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Sphingosine-1-Phosphate Phosphohydrolase Regulates Endoplasmic Reticulum-to-Golgi Trafficking of Ceramide‡

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Previous studies demonstrated that sphingosine-1-phosphate (S1P) phosphohydrolase 1 (SPP-1), which is located mainly in the endoplasmic reticulum (ER), regulates sphingolipid metabolism and apoptosis (H. Le Stunff et al., J. Cell Biol. 158:1039–1049, 2002). We show here that the treatment of SPP-1-overexpressing cells with S1P, but not with dihydro-S1P, increased all ceramide species, particularly the long-chain ceramides. This was not due to inhibition of ceramide metabolism to sphingomyelin or monohexosylceramides but rather to the inhibition of ER-to-Golgi trafficking, determined with the fluorescent ceramide analog *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-D-erythro-sphingosine (DMB-Cer). Fumonisin B1, an inhibitor of ceramide synthase, prevented S1P-induced elevation of all ceramide species and corrected the defect in ER transport of DMB-Cer, readily allowing its detection in the Golgi. In contrast, ceramide accumulation had no effect on either the trafficking or the metabolism of 6-([*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino] hexanoyl)-sphingosine, which rapidly labels the Golgi even at 4°C. Protein trafficking from the ER to the Golgi, determined with vesicular stomatitis virus ts045 G protein fused to green fluorescent protein, was also inhibited in SPP-1-overexpressing cells in the presence of S1P but not in the presence of dihydro-S1P. Our results suggest that SPP-1 regulates ceramide levels in the ER and thus influences the anterograde membrane transport of both ceramide and proteins from the ER to the Golgi apparatus.

All sphingolipids contain ceramide, which consists of a sphingoid base, typically sphingosine or dihydrosphingosine, and an amide linked acyl chain. Ceramide, a bioactive molecule (39), can be deacylated to sphingosine, which in turn can be phosphorylated to sphingosine-1-phosphate (S1P), also a potent lipid mediator that regulates vital biological processes (56). S1P is the ligand of a family of specific cell surface G protein-coupled receptors, hereafter referred to as S1PRs. These receptors couple to various G proteins to regulate cell migration, angiogenesis, vascular maturation, heart development, neurite retraction, and lymphocyte trafficking and immune responses (reviewed in references 49, 53, and 56). Dihydrosphingosine-1-phosphate (dihydro-S1P), which is identical to S1P but lacks the *trans* 4,5 double bond, binds to all of the S1PRs and activates them and yet does not mimic all of the effects of S1P, especially those related to cell survival and protection against apoptosis (56). Hence, S1P might also have intracellular functions independent of S1PRs. Indeed, S1P also regulates important functions in yeast and plant cells that lack S1PRs (reviewed in references 53 and 56).

In contrast to S1P, ceramide, a central molecule in sphingolipid metabolism, has been implicated in cell growth arrest, differentiation, and apoptosis (10, 22, 39). Abundant evidence indicates that the balance between ceramide and S1P is a critical factor that determines cell fate (22, 39, 56). Whereas little is known of the intracellular targets of S1P, several direct targets of ceramide have been identified in vitro, including serine/threonine protein phosphatases (PP1 and PP2A), cathepsin D, the kinase suppressor of Ras, Raf, PKCζ, and MEKK (reviewed in reference 39). Key elements in defining the role of ceramide in cell signaling are its hydrophobic nature and its inability to spontaneously move among different subcellular membranes where the enzymes of its metabolism and its molecular targets are located. As a consequence, the biological effects of ceramide may also depend on the generation of specific signaling pools and the regulation of its intracellular trafficking within the cell (10, 39).

Previous studies suggest that S1P phosphohydrolase 1 (SPP-1), which is located mainly in the endoplasmic reticulum (ER), plays an important role in regulating the balance of the sphingolipid metabolites, ceramide and S1P (20, 26). Overexpression of Lcb3p, the yeast homologue of SPP-1, results in ceramide accumulation and, conversely, its deletion results in the accumulation of phosphorylated long chain sphingoid bases and reduced ceramide levels (33, 35, 36). Moreover, overexpression of SPP-1 in mammalian cells increased ceramide levels, particularly in the presence of exogenous S1P (26). Surprisingly, however, dihydro-S1P, which is also readily de-

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phosphorylated by SPP-1 in vitro, did not cause the elevation of ceramide levels. However, fumonisin B1 (FB1), an inhibitor of ceramide synthase, induced accumulation of sphingoid bases when cells were treated with either S1P or dihydro-S1P, suggesting that sphingosine and dihydrosphingosine produced in these cells are indeed substrates for ceramide synthase. Utilizing the (D-threo)-isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an analogue of glucosylceramide (GlcCer) and a potent inhibitor of GlcCer synthase (55), we demonstrated that it is unlikely that decreased utilization of ceramide for the formation of GlcCer was responsible for ceramide accumulation. PDMP inhibits anterograde membrane transport to the Golgi complex and from the Golgi complex to the plasma membrane (30, 50). A similar effect was observed when cells were incubated with short-chain C6-ceramide (51). SPP-1-overexpressing cells enabled us to determine whether the elevation of ceramide in the ER affects ER-to-Golgi trafficking of ceramide and whether it also regulates anterograde membrane transport.

MATERIALS AND METHODS

Materials. Dihydro-S1P, S1P, FB1, sphingosine, and PDMP were obtained from Biomol (Plymouth Meeting, PA). Serum and medium were obtained from Biofluids, Inc. (Rockville, MD). The internal standards for quantitation of the sphingolipids by electrospray ionization tandem mass spectroscopy (ESI-MS/MS) were obtained from Avanti-Polar Lipids (Alabaster, AL). *N*-(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-D-*erythro*-sphingosine (DMB-Cer), 6-([*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl)-sphingosine (NBD-Cer), and the Anti-Fade kit were from Molecular Probes (Eugene, OR). G418 was from Invitrogen (Gaithersburg, MD). [γ-^{32}P]ATP and [^{3}H]palmitic acid were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). A temperature-sensitive variant of vesicular stomatitis virus G protein (VSVG-ts045) tagged with green fluorescent protein (GFP) at the carboxy terminus was kindly provided by J. Lippincott-Schwartz.

Cell culture and transfection. Human embryonic kidney cells (HEK 293; ATCC CRL-1573) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Cells were stably transfected with vector or SPP-1 and cultured in medium containing 1 g of G418/liter as previously described (26). Lipofectamine Plus (Invitrogen) was used for other transfections as suggested by the manufacturer.

Treatments with fluorescent ceramide. Vector- or SPP-1-transfected HEK 293 cells (2×10^5 cells/well) were cultured on poly-D-lysine-coated six-well plates for lipid analyses or on glass coverslips for fluorescence microscopy. After 24 h, cells were incubated for 24 h at 37°C in DMEM in the presence of vehicle, S1P, or dihydro-S1P. Cells were then labeled with 5 μM DMB-Cer or NBD-Cer (as 1:1 complexes with fatty acid-free bovine serum albumin [BSA]) in DMEM at 4°C for 30 min as described previously (43). After three washes with DMEM containing 0.34 mg of fatty acid-free BSA/ml, cells were incubated for 30 min at 37°C in DMEM containing 0.34 mg of fatty acid-free BSA/ml.

Lipid analysis. Lipids were extracted with $CHCl_3$-methanol as described previously (43) and separated by thin-layer chromatography (TLC) using $CHCl_3$–methanol–15 mM $CaCl_2$ (60:35:8 [vol/vol/vol]) as the developing solvent. Fluorescence-labeled lipids were quantified with a Bio-Rad (Hercules, CA) FX fluorescence scanner (488-nm excitation, 560-nm emission). Values are presented as percentages of the total fluorescence recovered (the sum of DMB-Cer, DMB-SM, and DMB-GlcCer) as described previously (8).

Confocal fluorescence microscopy. Cells grown on coverslips were washed and then fixed with 1.5% paraformaldehyde solution in phosphate-buffered saline (PBS) for 10 min at room temperature. Coverslips were mounted on glass slides with an Anti-Fade kit, and images were collected a Zeiss LSM 510 laser confocal microscope (Thornwood, NY) with a ×63 objective lens. Fluorescent lipids were observed by using the settings for fluorescein isothiocyanate. For Golgi colocalization experiments, cells were transfected with the Golgi-localized fluorescent protein, DsRed-Monomer Golgi (Clontech), 48 h prior to labeling with DMB-Cer. No overlap was observed between DMB and DsRed fluorescence. Quantitative colocalization analysis was performed by using Zeiss LSM 5 version 3.5SP1.1 software, and Pearson correlation coefficients, a measure of colocalization, were derived as described previously (34). Alternatively, cells were grown on coverslips etched with a numbered grid (Bellco), labeled with DMB-Cer, and fixed, and images were collected as described above from known regions of the grid using a ×40 1.3 NA water immersion objective lens. The cells were then permeabilized with 0.5% Triton X-100 for 3 min and stained with antibodies to the Golgi protein GM130 (Stressgen) essentially as described previously (30) using Alexa 555-conjugated secondary antibody (Molecular Probes). Corresponding regions of the grid were then reimaged.

Mass spectrometry of sphingolipids and metabolites. Cells (2×10^6) were cultured in 10-cm dishes, serum starved, and incubated with vehicle, S1P, or dihydro-S1P for 24 h. Cells were then washed extensively with PBS and released by treatment with trypsin. An aliquot of cells was taken for protein determination; internal standards were added to the rest of the cells, lipids were extracted, and sphingolipid species were quantified by liquid chromatography and ESI-MS/MS as described previously (57).

Uptake of ^{32}P-labeled phosphorylated sphingoid bases. ^{32}P-labeled sphingoid bases were prepared with recombinant SphK1 as previously described (40). Cells (4×10^5) were seeded in poly-D-lysine-coated six-well plates in DMEM supplemented with 10% fetal bovine serum. The following day, cells were washed and incubated in 2 ml of DMEM containing 5 μM [^{32}P]S1P or 5 μM [^{32}P]dihydro-S1P, added as BSA complexes. At each time point, the medium was collected, and the cells were washed and then incubated with DMEM containing 0.5 mg of BSA/ml for 30 min at 37°C. The cells were then washed with PBS and scraped in 1 ml of methanol. Lipids were extracted by adding 1 ml of $CHCl_3$, 1 ml of NaCl (1 M), and 50 μl of concentrated HCl (7). Medium (2 ml) was extracted similarly by adding 2 ml of methanol, 2 ml of $CHCl_3$, and 100 μl of concentrated HCl. Lipids in the organic phases were separated by TLC on silica gel G60 with chloroform-acetone-methanol-acetic acid-water (10:4:3:2:1 [vol/vol/vol/vol/vol]) as a solvent (7), and the radioactive spots comigrating with authentic S1P and dihydro-S1P were quantified with the FX Molecular Imager. In some experiments, ^{32}P-labeled phospholipids were quantified similarly.

VSVG-GFP trafficking. Cells were transfected with VSVG-ts045 fused to GFP and cultured on poly-D-lysine-coated coverslips at 40°C. After 24 h, cells were incubated in DMEM in the presence of vehicle, S1P, or dihydro-S1P for 20 h at 40°C. The cells were then shifted to 32°C for the indicated times, fixed as described above, and imaged by confocal microscopy.

Statistical analysis. Experiments were repeated at least three times with consistent results. For each experiment, data from triplicate samples were calculated and expressed as means ± standard deviations (SD). Differences between groups were determined with paired Student *t* test; a *P* value of <0.05 was considered significant.

RESULTS

SPP-1 preferentially increases long-chain ceramide species. Previous studies indicate that SPP-1 is a highly specific sphingoid base phosphate phosphohydrolase that is localized to the ER (20, 26, 32). In vitro, both S1P and dihydro-S1P are substrates for SPP-1 (32), and the products, sphingosine and dihydrosphingosine, can be further metabolized in the ER to ceramide and dihydroceramide, respectively. We previously reported that SPP-1 expression markedly increased ceramide levels and enhanced apoptosis after treatment with S1P but not after treatment with dihydro-S1P (26). To examine in more detail the effect of SPP-1 on sphingolipid metabolism and to follow the fate of the sphingoid base after S1P and dihydro-S1P are dephosphorylated in situ by SPP-1, we utilized ESI-MS/MS. As expected, when cells were treated with S1P or dihydro-S1P, which are readily taken up, there were large increases in the intracellular levels of these phosphorylated sphingoid bases (Fig. 1A and B). In addition, there were also large increases in the corresponding free sphingoid bases (Fig. 1C and D). The levels of both S1P and dihydro-S1P were somewhat lower in SPP-1-overexpressing cells (Fig. 1A and B) with concomitant increases in sphingosine (Fig. 1C) and dihydrosphingosine (Fig. 1D). Surprisingly, after 24 h of treatment, cellular levels of dihydro-S1P were much higher than S1P (Fig. 1A and B). To determine whether this was a result of differences in uptake or

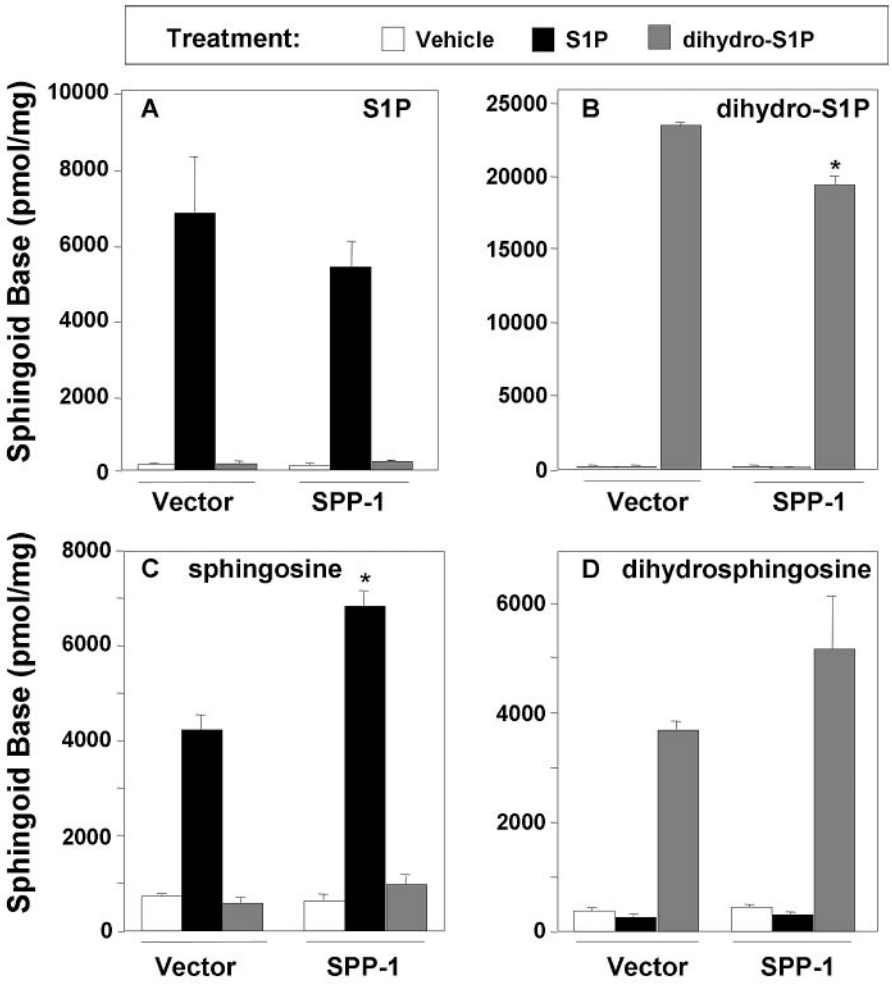

FIG. 1. ESI-MS/MS measurements of uptake and cellular levels of S1P and dihydro-S1P. HEK 293 cells expressing vector or SPP-1 were incubated in serum-free media with vehicle (□), 5 μM S1P (■), or 5 μM dihydro-S1P (▨) for 24 h as described in Materials and Methods. Lipids were extracted and phosphorylated (A and B) and free sphingoid bases (C and D) were analyzed by ESI-MS/MS. The data are averages of triplicate determinations and are expressed as pmol lipid per mg of protein. ❋, $P < 0.05$ compared to vector.

metabolism, we followed the fate of ^{32}P-labeled S1P and dihydro-S1P. No significant differences in the rate of uptake of these phosphorylated sphingoid bases were observed in the vector or in SPP-1-overexpressing cells, and by 12 h, less than 20% of the radioactive lipids remained in the medium (Fig. 2A and data not shown). Concomitantly, there was a gradual increase in ^{32}P-labeled S1P and dihydro-S1P in the cells. However, in agreement with mass measurements by mass spectrometry, cellular dihydro-S1P accumulated to a much greater extent than S1P (Fig. 2B), and levels were reduced by the overexpression of SPP-1. Interestingly, when the lipids were extracted after 24 h and resolved by TLC, three other bands appeared with higher R_f values than the phosphorylated sphingoid bases that comigrated with phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin (SM) standards (Fig. 2B, inset). These phospholipids were detected when vector cells were treated with S1P for 24 h and were less pronounced after dihydro-S1P treatment. It has been shown in *Drosophila melanogaster* and in mammalian cells that ethanolamine phosphate derived from the degradation of S1P by S1P lyase is utilized for the synthesis of PE (4, 6), and this conserves the [^{32}P]phosphoethanolamine intact that is derived from [^{32}P]S1P. The expression of SPP-1 not only decreased cellular ^{32}P-labeled sphingoid bases but also suppressed their conversion into these other phospholipids (Fig. 2B, inset), since it removes the labeled phosphate and decreases the [^{32}P]S1P available for degradation by the lyase.

ESI-MS/MS can also be used to simultaneously determine ceramide species with different degrees of saturation and fatty acids, and sphingolipids derived from ceramide, including SM and monohexosylceramides, and their various acyl chain length species (57). Treatment of SPP-1-overexpressing cells with S1P markedly increased the mass of all ceramide acyl chain species, although the very-long-chain C24 species was increased to a lesser extent (Fig. 3A). Importantly, although sphingosine was increased in vector-transfected cells treated with S1P (Fig. 1C), there was only a small increase in ceramide compared to the dramatic increase in SPP-1-expressing cells (Fig. 3A). It is also evident from comparisons of the subspecies distributions (see Fig. S1A in the supplemental material) that treatment with

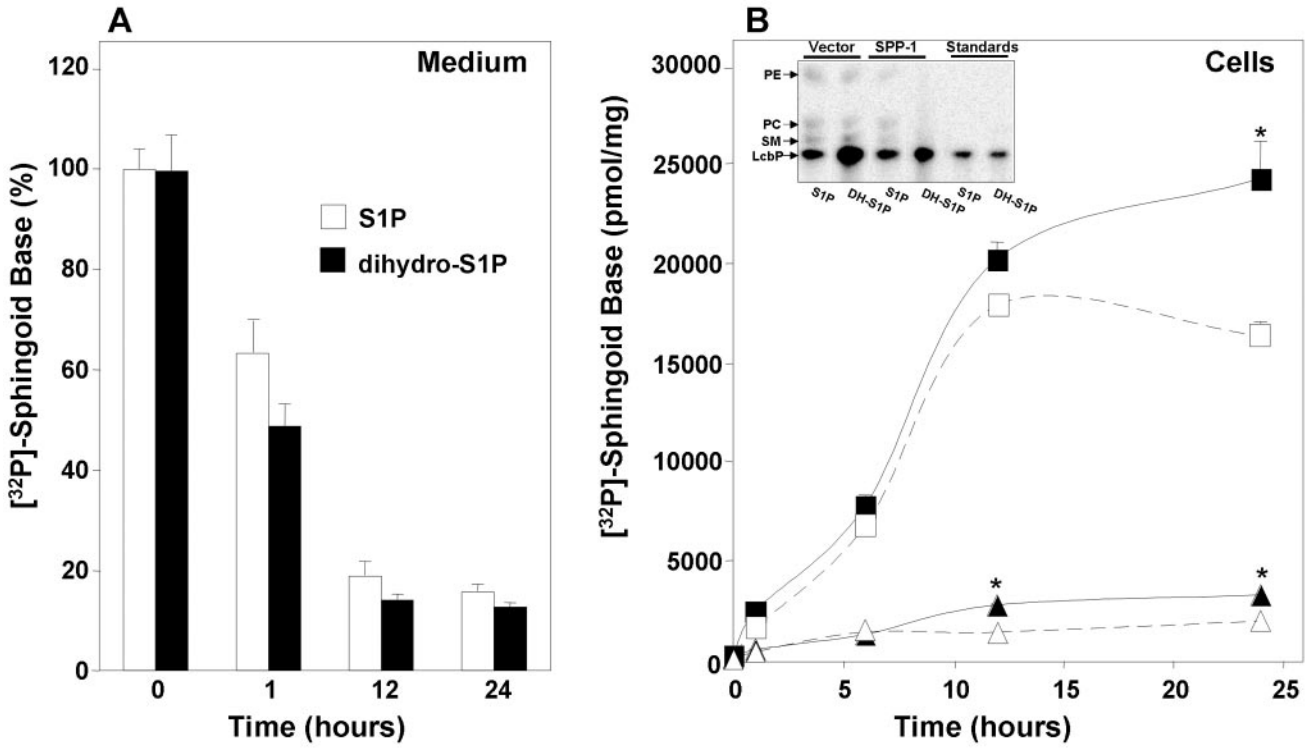

FIG. 2. Uptake and metabolism of dihydro-S1P and S1P. (A) HEK 293 cells overexpressing SPP-1 were incubated in serum-free media with 5 μM [^{32}P]S1P (□) or [^{32}P]dihydro-S1P (■) for the indicated times. Lipids were extracted and phosphorylated sphingoid bases separated by TLC as described in Materials and Methods. The data are expressed as the percentage of ^{32}P-labeled sphingoid base remaining in the medium. (B) HEK 293 cells expressing vector (solid lines) or SPP-1 (dashed lines) were incubated in serum-free media with [^{32}P]S1P (triangles) or [^{32}P]dihydro-S1P (squares) for the indicated times. Lipids were extracted from cells and analyzed by TLC. The data are expressed as pmol of phosphorylated sphingoid base per mg of protein and are means ± the SD of triplicate determinations. ✻, $P < 0.05$ compared to vector. (Inset) Cell-associated phospholipids from duplicate cultures after 24 h of treatment with [^{32}P]S1P or [^{32}P]dihydro-S1P (DH-S1P) were extracted and analyzed by TLC. Bands are labeled based on comigration with authentic unlabeled standards. LcbP, sphingoid base phosphates. A representative autoradiograph of a TLC plate is shown.

S1P increased the proportion of long-chain ceramides, particularly C16 and C18, in SPP-1-expressing cells. However, treatment with dihydro-S1P did not significantly alter any ceramide species. Of note, dihydro-S1P increased C16-dihydroceramide (Fig. 3; see Fig. S1A in the supplemental material). The increased ceramide levels caused by treatment of SPP-1 expressing cells with S1P were also reflected in the increased mass of SM (see Fig. S1B in the supplemental material) and monohexosylceramides (see Fig. S1C in the supplemental material). Interestingly, only the C16 and C18 species of these sphingolipids were elevated, with no significant changes detected in very-long-chain species (see Fig. S1B and C in the supplemental material).

SPP-1 decreases transport of ceramide from the ER to the Golgi. Ceramide is synthesized on the ER, and it must travel to the Golgi to be metabolized to SM and GlcCer (12). To examine whether aberrant trafficking of ceramide from the ER to the Golgi is involved in its accumulation in SPP-1 cells, we utilized a fluorescent ceramide analog, DMB-Cer, which has been widely used to monitor ceramide trafficking in intact cells (8, 43, 51). Since the critical micellar concentration of DMB-Cer is higher than that of natural ceramide (43), externally supplied DMB-Cer is readily incorporated into the plasma membrane in living cells even at 4°C and, after movement across the plasma membrane, fluorescently labels intracellular membranes, particularly the ER (8, 43, 51). DMB-Cer is then efficiently transported from the ER to the Golgi, where it can be metabolized to DMB-SM (8, 43, 51). When vector- and SPP-1-transfected HEK 293 cells were loaded with DMB-Cer at 4°C, the fluorescence was observed in the nuclear envelope and in a diffuse, reticular ER pattern. Upon warming the cells to 37°C for 30 min, the DMB fluorescence exited the ER and accumulated in juxtanuclear regions corresponding to the Golgi, in agreement with many previous studies (5, 8, 15, 24, 43, 45, 50–52), as indicated by colocalization with the fluorescently tagged Golgi marker, DsRed-Monomer Golgi (Fig. 4A). There were no obvious effects of overexpression of SPP-1 on the transport of DMB-Cer (Fig. 4B). However, while pretreatment with S1P or dihydro-S1P had no effect on the transport of DMB-Cer to the Golgi in vector-transfected cells, the DMB fluorescence in SPP-1-expressing cells treated with S1P, but not with dihydro-S1P, did not markedly change after 30 min at 37°C (Fig. 4B and C), remaining mainly localized to the nuclear envelope and in a diffuse reticular ER pattern (Fig. 4B). In agreement, the Golgi matrix polypeptide GM130 (54) colocalized with DMB-Cer in SPP-1-expressing cells treated with vehicle, but not when treated with S1P, even though these cells had normal Golgi staining (Fig. 4D).

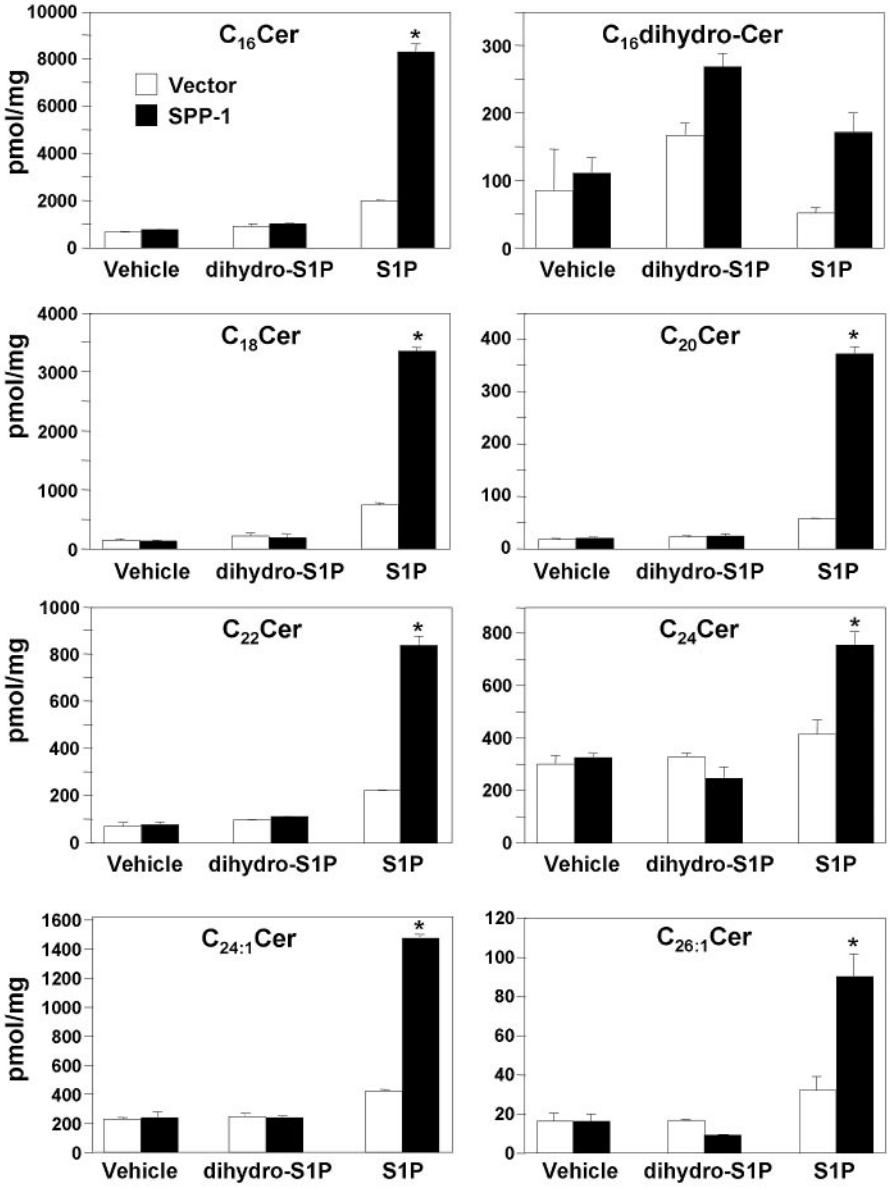

FIG. 3. S1P, but not dihydro-S1P, markedly increases specific ceramide species. HEK 293 cells expressing vector (□) or SPP-1 (■) were incubated in serum-free media with vehicle, S1P, or dihydro-S1P for 24 h. Lipids were extracted, and ceramide species were determined by ESI-MS/MS. The data are averages of triplicate determinations and are expressed as pmol of lipid per mg of protein. ✻, $P < 0.05$ compared to vector.

Because transport of DMB-Cer from the ER to the Golgi apparatus, the site of SM synthesis, in SPP-1-expressing cells treated with S1P was markedly reduced, it was of interest to examine whether its conversion to SM was also affected. TLC analysis revealed that DMB-Cer was converted at 37°C to DMB-SM in a time-dependent manner (Fig. 5), in agreement with other studies (8, 43). There were no significant differences in formation of DMB-SM in vector cells treated with S1P or dihydro-S1P (Fig. 5A). However, in SPP-1-overexpressing cells, S1P caused a 50% reduction in formation of DMB-SM (Fig. 5). To further confirm that this reduction is due to decreased translocation of DMB-Cer from the ER to the Golgi apparatus where SM synthase exists, cells were treated with brefeldin A (BFA), which induces fusion of the Golgi membrane with the ER (2). The rate of conversion of DMB-Cer to DMB-SM in SPP-1-expressing cells was restored to control levels by BFA (Fig. 5B), supporting the notion that ceramide accumulation in these cells is due to its decreased translocation from the ER to the Golgi apparatus.

SPP-1 does not alter SM or GlcCer synthase activity. To determine whether inhibition of SM synthase or GlcCer synthase might also contribute to the increase in ceramide in SPP-1-expressing cells, we utilized NBD-Cer, a fluorescently labeled ceramide derivative that is an efficient substrate for both GlcCer and SM synthases (11) and rapidly targets the Golgi in an ATP- and ceramide transporter (CERT)-indepen-

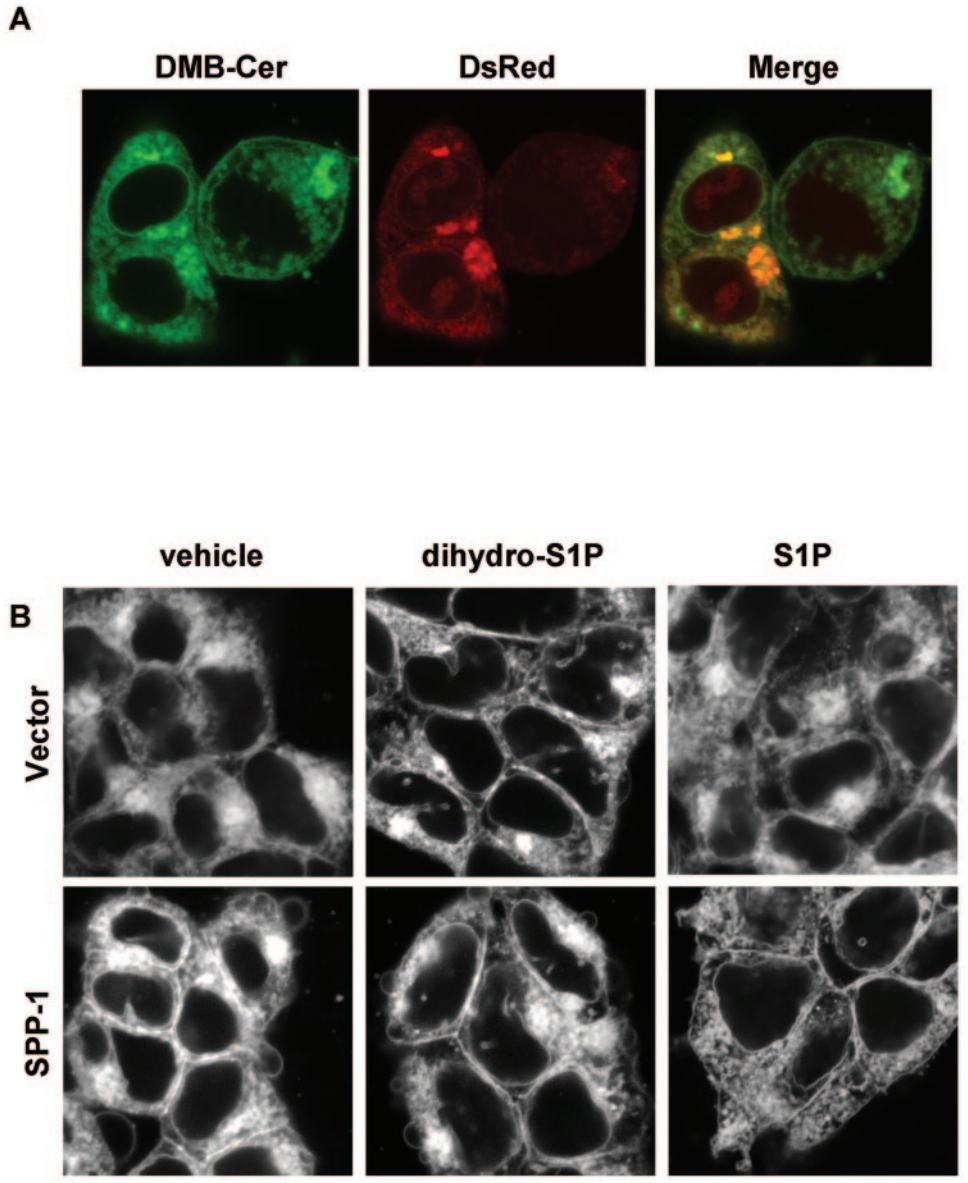

FIG. 4. S1P, but not dihydro-S1P, alters ER-to-Golgi trafficking of DMB-Cer in SPP-1-expressing cells. (A) HEK 293 cells overexpressing SPP-1 were transfected with DsRed-Monomer Golgi and incubated with DMB-Cer (5 μM) for 30 min at 4°C. After being washed, the cells were incubated for 30 min at 37°C in DMEM containing 0.34 mg of BSA/ml and fixed, and localization of fluorescence was then determined by confocal microscopy. The right panels show the superimposed merged pictures, with yellow indicating colocalization. The Pearson correlation coefficients for colocalization of DMB fluorescence with that of DsRed without and with S1P treatment were 0.91 ± 0.04 and 0.26 ± 0.1, indicating significant overlap and low overlap, respectively. (B) Vector- and SPP-1-expressing HEK 293 cells were preincubated in DMEM containing vehicle, S1P (1.5 μM), or dihydro-S1P (1.5 μM) for 24 h at 37°C and then incubated with DMB-Cer (5 μM) for 30 min at 4°C. After being washed, the cells were incubated for 30 min at 37°C in DMEM containing 0.34 mg of BSA/ml, fixed, and then examined by confocal fluorescence microscopy. (C) Duplicate experiments were quantified by scoring the number of cells that showed typical Golgi staining after pulse-labeling with DMB-Cer. A total of 90% ± 6% (n = 75) of vector- and SPP-1-transfected cells showed DMB-Cer targeting to the Golgi, even after treatment with dihydro-S1P, compared to less than 29% ± 5% (n = 75) of cells transfected with SPP-1 and treated with S1P for 30 min. (D) SPP-1-expressing cells grown on gridded coverslips were treated for 24 h without (control) or with 1.5 μM S1P, labeled with DMB-Cer as described in panel B, washed, and then warmed to 37°C for 30 min. The cells were then fixed, and DMB fluorescence images (green) were collected from known regions of the grid by confocal microscopy. The cells were then permeabilized, stained with antibodies to the Golgi protein GM130 (red), and reimaged. The right panels show the superimposed merged pictures, with yellow indicating colocalization.

dent manner (8, 25). In agreement with previous studies (8, 25, 44, 52), NBD-Cer localized to the nuclear envelope, as well as to a brightly stained juxtanuclear compartment indicative of the Golgi (Fig. 6A). However, in contrast to DMB-Cer (Fig. 4B and C), redistribution of NBD-Cer to the Golgi region was not affected by pretreatment of SPP-1 overexpressing cells with S1P (Fig. 6A); essentially 100% of the cells were Golgi positive, irrespective of treatment. Moreover, SPP-1 expression

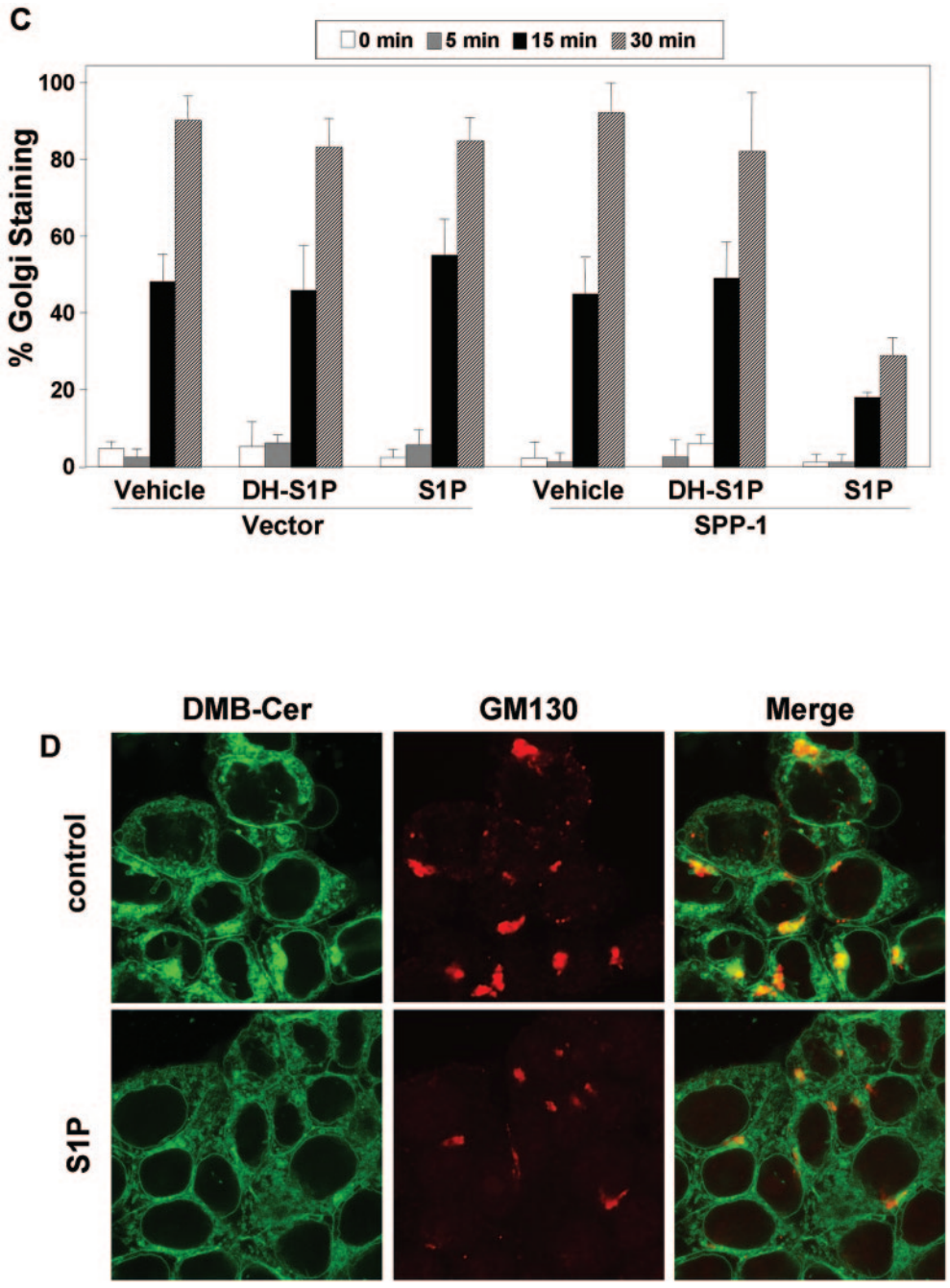

FIG. 4—*Continued.*

had no effect on the conversion of NBD-Cer to NBD-SM or NBD-GlcCer in the absence or presence of either S1P or dihydro-S1P (Fig. 6B). Taken together, these results indicate that ceramide accumulation in SPP-1-expressing cells treated with S1P is mainly due to a reduction in its rate of trafficking from the ER to the Golgi rather than to the inhibition of SM or GlcCer synthases.

To further substantiate that the elevation of ceramide in the ER is responsible for the reduction of its trafficking to the Golgi, we used FB1, an inhibitor of acyl-coenzyme A (CoA)-dependent dihydroceramide/ceramide synthase. In agreement with previous studies (26), FB1 prevented S1P-induced elevation of all ceramide species in SPP-1-expressing cells (Fig. 7A). Moreover, while DMB-Cer fluorescence remained mainly localized to the ER after 30 min at 37°C in SPP-1-overexpressing cells treated with S1P, FB1 corrected the defect in ER transport, and DMB-Cer fluorescence was readily detected in the Golgi (Fig. 7B and C). Moreover, FB1 restored the rate of conversion of DMB-Cer to DMB-SM in SPP-1-expressing cells treated with S1P (Fig. 7D).

Effect of ceramide accumulation on protein trafficking. Previous studies have suggested that the transport of proteins and

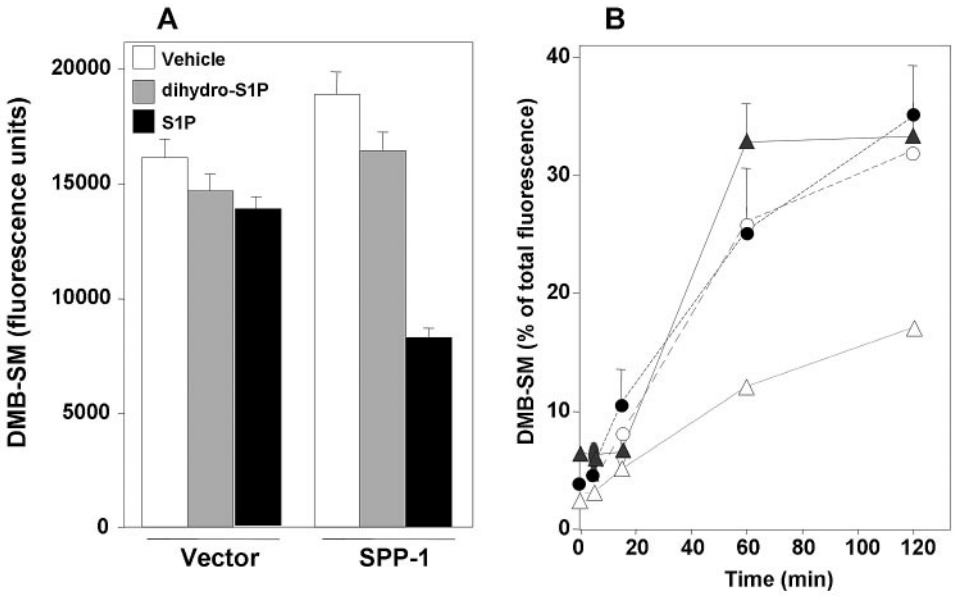

FIG. 5. Metabolism of DMB-Cer in SPP-1-expressing cells. (A) Lipids were extracted from Vector- and SPP-1-expressing HEK 293 cells treated as described in Fig. 5B and separated by TLC. DMB-SM was quantified as described in Materials and Methods. The data are means ± the SD. (B) Vector- and SPP-1-expressing cells were preincubated without or with 1 μg of BFA/ml for 20 min at 37°C before being labeled with 5 μM DMB-Cer for 30 min at 4°C. After being washed, the cells were further incubated in medium containing 0.34 mg of BSA/ml without or with 1 μg of BFA/ml for the indicated times at 37°C. Cellular lipids were extracted and DMB-SM quantified. Symbols: ○, vector cells treated with S1P; ●, SPP-1 cells treated with vehicle; △, SPP-1 cells treated with S1P; ▲, SPP-1 cells treated with BFA plus S1P.

sphingolipids through the secretory pathway may be coupled to sphingolipid synthesis and ceramide accumulation (30, 50, 51). To examine whether ceramide accumulation in SPP-1-overexpressing cells also affects protein trafficking, we used the well-studied GFP-tagged temperature-sensitive protein VSVG-ts045, which misfolds and is retained in the ER at 40°C (23, 46). VSVG-ts045 rapidly folds and exits the ER when the temperature is decreased to 32°C (23). Because the GFP tag does not alter protein transport or function, it is a convenient tool to track VSVG-ts045 as it moves through the secretory pathway (28). In agreement with previous studies, export of VSVG-ts045 to the Golgi was rapid and nearly complete within 5 min upon shifting vector transfected HEK 293 cells to 32°C, in the absence or presence of S1P (Fig. 8A), as revealed by confocal microscopy. However, treatment of SPP-1-expressing cells with S1P, but not with dihydro-S1P, caused a significant delay in the appearance of VSVG-ts045 in the Golgi, with a $t_{1/2}$ of around 15 min (Fig. 8B and C). These results suggest that SPP-1-expressing cells treated with S1P also have a defect in the ER-to-Golgi transport of proteins that is linked to the increased ceramide levels.

DISCUSSION

Trafficking of secretory proteins in eukaryotic cells is mediated by vesicular transport which loads a desired set of proteins and delivers them to specific compartments. In contrast, many types of lipids, including ceramide, that are synthesized in the ER, are sorted to other membranes and organelles also by nonvesicular mechanisms. Ceramide biosynthesis at the cytoplasmic face of the ER is initiated by the condensation of L-serine with palmitoyl CoA, catalyzed by serine palmitoyltransferase (12, 13, 37). In two rapid reactions, the product, 3-ketosphinganine, is reduced to dihydrosphingosine and subsequently acylated by (dihydro)ceramide synthases (the LASS family) to form dihydroceramide (reviewed in reference 12). Dihydroceramide is then converted to ceramide by desaturases (58). Ceramide and dihydroceramide can be translocated from the ER to the Golgi apparatus by vesicular trafficking but also mainly by nonvesicular transport mediated by CERT (15). It is then converted to SM by phosphatidylcholine-ceramide cholinephosphotransferases (SMS1) on the lumenal side of the Golgi. SM can also be formed at the plasma membrane by SMS2 (18). Ceramide is also translocated by mechanisms that are not well characterized to the cytosolic leaflet of the Golgi apparatus, where it is converted to GlcCer (9, 59) and, after translocation into the lumen, GlcCer is further metabolized to more complex glycosphingolipids. It is still unclear why GlcCer is synthesized on the cytosolic surface, whereas all other glycosphingolipids are synthesized on the lumenal surfaces of the ER or Golgi.

Previous studies with yeast and mammalian cells suggested that S1P phosphohydrolases regulate ceramide levels in the ER by a mechanism that is not well understood (reviewed in reference 27). Indeed, we observed that the treatment of cells overexpressing SPP-1 with S1P induced large increases in the mass of C16 and C18 ceramides but also significantly increased all of the more minor chain length ceramide species. Surprisingly, however, although most of the exogenous S1P that was taken up by the cells was degraded by SPP-1-independent pathways, it appears that the portion that was degraded by SPP-1 was mainly reutilized for the synthesis of ceramides and subsequently converted to C16 and C18 SM and monohexosylceramides. It is thus possible that sphingosine produced in

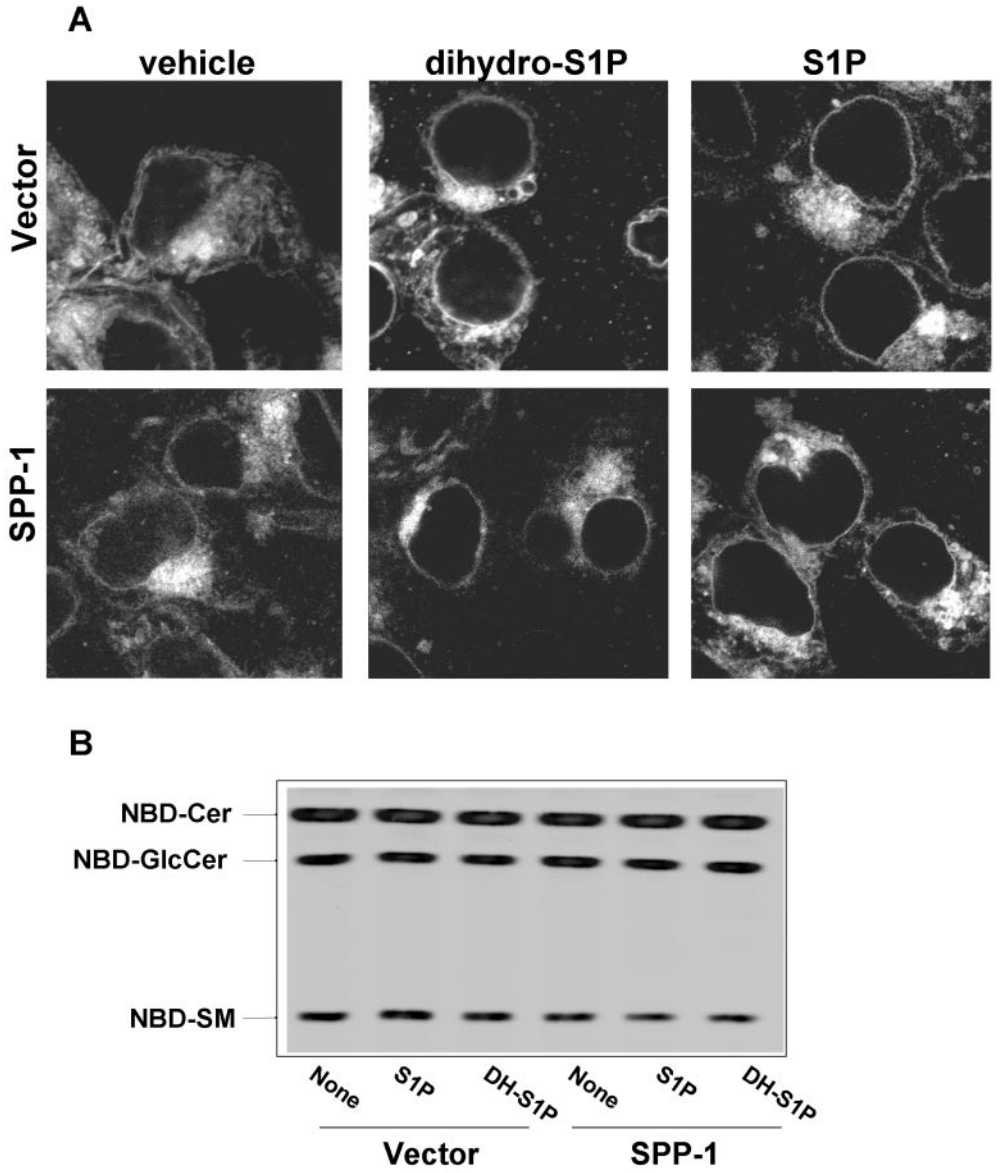

FIG. 6. Lack of effect of S1P and dihydro-S1P on intracellular trafficking and metabolism of NBD-Cer. (A) Vector- and SPP-1-expressing HEK 293 cells were preincubated in DMEM containing vehicle, S1P (1.5 μM), or dihydro-S1P (1.5 μM) for 24 h at 37°C and then incubated with NBD-Cer (5 μM) for 30 min at 4°C. After being washed, the cells were incubated for 30 min at 37°C in DMEM containing 0.34 mg of BSA/ml and then examined by confocal fluorescence microscopy. (B) Lipids were extracted from duplicate cultures and separated by TLC.

the ER by dephosphorylation of S1P is positioned to be converted to ceramide by a subset of acyl-CoA-specific LASS family ceramide synthases that are localized to the ER (48, 60). LASS1 and LASS4 expression preferentially increase C18 ceramide, LASS2 increases mainly C16 and C24:1 ceramide, and LASS5 and LASS6 produce C16 ceramide (38, 48, 60). Moreover, dihydrosphingosine, rather than sphingosine, was the preferred substrate for LASS5, whereas no preference was seen with LASS4. It is still not clear whether the formation of ceramide in the ER depends directly on the availability of its substrate sphingosine or whether sphingosine activates specific ceramide synthase(s). It is also possible that S1P functions at the ER to downregulate de novo and/or salvage pathways of sphingolipid synthesis (31) and, when degraded, these mechanisms are not operational. The molecular mechanisms by which this is accomplished remain to be elucidated, although we suggest that SPP-1 functions as a conduit for salvaged sphingoid bases to be reutilized.

Our results suggest that SPP-1 expression, in addition to altering the level of ceramide, may also regulate the availability of S1P for irreversible degradation by S1P lyase. It has been shown in *Drosophila* that phosphoethanolamine derived from S1P degradation by the lyase is incorporated into PE, which in turn controls the release of sterol regulatory element-binding protein from *Drosophila* cell membranes, exerting feedback control on the synthesis of fatty acids and phospholipids (6). Similarly, our results suggest that in HEK 293 cells, S1P can slowly be reutilized to form PE, as has been observed for other

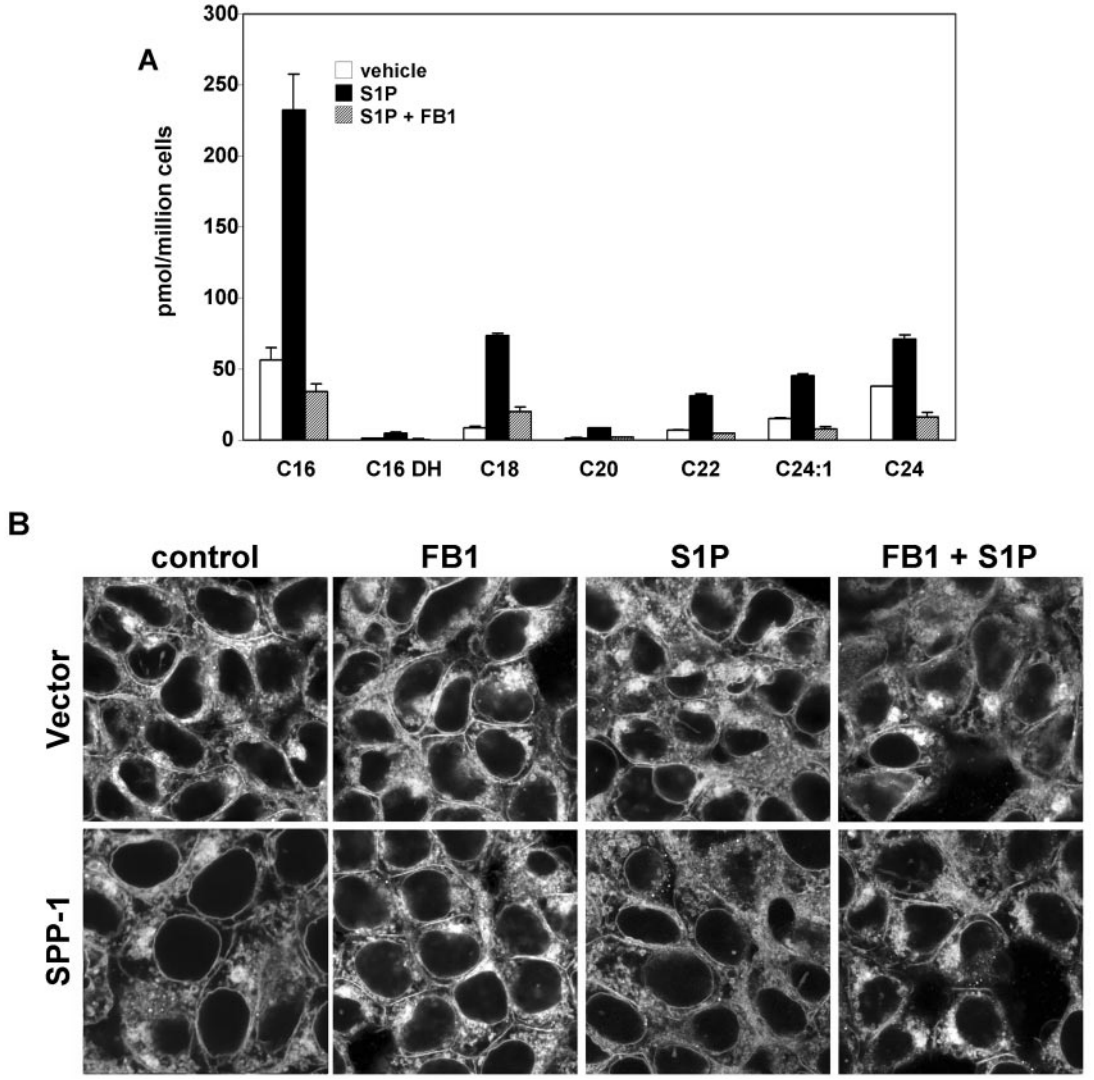

FIG. 7. Effects of FB1 on intracellular ceramide levels and ER-to-Golgi trafficking of DMB-Cer. (A) Vector- and SPP-1-transfected HEK 293 cells were incubated for 24 h without or with 1.5 μM S1P in the absence or presence of 25 μM FB1. Ceramide content was determined by mass spectrometry as described in Materials and Methods. The data are expressed as pmol per 10^6 cells and are means ± the SD of triplicate determinations. (B and C) Vector- and SPP-1-transfected HEK 293 cells were incubated for 24 h without or with 1.5 μM S1P in the absence or presence of 25 μM FB1. Cells were then incubated with DMB-Cer (5 μM) for 30 min at 4°C. After being washed, the cells were incubated for 30 min (B) or for the indicated times (C) at 37°C in DMEM containing 0.34 mg of BSA/ml and then examined by confocal fluorescence microscopy. For panel C, duplicate experiments were quantified by scoring the number of cells that showed typical Golgi staining after pulse-labeling with DMB-Cer. (D) Vector (circles)- and SPP-1 (triangles)-transfected cells were preincubated with 1.5 μM S1P in the absence (open symbols) or presence (filled symbols) of 25 μM FB1 for 24 h at 37°C and then labeled with 5 μM DMB-Cer for 30 min at 4°C. After being washed, cells were further incubated in medium containing 0.34 mg of BSA/ml for the indicated times at 37°C. Cellular lipids were extracted, separated by TLC and DMB-SM quantified. Symbols: ○, vector cells treated with S1P; △, SPP-1 cells treated with S1P; ▲, vector cells treated with S1P plus FB1; ▲, SPP-1 cells treated with S1P plus FB1.

mammalian cells (4). PE can be trimethylated to form PC, which in turn provides the head group for SM synthesis. Intriguingly, there was less radioactivity incorporated into phospholipids which comigrated with PE, PC, and SM, from [^{32}P]dihydro-S1P compared to [^{32}P]S1P, which correlates with reduced degradation of [^{32}P]dihydro-S1P. Because both S1P lyase and SPP-1 reside in the ER, it is conceivable that SPP-1 activity determines the level of S1P available as a substrate for S1P lyase, controlling the production of a specific pool of PE. It has been suggested that in yeast the active site of the SPP-1 homologue Lcb3p faces the lumen side of the ER (21), while that of the lyase faces the cytosol (19). If this topology applies to mammalian SPP-1, it implies that phosphorylated sphingoid bases must traverse the ER membrane to reach the active site of SPP-1. Therefore, our results suggest that either dihydro-S1P does not reach the ER to the same extent as S1P or that it cannot traverse the ER membrane to be degraded by SPP-1. Moreover, in contrast to S1P, much of the exogenous dihydro-S1P taken up by HEK 293 cells was not degraded by either SPP-1-dependent or -independent pathways. Of note, dihydro-

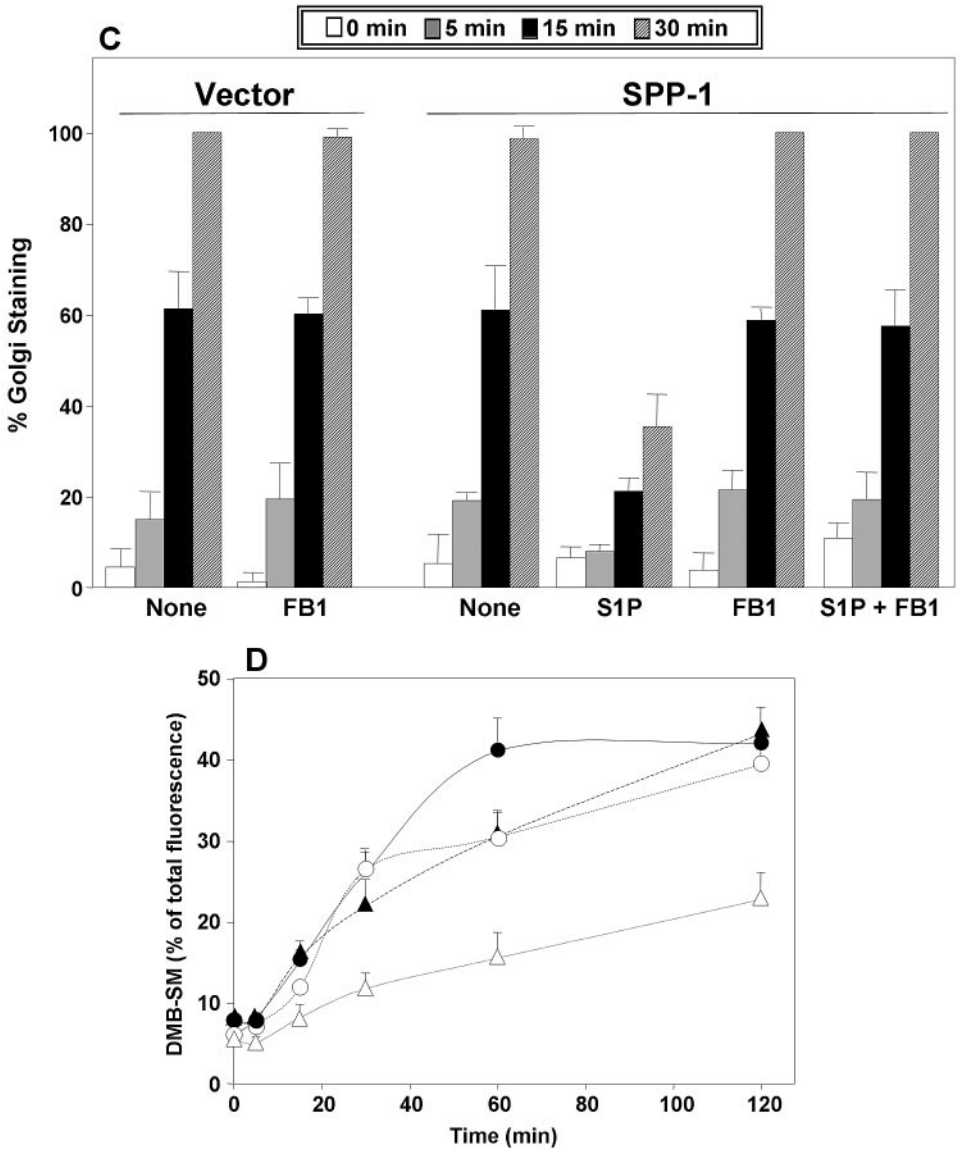

FIG. 7—*Continued.*

S1P treatment increased only the C16 dihydroceramide species in SPP-1-expressing cells, whereas treatment with S1P caused increases in all ceramide species. This suggests that dihydro-S1P or one of its metabolites, such as dihydroceramide, may feedback to inhibit de novo ceramide biosynthesis or that dihydro-S1P may not be effectively transported to the ER, where it is expected to be salvaged by SPP-1 back to ceramide. Another interesting observation noted here is that dihydro-S1P is indeed taken up and accumulated to a much greater extent than S1P. Therefore, its inability to mimic the survival effects of intracellular S1P (41, 42) suggests that dihydro-S1P is not recognized by the intracellular targets of S1P or may be sequestered away from them.

Redistribution of DMB-Cer from the ER to the Golgi is blocked in cells lacking CERT, and it has been suggested that DMB-Cer is a good probe for CERT-mediated pathways of ceramide trafficking in cells (15, 25). In the present study, we examined the consequences of the elevation of ceramide in the ER on its ability to traffic between the ER and Golgi. Redistribution of intracellular DMB fluorescence from intracellular membranes to the Golgi apparatus was slower when ceramide was accumulated at the ER of SPP-1-overexpressing cells, and there was a corresponding decrease in its conversion to DMB-SM. Conversely, inhibiting ceramide accumulation with FB1 restored trafficking of DMB-Cer from the ER to the Golgi. In contrast, ceramide accumulation did not impair the ER-to-Golgi redistribution of NBD-Cer, which undergoes spontaneous intramembrane transfer much faster than DMB-Cer and is able to move to the Golgi in an ATP- and CERT-independent manner (8, 15). This result also suggests that the Golgi ultrastructure was not altered in SPP-1 cells, a finding consistent with previous studies that reported little perturbation of the Golgi under conditions of ceramide accumulation (30, 50), whereas another study suggests that sphingosine, generated

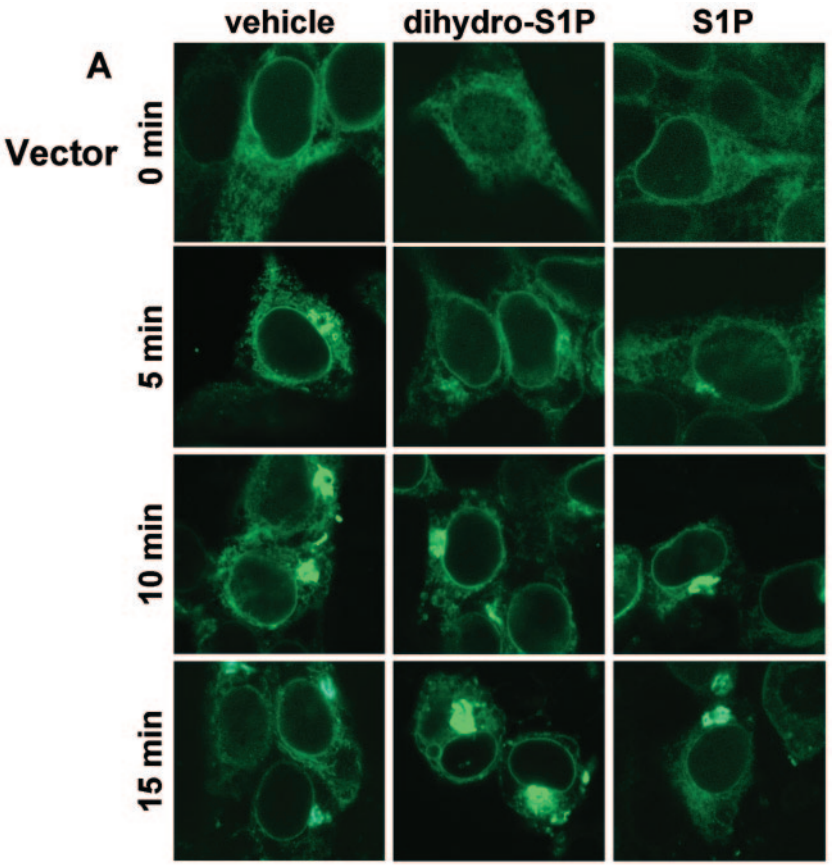

FIG. 8. S1P, but not dihydroS1P, alters the intracellular trafficking of VSVG-ts045 protein in SPP-1-overexpressing cells. HEK 293 cells stably expressing vector (A) or SPP-1 (B) were transfected with VSVG-ts045 and incubated in DMEM in the presence of vehicle, 1.5 μM S1P, or 1.5 μM dihydro-S1P for 20 h at 40°C, shifted to 32°C for the indicated times, and examined by confocal microscopy. (C) The percentage of cells with distinct Golgi staining was determined. The data are means ± the SD.

from the hydrolysis of ceramide, causes Golgi fragmentation (17). Moreover, this suggests that the traffic of ceramide from the ER to the Golgi is a rate-limiting step in the synthesis of sphingolipids.

Vesicles not only are important for lipid trafficking, they also play a central role in the directed movement of proteins between intracellular compartments. Integral membrane, lumenal, and secretory proteins are synthesized in the ER and trafficked via vesicles through compartments of the secretory pathway. It has been suggested that the glycerol phospholipid metabolite diacylglycerol is essential for the recruitment of a vesicle biogenesis factor, protein kinase D, a cytosolic serine-threonine kinase that binds to the trans-Golgi network and regulates the fission of transport carriers specifically destined to the cell surface (1). Interestingly, it was shown that in cells pretreated with FB1 (which also decreased diacylglycerol) for 24 h, the transport of VSV-G protein from the Golgi to the plasma membrane was delayed (1).

Previous studies indicated that C6 ceramide decreased the rate of anterograde (52) and retrograde (3) vesicular traffic. In agreement with this, the accumulation of endogenous ceramide induced by the inhibition of its conversion to sphingolipids also slowed anterograde traffic (30, 50). However, cells expressing a mutated CERT, which results in a defect in the transport of ceramide from the ER to the Golgi for SM synthesis, have normal ER-to-Golgi trafficking of proteins (8). Interestingly, in the present study, we found that accumulation of ceramide at the ER due to dephosphorylation of S1P by SPP-1 also reduced the rate of transport of VSVG from the ER to the Golgi, suggesting that SPP-1 may also play an important role in vesicular transport. Our results indicate that ceramide produced in the sphingolipid salvage pathway can also regulate anterograde vesicular traffic. It is tempting to speculate that ceramide generated by the de novo pathway and that formed in the salvage (reutilization) pathway may have different distributions within ER subcompartments. Alternatively, ceramide produced from these two pathways may differ in acyl chain lengths, leading to the formation of microdomains with distinct functions. In this regard, the treatment of SPP-1-overexpressing cells with S1P increased the proportion of long-chain ceramides, particularly the C16 and C18 species, compared to very-long-chain ceramides, which could alter the biophysical properties of the bilayer (16). In this regard, a genome-wide screen for proteins involved in cargo sorting and vesicle formation in yeast suggested that the fatty acid and long-chain base in ceramide are involved in the proper and efficient delivery of proteins to the cell surface (47). Intriguingly, recent studies suggest that a family of *trans*-Golgi net-

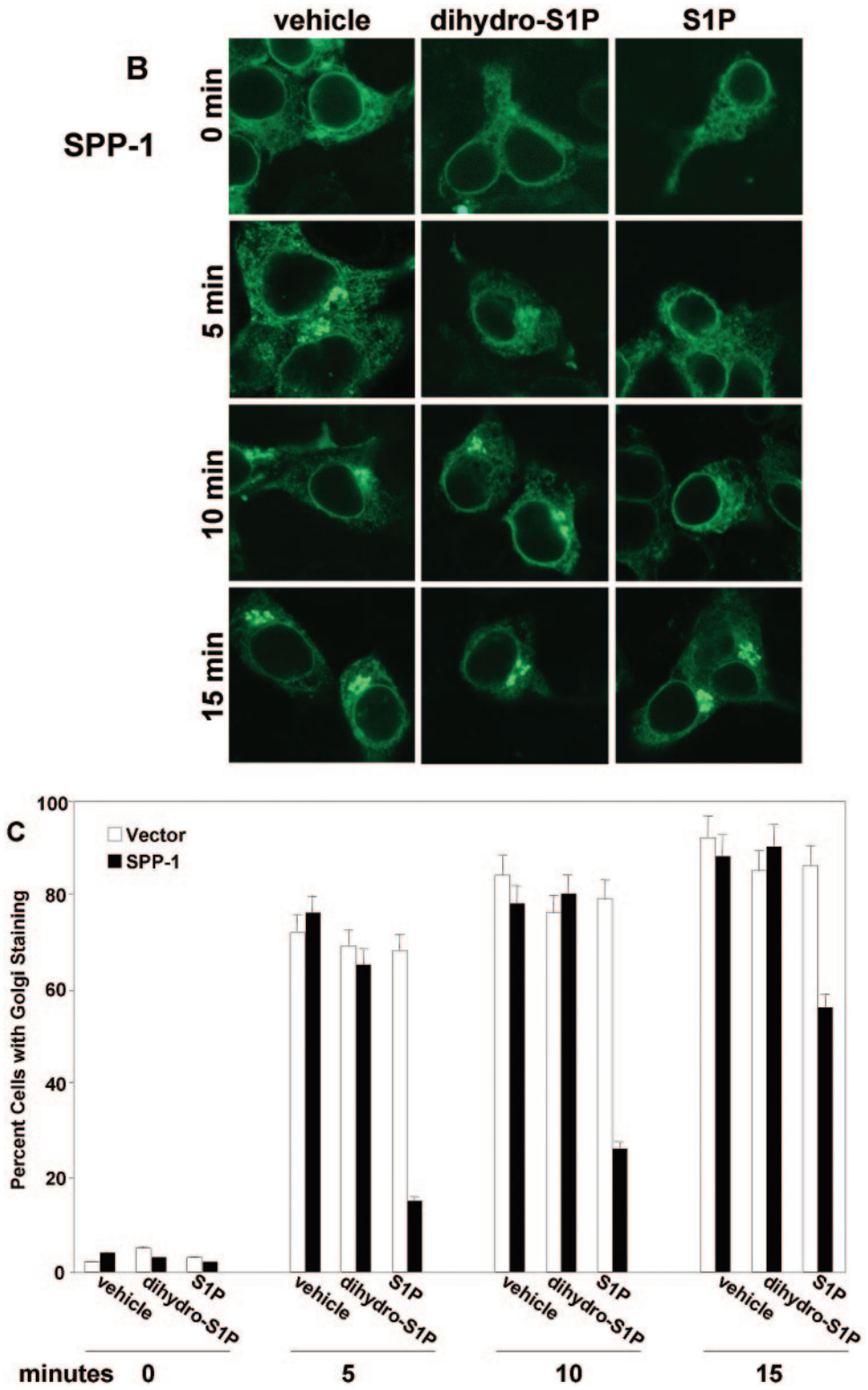

FIG. 8—*Continued.*

work (TGN)-associated proteins called FAPPs (the four-phosphate adaptor proteins), control Golgi-to-cell-surface membrane traffic by binding to $PtdIns_4P$ and a small GTPase ADP-ribosylation factor through their plekstrin homology domains. Although the precise mechanism of action of the FAPPs at the TGN is largely unknown, GPBP and FAPP2 have putative ceramide or GlcCer transfer and/or sphingolipid-binding domains that may selectively target them to separate

TGN domains (14). Thus, it has been proposed that short-range lipid transfer between the ER and the TGN may exert a specific and direct role in carrier biogenesis in addition to feeding the metabolic pathways for phospholipid biosynthesis (29).

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