Ceramide-mediated Macroautophagy Involves Inhibition of Protein Kinase B and Up-regulation of Beclin 1*

Received for publication, December 11, 2003, and in revised form, February 2, 2004
Published, JBC Papers in Press, February 17, 2004, DOI 10.1074/jbc.M313561200

Francesca Scarlatti‡§, Chantal Bauvy‡, Annamaria Venturi‡, Giusy Sala‡, Françoise Cluzeaud¶, Alain Vandewalle‡, Riccardo Ghidoni§, and Patrice Codogno‡**

From INSERM U504, 16 Avenue Paul-Vaillant-Couturier, 94807 Villejuif Cedex, France, ¶Laboratory of Biochemistry and Molecular Biology, San Paolo Medical School, 20142 Milan, Italy, and **INSERM U478, 75870 Paris Cedex 18, France

The sphingolipid ceramide is involved in the cellular stress response. Here we demonstrate that ceramide controls macroautophagy, a major lysosomal catabolic pathway. Exogenous C2-ceramide stimulates macroautophagy (proteolysis and accumulation of autophagic vacuoles) in the human colon cancer HT-29 cells by increasing the endogenous pool of long chain ceramides as demonstrated by the use of the ceramide synthase inhibitor fumonisin B1. Ceramide reverted the interleukin 13-dependent inhibition of macroautophagy by interfering with the activation of protein kinase B. In addition, C2-ceramide stimulated the expression of the autophagy gene product beclin 1. Ceramide is also the mediator of the tamoxifen-dependent accumulation of autophagic vacuoles in the human breast cancer MCF-7 cells. Monodansylecadaverine staining and electron microscopy showed that this accumulation was abrogated by myriocin, an inhibitor of de novo synthesis ceramide. The tamoxifen-dependent accumulation of vacuoles was mimicked by 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthase. 1-Phenyl-2-decanoylamino-3-morpholino-1-propanol, tamoxifen, and C2-ceramide stimulated the expression of beclin 1, whereas myriocin antagonized the tamoxifen-dependent up-regulation. Tamoxifen and C2-ceramide interfere with the activation of protein kinase B, whereas myriocin relieved the inhibitory effect of tamoxifen. In conclusion, the control of macroautophagy by ceramide provides a novel function for this lipid mediator in a cell process with major biological outcomes.

Macropautophagy or autophagy is an evolutionary conserved lysosomal pathway involved in the turnover of long-lived proteins and organelles (1–3). Autophagy starts with the formation of a multilayer membrane-bound autophagosome that sequesters fractions of the cytoplasm (4, 5). In mammalian cells, most of autophagosomes receive inputs from endocytic compartments before fusing with lysosomes where the degradation of the sequestered material is completed (6, 7).

The physiological importance of autophagy during starvation has been primarily highlighted in rat liver (8) and then in different cell types (reviewed in Refs. 9 and 10). At the same time, the term autophagic cell death or type II programmed cell death (PCD II) has been introduced (11) to describe a cell death different from apoptosis or type I programmed cell death (PCD I) (reviewed in Refs. 12 and 13). The recent progress made in characterization of the molecular mechanism controlling autophagy has brought a renewal of interest for this process (14).

There is now evidence for the role of autophagy during development (15–17), in the life span extension (15, 18), and in disease such as cancer (19, 20), neurodegenerative disease (21, 22), and myopathies (23, 24).

A family of autophagy-related genes discovered in yeast and almost entirely conserved in all eucaryotic phyla controls the formation of the autophagosome (25). Two conjugation systems (Atg5p-Atg12p and Atg8p lipidation) are involved in the formation of the autophagosome (26) together with a class III phosphatidylinositol 3-kinase (class III PI3K, the homologue of the yeast Vps34) complex (27, 28). In this complex, the protein beclin 1 (the orthologue of the yeast Atg6, see Ref. 29) is a tumor suppressor gene product (30, 31). The drug 3-MA commonly used to inhibit the autophagic pathway (32) interferes with the activity of class III PI3K to interrupt autophagy at the sequestration step (33, 34). Alternatively, the stimulation of the class I PI3K/PKB signaling pathway by growth factors and cytokines has an inhibitory effect on autophagy in many cell types (15, 34–36). This inhibitory effect is probably due to the class I PI3K/PKB-dependent activation of the kinase target of rapamycin which down-regulates autophagy (37–40).

Ceramide is a sphingolipid mediator with an essential role in cell growth, cell death, proliferation, and stress response (41–43). As all of these different situations are correlated with modulation of autophagy (14), we have investigated the potential role of ceramide in regulating autophagy.

Ceramide can be generated and consumed by different metabolic routes (44). Ceramide is generated by de novo synthesis in the endoplasmic reticulum or by the hydrolysis of sphingomyelin by acid sphingomyelinases, localized in acidic compartments and neutral sphingomyelinases, and localized in the plasma membrane and mitochondria. Ceramide is engaged in the biosynthesis of glucosylceramide (and other complex glyco-

* This work was supported in part by institutional funding from INSERM, by Association pour la Recherche sur le Cancer Grant 5831, and from Fondazione CaRiPLo, Milan, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by Ministero dell’Istruzione dell’Università e della Ricerca fellowships.

To whom correspondence should be addressed: INSERM U504 Glycobiologie et Signalisation Cellulaire, 16 Ave. Paul-Vaillant-Couturier, 94807 Villejuif Cedex, France. Tel.: 33-1-4559-5042; Fax: 33-1-4677-0233; E-mail: codogno@cfj.insERM.fr

The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; C2-Cer, C2-ceramide; C2-DH Cer, C2-dihydroceramide; DGR, diacylglycerol kinase; DMEM, Dulbecco’s modified Eagle’s medium; FB1, fumonisin B1; HBSS, Hanks’ balanced salt solution; IL-13, Interleukin-13; 3-MA, 3-methyladenine; MDC, monodansylecadaverine; Myriocin, Myriocin; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PKB, Protein kinase B; RT-PCR, reverse transcriptase-PCR; PCD, programmed cell death; TAM, tamoxifen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
sphingolipids) and of sphingomyelin. Ceramide can also generate ceramide 1-phosphate, sphingo sine, and sphingosine 1-phosphate. Sphingosine 1-phosphate is a second messenger that often has an opposite effect to ceramide on biological outcomes (46).

Here we show that, in two different cell lines, ceramide stimulates autophagy by two non-exclusive mechanisms. In human colon cancer HT-29 cells, C₂-Cer, a cell-permeable ceramide analogue, stimulates autophagy by increasing the intracellular pool of long chain ceramides. Ceramide reverts the inhibition of the class I PI3K signaling pathway on autophagy by interfering with the IL-13-dependent activation of protein kinase B (PKB) (34) and stimulates the expression of beclin 1. Ceramide also mediates the TAM-dependent accumulation autophagic vacuoles observed in human breast cancer MCF-7 cells (47). The ceramide-dependent expression of beclin 1 in TAM-treated cells was impaired in the presence of Myriocin, an inhibitor of the serine palmitoyltransferase, the rate-limiting enzyme of de novo synthesis of ceramide. By contrast, the expression of beclin 1 was stimulated when MCF-7 cells were treated with PDMP, an inhibitor of glucosylceramide synthase. In addition, tamoxifen and C₂-Cer inhibit the activation of protein kinase B, whereas myriocin antagonizes the inhibitory effect of tamoxifen. These data suggest a novel function for ceramide in controlling a major lysosomal pathway and provide a novel molecular link between autophagy and cell response to stress.

EXPERIMENTAL PROCEDURES
Reagents—C₂-Cer and C₂-DHCer were from Calbiochem and were dissolved in ethanol before use. FB1, Myri, TAM, 3-MA, MDC were purchased from Sigma. Cell culture medium and fetal bovine serum were from Invitrogen. Nitrocellulose membranes were from Schleicher & Schuell. Ceramide from porcine brain, used for internal standard, was from Avanti Polar Lipids Inc. (Alabaster, AL). The radioisotopes t-[U-¹⁴C]valine (256 mCi/mmol) and γ-³²P]ATP (3Ci/μl), the ECL™ Western blotting detection kit, and the donkey anti-rabbit antibody were purchased from Amersham Biosciences. The polyclonal rabbit anti-phospho-PKB Ser473 was from Cell Signaling, and goat anti-PKB was from Santa Cruz Biotechnology. IL-13 was kindly provided by Adrian Minty (Sanofi-Synthelabo, France). The rabbit anti-beclin 1 was kindly provided by Tamotsu Yoshimori (National Institute of Genetics, Shizukuizu, Japan). The monoclonal mouse anti-actin was from Chemicon International Inc. (Temecula, CA). Goat anti-mouse and swine anti-rabbit antibodies were obtained from Bio-Rad and Caltag (Burlingame, CA), respectively.

Cell Culture and Viability—Human breast cancer cell line MCF-7 was maintained at 37 °C in 10% CO₂ in DMEM, supplemented with 5% fetal bovine serum and 100 ng/ml each of penicillin and streptomycin as reported previously (34). Human colon cancer cell line HT-29 was maintained at 37 °C in 10% CO₂ in DMEM, supplemented with 10% fetal bovine serum and 100 ng/ml each of penicillin and streptomycin as reported previously (34).

Cell viability was determined by the trypan blue exclusion test.

Analysis of Protein Degradation—HT-29 cells were incubated for 24 h at 37 °C with 0.2 μCi/ml of t-[¹⁴C]valine. Three hours before the end of the radiolabeling period, cells were treated with increasing concentrations of C₂-Cer or C₂-DHCer, and when required 100 μM FB1 was added at the same time. At the end of radiolabeling period, cells were washed three times with phosphate-buffered saline, pH 7.4, and then incubated in nutrient-free medium (HBSS plus 0.1% of bovine serum albumin and 10 mM cold valine). After 16 h of incubation, at which time the damaged proteins were degraded, the medium was replaced with fresh nutrient-free medium, and when required 10 mM 3-MA or 30 ng/ml of IL-13 was added, and the incubation was continued for an additional 4 h period. Cells and radiolabeled proteins from the 4-h chase medium were precipitated in trichloroacetic acid at a final concentration of 10% (w/v) at 4 °C. The precipitated proteins were separated from the soluble radioactivity by centrifugation at 600 × g for 10 min and then dissolved in 0.5 ml of NaOH. Radioactivity was determined by liquid scintillation counting. Protein degradation was calculated by dividing the acid-soluble radioactivity recovered from both cells and medium by the radioactivity contained in precipitated proteins from both cells and medium (34).

MDC Staining of Autophagic Vacuoles—MCF-7 cells were incubated for 4 days with 1 μM of TAM dissolved in Me₃SO/EtOH (1:1, v/v). When required 100 μM Myriocin dissolved in methanol was added together with TAM. Alternatively, cells were incubated for 4 days with 20 μM PDMP in the absence of TAM. MDC staining was carried out essentially as described previously (48). An MDC stock solution (0.1 ml in Me₃SO) was diluted 1:1000 in DMEM and applied to the cells for 30 min at 37 °C. After washing with phosphate-buffered saline, cells were examined by fluorescence microscopy (Axioplan, Zeiss).

Electron Microscopy and Morphometry—Electron microscopy and morphometric analysis were performed as described previously (49). Cells were fixed for 30 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy by standard procedures. For morphometric analysis, 20 micrographs per condition were used.

Quantification of Endogenous Ceramide—Total endogenous ceramide levels were measured using the DGR assay as described previously (50, 51). Cells were collected, and lipids were extracted according to Bligh and Dyer (52). [³²P]Ceramide from porcine brain, used for internal standard, was separated from the soluble radioactivity by centrifugation at 600 × g for 10 min at 4 °C, and 10 μM of TAM dissolved in Me₃SO/EtOH (1:1, v/v) was added, and the incubation was continued with [³²P]Cer(C₂) and [³²P]Cer(C₄) at 37 °C for 1 h. At the end of the reaction, lipids were extracted according to Bligh and Dyer (52). [³²P]Ceramide 1-phosphate was determined by TLC separation in chloroform/methanol/acetic acid/water, (10:4:3:2:1 by volume). The radioactivity associated with ceramide 1-phosphate spots was determined after scraping and counting in a scintillator counter. Ceramide levels were referred to the level of total phospholipids.

Immunoblotting—Detection of total and phospho-PKB in HT-29 cells was performed as described previously (54). After being resolved by 10% SDS-PAGE, proteins were transferred onto nitrocellulose membrane. The ceramide-dependent expression of beclin 1 in TAM-treated cells was impaired in the presence of Myriocin, an enzyme of DGK containing membranes (Calbiochem), and 1 μM ATP mixed with [γ-³²P]ATP (13 μCi/ml) in a final volume of 0.1 ml. At the end of the reaction, lipids were extracted according to Bligh and Dyer (52). [³²P]Ceramide 1-phosphate was determined by TLC separation in chloroform/methanol/acetic acid/water, (10:4:3:2:1 by volume). The radioactivity associated with ceramide 1-phosphate spots was determined after scraping and counting in a scintillator counter. Ceramide levels were referred to the level of total phospholipids.
RESULTS

C2-Cer Has a Stimulatory Effect on Autophagy in HT-29 Cells—We have shown previously (55) that hallmarks of apoptosis are detectable after 8 h of incubation of HT-29 cells in the presence of 100 μM C2-Cer. In order to investigate the effect of C2-Cer on the control of autophagy in viable cells, HT-29 were incubated with an increasing amount of C2-Cer (from 25 to 100 μM) for a limited time. Viability was minimally affected when cells were cultured for 3 h in complete medium with increasing concentrations of C2-Cer and then transferred to a nutrient-free medium in the presence or absence of 3-MA (Fig. 1A, upper panel). In addition, no signs of apoptosis were detectable (changes in nuclear morphology or activation of caspases, data not shown) under these conditions. A dose-dependent increase in the rate of protein degradation was observed in C2-Cer pre-treated cells (Fig. 1A, lower panel). In contrast, no significant change in proteolysis was detected when the same experiment was performed in cells pre-treated with the inactive analogue C2-DHCer. The increase in proteolysis induced by C2-Cer was essentially due to the augmentation in the fraction of protein degradation sensitive to 3-MA (Fig. 1A). This result suggested that C2-Cer stimulates autophagy in HT-29 cells. This conclusion was supported by morphometric analysis showing that the fractional volume occupied by autophagic vacuoles is significantly increased in cells treated with C2-Cer and then incubated either in nutrient-free medium or in complete medium (Fig. 1B).

The Endogenous Ceramide Is Responsible for the Stimulatory Effect of C2-Cer on Autophagy in HT-29 Cells—Next we wanted to characterize the mechanism responsible for the effect of C2-Cer on autophagy. The first step toward this goal was to investigate whether the conversion of C2-Cer into endogenous long chain ceramides is critical to stimulate autophagy. It has been shown recently that short chain ceramides can be converted into long chain ceramides via the sequential deacylation of ceramide analogues into sphingosine and the subsequent reacylation with long chain fatty acids by ceramide synthase (56). In a first set of experiments we have analyzed changes in the endogenous pool of long chain ceramides in response to C2-Cer treatment. To this end, HT-29 cells were incubated for 3 h in complete medium with 100 μM C2-Cer or 100 μM C2-DHCer in the presence or absence of 100 μM fumonisin (FB1), an inhibitor of the activity of ceramide synthase (44). Thereafter, cells were incubated in nutrient-deprived medium with 10 mM 3-MA when required. Finally, the level of endogenous long chain ceramides was analyzed by the DGK assay. C2-Cer treatment induced a 1.5-fold increase in the endogenous pool of ceramide, whereas no change was observed in C2-DHCer-treated cells (Fig. 2A). The C2-Cer-dependent increase in endogenous long chain ceramides was totally blocked in the presence of FB1, whereas the autophagic inhibitor 3-MA has no effect on the accumulation of ceramide. From these results we conclude that the fractional volume occupied by autophagic vacuoles is expressed as a percentage of the cell volume. *, p < 0.05; **, p < 0.01 represents statistical differences between groups.

Effect of C2-Cer on Autophagy in HT-29 Cells—Next we wanted to characterize the mechanism responsible for the effect of C2-Cer on autophagy. The first step toward this goal was to investigate whether the conversion of C2-Cer into endogenous long chain ceramides is critical to stimulate autophagy. It has been shown recently that short chain ceramides can be converted into long chain ceramides via the sequential deacylation of ceramide analogues into sphingosine and the subsequent reacylation with long chain fatty acids by ceramide synthase (56). In a first set of experiments we have analyzed changes in the endogenous pool of long chain ceramides in response to

C2-Cer treatment. To this end, HT-29 cells were incubated for 3 h in complete medium with 100 μM C2-Cer or 100 μM C2-DHCer in the presence or absence of 100 μM fumonisin (FB1), an inhibitor of the activity of ceramide synthase (44). Thereafter, cells were incubated in nutrient-deprived medium with 10 mM 3-MA when required. Finally, the level of endogenous long chain ceramides was analyzed by the DGK assay. C2-Cer treatment induced a 1.5-fold increase in the endogenous pool of ceramide, whereas no change was observed in C2-DHCer-treated cells (Fig. 2A). The C2-Cer-dependent increase in endogenous long chain ceramides was totally blocked in the presence of FB1, whereas the autophagic inhibitor 3-MA has no effect on the accumulation of ceramide. From these results we conclude that the fractional volume occupied by autophagic vacuoles is expressed as a percentage of the cell volume. *, p < 0.05; **, p < 0.01 represents statistical differences between groups.
was totally blocked by 100 μM FB1 in C2-Cer-treated cells (Fig. 2B). In fact, FB1 was as potent as 3-MA in inhibiting proteolysis. In contrast to FB1, the inhibitory effect of 3-MA on proteolysis was achieved independently of the accumulation of endogenous long chain ceramides (Fig. 2, A and B). Both drugs have no cumulative effect on the inhibition of proteolysis (data not shown), suggesting that 3-MA, which interferes with the activity of class III PI3K (34), acts downstream FB1 in the same pathway to inhibit autophagy.

Ceramide Interferes with the Class I PI3K Signaling Pathway to Stimulate Autophagy in HT-29 Cells—C2-Cer is able to stimulate the accumulation of autophagic vacuoles, whereas HT-29 cells were cultured in complete medium (Fig. 1B, b) suggesting that ceramide can relieve the inhibitory effect induced by growth factors. We have shown previously (34, 35) that the stimulation of the class I PI3K/PKB signaling pathway by IL-13 has a strong inhibitory effect on autophagy. In addition, ceramide is known to interfere with the activation of PKB in different cell types (57–61).

In a first series of experiments, we have investigated if C2-Cer can revert the inhibitory effect of IL-13 on autophagy (Fig. 3A). As reported previously, IL-13 is as potent as 3-MA in inhibiting autophagy in HT-29 cells (34, 35). However, in C2-Cer-treated cells, the inhibitory effect of IL-13 was significantly reverted, whereas the sensitivity to 3-MA was not altered.

In order to assay directly the effect of C2-Cer on the IL-13-dependent activation of PKB, we have analyzed the phosphorylation at position Ser473 which is required for the activation of PKB (59). As shown previously, IL-13 induced a phosphorylation of PKB on Ser473 (54). Phosphorylation at Ser473 was severely impaired in the presence of 100 μM C2-Cer either after IL-13 stimulation or in basal conditions (Fig. 3B). In contrast C2-DHCer has only a marginal effect on the phosphorylation of PKB which is in agreement with its lack of effect on autophagy.

Ceramide Is Responsible for the Tamoxifen-dependent Accumulation of Autophagic Vacuoles in MCF-7 Cells—In order to generalize the role of ceramide in autophagy to other cell lines and also to uncover other possible targets for ceramide in the autophagic pathway, we have investigated the role of ceramide in the control of autophagy in MCF-7 cells. We have selected this cell line for several reasons. 1) The antagonist estrogen receptor TAM induces an accumulation of autophagic vacuoles (47). 2) TAM modulates the (glyco)sphingolipid metabolism (62). 3) The expression of the autophagy gene beclin 1 is stimulated by TAM treatment (20).

According to Bursch et al. (47), TAM treatment induced a time-dependent accumulation of autophagic vacuoles in MCF-7 cells that can be visualized by MDC staining and at the ultrastructural level (Fig. 4, A and B). During the accumulation of autophagic vacuoles, we observed a 2.5-fold increase in the content of endogenous ceramide after 4 days of treatment with 1 μM TAM (Fig. 4A). The TAM-dependent accumulation of autophagic vacuoles as well as the TAM-dependent increase in the intracellular pool of ceramides were severely impaired in the presence of 100 nM Myr04, a potent inhibitor of serine palmitoyltransferase, the key rate-limiting enzyme of the de novo synthesis of ceramide (44). From these results we reasoned that blocking the utilization of ceramide should mimic the TAM effect on the accumulation of autophagic vacuoles. In order to limit the utilization of endogenous ceramide, we have used the glucosylceramide synthase inhibitor PDMP. PDMP treatment increased the pool of endogenous ceramide by 1.5-fold and stimulated the accumulation of autophagic vacuoles (Fig. 4, A and B).

In order to correlate the TAM-dependent increase in the intracellular pool of ceramides with the relief of the inhibitory

---

**Fig. 2.** The effect of C2-Cer on autophagy is mediated by an increase of the endogenous pool of long chain ceramides. A, endogenous long chain ceramides were quantified by the DGK assay as described under “Experimental Procedures.” The amount of ceramide (6.2 pmol of Cer/nmol of P) in HT-29 cells cultured in complete medium was set to 1. HT-29 cells were cultured for 3 h in complete medium with 100 μM C2-Cer in the presence or absence of 100 μM FB1 or with 100 μM C2-DHCer. 3-MA (10 mM) was added during the chase period in nutrient-free medium. After 20 h of incubation in nutrient-free medium, cells were harvested, and the ceramide content was analyzed by the DGK assay as described under “Experimental Procedures.” Values are the mean ± S.D. of four independent experiments. B, the rate of degradation of [14C]valine-labeled long lived proteins was measured in cells incubated in nutrient-free medium (HBSS) in the presence or absence of 10 mM 3-MA. C2-Cer (100 μM) or C2-DHCer (100 μM) was added during the last 3 h of incubation in complete medium. FB1 (100 μM) was added together with C2-Cer in complete medium. Values reported are the mean ± S.D. of four independent experiments. *p < 0.05 versus untreated cells.

concluded that C2-Cer induces an accumulation of endogenous long chain ceramides in HT-29 cells, and this accumulation is dependent on the recycling of the sphingosine moiety and the activity of ceramide synthase.

In order to investigate whether the accumulation of long chain ceramides is responsible for the C2-Cer-induced autophagy, we have evaluated the effect of FB1 on protein degradation in C2-Cer-treated cells. We observed that in cells exposed to 100 μM C2-Cer for 3 h, the rate of proteolysis was increased by 30–35% when compared with untreated cells or to cells treated with C2-DHCer (Fig. 2B). The increase of protein degradation activity of ceramide synthase.

In order to determine the key rate-limiting enzyme of the de novo synthesis of ceramide (44). From these results we reasoned that blocking the utilization of ceramide should mimic the TAM effect on the accumulation of autophagic vacuoles. In order to limit the utilization of endogenous ceramide, we have used the glucosylceramide synthase inhibitor PDMP. PDMP treatment increased the pool of endogenous ceramide by 1.5-fold and stimulated the accumulation of autophagic vacuoles (Fig. 4, A and B).

In order to correlate the TAM-dependent increase in the intracellular pool of ceramides with the relief of the inhibitory...
Ceramide Controls the Expression of Beclin 1—In MCF-7 cells, a 4-day TAM treatment induced 4.2- and 5.4-fold increases in beclin 1 expression at mRNA and protein levels, respectively (Fig. 6A). The TAM-dependent increase of beclin 1 expression was dramatically impaired in Myrio-treated cells. According to its TAM-like effect on the accumulation of autophagic vacuoles and in the content of long chain ceramides, PDMP stimulated the expression of beclin 1 (Fig. 6A). In order to address directly the role of ceramide in the control of beclin 1 expression, we have repeated the same experiment on C2-Cer-treated cells. After 1 h of treatment of MCF-7 cells with 75 μM C2-Cer, an accumulation of beclin 1 was observed. This accumulation was dependent on the increase in the endogenous pool of long chain ceramides (1.6-fold when compared with control cells). No such increase in beclin 1 expression was observed in C2-DH Cer-treated cells (data not shown). Longer times of incubation with C2-Cer were not considered due to a pronounced cytotoxicity. The effect of C2-Cer on the expression of beclin 1 has been also investigated in HT-29 cells (Fig. 6B). Although the basal expression of beclin 1 is higher in HT-29 cells than in MCF-7 cells, a time-dependent increase in its expression was observed in response to C2-Cer treatment at both mRNA and protein levels (Fig. 6B).

**DISCUSSION**

We report here that ceramide is involved in the control of autophagy by two non-mutually exclusive mechanisms. Ceramide interferes with the inhibitory class I PI3K signaling pathway and stimulates the expression of the autophagy gene beclin 1. These results uncover a novel function for ceramide and provide a new molecular basis for the relationship between autophagy and the cell response to stress, cell death, proliferation, and cell growth stimuli.

As a major effect of ceramide and its cell-permeable analogue is to trigger cell death (reviewed in Ref. 43), all the experiments reported here were performed under conditions where the viability is minimally affected in the cell lines used in this study (HT-29 and MCF-7). In HT-29 cells, hallmarks of apoptosis (activation of caspases, DNA ladder, and condensation of chromatin) occur after 8 h in response to C2-Cer treatment (55). The short incubation time (3 h in complete medium) used in the present study was sufficient to observe an increase in the intracellular concentration of long chain ceramides and a stimulation of autophagy. The stimulation of autophagy by the ceramide analogue cannot be ascribed to membrane perturbation, because C2-DH Cer is ineffective toward autophagy, even being a membrane-perturbing agent (63). In addition, FB1 blocked the C2-Cer-induced autophagy demonstrating that C2-Cer has no effect on autophagy per se but requires its metabolic transformation into long chain ceramides.

The inhibition of ceramide synthesis by Myrio impairs the accumulation of autophagic vacuoles in response to TAM treatment in MCF-7 cells. Accordingly, Myrio has an inhibitory effect on the TAM-dependent increased expression of beclin 1. These data suggest that ceramide is involved in the TAM-dependent expression of beclin 1 (this study and Ref. 20).

**FIG. 3.** Ceramide relieves the inhibitory effect of the class I PI3K/PKB pathway on autophagy. A, the rate of degradation of [14C]valine-labeled long lived proteins was measured in cells incubated in nutrient-free medium in the presence or absence of 10 mM 3-MA or 30 ng/ml IL-13. When required C2-Cer (100 μM) was added during the last 3 h of incubation in complete medium (see “Experimental Procedures”). Values reported are the mean ± S.D. of four independent experiments. *, p < 0.05 versus untreated cells; **, p < 0.05 versus IL-13-treated cells in the absence of C2-Cer. B, HT-29 cells were cultured in the presence or absence of 100 μM C2-Cer or C2-DH Cer for 3 h in serum-free medium. When required 30 ng/ml IL-13 was added. Cell lysates were analyzed by Western blotting for PKB phosphorylation at Ser473 (top panel) using a phosphospecific antibody. The expression of total PKB was detected by using an anti-PKB antibody (lower panel). Densitometry was performed on the original blots, and the ratio of phospho-Ser473/total PKB in IL-13-treated cells was set to 100%.

Effect of the class I PI3K/PKB pathway on autophagy, we have investigated the phosphorylation state of PKB in TAM-treated cells (Fig. 5A). TAM treatment induced a dramatic decrease of PKB phosphorylation at position Ser473, and this inhibitory effect was antagonized in the presence of 100 nM Myrio (Fig. 5A). According to these results, C2-Cer strongly inhibited the phosphorylation of PKB. In contrast, the inactive analogue C2-DH Cer did not inhibit the phosphorylation of PKB.

However, stimulation of autophagy is not only dependent on the inactivation of class I PI3K signaling pathway but also on the expression of autophagy genes such beclin 1. It has been shown previously that the expression of beclin 1 is greatly reduced in breast tumors and breast carcinoma cell lines including MCF-7 cells (see Ref. 29 and Fig. 6A) and that the forced expression of beclin 1 restores autophagic capacities in MCF-7 cells (29). On the basis of these data and on the observation that TAM was reported to stimulate the expression of beclin 1 (20), we have investigated the effect of ceramide on the expression of beclin 1.
phagy is consistent with previous data showing that TAM is able to reduce the activity of glucosylceramide synthase (62). The intimate mechanism of the effect of ceramide on the expression of beclin 1 remains to be investigated. However, our results suggest that ceramide acts on beclin 1 expression at the transcriptional and/or post-transcriptional level. The promoter of beclin 1 gene has several characteristics including an SP1-binding site and affinity for E2F (64) relevant to ceramide control. Ceramide has been shown to stimulate the SP1 binding to the promoter of acid sphingomyelinase (65) and to modulate the transcriptional activity of a reporter gene driven by the E2F-binding sites containing c-myc promoter (66). Whether beclin 1 is the only autophagy gene whose expression is controlled by ceramide remains to be investigated. It is noteworthy that the expression of death associated protein kinase, a kinase involved in the autophagic response to cytokine (67), is increased by ceramide treatment (68).

Ceramide controls autophagy by interfering with two pathways encompassing PI3Ks. Class I PI3K and class III PI3K products have been reported to inhibit and stimulate autophagy, respectively. PDMP (20 μM), and PDMP (20 μM). The endogenous ceramide was quantified by the DGK assay as described under “Experimental Procedures.” The amount of ceramide (4.1 pmol of Cer/nmol of Pi) in MCF-7 cells cultured in complete medium for 4 days was set to 1. Values reported are the mean ± S.D. of four independent experiments. Lower panel, fluorescence detection of MDC-positive vacuoles in cells cultured for 4 days in complete medium supplemented with Me2SO/EtOH (1:1, v/v, control) (a), TAM (1 μM) (b), TAM (1 μM) + Myrio (100 nM) (c), and PDMP (20 μM) (d). The bar represents 10 μm. Arrowheads indicate the presence of autophagic vacuoles. The bar represents 2 μm.

FIG. 4. Ceramide is responsible for the accumulation of autophagic vacuoles in MCF-7 cells. A, upper panel, accumulation of MDC-positive vacuoles in MCF-7 cells after a 4-day treatment with MeSO/EtOH (1:1, v/v, control), TAM (1 μM), TAM (1 μM) + Myrio (100

FIG. 5. Ceramide inhibits PKB phosphorylation in MCF-7. A, cell lysates of MCF-7 cells after a 4-day treatment with MeSO/EtOH (1:1, v/v, control), TAM (1 μM), and TAM (1 μM) + Myrio (100 nM) were analyzed by Western blotting for PKB phosphorylation at Ser473 (top panel) using a phosphospecific antibody. The expression of total PKB was detected by using an anti-PKB antibody (lower panel). Densitometry was performed on the original blots and the ratio of phospho-Ser473/total PKB in control cells was set to 100%. B, MCF-7 cells were cultured for 16 h in the absence of serum. Thereafter, 10% serum was added in the absence (control) or presence of 75 μM Cer or Cer-DHCer for 1 h. Western blotting was performed as described above. Densitometry was performed on the original blots and the ratio of phospho-Ser473/total PKB in control cells was set to 100%.

The intimate mechanism of the effect of ceramide on the expression of beclin 1 remains to be investigated. However, our results suggest that ceramide acts on beclin 1 expression at the transcriptional and/or post-transcriptional level. The promoter of beclin 1 gene has several characteristics including an SP1-binding site and affinity for E2F (64) relevant to ceramide control. Ceramide has been shown to stimulate the SP1 binding to the promoter of acid sphingomyelinase (65) and to modulate the transcriptional activity of a reporter gene driven by the E2F-binding sites containing c-myc promoter (66). Whether beclin 1 is the only autophagy gene whose expression is controlled by ceramide remains to be investigated. It is noteworthy that the expression of death associated protein kinase, a kinase involved in the autophagic response to cytokine (67), is increased by ceramide treatment (68).

Ceramide controls autophagy by interfering with two pathways encompassing PI3Ks. Class I PI3K and class III PI3K products have been reported to inhibit and stimulate autophagy.
Both pathways contain proteins with tumor suppressor properties that positively regulate autophagy (PTEN and beclin 1). The tumor suppressor PTEN, which hydrolyzes the products of class I PI3K (69, 70), has a stimulatory effect on autophagy (35). The tumor suppressor beclin 1 (30, 31) forms a complex with the class III PI3K to control the formation of the autophagosome (28). Loss of function or reduced expression of beclin 1 and/or PTEN provokes a default in autophagy that is intimately associated with the progression of tumor cells (19, 20, 30, 31). Accordingly, ceramide, a lipid with tumor suppressor properties, has a stimulatory effect on autophagy. Most interesting, the level of ceramide is lower in cancer cells than in normal cells (71) suggesting that ceramide is a factor to be considered in the down-regulation of autophagy in cancer cells. However, as discussed elsewhere, cancer cells are able stimulate the autophagic pathway in response to different stimuli (19). Several studies have shown that radiation treatment of cancer cells stimulates the generation of ceramide that is followed by a ceramide-induced cell death (reviewed in Ref. 72). Most interesting, low irradiation treatment induced a protective autophagic response in breast cancer cells (73). From these results, it is tempting to speculate that ceramide can be involved in triggering an autophagic response to protect cells, whereas a more intense stimulus would induce cell death.

The metabolic origin and the membrane localization of ceramide that controls autophagy remain to be firmly established. The inhibitory effect of Myrio on the accumulation of autophagic vacuoles suggests that the de novo pool of ceramide is involved in the stimulation of autophagy. However, a role for ceramide generated by the breakdown of sphingomyelin cannot be excluded. Preliminary experiments have shown that the incubation of HT-29 cells with bacterial sphingomyelinase has no effect on autophagy (data not shown). However, from this negative result no firm conclusion can be drawn because several studies report the lack of effect of exogenously added bacterial sphingomyelinase despite the involvement of sphingomyelinases in ceramide-dependent processes (74). Recently, by targeting bacterial sphingomyelinase to different subcellular localizations, Obeid and co-workers (75) have stressed the importance of the mitochondrial pool of ceramide in triggering apoptosis. Whether or not this pool and other pools of ceramide (plasma membrane, endosome/lysosome, and endoplasmic reticulum/Golgi apparatus) are able to stimulate autophagy is an important question for future investigations to delineate the role of ceramide in controlling autophagy and apoptosis.

We have shown previously that (glyco)sphingolipids are substrates for the autophagic pathway in HT-29 cells (76). The accelerated lysosomal catabolism of (glyco)sphingolipids during autophagy increases the acidic pool of ceramide. Whether or not this lysosomal pool of ceramide could have a positive feed-
back on autophagy remains to be investigated. This possibility is attractive to explain how stimuli, depending on their duration and intensity, could shift autophagy from its cell protective function to a role in cell dismantlement because of the presence of a cell death signaling sphingomyelinase in the lysosomal compartment (77).

In conclusion, ceramide stimulates autophagy by interfering with class I PI3K signaling pathway and increasing the expression of an autophagy gene. This adds a novel function for this second messenger engaged in stress response and brings an explanation for the stimulation of autophagy in many stress situations.

Acknowledgments—We are grateful to Tamotsu Yoshimori (National Institute of Genetics, Shizuku, Japan) and Adrian Minty (Santio-Synthelabo, France) for providing us with the anti-beclin 1 antibody and Lc3, respectively. We thank Isabelle Chantret (INSERM U504) for assistance in RT-PCR.
