Inhibition of Glycosphingolipid Biosynthesis Reduces Secretion of the β-Amyloid Precursor Protein and Amyloid β-Peptide

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Alzheimer disease is associated with extracellular deposits of amyloid β-peptides in the brain. Amyloid β-peptides are generated by proteolytic processing of the β-amyloid precursor protein by β- and γ-secretases. The cleavage by secretases occurs predominantly in post-Golgi secretory and endocytic compartments and is influenced by cholesterol, indicating a role of the membrane lipid composition in proteolytic processing of the β-amyloid precursor protein. To analyze the role of glycosphingolipids in these processes we inhibited glycosyl ceramide synthase, which catalyzes the first step in glycosphingolipid biosynthesis. The depletion of glycosphingolipids markedly reduced the secretion of endogenous β-amyloid precursor protein in different cell types, including human neuroblastoma SH-SY5Y cells. Importantly, secretion of amyloid β-peptides was also strongly decreased by inhibition of glycosphingolipid biosynthesis. Conversely, the addition of exogenous brain gangliosides to cultured cells reversed these effects. Biochemical and cell biological experiments demonstrate that the pharmacological reduction of cellular glycosphingolipids limits maturation and cell surface transport of the β-amyloid precursor protein. In the glycosphingolipid-deficient cell line GM95, cellular levels and maturation of β-amyloid precursor protein were also significantly reduced as compared with normal B16 cells. Together, these data demonstrate that glycosphingolipids are implicated in the regulation of the subcellular transport of the β-amyloid precursor protein in the secretory pathway and its proteolytic processing. Thus, enzymes involved in glycosphingolipid metabolism might represent targets to inhibit the production of amyloid β-peptides.

The deposition of amyloid β-peptides (Aβs)1 in extracellular plaques is an invariant neuropathological feature of Alzheimer disease (AD) (1, 2). Aβ derives from the β-amyloid precursor protein (APP) by proteolytic processing, which involves sequential cleavages by proteases called β- and γ-secretases (1, 3, 4). APP is a type I membrane protein that is transported from the endoplasmic reticulum via the Golgi compartment to the cell surface and undergoes maturation by N'- and O'-glycosylation (1, 3, 4). Within the secretory pathway and at the cell surface APP is predominantly cleaved by α-secretase, resulting in the secretion of soluble APP (APPs) (5). Because α-secretase cleaves APP within the Aβ domain, this cleavage precludes the generation of Aβ. Alternatively, APP can be cleaved by β-secretase. The cleavage of APP by β-secretase occurs predominantly in endosomal and lysosomal compartments after internalization from the cell surface (6, 7). The C-terminal fragments (CTFs) of APP resulting from α- or β-secretase cleavage can be cleaved within the transmembrane domain by γ-secretase to release p3 and Aβ, respectively (1, 3, 8).

The proteolytic processing of APP is influenced by the lipid composition of cellular membranes, as demonstrated by the pharmacological modulation of cellular cholesterol levels (9–12). In addition, the inhibition of acyl-coenzyme A cholesterol acyltransferase (ACAT) also led to strong reduction of Aβ generation in cultured cells and transgenic mice, indicating that cholesterol esters also influence proteolytic processing of APP (13, 14). These effects on APP processing might involve altered cleavage of APP by α-secretase and/or β-secretase, probably by redistribution of APP and secretases between distinct membrane microdomains (15–18).

Apart from cholesterol, glycosphingolipids (GSLs) have also been implicated in the pathogenesis of AD. It has been shown that the levels of several gangliosides are altered in AD brains (19). In addition, the ganglioside GM1 binds to Aβ and might contribute to early deposition of the peptide in amyloid plaques (20–22).

The biosynthesis of GSLs starts with the generation of glucosylceramide from UDP-glucose and ceramide by glucosylceramide synthase (Fig. 1A). Glucosylceramide represents the precursor of a large variety of GSLs (23) that are transported in the secretory pathway from the Golgi to the cell surface (11, 24). The physiological functions of GSLs include the regulation of cell adhesion, cell differentiation, and signal transduction (24–26). Dysfunction of GSL degradation is associated with several inherited diseases that are characterized by the accumulation of GSLs in endosomal/lysosomal compartments (27, 28).

Here we sought to analyze the role of GSLs in the proteolytic processing of APP and the generation of Aβ. By using different cell types that express endogenous APP, we demonstrate that depletion of cells from GSLs results in reduced secretion of glycosphingolipid; HEG, human embryonic kidney; PDMP, n-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; TRITC, tetramethylrhodamine isothiocyanate; WGA, wheat germ agglutinin.
soluble APP and Aβ. Our data indicate that GSLs are implicated in the transport of APP in the secretory pathway and its expression at the cell surface, thereby altering the proteolytic processing by secretases.

MATERIALS AND METHODS
Reagents and Antibodies— α- and β-Three-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), C6-ceramide, and TRITC-labeled wheat germ agglutinin (WGA) were obtained from Sigma. Purified gangliosides from bovine brain were obtained from Fidia Research Laboratories (Abano, Italy). Antibodies 5513 and 6687, recognizing the N- and C-terminal domains of APP, respectively, were described earlier (29) and generously provided by Dr. C. Haass. Polyclonal antibodies 2964 against Aβ were raised by inoculation of rabbits with synthetic Aβ40. Monoclonal antibody 6E10 was obtained from Signet Inc.

Cell Culture and Treatment—B16 and GM95 cells were obtained from the RIKEN cell bank (Tokyo, Japan) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Human embryonic kidney (HEK) 293 and HeLa cells were also cultivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. SH-SY5Y cells were maintained in RPMI supplemented with 10% fetal calf serum and excess amounts of 15% fetal calf serum. PDMP was dissolved in water at concentration of 10 μM PDMP for 48 h. Cellular membranes were separated by SDS-PAGE, and GM1 was detected by Western immunoblotting with cholera toxin. C, HEK293 cells were cultured in the absence (−) or presence (+) of 10 μM PDMP for 48 h. APP was immunoprecipitated from conditioned media (top) and cell lysates (bottom) and separated by SDS-PAGE. Secreted (APPs) and cellular APP were detected by Western immunoblotting. Migration of APPs and mature (m) and immature (im) APP is indicated by arrowheads. D, secretion of APPs was quantified by ECL imaging and normalization to cellular APP expression. Values represent the means of three independent experiments ± S.D. (solid bar, no PDMP; open bar, 10 μM PDMP). E and F, a similar experiment as that described for panels C and D was carried out with HeLa cells.

FIG. 1. Inhibition of GSL biosynthesis decreases the secretion of APPs. A, schematic showing the biosynthesis pathway of glycosphingolipids and the targeted inhibition of glucosyl ceramide synthase (GCS) by PDMP. B, detection of GM1. HEK293 (top) or HeLa (bottom) cells were cultured in the absence (−) or presence (+) of 10 μM PDMP for 48 h. Cellular membranes were separated by SDS-PAGE, and GM1 was detected by Western immunoblotting with cholera toxin. C, HEK293 cells were cultured in the absence (−) or presence (+) of 10 μM PDMP for 48 h. APP was immunoprecipitated from conditioned media (top) and cell lysates (bottom) and separated by SDS-PAGE. Secreted (APPs) and cellular APP were detected by Western immunoblotting. Migration of APPs and mature (m) and immature (im) APP is indicated by arrowheads. D, secretion of APPs was quantified by ECL imaging and normalization to cellular APP expression. Values represent the means of three independent experiments ± S.D. (solid bar, no PDMP; open bar, 10 μM PDMP). E and F, a similar experiment as that described for panels C and D was carried out with HeLa cells.

Analysis of Total Protein Secretion—Whatman 3MM paper was cut into squares (2 × 2 cm), soaked with 10% trichloroacetic acid (w/v), and dried. Conditioned chase media (25 μl) of radiolabeled cells was applied to the paper and left to dry. The filter papers were washed in 5% trichloroacetic acid (w/v), absolute ethanol, and acetone (twice each). Subsequent to drying, the filter papers were transferred to scintillation counter vials containing 5 ml of Optiphase Highsafe II scintillation mixture. The activities of 35S in the samples were counted on a scintillation counter using a 60-s time window.

Isolation of Membranes and Detection of GM1—Cells were scraped from the culture dishes and incubated in hypotonic buffer (10 mM Tris, pH 7.3, 10 mM MgCl2, 1 mM EDTA, and 1 mM EGTA) for 10 min on ice. Cells were then homogenized by passing 15 times through a 21-gauge needle and centrifuged for 10 min at 1000 rpm to pellet nuclei. The resulting supernatant was centrifuged 30 min at 16000 × g. Pelleted membranes were separated by SDS-PAGE, and GM1 was detected by Western immunoblotting with horseradish peroxidase-conjugated cholera toxin (Sigma).
GM95 cells using TRIzol, followed by reverse transcription to obtain cDNA. Semi-quantitative PCR was then performed for APP and actin cDNA with 18 cycles. Primer pairs were 5′-GGTGAGACCTGTCGCA-
CACG-3′ and 5′-TCCCTCCTGCTGACATTTGG-3′ for APP and 5′-
TGCGTGACATCAGAGAAG-3′ and 5′-GCTCATACCTCCTTCCAG-
GG-3′ for actin.

Data Analysis and Statistics—In metabolic labeling experiments, band intensities were analyzed with a phosphorimaging device (FLA2000, Fuji) and the Fuji Image Gauge 3.0 software. For enhanced chemiluminescence detection, signals were measured and analyzed using an ECL imager (ChemDoc™XRS; Bio-Rad) and the Quantity One software package (Bio-Rad). For quantitations, three independent experiments (n = 3) were carried out. Statistical analysis was carried out using Student’s t test. Significance values are as follows: *, p < 0.05; **, p < 0.01 (Figs. 3 and 4).

RESULTS

To analyze the role of GSLs in the proteolytic processing of APP and Aβ generation, GSL biosynthesis was inhibited with PDMP, a competitive inhibitor of glucosylceramide synthase that has been shown previously to efficiently decrease GSL biosynthesis in cultured cells (30–33). Treatment of HEK293 or HeLa cells with PDMP for 48 h led to a significant decrease in GSL biosynthesis as demonstrated by a strong reduction of GM1 levels (Fig. 1B). The treatment of cells with PDMP at the concentrations used did not affect cell viability (Supplemental Fig. 1, available in the on-line version of this article). We therefore used this approach to investigate the role of GSLs in APP processing. HEK293 or HeLa cells were treated with PDMP, and APP was immunoprecipitated from conditioned media and lysates. As demonstrated in Fig. 1, the secretion of APPs into conditioned media was markedly decreased in both HEK293 and HeLa cells that were treated with PDMP (Fig. 1, C–F). The decrease in APP secretion was observed for both variants APPs-α and APPs-β (Supplemental Fig. 2, available in the on-line version of this article). We also assessed the effect of PDMP on the precursor-product relationship of cellular and secreted APP in a pulse-chase experiment. In control cells, ~8% of 35S-labeled cellular APP was secreted into the conditioned media after 2 h. In the same time period, only ~2.5% of cellular APP was secreted in PDMP-treated cells (not shown). Together, these data demonstrate that PDMP treatment significantly decreases the secretion of APP. It should be noted that the effects of PDMP on APP secretion were selectively observed in non-transfected cells, but not in cells that stably overexpress APP (data not shown). This might be due to a tight regulation of the interaction of APP with membrane lipids (see “Discussion”). We therefore used exclusively non-transfected cells for the further experiments.

To prove that the effects of PDMP are due to decreased levels of GSLs, we first analyzed the effect of short term treatment with PDMP. In contrast to long term treatment (48 h), incubation of cells with PDMP for only 2 h did not significantly reduce levels of GSLs (data not shown). Under these conditions, the secretion of APPs was not significantly changed (Fig. 2A). We also assessed the effect of the inactive enantiomer L-PDMP that does not inhibit glucosylceramide synthase. Long term treatment of cells for 48 h with L-PDMP did not reduce APPs secretion (Fig. 2B). Together, these control experiments indicate that the reduced secretion of APPs observed upon cell treatment with PDMP is due to decreased GSL levels in cellular membranes.

We next tested the effect of exogenous GSLs on proteolytic processing of APP. Cells were cultured in the presence or absence of purified bovine brain gangliosides, and levels of cellular and secreted APP were analyzed. The addition of exogenous GSLs significantly increased the cellular levels of endogenous APP as well as the secretion of APPs (Fig. 2C). Together, these data demonstrate a regulatory role of GSLs in the cellular metabolism of APP. The levels of endogenous Aβ in the conditioned media of HEK293 were below the detection limits (data not shown), likely due to efficient cleavage of APP by α-secretase in this cell type (34) (see Supplemental Fig. 2).
Because neuronal cells secrete higher levels of Aβ, we used human neuroblastoma SH-SY5Y cells to prove a role of GSLs in the processing of APP by pulse-chase experiments. Two variants of endogenously expressed APP were detected after pulse labeling in cell lysates that represent distinct APP splice forms, including the neuron-specific APP695 form (Fig. 3A). The presence of distinct splice variants in SH-SY5Y cells was also confirmed by reverse transcription PCR using isoform-specific primers (Supplemental Fig. 3, available in the on-line version of this article). PDMP treatment did not affect the expression of the distinct APP variants, as demonstrated by the similar levels of cellular APP after pulse labeling (Fig. 3A). Also, no effect on cell viability was detected (Supplemental Fig. 1C). As observed in HEK293 and HeLa cells, PDMP resulted in a significant reduction of APP5 secretion into the conditioned media of SH-SY5Y cells (Fig. 3, A and B). The reduction was observed for the APP5/770 as well as for the neuron-specific APP695 splice variants. In contrast to HEK293 cells, SH-SY5Y cells predominantly secrete APP5-β (not shown). Together, these data indicate that PDMP treatment inhibits secretion of both APP5-α and APP5-β. However, total protein secretion was not reduced upon PDMP treatment, indicating a selective role of GSLs in the secretion of APP (Fig. 3C).

Because the inhibition of glucosylceramide synthase might lead to accumulation of its substrate ceramide, which was shown to alter the proteolytic processing of APP by stabilizing the β-site APP-cleaving enzyme BACE1 (35), we tested the effect of C6-ceramide on the secretion of APP5-β. The treatment of cells with ceramide at concentrations of 10 μM, which was shown to stabilize BACE1 (35), did not inhibit the secretion of APP5-β, indicating that the inhibition of APP secretion observed after PDMP was due to decreased levels of GSLs (Fig. 3D).

To investigate the role of GSLs in the generation of Aβ, SH-SY5Y cells were incubated in the presence or absence of PDMP for 48 h, and Aβ was immunoprecipitated from conditioned media and cell lysates and detected by Western blotting. The band marked by an asterisk likely represents mature APP695.
sulfo-N-hydroxysuccinimide-biotin. In SH-SY5Y cells, biotinylated APP could not be detected (not shown), probably due to very efficient proteolytic processing and secretion in this cell type that results in low levels of surface APP. In contrast, biotin-labeled APP could be readily detected in HEK293 cells (Fig. 5A). In GSL-depleted cells, the levels of biotin-labeled APP were markedly reduced, demonstrating that suppression of GSL biosynthesis reduces the expression of APP at the cell surface. In contrast, the cell surface expression of the endogenous Fas receptor, also a type I membrane protein, was not decreased upon GSL depletion (Fig. 5B). We also assessed the effect of PDMP on the general expression of cell surface proteins by cell staining with TRITC-labeled WGA, a lectin that binds to glycoproteins. No significant difference in the cell surface expression of glycoproteins was observed between treated and non-treated cells (Fig. 5C). In addition, the levels of total biotinylated proteins detected by streptavidin-conjugated horseradish peroxidase were very similar in treated and non-treated cells (not shown). Together, these experiments demonstrate that GSL depletion selectively reduced the cell surface expression of APP without generally affecting other membrane proteins. However, the data do not exclude the possibility that cell surface expression of other selected membrane proteins is also affected by GSL depletion.

The decreased expression of APP at the cell surface and reduced secretion of APPβ-0 and APPβ-γ after GSL depletion suggested that GSLs might be implicated in the forward transport of APP in the secretary pathway. To address this possibility, we performed pulse-chase experiments and analyzed the maturation of APP that occurs in the Golgi compartment. Cells were labeled with [35S]methionine for 10 min and then chased for various time periods. After pulse labeling, a prominent band was detected representing endogenous immature (N- and O-glycosylated) APP. After 30 min of chase, a slower migrating form appeared that represents mature (N- and O-glycosylated) APP. The mature form becomes predominant after 60 and 90 min (Fig. 6A). GSL depletion reduced the transport of APP to or within the Golgi compartment as indicated by decreased maturation of APP in PDMP-treated cells (Fig. 6A and B). We also observed decreased levels of total APP in GSL-depleted cells.
after chase periods of 60 and 90 min (Fig. 6, A and C). Because PDMP also reduced the secretion of APPS (see Figs. 1 and 3), these data indicate an increased degradation of cellular APP in GSL-deficient cells. Indeed, when cells were cultured in the presence of PDMP for 2 weeks we also observed a marked decrease in the levels of mature APP under steady state conditions (data not shown).

To prove these findings in an independent cellular model, we used the mouse melanoma cell lines B16 and GM95. Whereas B16 cells produce GSLs, GM95 cells are defective in GSL biosynthesis due to decreased activity of glucosylceramide synthase and are commonly used as a model of GSL-deficient cells (39–45). As expected, very little if any GM1 could be detected in GM95 cells, whereas B16 cells express robust amounts of GM1 (Fig. 7A). To investigate the maturation of APP in both cell types, we performed pulse-chase experiments. In B16 cells, endogenous APP undergoes maturation as indicated by the appearance of a slower migrating band during the chase period (Fig. 7B, left). In contrast, the GSL-deficient GM95 cell line revealed significantly reduced maturation of APP (Fig. 7B, right), which is consistent with the data obtained with pharmacological inhibition of GSL biosynthesis (see Fig. 6). In addition, steady state levels of cellular APP were strongly decreased in GM95 cells as compared with B16 cells (Fig. 7C), whereas expression of APP mRNA was similar in both cell lines (Fig. 7D). Together, these data indicate that GSLs facilitate the maturation and stabilization of APP.

DISCUSSION

We demonstrate that GSLs are implicated in the regulation of proteolytic processing and subcellular transport of APP. The inhibition of GSL biosynthesis reduced the secretion of APPS and Aβ in different cell types, whereas the addition of exogenous brain gangliosides reversed these effects. As demonstrated by pulse-chase experiments and cell surface biotinylation, the reduction of cellular GSLs decreased the maturation of APP in the secretary pathway and its expression at the cell surface. These effects were observed in cells with pharmacologically or genetically altered GSL biosynthesis. Of note, the effects are observed selectively for endogenous APP, whereas overexpressed APP revealed unaltered maturation and secretion (data not shown). This might be due to tightly regulated interactions of APP with specific lipids within cellular membranes. The overexpression of membrane proteins might therefore lead to altered interactions with lipids, probably by saturation effects (46).

Recent evidence suggests that GSLs are implicated in the
pathogenesis of AD. The levels of individual GSLs are altered in AD brains (19). Moreover, the ganglioside GM1 has been detected in amyloid plaques and binds specifically to β-secretase (20, 21). GM1 might also facilitate the aggregation of αβ into fibrils and deposition in amyloid plaques (22). Interestingly, treatment of APP transgenic mice with GM1 efficiently reduced plaque load in the brains, likely due to sequestering peripheral αβ (47).

The precise role of GSLs in the proteolytic processing of APP and αβ production is not well understood. The inhibition of ceramide synthesis in Chinese hamster ovary cells that overexpress human APP resulted in elevated secretion of APPα, likely due to an increase in α-secretase cleavage (48). Here, we observed a significant decrease in the secretion of endogenous APP in different cell types upon the depletion of GSLs. This discrepancy might be explained by overexpression of APP in the former study (48) that might mask some effects on transport and/or processing of APP (see above). Moreover, in our approach GSL biosynthesis was targeted selectively, whereas the inhibition of ceramide synthesis led also to a strong decrease in the biosynthesis of sphingomyelin that might serve additional functions in APP metabolism (48). In another study, the direct addition of GM1 to cells that overexpress human APP led to increased secretion of APPα, whereas secretion of APPα-β was attenuated (49). Because GM1 incorporates into the plasma membrane, these effects might involve alterations in α- and γ-secretase cleavage of APP at the cell surface without affecting transport of APP in the secretory pathway. By targeting GSL biosynthesis, which occurs in the secretory pathway, we could demonstrate that GSLs are also implicated in the maturation of APP in the Golgi and the further transport to the cell surface. However, our data do not rule out the possibility that GSLs might have additional effects on the proteolytic processing of APP at the cell surface, such as direct or indirect modulation of secretase activities (48, 49).

Apart from their role in adhesion processes and signal transduction at the cell surface (24, 50), GSLs have been implicated previously in subcellular protein transport (24, 27, 51). Studies with yeast cells have shown that the inhibition of GSL biosynthesis affects forward transport and stable membrane association of glycosylphosphatidylinositol-anchored proteins (52, 53). Less is known about the role of GSLs in protein transport in the secretory pathway in mammalian cells. Recently, it has been shown that GSLs are involved in the sorting of tyrosinase from Golgi compartments to melanosomes in mouse melanoma cells (40). In agreement with our data, these studies in both yeast and mouse melanocytes also demonstrated that inhibition of GSL biosynthesis does not generally impair protein transport or secretion (40, 52, 53). Thus, GSLs appear to mediate the transport of individual proteins, probably at distinct steps in the secretory pathway. The attenuated transport of APP to the cell surface in GSL-deficient cells is consistent with the decreased secretion of APP by α-secretase, which is known to occur during transport to or directly at the cell surface (54, 55). In contrast, β-secretase cleavage likely occurs predominantly in endocytic compartments after re-internalization of APP from the cell surface (6, 38). Thus, the decreased generation of Aβ upon the depletion of cells from GSLs might be due to decreased access of β-secretase to APP in endocytic compartments. The decreased levels of CTB-β in GSL-depleted SH-SY5Y cells are in agreement with this notion. Taken together, our data indicate that GSLs and the respective enzymes involved in their biosynthesis might represent targets to decrease formation of Aβ in therapeutic strategies for AD.

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