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REVIEW

The ceramide transporter and the Goodpasture antigen binding protein: one protein – one function?

Chiara Mencarelli,* Mario Losen,* Caroline Hammels,* Jochen De Vry,* Matthijs K. C. Hesselink,† Harry W. M. Steinbusch,* Marc H. De Baets*,‡ and Pilar Martínez-Martínez*

*Department of Neuroscience, School for Mental Health and Neuroscience, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, The Netherlands

†NUTRIM School for Nutrition, Toxicology and Metabolism, Department of Human movement Sciences, Maastricht University, The Netherlands

‡Neuroimmunology Group, Biomedical Research Institute (BIOMED), Hasselt University, Diepenbeek, Belgium

Abstract

The Goodpasture antigen-binding protein (GPBP) and its splice variant the ceramide transporter (CERT) are multifunctional proteins that have been found to play important roles in brain development and biology. However, the function of GPBP and CERT is controversial because of their involvement in two apparently unrelated research fields: GPBP was initially isolated as a protein associated with collagen IV in patients with the autoimmune disease Goodpasture syndrome. Subsequently, a splice variant lacking a serine-rich domain of 26 amino acids (GPBPΔ26) was found to mediate the cytosolic transport of ceramide and was therefore (re)named CERT. The two splice forms likely carry out different functions in specific sub-cellular localizations. Selective GPBP knockdown induces extensive apoptosis and tissue loss in the brain of zebrafish. GPBP/GPBPΔ26 knockout mice die as a result of structural and functional defects in endoplasmic reticulum and mitochondria. Because both mitochondria and ceramide play an important role in many biological events that regulate neuronal differentiation, cellular senescence, proliferation and cell death, we propose that GPBP and CERT are pivotal in neurodegenerative processes. In this review, we discuss the current state of knowledge on GPBP and CERT, including the molecular and biochemical characterization of GPBP in the field of autoimmunity as well as the fundamental research on CERT in ceramide transport, biosynthesis, localization, metabolism and cell homeostasis.

Keywords: autoimmunity, ceramide, GPBP/CERT, neurodegenerative diseases.

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Address correspondence and reprint requests to Dr. Pilar Martínez-Martínez, Department of Neuroscience, School for Mental Health and Neuroscience, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. E-mail: p.martinez@maastrichtuniversity.nl

Abbreviations used: Aβ, β-amyloid; aa, amino acids; AD, Alzheimer's disease; APP, amyloid precursor protein; A-SMase, acidic SMase; CERT, ceramide transporter; $CERT_L$, ceramide transporter long isoform; ER, endoplasmic reticulum; FAPP2, four-phosphate-adaptor protein 2; FFAT, two phenylalanine amino acids 'FF' in an acidic tract motif; GlcCer, glucosylceramide; GPBP, Goodpasture antigen-binding protein; GSLs, glycosphingolipids; NFkB, nuclear factor kappa B; NGF, nerve growth factor; N-SMase, neutral-SMase; OLGs, oligodendrocytes; OSBP, oxysterol-binding protein; PD, Parkinson's disease; PH, pleckstrin homology; PIP, phosphatidylinositol phosphates; Pol K, DNA polymerase k; SM, sphingomyelin; SR, serine repeat motif; START, steroidogenic acute regulatory protein (StAR)-related lipid transfer; TNF, tumor necrosis factor; VAPs, vesicle associated-ER proteins.

CERTain facts about Goodpasture antigen-binding proteins

Goodpasture antigen-binding proteins (GPBPs) are widely distributed in the central nervous system (CNS), where they are involved in brain development and homeostasis. Different GPBP isoforms are expressed in the cell. GPBP/ceramide transporter long isoform ($CERT_L$) with 26 additional amino acids (aa) (Ex11+) (Raya *et al.* 1999) and GPBPΔ26/ without 26 aa (Ex11−) (Hanada *et al.* 2003) have been studied in detail.

These proteins were characterized in the context of two different biological processes: GPBP/$CERT_L$ was initially isolated in 1999 by Raya and colleagues and named as Goodpasture antigen-binding protein (Raya *et al.* 1999). Goodpasture's syndrome is a strictly human disorder caused by antibodies directed against the non-collagenous domain of the α3-chain of collagen type IV [α3 (IV) NCI]. Collagen is a major component of the extracellular matrix. The autoantibodies cause rapid functional disruption of the basement membrane of lungs, kidneys and the choroid plexus (Salama *et al.* 2001). GPBP is a soluble extracellular protein which binds and phosphorylates the antigen in this syndrome. Its expression is increased in other spontaneous autoimmune disorders including Goodpasture's syndrome, lupus erythematosus, pemphigoid and lichen planus (Raya *et al.* 2000), suggesting that GPBP might be an important protein in autoimmune disorders, where autoantigens arise from abnormal protein domain organization.

Subsequently, in 2003, Hanada and colleagues showed that a spliced variant of GPBP which lacks a serine-rich domain composed of 26 aa residues was responsible for the cytosolic trafficking of ceramide from the endoplasmic reticulum (ER), to the Golgi apparatus. Hence, this isoform was termed as CERT (Hanada *et al.* 2003). Since the longer isoform also has ceramide transport properties *in vitro*, GPBP was renamed as $CERT_L$ (Hanada *et al.* 2003).

Thus far it is not clear how the extracellular function of GPBP/$CERT_L$, which is related to immune complex-mediated pathogenesis, can be conciliated with the critical role of the protein as a cytosolic ceramide transporter. Much of the literature involving GPBPs does not distinguish between the different protein variants and generally refers solely to GPBPΔ26/CERT. This lack of differentiation between the isoforms hampers the interpretation and understanding of results and poses the risk of neglecting genuine functional differences between them. Here, we will refer to both splice isoforms as GPBPs, to the longer splicing variant as GPBP/$CERT_L$ and to the shorter splicing variant as GPBPΔ26/CERT. Despite the difference of 26 amino acids, GPBP/$CERT_L$ and GPBPΔ26/CERT have undistinguishable molecular weights when analyzed by electrophoresis. However, they are not the only isoforms found in the cell. Smaller and bigger molecular weight isoforms have been described, which result from an alternative initiation translation site and post-translational modifications. Each of these isoforms is addressed specifically below.

In this review, we comprehensively summarize the current knowledge about GPBPs including their structure, properties and functions. Finally, we will focus on recent findings which delineate an intriguing picture of biological and pathological processes, defining GPBPs as pivotal proteins for brain homeostasis and disease processes.

Molecular and biochemical characterization of GPBPs

Two splicing variants: 26 amino acids of difference

GPBP/$CERT_L$ and GPBPΔ26/CERT are alternatively spliced variants of 624 and 598 aa respectively that are identical in sequence, except for the 26 aa serine rich domain (SR2) that is not present in the shorter isoform (Raya *et al.* 2000). GPBPs are phylogenetically highly conserved during evolution between lower vertebrates and mammals at the amino acid level (Fig. 1). This holds true also for the 26 aa of exon 11 as such, suggesting that the two isoforms perform crucial and distinct physiological functions.

Goodpasture antigen-binding protein has three distinct functional domains; the pleckstrin homology (PH) domain, the MIDDLE REGION and the START (steroidogenic acute regulatory protein-related lipid transfer) domain (Fig. 2). The amino terminal ~120 aa region contains the PH domain that targets the protein to the Golgi apparatus (Hanada *et al.* 2003). The PH domain recognizes a specific isomer of phosphatidylinositol phosphates (PIP), namely PI4P which confers Golgi targeting in mammalian cells (Levine and Munro 2002).

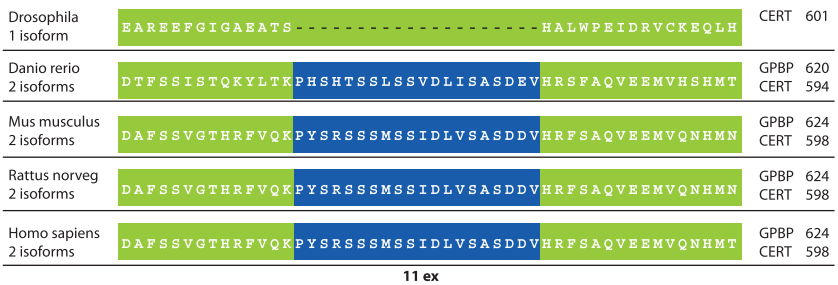

Fig. 1 Aminoacid sequence aligment of GPBPs in various species. In *Drosophila* only the GPBPΔ26/CERT exists.

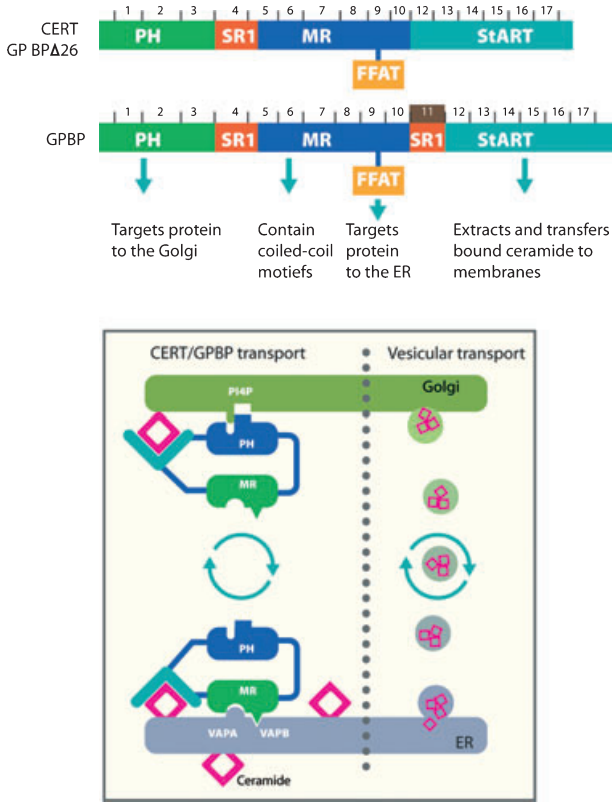

Fig. 2 GPBP/CERT$_L$ and GPBPΔ26/CERT domains: N-terminal Pleckstrin homology (PH) domain. Middle region (MD) with two serine-rich domains (SR1, SR2) and FFAT motif (double phenylalanine in an acidic tract); the 11th exon, encoding SR2, is deleted in GPBPΔ26/CERT. C-terminal steroidogenic acute regulatory protein related lipid transfer domain (START). Molecular transfer model of ceramide from ER to the *trans*-Golgi region by CERT mediated pathway and by vesicular-dependent minor pathway.

The mutation G67E in the PH domain present in Chinese hamster ovary mutant cell line LY-A, destroys the PI4P binding activity of this domain, resulting in an impaired ER-Golgi ceramide transport (Hanada *et al.* 2003).

The MIDDLE REGION of 250 aa contains a coiled-coil motif that might play a role in self-oligomerization (Raya *et al.* 2000). Moreover, the presence in this domain of a FFAT motif (comprising two phenylalanine aa 'FF' in an acidic tract) is essential for GPBPs' ER targeting (Kawano *et al.* 2006). FFAT interaction with vesicle associated-ER proteins (VAPs) is a common mechanism where proteins, most of which are involved in lipid metabolism, target ER membranes (Loewen *et al.* 2003). The GPBPs' FFAT motif interacts with the ER resident type II membrane protein VAP, VAP-A and VAP-B. Mutations in the FFAT motif of GPBPΔ26/CERT destroy the VAP-GPBPΔ26/CERT interaction and, consequently, the GPBPΔ26/CERT-mediated ER to Golgi transport of ceramide in cells (Kawano *et al.* 2006).

The carboxyl terminal of 230 aa is a START domain. This is a structural region that forms a deep lipid-binding pocket that can extract ceramide from membranes and transfer the bound ceramide to other membranes (Tsujishita and Hurley 2000; Alpy and Tomasetto 2005). GPBPs can transfer natural ceramide isoforms (C16-dihydroceramide, C16-phytoceramide) and various ceramide molecular species having C14–C20 amide-acyl chains (Kumagai *et al.* 2005). However, GPBPs can not extract any other lipid type such as sphingosine, sphingomyelin (SM), phosphatidilcholine or cholesterol (Hanada *et al.* 2003).

GPBP/CERTL receive multiple phosphorylations at a serine repeat motif (SR1) that is also present in the GPBPΔ26/CERT isoform and is located after the PH domain (Kumagai *et al.* 2007). The hyper-phosphorylation at the SR1 domain down-regulates the ER to Golgi transport by repressing the PI4P binding ability of the PH domain and the ceramide transfer activity of the START domain (Kumagai *et al.* 2007).

Loss of SM and cholesterol from the plasma membrane induces the dephosphorylation of the SR1 motif to activate GPBPΔ26/CERT to target both the ER and the Golgi membranes (Kumagai *et al.* 2007). However, further studies are needed to elucidate how SM/cholesterol rafts affect the phosphorylation of GPBPΔ26/CERT. The serine rich domain SR2 located before the START domain is only present in the GPBP/CERT$_L$ isoform and constitutes the crucial site that differentiates the two isoproteins (Raya *et al.* 2000).

One gene, two splicing variants, many isoproteins

The human gene encoding GPBPs, *COL4A3BP*, is located in the chromosomal 5q13.3 region and consists of 17 exons. The controversy over GPBP isoforms undoubtedly reflects the complex arrangement of this gene: its mRNA undergoes alternative processing either of exon splicing or rare protein translation initiation, giving rise to multiple proteins (Fig. 3).

GPBP/CERT$_L$ and GPBPΔ26/CERT which are derived from alternative splicing as mentioned above, exist in turn as different isoforms resulting from the use of two translation initiation codons: the classical AUG codon and the rare in-frame ACG codon located upstream (−83) of the known AUG start (Revert *et al.* 2008). We will refer to the additional GPBP/CERT$_L$ isoprotein resulting from ACG translation initiation as GPBP/CERT$_L$Σ128, since 128 additional amino acids are present at the amino terminus of the protein. It is not yet known if also the GPBPΔ26/CERT mRNA is subject to the alternative translation initiation.

A small number of mammalian mRNAs initiate translation from a non-AUG codon, in addition to initiating at a downstream in-frame AUG codon. Such mRNAs encode regulatory proteins such as proto-oncogenes, transcription factors, kinases, or growth factors. Their translation initiation

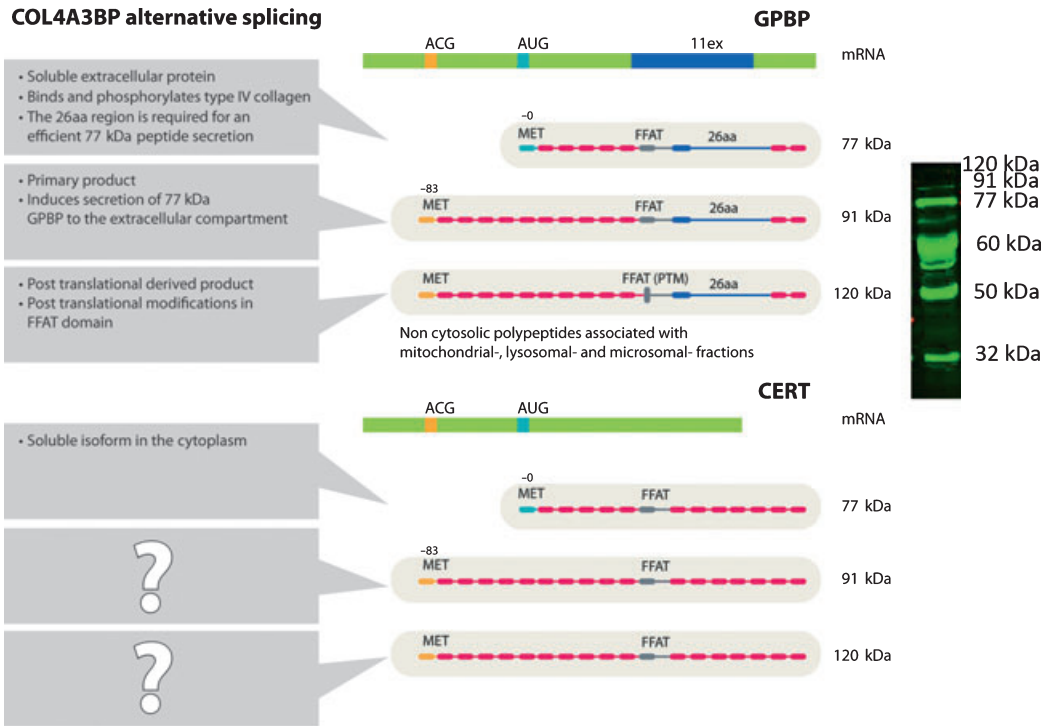

Fig. 3 GPBP/CERT$_L$ is expressed as multiple isoforms because of an alternative translation initiation. GPBP mRNA, in addition to the traditional initiation codon, has a non-canonical translation initiation site (ACG) located upstream of the known AUG. These conventional and alternative start sites result, respectively, in the synthesis of two polypeptides of 77 and 91 kDa, harboring different amino terminal domains that confer distinct functions to the isoforms and control their localizations. The GPBP/CERT$_L$ 91 kDa product (GPBPS128) partly enters the secretory pathway where undergoes covalent modifications to yield a 120 kDa polypeptide. Similarly, GPBPΔ26/CERT possibly exists as different isoforms resulting from canonical (77 kDa) and non-canonical (91 kDa and 120 kDa) mRNA translation initiation. Western blot analysis for GPBP proteins (modified from Mencarelli *et al.* 2009) in rat cortical extract shows the presence of high molecular weight isoforms together with other lower molecular bands products previously described, probably generated from the proteolysis of the higher molecular weight isoforms (Revert *et al.* 2008).

results in the synthesis of proteins harbouring different amino terminal domains potentially conferring on these isoforms distinct functions (Touriol *et al.* 2003).

GPBP/CERT$_L$ alternative initiation codons generate two primary polypeptides of 77 and 91 kDa, respectively (Fig. 3), leading to the synthesis of two protein isoforms with differential transcriptional activities and different subcellular localizations as has been described previously for proteins of the c-myc, fibroblast growth factor and Vascular endothelial growth factor (VEGF) families (Prats *et al.* 1989; Meiron *et al.* 2001; Touriol *et al.* 2003). The 77 kDa polypeptide, resulting from canonical translation, is secreted extracellularly. It is soluble and interacts with type IV collagen in the extracellular matrix. The SR2 region is critical for GPBP/CERT$_L$ secretion (Revert *et al.* 2008) and the FFAT domain is essential for GPBP/CERT$_L$ to enter the secretory pathway (Revert *et al.* 2008).

The GPBP/CERT$_L$Σ128 isoform resulting in the 91 kDa isoprotein remains associated to membranes and it has been suggested that it regulates the secretion of 77 kDa GPBP/CERT$_L$ (Revert *et al.* 2008).

GPBP/CERT$_L$Σ128 enters the secretory pathway where it undergoes covalent modifications to yield a 120 kDa polypeptide. However, the type(s) of post-translational modification(s) that this isoform harbors is not yet known.

In eukaryotes, the existence of bifunctional genes where a single transcript serves as the template for synthesis of both a cytoplasmic isoform and a mitochondrial isoform has also been postulated (Wang *et al.* 2003). Each of these genes encodes mRNAs with distinct 5-ends which are generated from two alternative in-frame initiation codons. Interestingly, the mitochondrial forms can be translated from non-AUG codons on the long mRNA, whereas the cytosolic forms are translated from the second in-frame AUG on the short mRNA. As a consequence, the mitochondrial enzymes have the same polypeptide sequences as their cytosolic counterparts, except for a short amino-terminal mitochondrial targeting sequence (Tang *et al.* 2004; Chen *et al.* 2009). Although so far no direct evidence has been obtained to verify this hypothesis, recent discoveries indicated a possible role of GPBPs in mitochondrial function (Wang *et al.* 2009).

So far only a cytoplasmic 77 kDa iosform of GPBPΔ26/CERT has been described; however it is possible that also alternative translation initiation isoforms with the amino acids encoded by exon 11 exist.

A busy gene promoter

COL4A3BP has been found to localize head-to-head with *POLK*, the gene encoding DNA polymerase k (Pol K) (Granero *et al.* 2005). Thus, the promoters of *COL4A3BP* and Pol K are overlapping and bidirectional. Pol K is a DNA polymerase that enhances the frequency of spontaneous mutations and it plays a role in repairing DNA damage by facilitating base pairing at aberrant replication forks (Ohashi *et al.* 2000; Zhang *et al.* 2000). These mutations are important when they are introduced into the immunoglobulin genes of B cells, because they lead to a variety of mutant antibodies with can be selected for high affinity binding to antigens (Faili *et al.* 2004). This specific arrangement suggests the possibility that Pol K and GPBPs are partners in specific cell programs. An augmented expression of both *COL4A3BP* and *POLK* has been found in patients suffering from skin autoimmune processes (Raya *et al.* 2000; Granero *et al.* 2005).

The bidirectional *POLK*/*COL4A3BP* promoter can be activated at three different regulatory elements; an Sp1 site, a TATA-like sequence and a nuclear factor kappa B (NFkB)-like site. When Sp1 and NFkB form a complex containing predominantly Sp1 compared to NFkB, the transcription is more efficient in the *POLK* direction. It was shown that tumor necrosis factor (TNF) induced the transcription of this promoter in the *COL4A3BP* direction (Granero *et al.* 2005). TNF is a crucial pro-inflammatory cytokine mediating many aspects of immunity (McCoy and Tansey 2008). Thus, the interaction of TNF with the promoter of *COL4A3BP* might explain the increased expression of GPBPs found in several autoimmune disorders. *COL4A3BP* promoter activation by other cytokines has not been reported to our knowledge.

Functional differences between GPBP/CERT$_L$ and CERT/GPBPΔ26

Despite the similarity of GPBP/CERT$_L$ and CERT/GPBPΔ26 with respect to their domain organization, several observations suggest important functional differences between these two splice variants.

Both GPBP/CERT$_L$ and CERT/GPBPΔ26 exist as oligomers under native conditions: they self-associate *in vivo* to form high molecular weight aggregates mainly stabilized by non-covalent bonds. The presence of the 26-residue-serine rich motif in GPBP/CERT$_L$ increases this interaction and the specific kinase activity. Moreover, it allows GPBP/CERT$_L$ to be secreted extracellularly where it exists as a soluble form (Revert *et al.* 2008). GPBP/CERT$_L$ is able to bind collagen type IV with high affinity, in contrast to GPBPΔ26/CERT which has low-affinity binding to collagen (Raya *et al.* 2000). It was demonstrated that GPBP/CERT$_L$ is a non-conventional serine/threonin kinase active towards extracellular matrix proteins that might contribute to their proper organization and supramolecular assembly.

GPBPΔ26/CERT is the most common transcript in cells and widely expressed in normal human tissues, whereas GPBP/CERT$_L$ shows preferential expression in brain, skeletal muscle and heart. Moreover, GPBP/CERT$_L$ is expressed in tissues targeted by autoimmune responses including human alveolar and glomerular basement membrane, the biliary ducts and the Langerhans islets (Raya *et al.* 2000). Over-expression of GPBP/CERT$_L$ in mice causes dissociation of the glomerular basement membrane and subsequent accumulation of IgA in the absence of an evident autoimmune response (Revert *et al.* 2007).

Both isoforms can mediate ceramide transfer from the ER to the Golgi complex (Hanada *et al.* 2003), a process critical for the synthesis and maintenance of normal levels of sphingolipids in mammalian cells. However, the question remains if GPBP/CERT$_L$ is able to transport ceramide to other subcellular localizations including the extracellular space and if the function as a ceramide transporter is related to collagen interaction.

Various animal models have clearly pointed the different role of the two splicing variants. In zebrafish GPBP/CERT$_L$ is mostly expressed at early stages of embryogenesis compared to GPBPΔ26/CERT, and its knockdown selectively induces apoptosis in muscle and brain during early development (Granero-Molto *et al.* 2008). In *Drosophila* only the GPBPΔ26/CERT isoform is present (Fig. 3). Loss of functional GPBPΔ26/CERT in these flies resulted in changes in membrane properties and decreased physiological functions caused by increased oxidative stress. In contrast they did not develop neuronal degeneration in the brain and the aged phenotype that the animals show is not because of specific damage in the nervous system (Rao *et al.* 2007). Homologs of key enzymes belonging to the sphingolipid metabolic pathway have been discovered in *Drosophila melanogaster*, making this an optimal model to study the effects of dysregulation of sphingolipid metabolism on nervous system development, function and integrity. Therefore, the presumed lack of GPBP/CERT$_L$ isoform here would indeed merit further investigation.

In rat brain, GPBPs are widely distributed and have higher expression levels in neurons compared to other cell types (Mencarelli *et al.* 2009). The presence of high levels of ceramide and its related metabolic enzymes in neurons throughout the brain might require a strong expression of its transporter. Which specific isoform is responsible for this generalized brain expression still remains to be investigated.

GPBPs, FAPP2 and OSBP1: a structure in common

The intense interest elicited by the lipid-transfer proteins over the last few years has been driven by the crucial role of lipids in cell, tissue and organ physiology as demonstrated by many human diseases that involve the disruption of lipid metabolism. Like GPBPs, other proteins with putative lipid transfer activity are potentially able to mediate lipid metabolic pathways and membrane trafficking events in the cell. Specifically, two of them share similar protein domain organizations with GPBPs: oxysterol-binding protein 1 (OSBP1) (Ridgway *et al.* 1992) and phosphatidylinositol four-phosphate-adaptor protein 2 (FAPP2) (Godi *et al.* 2004) which are both characterized by a highly homologous N-terminal plecktrin homology (PH) domain that binds PI4P at the Golgi complex. Beside the PH domain, they also have a distinct lipid binding/transfer domain at their C-terminus (Fig. 4).

OSBP1 is a member of a family of sterol-binding proteins with roles in lipid metabolism, regulation of secretory vesicle generation and signal transduction (Lagace *et al.* 1997, 1999). It is an 807 aa protein that comprises, besides an N-terminal PH domain, a FFAT motif that binds the integral ER membrane proteins VAP-A and VAP-B, and an oxysterol-binding domain. The other members of the family are referred to as OSBP-related proteins, with a highly conserved OSBP-type sterol-binding domain at the C-terminus (Lehto and Olkkonen 2003), each subject to tissue-specific transcriptional regulation (Lehto *et al.* 2001).

OSBP1 acts at the interface between the ER and the Golgi complex and could be a mammalian sterol-transfer protein (Ridgway *et al.* 1992). Consistent with the high cholesterol content of the brain, OSBP1 and many other OSBP-related proteins are highly expressed in the CNS (Laitinen *et al.* 1999).

FAPP2 is a 507 aa protein, with an N-terminal PH domain, a proline rich domain and a glycolipid-transfer-protein homology domain at its C-terminus. The presence of this glycolipid-transfer-protein domain raises the interesting possibility that FAPP2 itself is a glucosylceramide (GlcCer) transporter towards appropriate sites for the synthesis of complex glycosphingolipids (GSLs) (D'Angelo *et al.* 2007). GSLs constitute a major component of neuronal cells and are thought to be essential for brain function, as GSL deficiency has been shown to lead to a down-regulation of gene expression sets involved in brain development and homeostasis. This trafficking function of FAPP2 indicates a crucial role for this protein in determining the lipid composition of the neuronal plasma membrane.

Considering that these proteins have similar binding partners at the Golgi apparatus (the PI4P through their PH domain) and at the ER (VAPs through FFAT domain of GPBPΔ26/CERT and OSBP1), a cross-interaction between them is possible.

According to this, it has been shown that OSBP1 can regulate sphingomyelin synthesis by interacting with GPBPΔ26/CERT, promoting its binding with VAPs and consequently enhancing GPBPΔ26/CERT-dependent ceramide transport to the Golgi complex (Perry and Ridgway 2006). A mutant of OSBP1 was found to limit transport of a fluorescent ceramide analogue to sites in the ER, suggesting that the function of OSBP1 involves the transport of ceramide from ER to the Golgi sites where sphingomyelin synthase is active (Wyles *et al.* 2002). The shift of OSBP1 to a Golgi location upon 25OH (hydroxycholesterol) treatment of cells coincides with Golgi translocation and activation of GPBPΔ26/CERT (Perry and Ridgway 2006). Thus, localization of GPBPΔ26/CERT at the Golgi apparatus seems to be OSBP-dependent and correlates with increased SM synthesis and ceramide transport (Perry and Ridgway 2006). Taken together, these observations suggest that OSBP1 acts as a sterol sensor whose function is to integrate, possibly via regulation of GPBPΔ26/CERT function, the cellular sterol

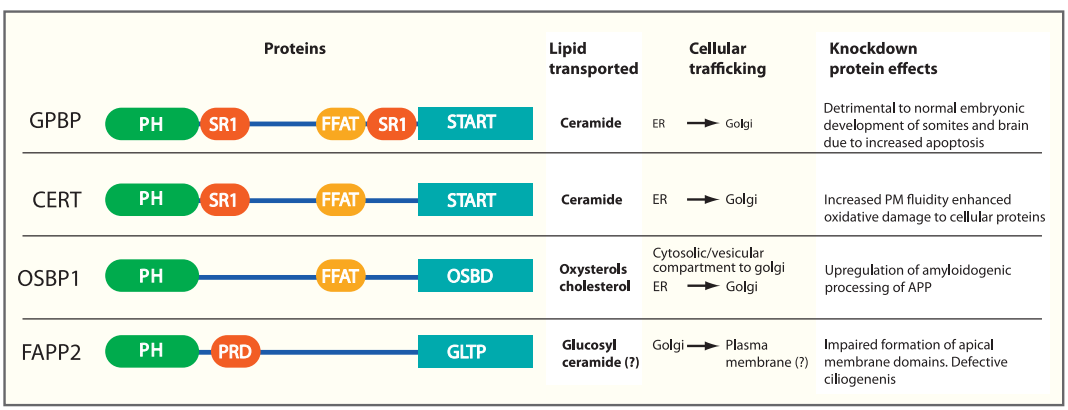

Fig. 4 Domain organization of lipid binding/transfer proteins GPBPs, OSBP1 and FAPP2. PH (pleckstrine homology domain), SR1/SR2 (serine rich domain), PRD (proline rich domain), FFAT (two phenylalanine in an acidic tract), GLTP (glycolipid transfer protein domain), START (StART related lipid transfer domain), OSBP (oxysterol binding domain).

status with sphingomyelin metabolism (Olkkonen *et al.* 2006).

A pleiotropic ceramide

Ceramide is a precursor for the biosynthesis of all complex sphingolipids, as it is a product of their degradation. It is composed of an *N*-acylated (14–26 carbons) sphingosine (18 carbons). It has been suggested that ceramide and other sphingolipids found in the phospholipid bilayer membranes are not only building blocks that confer structural rigidity by virtue of their ability to form strong hydrogen bonds (Zhang *et al.* 2009). Besides their structural role, sphingolipids participate in a variety of activities in diverse cellular and developmental events and they seem to act as lipid second messengers involved in many biological events spanning from cell growth (Jayadev and Hannun 1996; Spiegel and Merrill 1996; Schwarz and Futerman 1997), apoptosis (Bose *et al.* 1995; Gulbins *et al.* 1995; Hannun and Obeid 1995; Haimovitz-Friedman *et al.* 1997), lifespan (Hannun 1996; Wang *et al.* 1999; Cutler and Mattson 2001), and vesicular trafficking to neuronal differentiation and functioning (Toman *et al.* 2000; Bieberich *et al.* 2001).

Moreover, data in the fields of immunology, endocrinology and neurobiology also suggest a fundamental involvement of ceramide in the onset of several diseases (Venable *et al.* 1995; Merrill *et al.* 1997; Mathias *et al.* 1998; Pandey *et al.* 2007). These aspects will be discussed more extensively later on.

Ceramide production can occur in different ways: *de novo* synthesis by a synthase, SM hydrolysis by various SMases or recycling of sphingolipids by different hydrolases (Perry and Hannun 1998). Enzymes involved in each of these ceramide generation pathways are located in distinct subcellular compartments. Since ceramide is very hydrophobic compared to other lipid species, its concentrations in the cytosol are extremely low and its molecule is usually trapped in the membrane where it is formed (Venkataraman and Futerman 2000). This hydrophobicity tends to isolate pools of ceramide in different subcellular compartments. It has been proposed that the compartmentalization of ceramide might have regulatory functions in the cell (Liu and Anderson 1995; Kolesnick *et al.* 2000; Bionda *et al.* 2004). The discovery of ceramidase enzymes in most of the organelles (ER, Golgi, mitochondria, lysosome and plasma membrane) (Li *et al.* 1999; El Bawab *et al.* 2000; Mao *et al.* 2003; Tani *et al.* 2005; Xu *et al.* 2006) support this view. To better understand the involvement of GPBPs in cellular biology it is important to review all the different ways of ceramide production inside the cell.

De novo synthesis of ceramide

De novo synthesis of ceramide requires coordinate action of different enzymes along a subsequent series of reactions which occur both at the cytosolic surface of the ER (Perry and Hannun 1998) [Fig. 5(1)] and in the mitochondrial outer and inner membranes [Fig. 5(1)] (Shimeno *et al.* 1998; Bionda *et al.* 2004). The *de novo* ceramide biosynthesis, which starts with the condensation of a serine and a fatty acyl-coA, involves the key enzyme ceramide synthase (Ichikawa *et al.* 1996) which catalyzes the acylation of either sphinganine to form dihydroceramide or sphingosine to form ceramide. In mammals ceramide synthase, in contrast to the rest of the enzymes of sphingolipid metabolism, exists as a family of multiple isoforms, six in humans and mice and each isoform exhibits varying substrate selectivity and cell specificity (Venkataraman and Futerman 2000). This pathway needs several hours to generate detectable amounts of ceramide (Bose *et al.* 1995). Once formed, ceramide is delivered to the luminal surface of the Golgi membrane for sphingomyelin and glycosphingolipid synthesis (Fukasawa *et al.* 1999; Kumagai *et al.* 2005). There are at least two pathways by which ceramide is transported from the ER to the Golgi compartment: an ATP and cytosol dependent major pathway (non-vesicular, GPBPΔ26/CERT mediated) (Hanada *et al.* 2003) and an ATP or cytosol independent minor pathway (vesicular transport) (Brugger *et al.* 2000). Once in the Golgi apparatus, ceramide can serve as a precursor for glycosphingolipids or it can be converted to SM by SM synthase 1 (SMS1), that is localized in the *trans*-Golgi region [Fig. 5(2)] and catalyzes the transfer of phospatidylcholine to ceramide to produce SM (Yamaoka *et al.* 2004).

The down-regulation of CERT by RNA interference resulted in a significant but incomplete reduction of basal SM synthesis but it has no effect on glycosphingolipid synthesis (Hanada *et al.* 2003; Huitema *et al.* 2004). Ceramide destined for the formation of SM reaches the Golgi by an ATP-dependent, vesicle-independent transport pathway mediated by GPBPΔ26/CERT and a minor ATP-independent transport vesicular pathway (Fukasawa *et al.* 1999; Funakoshi *et al.* 2000; van Meer and Holthuis 2000; Hanada *et al.* 2003). On the other hand ceramide destined for conversion to GlcCer [Fig. 5(3)] appears to reach the Golgi only by vesicular transport (Huwiler *et al.* 2000). This suggests separate ceramide transport pathways for GlcCer and SM synthesis. GlcCer synthase has been detected in both the ER and Golgi compartments (Schweizer *et al.* 1994); therefore the synthesis of GlcCer may not depend exclusively on the ER-to-Golgi transport of ceramide. In this regard it is noteworthy that GPBPΔ26/CERT transfer activity varies according to the acyl chain lengths of the ceramides. It preferentially transfers C16–18 ceramides rather than longer ceramides (Kumagai *et al.* 2005). This correlates with the presence of a C16–18 acyl chain SM in many tissues and cell lines. Through vesicular transport, SM and glycosphingolipids reach the plasma membrane where resides the enzyme SM synthase 2 (SMS2) [Fig. 5(4)], required for SM homeo-

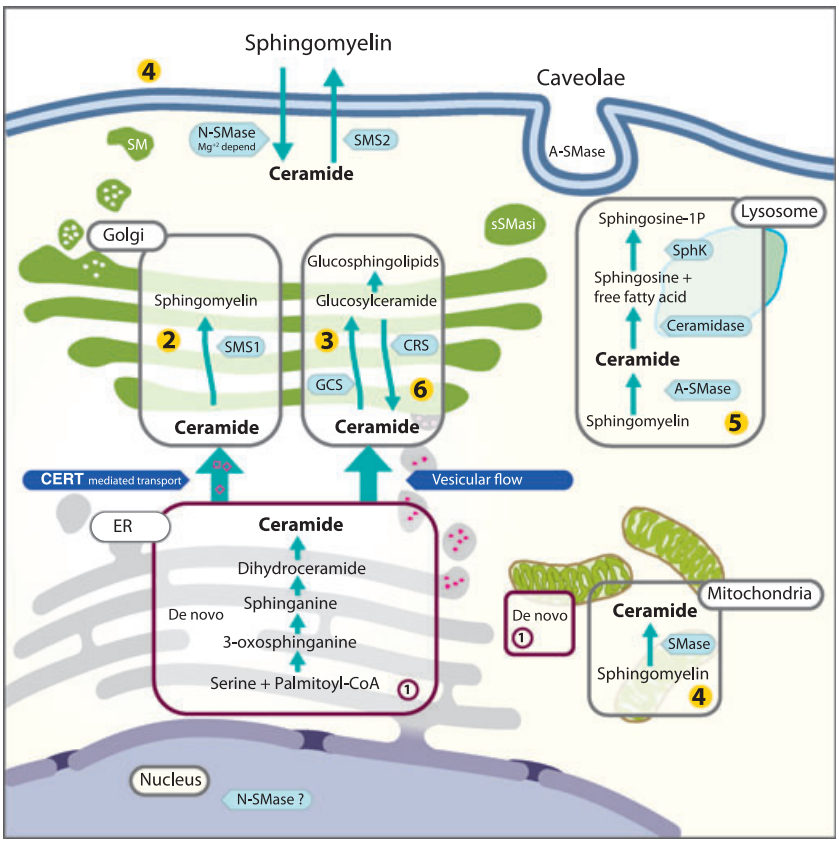

Fig. 5 Ceramide can be generated by the activity of two general metabolic pathways. (i) The anabolic pathway that occurs at the cytosolic surface of the ER from condensation of serine with acyl CoA and acylation of the sphingoid base [*de novo* synthesis (1)]; once formed, ceramide is delivered to the luminal side of the Golgi apparatus and converted to sphingomyelin by SMS1 (2) that catalyzes the transfer of phosphocholine involving the hydrolysis of phosphotidylcholine (PPC) to diacylglycerol (DAG); ceramide is also converted to glycosylceramide by (GlcCer) glucosylceramide synthase (GCS) (3). GlcCer is further converted to more complex glycosphingolipids. There are separate ceramide transporters for the SM and GlcCer synthesis, GPBPs for the former and vesicular flow for the latter. (ii) The catabolic pathway, that takes place at the membrane of different organelles in which ceramide is formed by the degradation of sphingomyelin by different sphingomyelinases (SMases) (4, 5). Ceramide is also formed by the degradation of glycosphingolipids that occur in lysosome compartments: hydrolisis of ceramide by ceramidases releases sphingosine, which can be further converted to sphingosine1-phosphate by sphingosine kinase (Sphk) (5). Metabolism of cerebrosides by cerebrosidase (CRS) also generates ceramide (6). Ceramide is also present in mitochondria where it is synthesized via *de novo* pathway and/or activity of the acid sphingomyelinase (1, 4).

stasis regulation of the intracellular levels of ceramide in plasma membranes (Huitema *et al.* 2004).

From there, these lipids travel towards the outer, non-cytosolic surface of the plasma membrane and all membranes of the endocytic system, where they are eventually degraded.

Ceramide catabolic pathway

In contrast to the multi-step *de novo* pathway, the catabolic pathway for ceramide generation involves the action of a single class of enzymes namely SMases. These enzymes are homologues of phospholipase C, which hydrolyses the phosphodiester bond of SM yielding ceramide and phosphocholine (Levade *et al.* 1986). Moreover, if the *de novo* synthesis is found in the ER and in the mitochondria, the sphingomyelin hydrolysis occurs in a variety of membranous systems (Levade and Jaffrezou 1999).

Currently five different enzymes have been identified and categorized in acid, neutral and alkaline SMases that differ in catalytic properties, subcellular localization, biological effects and probably in their mode of regulation (Huwiler *et al.* 2000).

Acidic SMase (A-SMase) [Fig. 5(5)] was the first sphingomyelin-hydrolysing enzyme to be purified and it was originally described as an endosomal/lysosomal hydrolase (pH 4.5–5) required for the turnover of cellular membranes (Gatt *et al.* 1966). Sphingolipids, that reach lysosomes via caveolar and or clathrin-dependent endocytosis, are mainly catabolised within these organelles.

Impaired sphingolipid degradation, besides affecting a large variety of cellular processes, has drastic effects on the nervous system, leading to neurodegeneration, shortened lifespan and early death. In line with this, several lipid

storage disorders are associated with significant progressive brain degeneration, as discussed below.

Ceramide and GPBPs in mitochondria

Mitochondria contain ceramide with a three-fold higher concentration in the outer membranes than the inner membranes (Ardail *et al.* 2001). These organelles also contain the enzymes responsible for the synthesis (Shimeno *et al.* 1998) and hydrolysis of ceramide (El Bawab *et al.* 2000). The synthesis of ceramide occurs in the mitochondrial outer and inner membranes [Fig. 5(1 and 4)] (Bionda *et al.* 2004). Thus, mitochondria have mechanisms for regulating the level of ceramide. Importantly, mitochondrial ceramide levels have been shown to be elevated prior to the induction phase of apoptosis (Thomas *et al.* 1999; Rodriguez-Lafrasse *et al.* 2001; Birbes *et al.* 2005; Siskind 2005). In fact, many death signals, including TNF-α, influence mitochondria function through the activation of pro-apoptotic members of the Bcl-2 family (Gross *et al.* 1998).

Tumor necrosis factor treatment of cells resulted in increased mitochondrial ceramide levels that were associated with Bax translocation to mitochondria (Birbes *et al.* 2005). Interestingly TNF is also able to increase the mRNA levels of GPBPs (Granero *et al.* 2005).

It is important to mention that most of the experiments with cells and isolated mitochondria have been carried out using short chain permeable ceramides in which an acetyl or hexanoyl group replaces the natural long-chain fatty acid (C2- and C6-ceramide). Ceramides have been reported to have numerous effects on mitochondria, including the inhibition and/or activation of the activities of various components of the mitochondrial electron transport chain (Gudz *et al.* 1997; Siskind and Colombini 2000), the enhanced generation of reactive oxygen species (Zamzami *et al.* 1995) and release of intermembrane space proteins (Ghafourifar *et al.* 1999), probably by forming large protein permeable channels in planar phospholipid and mitochondrial outer membranes (Siskind 2005).

For GPBPs also a function in mitochondria integrity has been described: mice lacking both GPBP/CERT$_L$ and GPBPΔ26/CERT die early in embryogenesis as a result of structural and functional defects not only in the ER but also in the mitochondria: both organelles show proliferation, vesiculation and engorgement appearing aberrant with gross structural changes and functional defects (Wang *et al.* 2009). The impact of deficient ceramide transporters on mitochondrium integrity and functioning is a surprising finding. These results point to GPBPs as important effectors for mitochondrial homeostasis. They could exert their action in principally two different, albeit interconnected, mechanisms: either transferring directly ceramide outside the mitochondrium or shuttling this hydrophobic lipid within its complex structure. In both views the loss of GPBPs could lead to the detected changes in the physiological state of this organelle. The double-membrane system divides the mitochondrium in two aqueous compartments, the intermembrane space and the matrix. Because of the highly insoluble nature of ceramide, there is a large energy barrier inhibiting the movement of the molecule in these compartments that could give an explanation for the presence of the ceramide transporters (Small 1970; Sot *et al.* 2005; Goni and Alonso 2006). Ceramide and its related enzymes could presumably affect membrane dynamics and contribute to crista reorganization (Veiga *et al.* 1999; Montes *et al.* 2002; Scorrano *et al.* 2002). Mitochondrial membrane reassembly is required to control many metabolic processes. Maintenance of normal crista structure is essential for mitochondrial function (Mannella 2006). Generation of the characteristic cristae appearance has been linked to the conduction of protons that participate indirectly in ATP synthesis in mitochondrial respiration (Paumard *et al.* 2002; Arnoult *et al.* 2005). In all these dynamic events GPBPs could quickly redistribute ceramide.

The lack of CERT/GPBPΔ26 in *Drosophila*, which as aforementioned does not have the CERT/GPBP$_L$ isoform, affects plasma membrane fluidity and initiates oxidative stress. Flies are viable and fertile even though they die prematurely as a result of accelerated aging (Rao *et al.* 2007). The impact of GPBPs knock-out on mouse embryos causes a series of pathological changes in ER and mitochondria. This different development in mice supports the idea that it is the lack of the GPBP/CERT$_L$ isoform which is responsible for this phenotype rather than CERT/GPBPΔ26 which is mostly involved in the transfer of lipids in different intracellular compartments to ensure correct lipid synthesis and distribution (Hanada *et al.* 2007).

Chinese hamster ovary cells (Ly-A) which carry a missense mutation in the PH domain of CERT/GPBPΔ26 do not show mitochondrial defects but only impairment of SM synthesis (Funakoshi *et al.* 2000) since the latter is strictly dependent on CERT transport of ceramide (Hanada *et al.* 2003). So it is intriguing to speculate that as these cells do not have mitochondrial impairments, maybe the GPBPs isoforms related to mitochondria do not need the PH domain to be functional in these specific organelles. This would also explain why GPBPs are expressed as different isoforms (Revert *et al.* 2008).

These studies suggest intimate and direct connections between ceramide, GPBPs and mitochondria. Interestingly, tissues that show a higher expression of GPBP/CERT$_L$ are brain, muscle and testis, organs that are by definition highly dependent on aerobic metabolism. The CNS has an immense metabolic demand because neurons are highly differentiated cells that need large amounts of ATP for maintenance of ionic gradients across the cell membranes and for neurotransmission (Kann and Kovacs 2007). Since most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply (Kann

and Kovacs 2007). In concordance with this neurons contain high levels of GPBPs (Mencarelli *et al.* 2009).

Ceramide and GPBPs in the CNS

Among the tissues of the body, the nervous system is one of the richest in lipid content. Sphingolipids and other complex glycosphingolipids are highly represented in the brain where they were first discovered more than a century ago by J. L. W. Thudichum (1884) (van Echten-Deckert and Herget 2006). They do not only have a structural function but they act as important molecular tools in regulating multiple biochemical cell activities (Tepper *et al.* 1999; Hannun and Obeid 2002; Radin 2003).

Ceramide has been implicated as a prominent mediator in a remarkable array of cellular processes through a variety of complex pathways induced by numerous agonists and environmental stimuli. Neurons, as might be expected, share with other cell types the same wide range of responses mediated by this pleiotropic lipid.

The nervous system comprises many events of morphological and biochemical changes during the entire adult life. Generation, shaping and reshaping of the complex spatial organization, neuronal and glia differentiation, processes of synaptogenesis and neuritogenesis are typical phenomena characterizing the nervous system (Nieto-Sampedro and Nieto-Diaz 2005). In all these regulated events, of primary importance is the membrane lipid composition which determines cell surface properties (Simons and Ehehalt 2002). Ceramide modulates membrane structure and dynamics, associates with cholesterol and other sphingolipids and forms microdomains called lipid rafts. In neurons, lipid rafts accumulate preferentially on somal and axonal membranes and in the post-synaptic sites (Suzuki 2002). Lipid rafts play an important role in neuronal adhesion and in the modulation of neuronal ion channels, neurotransmitter receptors and in neurotransmitter release (Chamberlain *et al.* 2001).

Besides its structural role, ceramide involvement in cellular signaling has been extensively studied in the CNS and it has become clear that ceramide content and its spatial/temporal distribution change to some extent during aging, neuronal survival and apoptosis. Interestingly, information is beginning to emerge pointing to ceramide levels as triggers of neural development, differentiation, rate of growth and other cellular physiological events.

In neurons an increase in ceramide level, originating from neosynthesis and/or SM degradation, inhibits their proliferation and concomitantly induces differentiation (Riboni *et al.* 1995). In glioma cells, elevated ceramide levels cause growth arrest and process formation (Dobrowsky *et al.* 1994). The raised intracellular ceramide levels occur very early and persist during the differentiation of the cell. Normal ceramide levels are reestablished after cell differentiation indicating a key role for ceramide in this process (Riboni *et al.* 1995).

While ceramide stimulates cell differentiation in unspecialized cells, in post-mitotic neurons ceramide acts preferentially by accelerating the transition of neuronal development stages.

Maturation of cultured granule neurons is associated with an increase of ceramide levels in sphingolipid rich domains (Prinetti *et al.* 2001) and ceramide seems to be essential for cell axon and dendritic growth of neurons. Ceramide signaling in hippocampal neurons in culture elicits the early transition of a rounded cell lacking neurites towards one exhibiting small extended lamellopodia and minor processes (Brann *et al.* 1999). Ceramide is also required for the subsequent axonal elongation of neurons in order to acquire the normal mature phenotype (Brann *et al.* 1999; Ledesma *et al.* 1999; Mitoma *et al.* 1999). This effect of ceramide is dose-dependent; lower concentrations of the lipid stimulate cellular growth whereas higher concentrations induce cell death (Furuya *et al.* 1998; Irie and Hirabayashi 1998). However, ceramide synthesized *de novo* is not involved in axonal outgrowth since the development of hippocampal neurons is not altered by treatment with fumonisin B1, an inhibitor of the ceramide *de novo* synthesis. In contrast, the ceramide responsible for neuronal development is generated via a neutral-SMase (N-SMase) in response to nerve growth factor (NGF) (Brann *et al.* 1999), a member of the neurotrophin family that controls the development of the nervous system in the embryo and the maintenance of nervous tissue and neural transmission in the adult (Levi-Montalcini 1987). The binding of the NGF to the p75 receptor induces N-SMase activation (Brann *et al.* 1999). N-SMase is the major form of SMase found in the brain and its expression increases in rat brain with neuronal maturation (Spence and Burgess 1978). A-SMase (reviewed in section 'Ceramide catabolic pathway') is highly expressed in brain cells but its expression level does not change significantly during development (Spence and Burgess 1978). A-SMase activation appears to mediate signaling pathways that induce programmed cell death. Many studies have presented evidence of ceramide production via A-SMase in response to apoptotic signals, most notably generated by members of the NGF/tumor necrosis factor receptor family (Verheij *et al.* 1996; Kronke 1999). A-SMase-deficient mice mimic the lethal, neurovisceral form of the human sphingomyelin storage disease, known as Niemann-Pick disease (Otterbach and Stoffel 1995) (review in the next section).

Excitotoxic cell death, induced after an intense exposure of the neuronal cell to glutamate or related excitatory amino acids (Choi 1992), is partially abolished in A-SMase-deficient neurons. On the other hand, A-SMase-deficient and wild type neurons are both susceptible to NGF induced apoptosis (Furuya *et al.* 1998; Irie and Hirabayashi 1998). It has been suggested that N-SMase responds also to high ceramide levels inducing neuronal death rather than accelerating axonal outgrowth (Brann *et al.* 2002).

The fate of the cell seems to depend exclusively on intracellular ceramide levels (Brann *et al.* 1999). Interestingly, in immature neurons apoptosis is induced even at lower doses of ceramide than in mature neurons (Furuya *et al.* 1998; Mitoma *et al.* 1998). Probably, at certain levels and depending on developmental stages, ceramide reaches a threshold required for an apoptotic response.

Experimental manipulations that increase intracellular ceramide level (e.g. exposure to exogenous ceramide analogs or treatment with bacterial SMases) potently induce apoptosis in differentiating mammalian cells (Hannun and Obeid 1995).

There is good evidence that ceramide synthesized *de novo* also plays an important role in neuronal death with the same apoptotic features as ceramide generated from SM. Indeed, the inhibition of the *de novo* biosynthesis by fumonisin B1 stops apoptosis induced by DNA-damaging agents and TNF.

A considerable amount of programmed cell death takes place in the nervous system during development. In neurodegenerative diseases neuronal death also occurs extensively. Neurons may use different apoptotic mechanisms during development or pathological conditions and ceramide metabolism seems to be a crucial target in both physiological death and disease.

In summary, ceramides have both a structural and a signaling function in the brain. At present the potential role of ceramide metabolism points to questions yet to be addressed and more in depth studies of the ceramide transporters in the CNS will stimulate new avenues of research in this exciting area.

Ceramide in neurodegenerative diseases

A balance between specific sphingolipids is essential for neuronal function (Buccoliero and Futerman 2003). When the sphingolipid metabolism is dysregulated as in Gaucher, Krabbe or Niemann-Pick disease, neurodegeneration occurs (Jeyakumar *et al.* 2002).

Gaucher disease is caused by mutations in the gene encoding for glucosylceramidase, an enzyme that catalyzes the hydrolytic cleavage of glucose from glucocerebrosides to form ceramides during lysosomal degradation of sphingolipids (Brady *et al.* 1965). Without glucosylceramidase, glucocerebroside and related lipids can build up to toxic levels within cells. Abnormal accumulation and storage of these lipids could affect calcium homeostasis and contribute to synuclein aggregation, neurodegenerative changes and neuronal loss (Pelled *et al.* 2005; Manning-Bog *et al.* 2009).

Krabbe disease is caused by a deficiency of the lysosomal enzyme galactosylceramidase, which is needed for the conversion of galactosylceramide to ceramide. The disease is characterized by myelin disorganization (Pastores 2009) resulting from accumulation of incompletely metabolized galactosylceramide.

Niemann-Pick disease refers to a group of lipid storage disorders as well. In the A and B subtype, SMase is deficient with the consequent accumulation of sphingomyelin (Brady *et al.* 1966). Type C is characterized by excessive intracellular accumulation of unesterified cholesterol (Blanchette-Mackie *et al.* 1988). The subtypes of this disease have an extremely varied clinical presentation, but all are characterized by a range of progressive neurological manifestations (Futerman and van Meer 2004).

In Farber disease, an increase in ceramide levels, resulting from ceramidase deficiency, leads to mental retardation and motor dysfunction (Bar *et al.* 2001). It is characterized by an accumulation of lipids and patients frequently die before the age of 2 years.

Beside genetic mutations that affect the functionality of the enzymes involved in the sphyngolipid metabolism, many stress signals can induce ceramide overproduction, like cytokines (TNFα, interleukin 1β, Fas ligand), nitric oxide, and environmental stresses (UV/ionizing radiation, heat shock and oxidative stress) (Hannun and Obeid 1995; Jayadev *et al.* 1995; Venable *et al.* 1995; Hannun 1996; Verheij *et al.* 1996). Interestingly, all the pathways involved in ceramide generation participate in response to these agents (Fig. 5) (Kolesnick and Kronke 1998; Hannun and Luberto 2000). The precise nature of ceramide-intracellular targets is controversial and varies depending on the cell types (Hannun 1996; Okazaki *et al.* 1998; Kolesnick and Hannun 1999; Pettus *et al.* 2002).

Many reports have emphasized the role of ceramide in neurodegenerative disorders to the extent that serum ceramides have been indicated as early predictors of cognitive impairment (Mielke *et al.* 2008). Alterations in ceramide levels were also detected in the cerebrospinal fluid of Alzheimer's disease (AD) patients because of inhibition of the ceramide-metabolizing enzyme GlcCer synthase (Satoi *et al.* 2005). It has been found that AD brains contain approximately three fold more ceramide when compared to age matched controls (Cutler *et al.* 2004). Brain regions with extensive β-amyloid (Aβ) plaques (cortex and hippocampus) were characterized by higher ceramide levels and decreased SM content (Cutler *et al.* 2004) and the activity of the enzyme ceramidase was found to be elevated, perhaps in response to high ceramide concentration (Huang *et al.* 2004). A shift in sphingolipid metabolism towards up-regulation of gene expression of the enzymes controlling *de novo* synthesis of ceramide and down-regulation of the enzymes involved in glycosphingolipid synthesis was also evident in early stages of AD.

In vitro exposure of hippocampal neurons to Aβ peptide induces membrane oxidative stress resulting in perturbed ceramide metabolism. Several studies have shown that Aβ induces apoptosis via the SM/ceramide pathway in various

brain cells including human and rat primary neurons (Jana and Pahan 2004; Malaplate-Armand *et al.* 2006), rat oligodendrocytes (Cheng *et al.* 2003; Lee *et al.* 2004; Zeng *et al.* 2005; Malaplate-Armand *et al.* 2006), rat astrocytes and glial cells (Ayasolla *et al.* 2004) and murine neuroblastoma cells (Obeid *et al.* 1993; Satoi *et al.* 2005). The composition of lipid rafts including SM, cholesterol and ceramide controls amyloid processing and aggregation. It was shown that β- and γ-cleavage of amyloid precursor protein (APP) occurs inside rafts and both β- and γ-secretase are lipid raft associated (Simons and Vaz 2004; Vetrivel *et al.* 2004; Rajendran and Simons 2005). The accumulation of Aβ peptide derived from this process starts in early endosomes and has been shown to be subsequently released into the extracellular space in association with exosomes (Rajendran *et al.* 2006). Ceramide is highly enriched in exosomes and -possibly thanks to its cone-shaped structure- it regulates biogenesis and dynamics of membrane budding (Trajkovic *et al.* 2008). Whether ceramide *per se* also plays a role in mediating Aβ aggregation/disaggregation remains unknown.

OSBP1, which regulates ceramide transport and SM synthesis, has been found to be involved in the regulation of APP processing (Zerbinatti *et al.* 2008). Over-expression of OSBP1 down-regulates the amyloidogenic processing of APP: the presence of OSBP1 at the Golgi complex triggers ceramide transporter activity leading to increased SM synthesis and consequently reducing ceramide levels. Knockdown of OSBP1 has the opposite effect, thus an increase of ceramide and reduced SM synthesis, leading to increased Aβ production and aggregation (Zerbinatti *et al.* 2008). However, the role of OSBP1 in amyloid processing and regulation of ceramide levels is still under discussion.

Together with the abovementioned processing of APP, also post-translational refolding of normal prion protein is affected by the content of sphingomyelin, cholesterol and ceramide in lipid rafts at the level of the plasmatic membrane (Taraboulos *et al.* 1995; Kivipelto *et al.* 2001; Simons *et al.* 2001; Baron *et al.* 2002). If this is the case, abnormal composition of the lipid rafts could cause defective protein folding and trigger consequently the protein aggregation process, a hallmark of many neurodegenerative diseases.

Ceramides have also been implicated in the pathological death of neurons that occurs in Parkinson's disease (PD) (Brugg *et al.* 1996). Dopaminergic neurons in primary cultures derived from the mesencephalon, a primary region of neuronal degeneration in PD, undergo apoptosis through a ceramide-dependent mechanism (Brugg *et al.* 1996).

Tumor necrosis factor α receptors have been found on dopaminergic neurons that degenerate in PD and TNFα-immunoreactive glia have been detected in close proximity to degenerating neurons (Boka *et al.* 1994). Importantly, TNF induces *COL4A3BP* expression by regulating the interaction of NFkB with the *COL4A3BP* promoter (Granero *et al.* 2005). NFkB has also been found to be augmented in the nucleus of dopaminergic neurons in parkinsonian patients. Thus, it is expected that the augmented levels of TNF and of NFkB will activate *COL4A3PB* leading to an increase in GPBPs levels. Higher levels of the ceramide transporter in this context might help to reduce the abnormal ceramide concentration and therefore attenuate downstream effects of ceramide.

Moreover, some of the genes involved in the genetics of Lewy body disease are strictly linked to ceramide metabolism (for review, see Bras *et al.* 2008). In this regard it has been reported that a knockdown of the ceramide synthase LASS2 in *Caenorhabditis elegans*, results in increased alpha-synuclein inclusions (van Ham *et al.* 2008). Therefore, ceramide and its transporters could play a role in protein inclusions formation.

Moreover, Sidransky and colleagues found that heterozygous mutations that occur in the enzyme glucosylcerebrosidase pre-dispose to PD (Aharon-Peretz *et al.* 2004; Bras *et al.* 2009) and Lewy body disorders (Mata *et al.* 2008) (Sidransky *et al.* 2009). Glucosylcerebrosidase is an enzyme that catalyzes the breakdown of the lipid glucosylcerebroside, which is highly enriched in the brain, to ceramide and glucose. This correlation between an increase of ceramide content and the onset of the disease reveals a strong association between ceramide metabolism and PD.

Cellular ceramide levels are also important in the regulation of cellular senescence (Venable *et al.* 1995). Endogenous levels of ceramide increase considerably if compared with other lipids as cells enter the senescence phase (Mouton and Venable 2000) and exogenous doses of ceramide are able to induce a senescent phenotype in young cultured cells (Lightle *et al.* 2000; Mouton and Venable 2000). Age-related increases in brain ceramide and neutral SMase levels have also been reported (Palestini *et al.* 1993).

The expression of the ceramide transporter in the CNS is widespread, with high levels of GPBPs immunoreactivity in neurons of the cortex, hippocampus, the basal ganglia, the olfactory bulb and some nuclei of the thalamus, the hypothalamus and the septal area (Mencarelli *et al.* 2009). Interestingly, glial cells do not show immunoreactivity for GPBPs suggesting that in this cell type GPBPs are present at lower levels. In zebrafish, specific knockdown of GPBP/$CERT_L$ is detrimental to normal embryonic development. In this model the brain is affected, showing a clear reduction of the myelinated tracts, thin axons, hydrocephaly of the four ventricles and apoptosis leading to brain tissue loss (Granero-Molto *et al.* 2008).

Taken together, these findings document the significance of ceramides in the pathophysiology of diverse neurological diseases. GPBPs are regulators of ceramide which could contribute actively to induce profound changes in cellular metabolism. The pathways that link this putative second

messenger to neuronal dysfunction remain elusive. Sorting through these complex interrelationships will be essential to better understand neurodegeneration in the CNS.

Conclusion

The present review brings together our current knowledge concerning the isoproteins GPBP/CERT$_L$ and CERT/GPBPΔ26, that have generated controversy regarding their roles in several cellular processes. Although our understanding on their nature is still incomplete, the existing data allow us to distinguish the two isoforms by diverse functions in the nervous system. A deeper knowledge of the complexity of ceramides and GPBPs in cell signaling will increase our understanding of cell dynamics in various CNS disorders, opening new opportunities for drug development and therapies for neurodegenerative diseases.

Take home messages

GPBP/CERT$_L$ and GPBPΔ26/CERT are two splice variants of 624 and 598 aa respectively. GPBP/CERT$_L$, in turn exists in two alternative translation initiation isoforms, i.e. GPBP/CERT$_L$ and GPBPΣ128.

The isoproteins have identical amino acid sequences, except for the presence of a 26 amino acid serine-rich domain in the GPBP/CERT$_L$ isoforms and 128 additional amino acids in the GPBPΣ128 isoform.

All isoforms function as ceramide transfer proteins.

GPBP/CERT$_L$ is expressed in tissues targeted by autoimmune responses.

Increased expression of GPBP/CERT$_L$ has been associated with immune complex-mediated pathogenesis.

Increased GPBP/CERT$_L$ expression induces type IV collagen disorganization and deposits of immunoglobulin A in glomerular basement membrane.

GPBPΔ26/CERT is a more common splice variant widely expressed throughout the body.

GPBP/CERT$_L$ and GPBPΔ26/CERT, in turn, exist as different isoforms resulting from canonical (77 kDa) and non-canonical (91 kDa) mRNA translation initiation.

The 77 kDa GPBP/CERT$_L$ behaves as a soluble secretable protein and the 91-kDa GPBP/CERT$_L$ as a membrane-bound protein. The 77 GPBPΔ26/CERT is a cytosolic isoform.

TNF increases the mRNA levels of GPBPs.

GPBP/CERT$_L$ and GPBPΔ26/CERT are differentially expressed during embryogenesis in zebrafish with GPBP/CERT$_L$ expressed at the earlier stage than GPBPΔ26/CERT.

Specific GPBP/CERT$_L$ knockdown results in loss of myelinated tracks in the CNS and to extensive apoptosis and tissue loss in the brain.

GPBPΔ26/CERT knockdown in *Drosophila* does not lead to neuronal degeneration in the brain but in changes in membrane properties that increase oxidative stress.

Mice lacking both GPBP/CERT$_L$ and GPBPΔ26/CERT die prematurely and show structural and functional defects in the ER and mitochondria.

Neurons express more GPBPs, than astroglial cells.

Expression levels of GPBPs were observed widely throughout the brain.

GPBPs share similar protein domain organization with two other lipid binding/transfer proteins, OSBP1 and FAPP2.

GPBPs are regulators of ceramide and could be involved in molecular mechanisms underlying ceramide-mediated signaling cascades.

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