets are the Cer-activated protein phosphatases PP1 and PP2A [1,68,127–131]. There is also some evidence for a Cer-activated PP2A in t-SNARE (target soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) dephosphorylation and exocytosis in yeast [129]. However, much of the evidence has remained indirect, and is based mainly on *in vitro* studies with high doses of natural Cer [130] or the use of exogenous short-chain Cer. Another potential Cer target is cathepsin D [132]. This protease, co-localizing with aSMase in endosomes, has been shown to bind Cer directly *in vitro*, leading to its activation. Cathepsin D as a putative Cer target is interesting, since this protease has been implicated in TNF-induced cell death signalling, downstream from aSMase [132], which produces Cer. However, this relatively new potential signalling pathway requires confirmation by other laboratories. Probably the best candidate to be a real physiological Cer target is PKCζ, as several groups have shown that natural Cer binds directly to and activates PKCζ *in vitro* [121,122,133], and is of possible relevance *in vivo* (to be discussed further in the next section).

An important property of a lipid second messenger is that it binds with high affinity to a well defined consensus motif in the primary sequence of its target protein(s). Such motifs have indeed been defined for phosphoinositides and DAG, but not for Cer. For example, we know in detail how DAG binds to the cysteine-rich domains of PKC [134–136]. Based on this knowledge and the structural similarity between Cer and DAG, we hypothesized several years ago that Cer might bind similarly to the single cysteine-rich domain in the regulatory domains of PKCζ, Raf-1 and KSR [136]. However, this hypothesis has not been proven, and in fact appears not to be true for Raf-1, since Cer appeared to bind to the catalytic domain rather than to the cysteine-rich domain of this protein [126]. Although there is some evidence for direct binding of natural Cer to PKCζ [122,133], the precise site where Cer binds to this protein remains unidentified.

PKCζ: a direct or indirect Cer target with multiple downstream effects

While binding to PKCζ *in vitro* has demonstrated for natural, long-chain Cer, the translocation and activation of PKCζ in cells have usually been induced by short-chain Cer, and is not found invariably in all cell types [137]; sometimes it is also induced by exogenous SMase [121,138]. The subcellular site to which PKCζ is translocated has not been determined in most cases, or appeared to be the perinuclear region rather than the plasma membrane [138,139]. This translocation to the nucleus and the involvement of PI 3-kinase in translocation [139] may suggest that this effect of Cer is indirect, possibly secondary to vesicular trafficking.

The cell biological consequences of Cer-induced PKCζ activation may be quite contrasting, depending on the cell system. This is also the case for the signalling function of PKCζ in general: the kinase has been implicated in mitogenic signalling [121,140] downstream of PI 3-kinase and PDK1 (3-phosphoinositide-dependent protein kinase 1) [141,142], but may also have the opposite effect by directly inhibiting the Akt/protein kinase B (PKB) survival pathway (Figure 3). Growth factors activate Akt/PKB via activation of PI 3-kinase, which may lead to phosphorylation and subsequent sequestering (by 14-3-3 protein) of pro-apoptotic Bad from the mitochondrion [143] (Figure 2). Short-chain Cer can counteract this effect of growth factors on PKB by inducing the binding of active PKCζ to PKB, which leads to inactivation of PKB [144,145] (Figure 3). This inactivation also depends on dephosphorylation of PKB by the Cer-activated protein phosphatase PP2A [146,147]. The data have been interpreted in terms of direct binding of Cer to, and activation of, PKCζ [144], but, again, the possibility cannot be excluded that the effect of Cer is the indirect consequence of the reorganization of membrane structure and/or altered vesicular trafficking. For example, Stratford et al. [148] have shown that Cer specifically prevents the membrane recruitment of PKB via its pleckstrin homology domain to the PI 3-kinase product PtdIns(3,4,5)P_3. This may alter complex formation among signalling proteins and may favour the dephosphorylation and inactivation of PKB [147], leading to growth suppression. Alternatively, in HEK-293 cells, Cer-activated PKCζ, formed a complex with and activated the MEKK1/SEK/SAPK pathway [where SAPK is stress-activated protein kinase, SEK is SAPK/ERK kinase and MEKK is mitogen-activated protein kinase (MAPK)/ERK kinase kinase], leading to cell cycle arrest [133]. It would be interesting to investigate whether this mechanism also applies to the early observation of the involvement of Cer in stress-induced apoptosis via the c-Jun N-terminal kinase (JNK)/SAPK pathway [149].

In marked contrast with the inhibition of Akt/PKB and the activation of SAPK/JNK, Cer-activated PKCζ can also stimulate proliferative/survival pathways. Wang et al. [150] showed Cer co-activation of nuclear factor κB/JNK and survival of PC12 cells. In alveolar macrophages, Cer-activated PKCζ has been proposed to play a central role in the activation of MEK (MAPK/ERK kinase) and subsequently of p42/44 MAPK/ERK by endotoxin (lipopolysaccharide) [151,152]. Moreover, in this system, (exogenous) Cer also activates PI 3-kinase and PKB, a survival pathway [153].

To conclude, there is much evidence that Cer can activate PKCζ, but it is not clear whether, *in vivo*, this activation is direct or indirect. Therefore the interpretation in most of these studies that short-chain Cer binds directly to and activates PKCζ in intact cells might be incorrect. Activation might well be a consequence of altered membrane organization/vesicular trafficking caused by this membrane-perturbing lipid. In addition, the signalling events downstream of PKCζ activation are diverse and even contrasting.

CONCLUDING REMARKS AND FURTHER PERSPECTIVES

Although Cer accumulation in cells is a general trait of apoptosis, the lipid has many other functions in cell biology. We emphasize here the important function of Cer production, both *de novo* and through SM hydrolysis, in membrane sub-structure, raft coalescence and intracellular vesicle transport. Vesicular trafficking (both anterograde and retrograde) is an essential element in many signalling pathways. From this perspective, it is not surprising that Cer can regulate diverse cell biological processes: in addition to growth arrest and apoptosis, it is implicated in necrosis [154–156], survival/proliferation [125,150,153,154,157], differentiation [158,159], senescence [160] and cytoskeletal

rearrangement (stress fibre formation [161] and axonal outgrowth [162]). Thus it should be stressed that Cer is not *a priori* an apoptotic lipid [163–165] and, in addition, that apoptosis does not necessarily require an elevation of Cer levels [71,166].

We emphasize that exogenous short-chain ceramides (e.g. C_2-Cer) are misleading experimental tools, because their physical properties in membranes and subcellular mobility are quite different from those of endogenous Cer. These synthetic ceramides enter the cell easily and interfere randomly with vital membrane-located processes, such as *de novo* phospholipid synthesis and the PKB survival pathway, which often results in growth arrest and/or cell death by apoptosis [1] or necrosis [155,156], but which may have other biological effects as well. This principle, on the other hand, has also led to a most valuable clinical application: C_6-Cer-coated balloon catheters prevent stretch-induced neointimal hyperplasia of vascular smooth muscle cells and secondary occlusion of coronary arteries [167].

The question of whether or not Cer is a *bona fide* second messenger still remains open. Clearly, rapid Cer formation at the cell surface after death receptor ligation has a membrane structural rather than a second messenger function. *De novo* Cer synthesis seems to be most important for complex sphingolipid synthesis (including lipid raft assembly) and intracellular vesicular trafficking. The minor pool of Cer in mitochondria, which increases during apoptosis, may have a membrane structural function as well, i.e. channel formation. It is possible that Cer generated from the minor pool of SM at the inner leaflet of the plasma membrane acts as a second messenger, but more rigorous proof is needed. The best candidate physiological target proteins for Cer identified to date are the protein phosphatases PP1 and PP2A, cathepsin D, and PKCζ. Although, for some of these proteins, direct binding of Cer and/or activation by Cer *in vitro* has been reported, definite proof would require the identification of a consensus motif for the high-affinity binding of Cer. However, such evidence is still lacking, and there is a reasonable possibility that many of the described effects of Cer on these proteins *in vivo* are indirect, and possibly the consequence of reorganizations in membrane fine structure or altered vesicular trafficking, as described.

We have not discussed here the idea that the putative 'apoptotic' messenger Cer may have its counterpart in sphingosine 1-phosphate, a mediator of cell proliferation. In this 'rheostat' model, put forward by Spiegel and co-workers [168], breakdown of 'apoptotic' Cer by a ceramidase leads to sphingosine, which, in turn, can be phosphorylated to form 'proliferative' sphingosine 1-phosphate, and vice versa [169–172]. The question here is how and where in the cell such a 'rheostat' would be regulated.

The role of Cer and the other sphingolipids in the structure and function of lipid rafts needs to be explored further. In order to understand signal initiation and lipid second-messenger formation from rafts, we need to know the lipid composition at the cytosolic side of the rafts, and the changes that occur at this location after cell stimulation. It will also be necessary to identify and characterize the different types of rafts, i.e. those that are located at the plasma membrane, and those in vesicles moving towards specific intracellular destinations. For example, an important question concerns the factors that determine the generation and routing of raft-containing endocytic vesicles towards mitochondria during apoptosis. Are SMase activity and certain glycolipids (such as G_{D3}) essential? What is the possible role of pro-apoptotic Bcl-2 family members and perhaps PKCδ [173] in this routing of membrane vesicles? How is the lipid–protein interaction in such vesicles defined? Certainly, the biophysical properties of Cer and other sphingolipids, and the enzymes that produce and convert these lipids at specific subcellular locations, are important modulating factors. Identification of these specific determinants is a major challenge for future research.

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