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Phosphatidylinositol 3-Kinase/AKT Pathway Regulates the Endoplasmic Reticulum to Golgi Traffic of Ceramide in Glioma Cells

*A LINK BETWEEN LIPID SIGNALING PATHWAYS INVOLVED IN THE CONTROL OF CELL SURVIVAL**

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Different lines of evidence indicate that both aberrant activation of the phosphatidylinositol 3-OH kinase (PI3K)/Akt survival pathway and down-regulation of the death mediator ceramide play a critical role in the aggressive behavior, apoptosis resistance, and adverse clinical outcome of glioblastoma multiforme. Furthermore, the inhibition of the PI3K/Akt pathway and the up-regulation of ceramide have been found functional to the activity of many cytotoxic treatments against glioma cell lines and glioblastomas as well. A reciprocal control between PI3K/Akt and ceramide signaling in glioma cell survival/death is suggested by data demonstrating a protective role of PI3K/Akt on ceramide-induced cell death in glial cells. In this study we investigated the role of the PI3K/Akt pathway in the regulation of the ceramide metabolism in C6 glioma cells, a cell line in which the PI3K/Akt pathway is constitutively activated. Metabolic experiments performed with different radioactive metabolic precursors of sphingolipids and microscopy studies with fluorescent ceramides demonstrated that the chemical inhibition of PI3K and the transfection with a dominant negative Akt strongly inhibited ceramide utilization for the biosynthesis of complex sphingolipids by controlling the endoplasmic reticulum (ER) to Golgi vesicular transport of ceramide. These findings constitute the first evidence for a PI3K/Akt-dependent regulation of vesicle-mediated movements of ceramide in the ER-Golgi district. Moreover, the findings also suggest the activation of the PI3K/Akt pathway as crucial to coordinate the biosynthesis of membrane complex sphingolipids with cell proliferation and growth and/or to maintain low ceramide levels, especially as concerns those treatments that promote ceramide biosynthesis in the ER.

Gliomas are the most frequent brain tumors derived from the transformation of glial cells or their precursors (1–3). The World Health Organization classifies gliomas according to their cellular features and their grade of malignancy (from I to IV) (4). Glioblastoma multiforme (GBM),[3] also referred to as grade IV astrocytoma, is the most frequent class of malignant primary brain tumor and one of the most aggressive forms of cancer. There is a poor prognosis for patients with GBM, the median survival being 9–12 months, and this does not significantly improve even after aggressive treatment with multiple approaches, including surgery, chemotherapy, and radiotherapy (5). This poor survival rate seems to be mainly due to the marked invasiveness and proliferation of GBM, as well as to their apoptotic resistance and aberrant angiogenesis. These malignancy features could be related to the varying mutations frequently found in GBM tumors that impact different key pathways involved in the control of cell proliferation, survival, differentiation, migration, and DNA repair (6–9).

Among the disregulated signaling pathways in GBMs, PI3K-Akt-PTEN has been identified as being an important oncogenic pathway (10–12). PI3Ks are members of a unique and conserved family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositol and phosphoinositides such as phosphatidylinositol(4,5)P_2 (PIP_2), which is converted to phosphatidylinositol(3,4,5)P_3 (PIP_3). The activation of PI3Ks can occur through growth factor receptors, G-protein-coupled receptors, cell adhesion molecules, and oncogenes such as Ras. The generated polyphosphoinositides bind to the pleckstrin homology (PH) domain of different proteins, including PDK1

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[3] The abbreviations used are: GBM, glioblastoma multiforme; ER, endoplasmic reticulum; PI3K, phosphatidylinositol-3-OH kinase; PIP_2, phosphatidylinositol(4,5)P_2; PIP_3, phosphatidylinositol(3,4,5)P_3; PH, pleckstrin homology; PTEN, phosphatase and tensin homologue; Cer, ceramide; Wm, wortmannin; BFA, brefeldin A; BSA, bovine serum albumin; CERT, ceramide transfer protein; [^{3}H]Sph, D-*erythro*-[3-^{3}H]sphingosine; [^{14}C]SM, [*choline-methyl*-^{14}C]sphingomyelin; [^{3}H]C_6-Cer, *N*-[*hexanoyl*-6-^{3}H]D-*erythro*-hexanoyl-sphingosine; HPTLC, high performance thin layer chromatography; NBD-C_6-Cer, 6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosine; FAPP, four phosphate adaptor protein; EGF-FAPP1-PH, PH domain of FAPP1 protein fused with enhanced green fluorescent protein; DN-AKT, dominant negative mutant of Akt; PBS, phosphate-buffered saline; siRNA, small interfering RNA; SMS, sphingomyelin synthase; GCS, glucosylceramide synthase; SMase, sphingomyelinase; PI4K, phosphatidylinositol 4-OH kinase; PI4P, phosphatidylinositol 4-phosphate; GlcCer, glucosylceramide; SM, sphingomyelin; SREBP, sterol regulatory element-binding protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; N-SMase, neutral SMase; A-SMase, acidic SMase.

(3′-phosphoinositide-dependent protein kinase 1) and the serine/threonine kinase Akt, thus facilitating the interaction between the two proteins and the activation of Akt by PDK1. Cell levels of phosphoinositides are controlled not only by PI3K but also by the tumor suppressor PTEN. which is a PIP_3 phosphatase. PTEN negatively regulates the PI3K/Akt pathway by converting PIP_3 back to PIP_2. The activation of Akt promotes cell growth and survival and inhibition of apoptosis and also regulates glucose and lipid metabolism at both the transcriptional and post-transcriptional levels; furthermore, Akt activation can exert some control over cell movement and vesicle trafficking within cells (13). The overexpression or the mutation of PI3K-related genes and the deletion or mutation of PTEN have been reported in many GBMs and glioblastoma-derived cell lines (12, 14, 15, 17, 18). Indeed, all the above-mentioned conditions determine high basal levels of PIPs and Akt activation, even in the absence of growth factor stimulation. In GBMs, reduced levels of PTEN and increased Akt activity have been correlated with more aggressive tumor behavior and reduced survival time in patients (19,20). The aberrant activation of PI3K-Akt-PTEN signaling has been identified as crucial to the malignant features of GBMs, such as rapid tumor growth, invasiveness, resistance to cytotoxic treatments, and massive neovascularization (12, 22–24).

Ceramide (Cer) has long been recognized as a ubiquitous mediator of cell death. Indeed, in GBMs, the possibility of disregulation in Cer-dependent signaling is strongly supported by the evidence of an inverse association between Cer levels and GBM malignancy progression, and the direct correlation of such levels with patient survival (25). Moreover, Cer up-regulation has been demonstrated as being crucial to many cytotoxic treatments of glioma cells (26–30), and exogenous Cer has been shown to induce glioma cell death by apoptosis, autophagic cell death, and necrosis (31–33). Cer-induced cell death, both apoptotic and non apoptotic, can be inhibited by an active Akt pathway (31, 34). Metabolic pathways involved in controlling Cer levels in glial cells include sphingomyelinases, other specific Cer metabolism enzymes such as serine palmitoyltransferase and sphingomyelin synthase, and Cer transport from the ER to the Golgi apparatus for the biosynthesis of complex sphingolipids (35–41). In addition, it has been demonstrated that the accumulation of *de novo* synthesized Cer is an early event in cannabinoid-triggered ER stress and apoptosis in glioma cells (40). This suggests that the modulation of Cer levels in the ER/Golgi compartment can be crucial to glioma cell decision toward survival or death. This study investigates the role of the PI3K-Akt-PTEN pathway in the regulation of the Cer metabolism in C6 glioma cells; this is a cell line in which the PI3K/Akt pathway is constitutively activated due to the lack of PTEN expression (42). We show that the PI3K/Akt pathway is capable of regulating the Cer metabolism and its levels in ER by promoting the biosynthesis of complex sphingolipids acting on the vesicular ER to Golgi transport of Cer.

EXPERIMENTAL PROCEDURES

Materials—All reagents were of analytical grade as follows: Dulbecco's modified Eagle's medium (DMEM), wortmannin (Wm), brefeldin A (BFA), bovine serum albumin (BSA), fatty acid-free BSA, and G418 were purchased from Sigma. Fetal calf serum (FCS) was from Cambrex (Walkersville, MD). LY294002 and LY303511 were from Cayman Chemical (Ann Arbor, MI). The antibodies recognizing phospho-Akt (Ser-473) were from Cell Signaling Technology, Inc. (Danvers, MA); polyclonal antibodies against Cer transfer protein (CERT) were from Bethyl Laboratories (Montgomery, TX). Lipofectamine 2000 and the Stealth RNAi were from Invitrogen. Complete Mini Protease inhibitor mixture tablets were from Roche Diagnostics. D-Erythro-[3-^{3}H]sphingosine ([^{3}H]Sph) (21.2 Ci/mmol) and [*choline-methyl*-^{14}C]sphingomyelin ([^{14}C]SM) (52 mCi/mmol) were from PerkinElmer Life Sciences; *N*-[*hexanoyl*-6-^{3}H]D-erythro-hexanoylsphingosine ([^{3}H]C_6-Cer) (60 Ci/mmol) was from ARC (St. Louis, MO). High performance thin layer chromatography (HPTLC) silica gel plates were from Merck. 6-((*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosine (NBD-C_6-Cer) and BODIPY-C_5-Cer were from Invitrogen. The plasmid encoding the dominant negative form of Akt (Thr-308 and Ser-473 mutated to Ala) was kindly provided by Prof. Stefanie Dimmeler, Department of Internal Medicine III, University of Frankfurt, Frankfurt, Germany. The plasmid encoding for the PH domain of four phosphate adaptor protein 1 (FAPP1) fused with enhanced green fluorescent protein (EGFP-FAPP1-PH) was kindly provided by Dr. Maria Antonietta De Matteis, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Chieti, Italy.

Cell Culture—The C6 glioma cell line was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy). Cells were routinely maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2 and 95% humidified air.

Plasmids and Transfection—C6 glioma cells were plated in 60-mm cell culture dishes and grown in DMEM supplemented with 10% FCS until they were 50–70% confluent. Then cells were transfected with expression plasmid encoding the dominant negative mutant of Akt (DN-AKT) or pcDNA3.1 empty vector using the Lipofectamine 2000 reagent according to the manufacturer's directions. Stable transfectants for DN-AKT and vector were selected in a 2–3-week period using G418 antibiotic (0.5 g/liter).

PH Domain Binding Assay—C6 glioma cells plated on coverslips at 1.5×10^4 cell/cm^2 were grown for 24 h in DMEM plus 10% FCS. Cells were transfected with 4 μg of plasmid encoding for the EGFP-FAPP1-PH domain using Lipofectamine 2000 reagent in accordance with the manufacturer's protocol. After 24 h the cells were treated for 1.5 h with or without 0.5 μM Wm or 5 μM Wm or 20 μM LY294002 at 37 °C. The cells were then washed (three times with PBS) and fixed with 0.5% glutaraldehyde solution in PBS for 10 min at 4 °C. The specimens were observed and analyzed with a fluorescence microscope (Olympus BX-50) equipped with a fast high resolution charge-coupled device camera (Colorview 12) and an image analytical software (analysis from Soft Imaging System GmbH).

RNA Interference—Small interfering RNA (siRNA) duplexes for rat CERT (GenBank™ accession number XM 345143.1) S87, S522, and control nontargeting siRNAs (scrambled

sequences of S87 and S522 oligonucleotides) described in Ref. 43 were used. C6 glioma cells plated at 3×10^4 cell/cm^2 were maintained 24 h in DMEM plus 10% FCS and then transfected in the same medium with a 1:1 (by mol) mix of S87 + S522 (si-CERT) or the nontargeting corresponding sequences (si-control) using Lipofectamine 2000 according to the manufacturer's protocol. All the experiments were performed 72 h after transfection.

Immunoblotting—Phospho-Akt immunoblotting were performed using C6 glioma cells lysed with Akt buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium fluoride, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and the protease inhibitor mixture). Solubilized proteins were centrifuged at 14,000 × *g* at 4 °C for 10 min. Supernatants were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 140 mM NaCl) containing 0.1% Tween 20 (TBS-T) and 5% skim milk, and then incubated with primary antibodies against phospho-Akt overnight at 4 °C. Membranes were washed in TBS-T, and bound antibodies were visualized with horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology) and chemiluminescent substrate. The relative intensities of bands were quantified by densitometry. CERT immunoblotting was performed using si-control and si-CERT transfected cells lysed with CERT buffer (10 mM Tris-HCl, pH 7.4, 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin), processed, and analyzed as described previously (43).

[^{3}H]Sphingosine and N-[^{3}H]Hexanoyl-sphingosine Metabolism—C6 glioma cells plated at 1.5×10^4 cell/cm^2 were grown 48 h in DMEM plus 10% FCS. Cells, preincubated for 30 min with 0.5 μM Wm, 20 μM LY294002, or 20 μM LY303511, in the presence or absence of 1 μg/ml BFA, were pulsed with [^{3}H]Sph (0.3 μCi/ml) or [^{3}H]C_6-Cer (0.6 μCi/ml) for 30 min or 1 h maintaining the pretreatment conditions. si-control and si-CERT cells, preincubated for 30 min with 0.5 μM Wm or 20 μM LY294002, were pulsed with [^{3}H]Sph (0.3 μCi/ml) for 1 h maintaining the pretreatment conditions. Stable transfectants for DN-AKT and vector were pulsed with [^{3}H]Sph (0.3 μCi/ml) for 1 h. All experiments were performed at 37 °C. Stock solutions of [^{3}H]Sph or [^{3}H]C_6-Cer in absolute ethanol were prepared and added to fresh medium. In all cases the final concentration of ethanol never exceeded 0.1% (v/v). At the end of pulse, cells were washed twice with PBS at 4 °C, harvested, and submitted to lipid extraction and partitioning as described previously (44). The methanolized organic phase and the aqueous phase were analyzed by HPTLC using chloroform/methanol/water (55:20:3 by volume) and butanol/acetic acid/water (3:1:1 by volume) as solvent system, respectively. Digital autoradiography of HPTLC plates was performed with Beta-Imager 2000 (Biospace, France), and the radioactivity associated with individual lipids was measured using the software provided with the instrument. The ^{3}H-labeled sphingolipids were recognized and identified as described previously (44).

In Vitro Enzyme Assays—Sphingomyelin synthase (SMS), glucosylceramide synthase (GCS), and sphingomyelinase (SMase) activities were assayed using as enzyme source a homogenate of the control (untreated cells), 0.5 μM Wm-, or 20 μM LY294002-treated cells. SMS activity was assayed as described previously (41), with minor modifications. The incubation mixture contained 50 mM Tris-Cl, pH 7.4, 25 mM KCl, 0.5 mM EDTA, 2 nmol (0.05 μCi) of [^{3}H]C_6-Cer (as 1:1 complex with fatty acid-free BSA), and 20 μg of cell protein in a final volume of 50 μl. After 15 min of incubation at 37 °C, the reaction was stopped by adding 150 μl of chloroform/methanol (1:2, by volume) at 4 °C. GCS activity was assayed as described previously (41) with minor modifications. The incubation mixture contained 50 mM Tris-Cl, pH 7.4, 25 mM KCl, 10 mM $MnCl_2$, 5 mM UDP-Glc, 2 nmol (0.05 μCi) of [^{3}H]C_6-Cer (as 1:1 complex with fatty acid-free BSA), and 15 μg of cell protein in a final volume of 50 μl. After 15 min of incubation at 37 °C, the reaction was stopped by adding 150 μl of chloroform/methanol (1:2, by volume) at 4 °C. In all cases, after lipid extraction and phase separation, the [^{3}H]lipids were resolved by HPTLC as described previously (41). Mg^{2+}-dependent neutral SMase (N-SMase) and acidic SMase (A-SMase) were assayed according to Ref. 41 with minor modifications. The N-SMase incubation mixture contained 20 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 0.1% Triton X-100, 250 μM [^{14}C]SM (0.1 μCi), and 5–20 μg of cell protein in a final volume of 25 μl. The A-SMase incubation mixture contained 200 mM acetate buffer (pH 5.0), 10 mM EDTA, 0.1% Triton X-100, 250 μM [^{14}C]SM (0.1 μCi), and 2–10 μg of cell protein in a final volume of 25 μl. After 30 min of incubation at 37 °C, the reactions were stopped by adding 100 μl of chloroform/methanol (2:1, by volume) at 4 °C; the radioactivity associated with the aqueous phase represented by [^{14}C]phosphocholine was measured.

Analysis of the Intracellular Distribution of Fluorescent Ceramides—C6 glioma cells or stable transfectants for DN-AKT and vector were plated at 1.5×10^4 cells/cm^2 and grown on a glass coverslip for 48 h in DMEM plus 10% FCS. The cells were then loaded with 5 μM BODIPY-C_5-Cer or NBD-C_6-Cer (1:1 complex with fatty acid free BSA) as described previously (43). After loading, the cells were incubated 30 min at 37 °C in DMEM plus 10% FCS with or without 0.5 μM Wm or 20 μM LY294002 or 20 μM LY303511. The cells were then washed (three times with PBS) and fixed with 0.5% glutaraldehyde solution in PBS for 10 min at 4 °C. The specimens were immediately observed and analyzed with a fluorescence microscope (Olympus BX-50), as described above.

Other Methods—Total protein amount was assayed with the Coomassie Blue- based reagent (Pierce), using BSA fraction V as standard. Radioactivity was measured by liquid scintillation counting. Statistical significance of differences was determined by the Student's *t* test.

RESULTS

Effect of PI3K/Akt on [^{3}H]Sph Metabolism—To investigate the effect of PI3K/Akt on Cer metabolism in C6 glioma cells, we first utilized the chemical inhibition of PI3K. As the commonly used PI3K inhibitors Wm and LY294002 can also inhibit type III PI4Ks, although at higher doses (45), and it has been shown that PI4KIIIβ plays a role in CERT-mediated Cer metabolism (46), we initially set up working concentrations to specifically inhibit PI3K. To do this we evaluated the effect of different

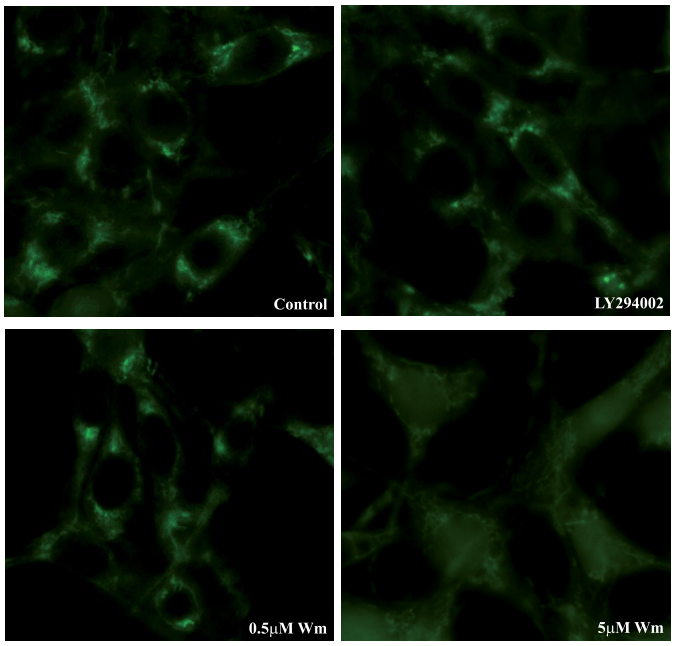

FIGURE 1. **Localization of EGFP-FAPP1-PH domain in C6 glioma cells.** Cells were plated at 3.0×10^4 cells/cm^2 on coverslips and grown for 24 h in DMEM plus 10% FCS. The cells were then transfected with a plasmid encoding the EGFP-FAPP1-PH domain as described under "Experimental Procedures." After 24 h, the cells were treated for 1 h in the presence or absence (*Control*) of 20 μM LY294002, or 0.5 μM Wm, or 5 μM Wm. Then the cells were fixed and processed for immunofluorescence microscopy (see "Experimental Procedures"). All images were processed and printed identically.

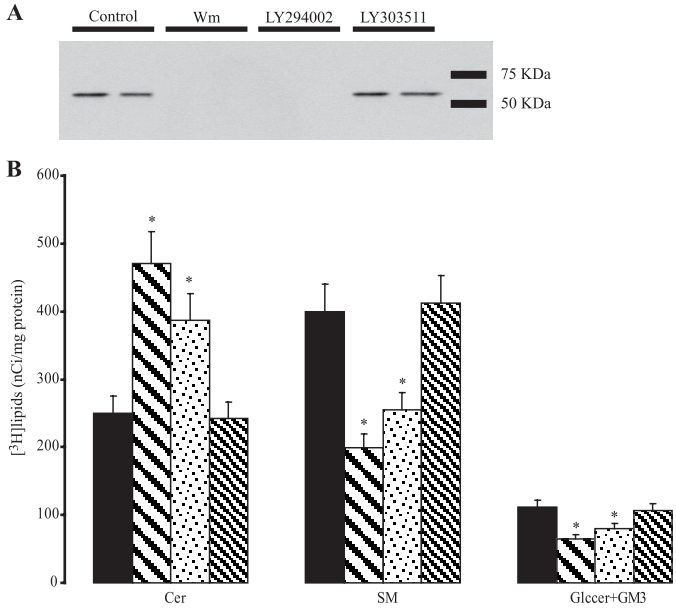

FIGURE 2. **Effect of PI3K inhibitors on AKT phosphorylation and [^{3}H]Sph metabolism in C6 glioma cells.** Cells were plated at 1.5×10^4 cells/cm^2 and grown for 72 h in DMEM plus 10% FCS and pretreated for 30 min with the different inhibitors. The cells were treated for 1 h with or without (*control*) 0.5 μM Wm, or 20 μM LY294002, or 20 μM LY303511 and harvested for immunoblot analysis of phospho-AKT levels (*A*), or pulsed 1 h with [^{3}H]Sph in the absence (*closed bars*) or presence of 0.5 μM Wm (*striped bars*), 20 μM LY294002 (*dotted bars*), or 20 μM LY303511 (*thin hatched bar*) (*B*). At the end of pulse, cells were harvested and submitted to lipid extraction. The [^{3}H]sphingolipid metabolites associated with the organic and aqueous phase (see "Experimental Procedures") were separated by HPTLC. Single lipids were identified by co-migration with pure standards and quantified by radiodensitometric analysis. All values are the mean ± S.D. of at least three individual experiments. *, $p < 0.01$ *versus* control.

concentrations of Wm and LY294002 on PI4K activity by staining cells with the EGFP-FAPP1-PH domain, which specifically interacts with phosphatidylinositol 4-phosphate (PI4P) (47). As shown in Fig. 1, the EGFP-FAPP1-PH domain in the control cells is almost exclusively present in the perinuclear region, representative of the Golgi complex, supporting the preferential localization of PI4P in this subcellular compartment (48). When C6 glioma cells expressing EGFP-FAPP1-PH domain were treated with 0.5 μM Wm or with 20 μM LY294002, most of the fluorescence accumulated in the Golgi region, thus indicating that the amount of PI4P in the Golgi complex was unchanged. Instead, for the cells treated with 5 μM Wm, which inhibits type III PI4Ks, there was almost the complete loss of association between the EGFP-FAPP1-PH domain and the Golgi complex (Fig. 1). Thus, in subsequent experiments, we used both 0.5 μM Wm and 20 μM LY294002 to specifically inhibit PI3K and evaluated their capacity to block the PI3K/Akt pathway. Immunoblot analyses with an antibody specific for Akt, phosphorylated at Ser-473, demonstrated that Wm and LY294002 strongly reduce Akt activation, because phospho-Akt is fairly detectable with both treatments (Fig. 2*A*). On the contrary, there was no change in the phospho-Akt levels when the cells were incubated with the LY294002-inactive analogue LY303511. Subsequently, we performed a metabolic study using [^{3}H]Sph, a sphingolipid precursor that is efficiently taken up by glial cells and rapidly incorporated first in Cer and then in sphingomyelin (SM) and glycosphingolipids (41). When C6 glioma cells were pulsed with [^{3}H]Sph in the presence or absence of Wm, LY294002, or LY303511, the radioactive precursor was rapidly and efficiently incorporated in both control and treated cells, and none of the treatments altered the [^{3}H]Sph uptake, determined as the radioactivity measured in the total lipid extract (data not shown). In all cases, [^{3}H]Sph was mainly metabolized to *N*-acylated compounds, most represented by [^{3}H]Cer, [^{3}H]SM, and, in lower amounts, [^{3}H]glucosylceramide ([^{3}H]GlcCer) and [^{3}H]GM3 (Fig. 2*B*). The extent of *N*-acylation (evaluated as the sum of tritiated Cer, SM, GlcCer, and GM3) was always very similar in the control and treated cells. However, treatment with Wm and LY294002 strongly modified the radioactivity distribution among the different Sph metabolites. In the Wm- and LY294002-treated cells, [^{3}H]Cer was higher than in the control cells, with a concomitant reduction of both [^{3}H]SM and [^{3}H]GlcCer + [^{3}H]GM3 (Fig. 2*B*) giving rise to a [^{3}H]SM/[^{3}H]Cer of 1.6, 0.42, and 0.66 and [^{3}H]GlcCer/[^{3}H]Cer ratios of 0.44, 0.14, and 0.20 in the controls, and the Wm- and LY294002-treated cells, respectively. Instead, treating the cells with LY303511 did not have any effect on the radioactivity distribution among the different *N*-acyl derivatives of [^{3}H]Sph (Fig. 2*B*). We then evaluated whether or not DN-Akt had effects on Cer metabolism similar to the chemical inhibition of PI3K. When cells expressed the DN-Akt, the radioactivity distribution among the different *N*-acyl derivatives of [^{3}H]Sph was also significantly different from that of the vector-expressing cells (Fig. 3). In particular, DN-Akt transfection promoted an increase in [^{3}H]Cer that was associated with the reduction of both [^{3}H]SM and [^{3}H]GlcCer, resulting in a [^{3}H]SM/[^{3}H]Cer ratio of 1.6 and

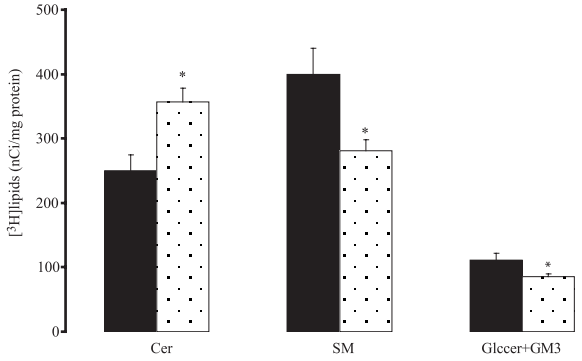

FIGURE 3. **Effect of DN-Akt expression on [^{3}H]Sph metabolism in C6 glioma cells.** Cells stably transfected with DN-Akt (*dotted bars*) or empty vector (*closed bars*) were plated at 1.5×10^4 cells/cm^2 and grown for 72 h in DMEM plus 10% FCS. Then cells were pulsed for 1 h with [^{3}H]Sph, harvested, and analyzed as described in Fig. 2. All values are the mean ± S.D. of at least three individual experiments. *, $p < 0.01$ *versus* control.

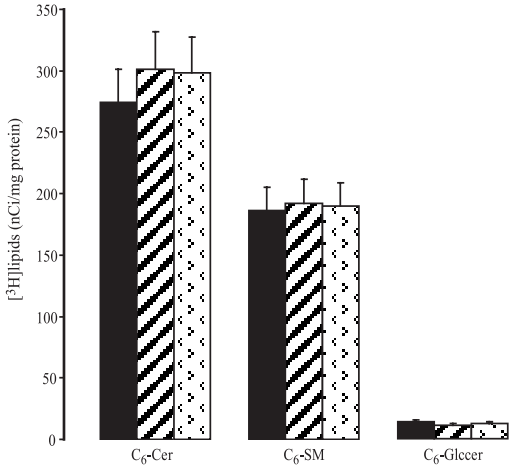

FIGURE 4. **Effect of PI3K/Akt inhibition on [^{3}H]SM synthase and [^{3}H]GlcCer synthase in C6 glioma cells.** Cells plated at 1.5×10^4 cells/cm^2 were grown for 72 h in DMEM plus 10% FCS and 30 min pretreated with the different inhibitors. Then cells were pulse for 1 h with [^{3}H]C_6-Cer in the absence (*closed bars*) or presence of 0.5 μM Wm (*striped bars*), or 20 μM LY294002 (*dotted bars*). At the end of pulse, cells were harvested and submitted to lipid extraction. The [^{3}H]C_6-Cer metabolites, associated with the organic phase, were separated and analyzed as described in Fig. 2. All values are the mean ± S.D. of at least three individual experiments.

0.79 and a [^{3}H]GlcCer/[^{3}H]Cer ratio of 0.45 and 0.22 in vector and DN-Akt-transfected cells, respectively. Also in this case we observed no differences in the uptake and the amount of *N*-acylated derivatives in vector- and DN-Akt-expressing cells (data not shown). Taken together, these results suggest that the PI3K/Akt pathway can regulate the metabolic utilization of Cer, favoring the maintenance of low Cer levels in C6 glioma cells.

Effect of PI3K/Akt on Sphingomyelin Synthase and Glucosylceramide Synthase Activity in C6 Glioma Cells—On the basis of these results, we investigated the possibility that the PI3K/Akt pathway can positively regulate the biosynthesis of complex sphingolipids. For this purpose we first evaluated the effect of Wm and LY294002 on the *in vitro* enzyme activity of SMS and GCS. Using the homogenate from control cells, the measured SMS activity was 0.24 ± 0.04 nmol, mg of protein^{-1} min^{-1} and that of GCS 0.25 ± 0.03 nmol, mg of protein^{-1} min^{-1}. No differences were observed in either SMS or GCS activities adding Wm or LY294002 to the enzyme assay or using the homogenate from Wm- and LY294002-treated cells as enzyme sources. We then evaluated the effect of Wm and LY294002 on the metabolic process of [^{3}H]C_6-Cer, a short chain, free diffusible analogue of Cer that allows the evaluation of the activity of SMS and GCS in living cells. Compared with the control cells, there was no modification of the uptake of [^{3}H]C_6-Cer in C6 glioma cells treated with Wm and LY294002. As shown in Fig. 4 when C6 glioma cells were pulsed for 1 h with [^{3}H]C_6-Cer, the amounts of synthesized [^{3}H]C_6-SM and [^{3}H]C_6-GlcCer were very similar in both control and treated cells, thus indicating that the PI3K/Akt pathway does not affect the activity of these enzymes in living cells. Taken together, these results indicate that the reduced utilization of Cer cannot be ascribed to a direct effect of PI3K/Akt on SMS and GCS. In addition, these results also indicate that PI3K/Akt inactivation did not influence the availability of phosphatidylcholine as a substrate for SMS.

Effect of PI3K/Akt on SMase Activity—To exclude any contribution of SM degradation to the increased [^{3}H]SM/[^{3}H]Cer ratio observed after PI3K/Akt inhibition, we investigated the effects of Wm and LY294002 on the activity of N-SMase and A-SMase. Using homogenates obtained from control cells and Wm- or LY294002-treated cells as enzyme source and adding Wm or LY294002 to the homogenate of control cells, no differences were observed in N-SMase or A-SMase activity (data not shown). This indicates that in C6 glioma cells, contrary to what was observed in oligodendrocytes (49), PI3K/Akt did not affect A-SMase activity.

Effect of PI3K/Akt on the Intracellular Distribution of BODIPY-C_5-Cer and NBD-C_6-Cer—To clarify the mechanism responsible for [^{3}H]Cer accumulation consequent to PI3K/Akt inhibition, we tested the possibility that the inhibition of this pathway affects the ER to Golgi transport of Cer. The transport of natural Cer from ER to the Golgi apparatus can be qualitatively evaluated from the analysis of BODIPY-C_5-Cer redistribution in cells (41, 50, 51); thus we first investigated the effect of 0.5 μM Wm, 20 μM LY294002, and 20 μM LY303511 on the distribution of BODIPY-C_5-Cer (Fig. 5*A*). After labeling the ER and other intracellular membranes, cells were chased in the presence or absence of Wm, LY294002, or its inactive analogue LY303511 to allow the redistribution of BODIPY-C_5-Cer. In the control and LY303511-treated cells, most of the fluorescence accumulated in the perinuclear region (Fig. 5*A*) representative of the Golgi apparatus (50), whereas in the Wm- and LY294002-treated cells, the accumulation of fluorescence in the Golgi region was strongly reduced (Fig. 5*A*). We then evaluated whether or not DN-Akt had similar effects on Cer traffic as the chemical inhibition of PI3K. When the cells expressed DN-Akt, the accumulation of fluorescence in the Golgi region was also strongly reduced (Fig. 5*A*). In contrast, the labeling of Wm- and LY294002-treated and DN-Akt-transfected cells with NBD-C_6-Cer (Fig. 5*B*), a specific marker of the Golgi complex, demonstrated that those treatments did not modify the accumulation of NBD fluorescence in the perinuclear region (Fig. 5*B*), thus allowing us to exclude the possibility that PI3K inhibition affects the organization of the Golgi apparatus. Taken together, these results strongly support the PI3K/Akt pathway regulation of Cer delivery to the Golgi apparatus.

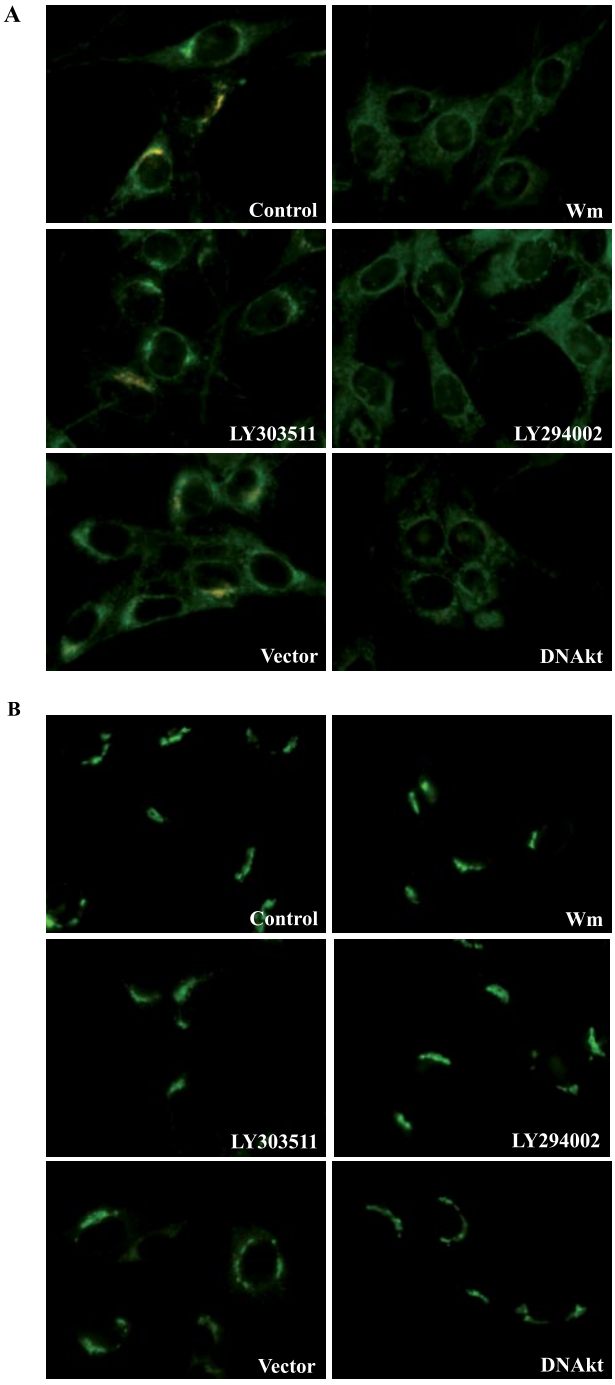

FIGURE 5. **Effect of PI3K/Akt inhibition on BODIPY-C_5-Cer and NBD-C_6-Cer intracellular distribution in C6 glioma cells.** Control cells and stable transfectant cells for DN-Akt or vector were plated at 1.5×10^4 cells/cm^2 on a coverslip and grown for 72 h in DMEM plus 10% FCS. Then the cells were incubated with 5 μM BODIPY-C_5-Cer (*A*) or NBD-C_6-Cer (*B*) for 30 min at 4 °C. Labeled cells were then incubated at 37 °C for 30 min in the absence (*Control, vector,* and *DN-Akt*) or presence of 0.5 μM Wm, 20 μM LY303511, and 20 μM LY294002, respectively. All images were processed and printed identically.

Effect of Wm and LY294002 on SM and GlcCer Formation from [^{3}H]Sph in BFA-treated Cells—To obtain further evidence for the effect PI3K/Akt inhibitors on Cer traffic, we investigated the effect of Wm and LY294002 on [^{3}H]Sph metabolism in C6 glioma cells treated with BFA, which leads to the fusion of the *cis*-Golgi membranes with the ER (52). Our working hypothesis was that, if Wm and LY294002 determine a defect in ER to the

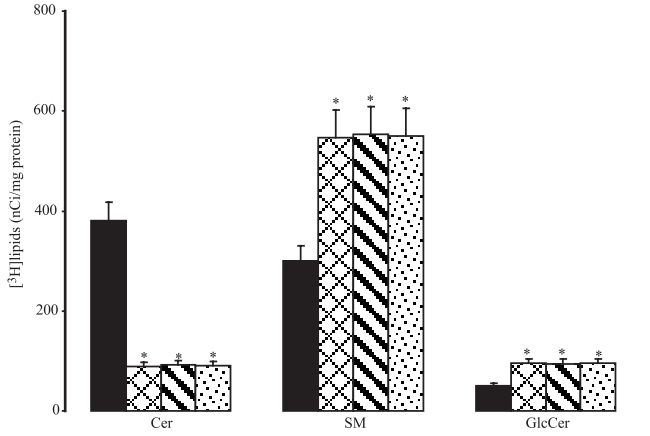

FIGURE 6. **Effect of PI3K/Akt inhibitors on [^{3}H]Sph metabolism in BFA-treated C6 glioma cells.** Cells were plated at 1.5×10^4 cells/cm^2 and grown for 72 h in DMEM 10% FCS and preincubated for 30 min with 1 μg/ml BFA in presence or absence of the different inhibitors. Then cells were pulsed with [^{3}H]Sph for 30 min in the absence (*closed bars*) or presence of 1 μg/ml BFA (*cross-hatched bars*), 0.5 μM Wm (*striped bars*), or 20 μM LY294002 (*dotted bars*) in BFA-containing medium. At the end, the cells were harvested and analyzed as described in Fig. 2. All values are the mean ± S.D. of at least three individual experiments. *, $p < 0.01$ *versus* control.

Golgi apparatus trafficking of Cer, treatment with BFA should make Cer metabolism insensitive to those inhibitors. Cells pretreated with BFA were pulsed 30 min with [^{3}H]Sph in the presence of BFA alone or with Wm or LY294002. Then the conversion of [^{3}H]Cer to [^{3}H]SM and [^{3}H]GlcCer was compared in control and BFA-treated cells (Fig. 6). In the BFA-treated cells, the amount of [^{3}H]SM and [^{3}H]GlcCer synthesized from [^{3}H]Cer was found to be about 87% higher than in the controls. In the presence of BFA, Wm and LY294002 were no longer able to impair [^{3}H]Cer metabolic utilization, thus implying that PI3K/Akt inhibitors affect the translocation of Cer from the ER to the Golgi apparatus.

Effect of PI3K/Akt on [^{3}H]Sph Metabolism in C6 Glioma Cells Down-regulated for CERT—Recent results obtained in our laboratories indicate that in C6 glioma cells, the ER to Golgi Cer transport, needed for Cer utilization in complex sphingolipid biosynthesis, involves both the Cer-binding protein CERT and a vesicle-mediated mechanism that can separately contribute to the control of sphingolipid metabolism and Cer levels in these cells, CERT being specific for SM biosynthesis (43). Based on our results that indicate a decrease of both [^{3}H]SM and [^{3}H]GlcCer after PI3K/Akt inhibition, and taking into account recent evidence that indicates a role for PI3K/Akt in the regulation of the ER to Golgi protein vesicular transport (53), we hypothesized that PI3K/Akt can also regulate the ER to Golgi vesicular traffic of Cer. Recently, we demonstrated (43) that the cells down-regulated for CERT represent a model for evaluating the contribution of vesicular traffic of Cer from ER to the Golgi apparatus, and thus we investigated the effect of Wm and LY294002 on [^{3}H]Sph metabolism in control and CERT down-regulated C6 glioma cells. As reported in Fig. 7 (*upper panel*), treatment with Wm and LY294002 induced a significant reduction in the biosynthesis of SM and GlcCer as it did in wild type cells. In fact, as shown in Fig. 7 (*upper panel*), si-control cell treatment with Wm and LY294002 determined an 83 and 43% increase in [^{3}H]Cer with a concomitant decrease of both

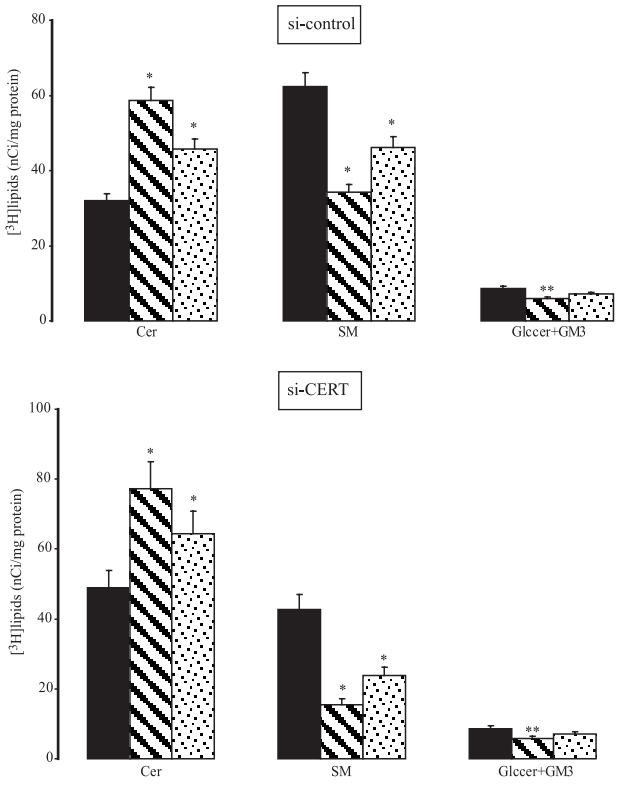

FIGURE 7. **Effect of PI3K/Akt inhibitors on [^{3}H]Sph metabolism in C6 glioma cells down-regulated for CERT.** Cells plated at 3×10^4 cell/cm^2 were maintained 24 h in DMEM plus 10% FCS before transfection with siRNA for CERT (*si-CERT*) or nontargeting sequences (*si-control*). After 72 h cells were pulsed with [^{3}H]Sph for 1 h in the presence or absence (*closed bars*) of 0.5 μM Wm (*striped bars*) or 20 μM LY294002 (*dotted bars*). At the end, cells were harvested and analyzed as described in Fig. 2. All values are the mean ± S.D. of at least three individual experiments. *, $p < 0.01$ *versus* control; **, $p < 0.05$ *versus* control.

[^{3}H]SM (45 and 26%) and [^{3}H]GlcCer (31 and 17%). As expected, in the CERT-silenced cells, the conversion of [^{3}H]Cer to [^{3}H]SM was strongly reduced, as demonstrated by the drop in the [^{3}H]SM/[^{3}H]Cer ratio from 1.95 in si-control cells to 0.87 in si-CERT cells, whereas [^{3}H]Cer utilization for glycosphingolipid biosynthesis was unaffected. In the si-CERT cells, the effect of PI3K/Akt inhibition on SM biosynthesis was higher than in si-control cells because, in si-CERT cells, Wm and LY294002 promoted a 64 and 34% decrease of [^{3}H]SM, respectively. In the same conditions the reduction of [^{3}H]GlcCer (30 and 15%, respectively) was very similar in control and down-regulated cells (Fig. 7, *lower panel*). These results strongly suggest the ER to Golgi vesicular transport as the major target of PI3K/Akt in the regulation of Cer metabolism.

DISCUSSION

Different lines of evidence indicate that both aberrant activation of the PI3K/Akt survival pathway (19, 20) and down-regulation of the death mediator Cer (25) play a critical role in the aggressive behavior, apoptosis resistance, and adverse clinical outcome of GBMs. Furthermore, now included in the therapeutic strategy design against such tumors is the inhibition of the PI3K/Akt pathway (12, 22, 24) and the up-regulation of Cer (26–30). Indeed, a reciprocal control between PI3K/Akt and Cer signaling in glioma cell survival/death is suggested by data indicating that Cer may induce apoptosis also by inactivation of the prosurvival kinase Akt (38, 54), possibly through the activation of a Cer-dependent protein phosphatase, and by data demonstrating a protective role of PI3K/Akt on Cer-induced cell death in glial cells (31, 34, 55). This led us to wonder whether the PI3K-Akt-PTEN pathway is able to regulate Cer metabolism; indeed, if such regulation does occur, some insight could be gained into crucial aspects that could, after further investigations, strongly influence our knowledge of the sensitivity of glioma cells to Cer-based cytotoxic treatments.

Here we report that in C6 glioma cells the PI3K/Akt pathway actually regulates Cer metabolism through a positive control of the vesicular ER to Golgi transport of Cer, which results in improved biosynthesis of both SM and glycosphingolipids. This conclusion is supported by several lines of evidence. Pulse experiments performed with [^{3}H]Sph indicate that PI3K inhibition by both LY294002 and Wm strongly reduces the metabolic utilization of newly synthesized Cer for the biosynthesis of both SM and glycosphingolipids. The drop in both the [^{3}H]SM/[^{3}H]Cer and [^{3}H]GlcCer/[^{3}H]Cer ratios, consequent to PI3K inhibition, was not because of reduced SMS and GCS activity; in addition, the amount of [^{3}H]C_6-SM and [^{3}H]C_6-GlcCer synthesized after pulse with [^{3}H]C_6-Cer, an experimental condition allowing an evaluation of the activity of these enzymes in living cells, was not affected by the LY294002 and Wm treatment. Moreover, treatment of C6 glioma cells with BFA, which causes Golgi disassembly and redistribution to the ER (52), and direct Cer availability for SMS and GCS, renders the glioma cell Cer metabolism insensitive to PI3K inhibitors. It emerges from these findings that the ER to Golgi transport of Cer is a PI3K regulated step of sphingolipid biosynthesis. An analysis of intracellular distribution of BODIPY-C_5-Cer, which mimics the intracellular movements of natural Cer (41, 50, 51), provides additional evidence that, in C6 glioma cells, PI3K inhibition strongly limits Cer traffic from ER to the Golgi apparatus without altering the Golgi apparatus organization, as demonstrated by data obtained with NBD-C_6-Cer, a specific marker of the Golgi complex (50). Moreover, as demonstrated by both metabolic and fluorescence microscopy studies, the effect of PI3K inhibitors on the ER to Golgi Cer traffic was mimicked by the expression of a dominant negative form of Akt in C6 glioma cells. Taken together, the data strongly support the involvement of the PI3K/Akt survival pathway in glioma cells in controlling the ER to Golgi Cer traffic. We recently demonstrated (43) that in C6 glioma cells the ER to Golgi transport of Cer is operated by the Cer-binding protein CERT, which specifically acts on SM biosynthetic process, and by a vesicle-mediated mechanism, functional to Cer utilization for both SM and GlcCer biosynthesis. Even if the concomitant decrease of both SM and GlcCer in PI3K/Akt-inhibited cells indicates the vesicular pathway as the most probable target of PI3K/Akt, very convincing evidence in this regard is given by results obtained in cells down-regulated for CERT, which represent a good model to study the vesicular transport of Cer. In fact, when the protein-mediated transport of Cer is knocked down, the PI3K/Akt inhibition still promotes a decrease in the biosynthesis of both GlcCer and SM, the efficiency of the PI3K/Akt blockade in inhibiting SM biosynthesis being higher in the control than in

the silenced cells, and the combined treatments having an additive effect on SM but not on GlcCer biosynthesis. Protein phosphorylation, known to modulate many membrane traffic processes throughout the cells, has also been implicated in the transport between the ER and Golgi (53, 56), and different protein kinases, including Akt (53), have been shown to regulate ER to Golgi traffic. To the best of our knowledge, this study is the first to show that the PI3K/Akt pathway can participate in controlling the sphingolipid metabolism and the regulation of Cer levels by acting on the vesicle-mediated movements of Cer in the ER-Golgi district, but the precise target of Akt still remains undisclosed. Because the activation of PI3K/Akt is downstream different proliferative/survival signals and the synthesis of new membrane are absolute requirements for cell growth and proliferation, the connection between PI3K/Akt and the biosynthesis of SM and glycosphingolipids could represent a mechanism to coordinate the production of crucial components of cell membrane with the proliferative attitude of the cells. In this context it is interesting to note that the PI3K/Akt pathway can positively regulate cholesterol biosynthesis by favoring the activating processing of SCAP/SREBP on its ER to Golgi movements (53). Furthermore, both Cer biosynthesis in the ER (57) and the biosynthesis of complex sphingolipids have been positively correlated with the activating processing of SREBP (58, 59), independently of cellular cholesterol concentration. Thus we can hypothesize a role for the PI3K/Akt pathway to coordinate the metabolism of SM and cholesterol, membrane lipids known to co-localize in cell membrane lipid rafts. The capacity of the PI3K/Akt pathway to regulate sphingolipid metabolism may also be pathologically relevant in gliomas if we consider that the more malignant phenotypes of these tumors are associated with the up-regulation of the PI3K/Akt pathway (19, 20), increased amounts of SM (60), and low Cer levels (25). Moreover, the SM/Cer mole ratio in PTEN-mutated C6 glioma cells (43) was significantly higher than in different glioma cell lines characterized by wild type PTEN (61). This suggests that, in gliomas, the PI3K/Akt pathway could contribute to Cer down-regulation by directing it toward SM synthesis, as a means of avoiding cell growth arrest and cell death. It is also tempting to speculate that Cer removal, as a consequence of a hyper-activated PI3K/Akt pathway, could somehow be involved in Cer-based cytotoxic treatment refractoriness in gliomas, especially as far as concerns those treatments that, in ER, act through Cer biosynthesis stimulation (16, 21). Based on recent evidence showing that PI3K inhibition synergizes with chemotherapy or death-inducing ligands to trigger apoptosis in glioblastoma cells (24), our findings could have important implications for Cer-based glioma therapy, and could provide a basis for further investigations into the overcoming of cell death resistance in glioblastomas.

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REFERENCES

1. Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D., and Dirks, P. B. (2004) *Nature* **432,** 396–401
2. Vescovi, A. L., Galli, R., and Reynolds, B. A. (2006) *Nat. Rev. Cancer* **6,** 425–436
3. Kondo, T. (2006) *Eur. J. Cancer* **42,** 1237–1242
4. Kleihues, P., Louis, D. N., Scheithauer, B. W., Rorke, L. B., Reifenberger, G., Burger P. C., and Cavenee, W. K. (2002) *J. Neuropathol. Exp. Neurol.* **61,** 215–225
5. Davis, F. G., and McCarthy, B. J. (2001) *Expert Rev. Anticancer Ther.* **1,** 395–401
6. Rao, R. D., and James, C. D. (2004) *Semin. Oncol.* **3,** 595–604
7. Guha, A., and Mukherjee, J. (2004) *Curr. Opin. Neurol.* **17,** 655–662
8. Brantley, E. C., and Benveniste, E. N. (2008) *Mol. Cancer Res.* **6,** 675–684
9. Hegi, M. E., Liu, L., Herman, J. G., Stupp, R., Wick, W., Weller, M., Mehta, M. P., and Gilbert, M. R. (2008) *J. Clin. Oncol.* **26,** 4189–4199
10. Vogt, P. K. (2001) *Trends Mol. Med.* **7,** 482–484
11. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M., and Abraham, R. T. (2000) *Cancer Res.* **60,** 3504–3513
12. Knobbe, C. B., Merlo, A., and Reifemberger, G. (2002) *Neuro-Oncol.* **4,** 196–211
13. Engelman, J. A., Luo, J., and Cantley, L. C. (2006) *Nat. Rev. Genet.* **7,** 606–619
14. Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., Bigner, D. D., and Bigner, S. H. (1997) *Cancer Res.* **57,** 4187–4190
15. Smith, J. S., Tachibana, I., Passe, S. M., Huntley, B. K., Borell, T. J., Iturria, N., O'Fallon, J. R., Schaefer, P. L., Scheithauer, B. W., James, C. D., Buckner, J. C., and Jenkins, R. B. (2001) *J. Natl. Cancer Inst.* **93,** 1246–1256
16. Morales, A., Lee, H., Goni, F. M., Kolesnick, R., and Fernandez-Checa, J. C. (2007) *Apoptosis* **12,** 923–939
17. Hartmann, C., Bartels, G., Gehlhaar, C., Holtkamp, N., and von Deimling, A. (2005) *Acta Neuropathol.* **109,** 639–642
18. Knobbe, C. B., Trampe-Kieslich, A., and Reifenberger, G. (2005) *Neuropathol. Appl. Neurobiol.* **31,** 486–490
19. Ermoian, R. P., Furniss, C. S., Lamborn, K. R., Basila, D., Berger, M. S., Gottschalk, A. R., Nicholas, M. K., Stokoe, D., and Haas-Kogan, D. A. (2002) *Clin. Cancer Res.* **8,** 1100–1106
20. Chakravarti, A., Zhai, G., Suzuki, Y., Sarkesh, S., Black, P. M., Muzikansky, A., and Loeffler, J. S. (2004) *J. Clin. Oncol.* **22,** 1926–1931
21. Ogretmen, B., and Hannun, Y. A. (2004) *Nat. Rev. Cancer* **4,** 604–616
22. Wong, M. L., Kaye, A. H., and Hovens, C. M. (2007) *J. Clin. Neurosci.* **14,** 301–308
23. Castellino, R. C., and Durden, D. (2007) *Nat. Clin. Pract. Neurol.* **3,** 682–693
24. Opel, D., Westhoff, M. A., Bender, A., Braun, V., Debatin, K. M., and Fulda, S. (2008) *Cancer Res.* **68,** 6271–6280
25. Riboni, L., Campanella, R., Bassi, R., Villani, R., Gaini, S. M., Martinelli-Boneschi, F., Viani, P., and Tettamanti, G. (2002) *Glia* **39,** 105–113
26. Sawada, M., Nakashima, S., Banno, Y., Yamakawa, H., Hayashi, K., Takenaka, K., Nishimura, Y., Sakai, N., and Nozawa, Y. (2000) *Cell Death Differ.* **7,** 761–772
27. Sawada, M., Kimono, T., Nakashima, S., Shinoda, J., Naganawa, T., Hara, S., Iwama, T., and Sakai, N. (2004) *Cell Death Differ.* **11,** 997–1008
28. Hara, S., Nakashima, S., Kiyono, T., Sawada, M., Yoshimura, S., Iwama, T., Banno, Y., Shinoda, J., and Sakai, N. (2004) *Cell Death Differ.* **11,** 853–861
29. Noda, S., Yoshimura, S., Sawada, M., Naganawa, T., Iwama, T., Nakashima, S., and Sakai, N. (2001) *J. Neurooncol.* **52,** 11–21
30. Guzman, M. (2005) *Handb. Exp. Pharmacol.* **168,** 627–642
31. Mochizuki, T., Asai, A., Saito, N., Tanaka, S., Katagiri, H., Asano, T., Nakane, M., Tamura, A., Kuchino, Y., Kitanaka, C., and Kirino, T. (2002) *J. Biol. Chem.* **277,** 2790–2797
32. Daido, S., Kanzawa, T., Yamamoto, A., Takeuchi, H., Kondo, Y., and Kondo, S. (2004) *Cancer Res.* **64,** 4286–4293
33. Kim, W. H., Choi, C. H., Kang, S. K., Kwon, C. H., and Kim, Y. K. (2005) *Neurochem. Res.* **30,** 969–979

34. Gomez del Pulgar, T., De Ceballos, M. L., Guzman, M., and Velasco, G. (2002) *J. Biol. Chem.* **277,** 36527–36533
35. Blasquez, C., Galve-Roperh, I., and Guzman, M. (2000) *FASEB J.* **14,** 2315–2322
36. Galve-Roperh, I., Sanchez, C., Cortes, M. L., Gomez del Pulgar, T., Izquierdo, M., and Guzman, M. (2000) *Nat. Med.* **6,** 313–319
37. Riboni, L., Viani, P., Bassi, R., Giussani, P., and Tettamanti, G. (2001) *J. Biol. Chem.* **276,** 12797–12804
38. Gomez del Pulgar, T., Velasco, G., Sanchez, C., Haro, A., and Guzman, M. (2002) *Biochem. J.* **363,** 183–188
39. Hinz, B., Ramer, R., Eichele, K., Weinzierl, U., and Brune, K. (2004) *Mol. Pharmacol.* **66,** 1643–1651
40. Carracedo, A., Morente, M., Egia, A., Blazquez, C., Garcia, S., Giroux, V., Malicet, C., Villuendas, R., Gironella, M., González-Feria, L., Piris, M. A., Iovanna, J. L., Guzman, M., and Velasco, G. (2006) *Cancer Cell* **9,** 301–312
41. Viani, P., Giussani, P., Brioschi, L., Bassi, R., Anelli, V., Tettamanti, G., and Riboni, L. (2003) *J. Biol. Chem.* **278,** 9592–9601
42. Kubiatowski, T., Jang, T., Lachyankar, M. B., Salmonsen, R., Nabi, R. R., Quesenberry, P. J., Litofsky, N. S., Ross, A. H., and Recht, L. D. (2001) *J. Neurosurg.* **95,** 480–488
43. Giussani, P., Colleoni, T., Brioschi, L., Bassi, R., Hanada, K., Tettamanti, G., Riboni, L., and Viani, P. (2008) *Biochim. Biophys. Acta* **1781,** 40–51
44. Riboni, L., Viani, P., and Tettamanti, G. (2000) *Methods Enzymol.* **311,** 656–682
45. Balla, A., and Balla, T. (2006) *Trends Cell Biol.* **16,** 351–361
46. Toth, B., Balla, A., Ma, H., Knight, Z. A., Shokat, K. M., and Balla, T. (2006) *J. Biol. Chem.* **281,** 36369–36377
47. Godi, A., Di Campli, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D. R., Kular, G. S., Daniele, T., Marra, P., Lucocq, J. M., and De Matteis, M. A. (2004) *Nat. Cell Biol.* **6,** 393–404
48. Cockcroft, S., and De Matteis, M. A. (2001) *J. Membr. Biol.* **180,** 187–194
49. Testai, F. D., Landek, M. A., Goswami, R., Ahmed, M., and Dawson, G. (2004) *J. Neurochem.* **89,** 636–644
50. Pagano, R. E., Martin, O. C., Kang, H. C., and Haugland, R. P. (1991) *J. Cell Biol.* **113,** 1267–1279
51. Fukasawa, M., Nishijima, M., and Hanada, K. (1999) *J. Cell Biol.* **144,** 673–685
52. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116,** 1071–1080
53. Du, X., Kristiana, I., Wong, J., and Brown, A. J. (2006) *Mol. Biol. Cell* **17,** 2735–2745
54. Zinda, M. J., Vlahos, C. J., and Lai, M. T. (2001) *Biochem. Biophys. Res. Commun.* **280,** 1107–1115
55. Pahan, K., Khan, M., and Singh, I. (2000) *J. Neurochem.* **75,** 576–582
56. Palmer, K. J., Konkel, J. E., and Stephens, D. J. (2005) *J. Cell Sci.* **118,** 3839–3847
57. Worgall, T. S., Juliano, R. A., Seo, T., and Deckelbaum, R. J. (2004) *Arterioscler. Thromb. Vasc. Biol.* **24,** 943–948
58. Scheek, S., Brown, M. S., and Goldstein, J. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11179–11183
59. Bijl, N., Scheij, S., Houten, S., Boot, R. G., Groen, A. K., and Aerts, J. M. F. G. (2008) *J. Pharmacol. Exp. Ther.* **326,** 849–855
60. Lou, H. O., Clausen, J., and Bierring, F. (1965) *J. Neurochem.* **12,** 619–627
61. Sullards, M. C., Wang, E., Peng, Q., and Merrill, A. H. (2003) *Cell. Mol. Biol.* **49,** 789–797

Phosphatidylinositol 3-Kinase/AKT Pathway Regulates the Endoplasmic Reticulum to Golgi Traffic of Ceramide in Glioma Cells: A LINK BETWEEN LIPID SIGNALING PATHWAYS INVOLVED IN THE CONTROL OF CELL SURVIVAL

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