Different modes of internalization of apoptotic alkyl-lysophospholipid and cell-rescuing lysophosphatidylcholine

Arnold H. VAN DER LUIT*, Marianne BUDDE*, Marcel VERHEIJ† and Wim J. VAN BLITTERSWIJK*¹

*Division of Cellular Biochemistry and †Department of Radiotherapy, The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands

The synthetic alkyl-lysophospholipid (ALP), Et-18-OCH₃ (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), can induce apoptosis in tumour cells. Unlike conventional chemotherapeutic drugs, ALP acts at the cell-membrane level. We have reported previously that ALP is internalized, and interferes with phosphatidylcholine (PC) biosynthesis *de novo*, which appeared to be essential for survival in lymphoma cells [Van der Luit, Budde, Ruurs, Verheij and Van Blitterswijk (2002) J. Biol. Chem. 277, 39541–39547]. Here, we report that, in HeLa cells, ALP accumulates in lipid rafts, and that internalization is inhibited by low temperature, monensin, disruption of lipid rafts and expression of a dominant-negative mutant of dynamin bearing a replacement of Lys⁴⁴ with alanine (K44A). Thus ALP is internalized via raft- and dynamin-mediated endocytosis. Dynamin-K44A alleviated the ALP-induced inhibition of PC synthesis and rescued the cells from apoptosis induction. Additional cell rescue was attained by exogenous lysoPC, which

after internalization serves as an alternative substrate for PC synthesis (through acylation). Unlike ALP, and despite the high structural similarity to ALP, lysoPC uptake did not occur via lipid rafts and did not depend on functional dynamin, indicating no involvement of endocytosis. Albumin back-extraction experiments suggested that (radiolabelled) lysoPC undergoes transbilayer movement (flipping). We conclude that ALP is internalized by endocytosis via lipid rafts to cause apoptosis, while exogenous cell-rescuing lysoPC traverses the plasma membrane outside rafts by flipping. Additionally, our data imply the importance of ether bonds in lyso-phospholipids, such as in ALP, for partitioning in lipid rafts.

Key words: CTP:phosphocholine cytidyltransferase, endocytosis, lysophosphatidylcholine, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH₃), phosphatidylcholine biosynthesis.

INTRODUCTION

The alkyl-lysophospholipid (ALP), Et-18-OCH (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), is an antitumour agent that can induce apoptosis. Unlike most conventional chemotherapeutic drugs, ALP acts at the cell-membrane level, where it accumulates due to its inherent resistance to lipases. ALP induces apoptosis by inhibition of CTP:choline phosphate cytidyltransferase (CT), a key enzyme in phosphatidylcholine (PC) biosynthesis [1,2]. In order to be activated, CT translocates from the cytosol to the ER (endoplasmic reticulum) [3]. Thus ALP needs to be internalized to inhibit CT at this location [4]. The precise mechanism by which ALP is internalized has been a matter of debate. Some investigators have argued that endocytosis is not significantly involved [5–7], whereas others reached the opposite conclusion [8,9]. Previously, we have demonstrated that, in the mouse T lymphoma cell line S49, ALP internalization occurs by vesicular routing that does not involve fluid-phase or receptor-mediated endocytosis but, instead, depends on intact lipid rafts. We suggested that ALP is internalized directly from these microdomains, but that additional evidence would be necessary to strengthen the notion that ALP is internalized by endocytosis. Furthermore, it was questionable whether this is a ubiquitous mechanism for cells to internalize the antitumour drug.

Endocytosis requires the activity of dynamin. This GTPase is involved in pinching off newly formed vesicles during membrane fission [10]. In the present study, we therefore expressed K44A (a dominant-negative mutant of dynamin bearing a replacement of Lys⁴⁴ with alanine) [11] in HeLa cells, to show that interference

with dynamin function inhibits ALP uptake and thus alleviates the ALP-induced blockade of PC synthesis, and prevents apoptosis initiation.

From previous work [1,2], it became clear that unrestrained *de novo* PC biosynthesis is crucial for cell survival, since lysoPC, as an alternative source for PC synthesis (by acylation), is able to rescue cells from ALP-induced apoptosis in S49 and HeLa cells. Previous work from our laboratory suggested that acylation of exogenous lysoPC to form PC occurs inside the cell [12]. However, the mechanism by which lysoPC is internalized has never been addressed to date. Considering the high structural similarity between lysoPC and ALP, one might surmise a similar mechanism of internalization. Surprisingly, however, we show here that this is not the case. Using the HeLa cell system, we found that lysoPC internalization and conversion into PC does not involve functional dynamin. Moreover, whereas ALP is internalized via lipid rafts, lysoPC is not. Instead, after rapid insertion into the outer leaflet of the plasma membrane, lysoPC traverses the lipid bilayer outside the rafts, prior to its conversion into PC.

EXPERIMENTAL

Reagents

[methyl-¹⁴C]Choline chloride (58 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). lyso-[choline-¹⁴C]PC (palmitoyl-linked; 55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St Louis, MO, U.S.A.).

Abbreviations used: ALP, alkyl-lysophospholipid; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; bSMase, bacterial sphingomyelinase; CT, CTP:phosphocholine cytidyltransferase; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; Et-18-OCH₃, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; lyso(PC), lyso(phosphatidylcholine); ER, endoplasmic reticulum; K44A, a dominant-negative mutant of dynamin bearing a replacement of Lys⁴⁴ with alanine; MβCD, methyl-β-cyclodextrin; SM, sphingomyelin.

¹ To whom correspondence should be addressed (e-mail w.v.blitterswijk@nki.nl).

The ALP Et-18-OCH₃ (or Edelfosine) was from BioMol (Plymouth Meeting, PA, U.S.A.). [³H]Et-18-OCH₃ (³H-ALP; 58 Ci/mmol; C₁₈) was synthesized by Moravsek Biochemicals (Brea, CA, U.S.A.). All these compounds were diluted in serum-free DMEM (Dulbecco's modified Eagle's medium). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 cm × 20 cm), were purchased from Merck (Darmstadt, Germany). LysoPC (from egg yolk, primarily containing palmitic and stearic acid groups), bacterial sphingomyelinase (bSMase; from *Bacillus cereus*), and M β CD (methyl- β -cyclodextrin) were from Sigma (Zwijndrecht, The Netherlands). BODIPY[®] (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-lactosylceramide was purchased from Molecular Probes (Leiden, The Netherlands). Monoclonal anti-dynamin antibody, recognizing Dyn1 and Dyn2, and anti-(haemagglutinin tag) (12CA5) were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Horseradish-peroxidase-conjugated rabbit anti-mouse secondary antibodies were from DAKO (Glostrup, Denmark). Signals were visualized using the ECL (enhanced chemiluminescence) system from Amersham Pharmacia Biotech.

Cell culture, transfection and cellular uptake of radiolabelled ALP and lysoPC, and of BODIPY-lactosylceramide

The human carcinoma cell line HeLa (mycoplasma-free) was cultured in DMEM (Gibco BRL Life Technologies, Inc., Paisley, Scotland, U.K.), supplemented with heat-inactivated, 7.5 % (v/v) fetal-calf serum, 50 units/ml penicillin and 50 μ g/ml streptomycin (Merck, Darmstadt, Germany). HeLa cells stably transfected with K44A-dynamin (element I mutant) were supplemented with 400 μ g/ml G418 (also known as Geneticin; Gibco BRL Life Technologies, Inc.), 1 μ g/ml tetracycline (Sigma) and 200 ng/ml puromycin (Sigma). In the presence of tetracycline, the dynamin construct is not expressed. For induction of the phenotype, cells were washed by six consecutive replacements of culture medium to deplete cells from tetracycline, and cells were grown for at least 2 days at 37 °C to induce expression.

Cells were incubated with ³H-labelled ALP (10 μ M; 0.1 μ Ci/2 × 10⁵ cells) or lyso-[¹⁴C]PC (50 μ M; 0.1 μ Ci/2 × 10⁵ cells) for the times indicated, washed three times with PBS, lysed in 0.1 M NaOH, and then radioactivity was quantified by liquid scintillation counting.

Cells were serum-starved overnight and incubated with BODIPY-lactosylceramide (5 μ M) for the times indicated. Cells were washed in PBS containing 1 % (w/v) BSA, and lysed in 1 % (v/v) Triton X-100. Fluorescence intensities were measured by a PerkinElmer Victor Wallac II fluorescence microplate reader, using 485 nm and 535 nm filters for excitation and emission wavelengths respectively.

PC biosynthesis

Cells were incubated with [methyl-¹⁴C]choline chloride (1 μ Ci/ml) or lyso-[choline-¹⁴C]PC (0.1 μ Ci/ml). At given time points, cells were washed with PBS or a mixture of BSA/PBS, lysed in 250 μ l of methanol, washed once with 250 μ l of methanol and lipids were extracted with 250 μ l of chloroform after phase separation induced by using a one-third volume of 1 M NaCl and a one-third volume of chloroform. The organic phase was washed with 1 vol. of a solution of methanol/H₂O/chloroform (235:245:15, by vol.), and lipids were separated by silica TLC using a chloroform/methanol/acetic acid/water mixture (60:30:8:5, by vol.). Radioactive lipids were visualized and quantified using a

Fuji BAS 2000 TR PhosphorImager, and identified using internal standards, which were stained with iodine.

DNA fragmentation assay using FACS analysis

Apoptosis was determined using FACS analysis. Cells were treated with trypsin, washed in PBS and lysed overnight at 4 °C in 0.1 % (w/v) sodium citrate, 0.1 % (v/v) Triton X-100 and 50 μ g/ml propidium iodide [13]. Fluorescence intensity of propidium-iodide-stained DNA was determined on a FACScan (Becton Dickinson Advanced Cellular Biology), and data were analysed using Lysis software.

Western blotting

Cells were washed once with PBS and lysed in lysis buffer [10 mM triethanolamine/HCl (pH 7.8)/150 mM NaCl/5 mM EDTA/1 mM PMSF/1 % (v/v) Nonidet P40/20 μ g/ml aprotinin/20 μ g/ml leupeptin]. Lysates were incubated for 30 min at 4 °C and clarified by centrifugation (12 000 g for 20 min), before being normalized for protein content. Before Western blot analysis, samples were boiled for 3 min in sampling buffer [200 mM Tris/HCl (pH 6.8)/8 % (v/v) SDS/20 % (v/v) 2-mercaptoethanol/40 % (v/v) glycerol] and subjected to SDS/PAGE [12 % (w/v) gels]. Separated proteins were transferred to nitrocellulose membranes and blocked for 1 h with 5 % (w/v) Nutrilon Premium 1 (Nutricia, Zoetermeer, The Netherlands) in TBST buffer [10 mM Tris/HCl (pH 7.5)/0.5 M NaCl/0.05 % (v/v) Tween 20]. Blots were incubated overnight at 4 °C with anti-dynamin monoclonal antibody, or with anti-(haemagglutinin tag) (12CA5) in 1 % (w/v) Nutrilon Premium 1 in TBST. After incubation with the appropriate horseradish-peroxidase-conjugated rabbit anti-mouse secondary antibody, proteins were detected using ECL.

Isolation of lipid rafts

A lipid-raft fraction was prepared by detergent extraction of cells and sucrose-gradient centrifugation, essentially as described previously [14]. For one raft isolation, 10⁸ cells were trypsin-treated, centrifuged and washed twice with 10 ml of ice-cold PBS. Cells were solubilized in 2 ml of ice-cold MBST buffer [25 mM Mes/150 mM NaCl/1 % (w/v) Triton X-100/1 mM Pefabloc] and homogenized with a tight-fitting Dounce homogenizer (10 strokes). The extract was adjusted to 40 % (w/v) sucrose by the addition of 2 ml of 80 % (w/v) sucrose in MBS (lacking Triton X-100), and then put on the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was prepared by overlaying 5 ml of 40 % (w/v) sucrose and 3 ml of 5 % (w/v) sucrose (both in MBS) respectively. The tubes were centrifuged at 39 000 rev./min in an SW41 rotor for 16–18 h at 4 °C, and 12 × 1.0 ml fractions were collected manually from the top of the gradient.

RESULTS

ALP-induced apoptosis in HeLa cells can be rescued by lysoPC

In active proliferating cells, ALP is cytotoxic, and has the potential to be used as anticancer drug. Figure 1(A) shows that, in HeLa cells, ALP induces apoptosis with an EC₅₀ of 12 μ M, identical with what we reported previously in the murine lymphoma S49 cells [2]. The first signs of apoptosis already appeared after 12 h of incubation, and maximal apoptosis was seen after 36 h (results not shown). We have previously argued that ALP does

Figure 1 ALP-induced apoptosis is counteracted by lysoPC

(A) HeLa cells were treated with Et-18-OCH₃ (ALP; concentrations indicated) for 16 h. Apoptotic nuclear fragmentation was measured by FACScan analysis (see the Experimental section and below). (B) ALP (10 μM)-induced apoptosis in HeLa cells, visualized as nuclear fragmentation (appearance of subdiploid fragments in front of the G₁/S/G₂ doublet peak) after propidium iodide (PI) staining and FACScan analysis, is rescued by 100 μM lysoPC (LPC), whereas lysoPC itself does not affect cell survival. (C) Dose-dependency of lysoPC to rescue ALP (15 μM)-induced apoptosis. Data are the means ± S.D. for four experiments.

not activate a G-protein-coupled receptor that might signal to apoptosis [15]. Barburina and Jackowski [1] have suggested that in HeLa cells, similarly to S49 lymphoma cells [2], the CT step of PC synthesis is a major target of ALP. These authors also showed that lysoPC supplementation to these cells prevented ALP-induced cytotoxicity (apoptosis was not accounted for). Figure 1 shows that exogenous lysoPC can rescue ALP-induced nuclear fragmentation in HeLa cells that were analysed as subdiploid fragments by FACS, being a hallmark for apoptosis, in a dose-dependent fashion. Figure 1(B) shows that lysoPC can overcome the cytotoxic, but not the cytostatic, effect, since the G₂ arrest persisted after combined treatment with lysoPC and ALP. This is in line with published results in macrophage-like BAC1.2F5 cells [16], but not with our previous results in the S49 lymphoma system [2]. The rescuing effect of lysoPC is unlikely to be the result of a signalling pathway downstream from a G-protein-coupled receptor, since lysoPC application did not elicit a calcium response (results not shown).

ALP is internalized by endocytosis, depending on dynamin

ALP must be internalized to inhibit the enzymic activity of CT, as CT translocates from the cytoplasm to the ER to be activated [3,4]. In order to investigate whether the internalization of ALP occurs via endocytosis, either we kept the cells on ice to arrest vesicular trafficking, or we added monensin, which disrupts the membrane electropotential and the pH [17]. Figure 2(A) shows that both treatments significantly reduced ALP internalization (by up to 50%). To provide additional and definitive proof that ALP is internalized via endocytosis, we examined the dependency on dynamin. Figure 2(B) shows that expression of K44A, the dominant-negative dynamin mutant, in HeLa cells resulted in a 40% decreased internalization of ALP, corroborating our previous observation that ALP is endocytosed. We have shown

Figure 2 Uptake of ALP and BODIPY-lactosylceramide by HeLa cells is inhibited in the cold by monensin and dominant-negative dynamin

(A) Uptake of ³H-ALP (10 μM; 37 °C) by HeLa cells in the absence (closed circles) or the presence (open triangles) of monensin, or on ice (open squares). (B) Uptake of ³H-ALP (10 μM; 37 °C) by HeLa cells that are non-induced (control; closed circles) or induced to express K44A dominant-negative dynamin (open squares). (C) Internalization of BODIPY-LacCer in time in HeLa cells that are non-induced (control; closed circles) or induced to express K44A dominant-negative dynamin (open squares). A.U., arbitrary units. All data are means ± S.D. for three experiments.

previously that endocytosis of ALP occurred through lipid rafts [2]; also see Figure 6). Pagano and colleagues showed that BODIPY-lactosylceramide is exclusively internalized by a clathrin-independent, raft/caveolae-dependent pathway [18]. As an independent raft-constituent control, we therefore show in Figure 2(C) that this fluorescent glycosphingolipid is internalized in the same dynamin-dependent fashion as is ALP.

Apoptosis induction by ALP is dependent on dynamin

Because dynamin is involved in internalization of ALP, interference with its function should therefore prevent the induction of apoptosis. When dynamin-K44A expression is induced in HeLa cells (after 24 h; Figure 3A), we observed a decline in the level of ALP-induced apoptosis compared with that of the wild-type (control) HeLa cells (Figures 3B and 3C), indicating that, indeed, dynamin is actively involved in ALP-induced apoptosis. It is noteworthy that, while levels of apoptosis were diminished by dynamin-K44A expression, the kinetics of apoptosis were not affected (results not shown). Figure 3(B) shows that ALP-induced apoptosis was already somewhat reduced before expression induction of the K44A construct (zero time). This can be explained by the fact that tetracycline suppression of the mutant (at zero time) is not absolute.

Under the conditions of impaired dynamin function, the reduced levels of ALP-induced apoptosis could still be diminished further by exogenous lysoPC (from 46% to 36%; Figure 3C). This suggests that lysoPC internalization, which rescues cells from apoptosis, is not mediated via an endocytic route that involves dynamin.

ALP inhibition of *de novo* PC synthesis is dynamin-dependent

From the results described thus far, we can conclude that ALP must be internalized via an endocytic pathway that depends on

Figure 3 Dynamin-K44A expression inhibits ALP-induced apoptosis; cell rescue by lysoPC is independent of dynamin

(A) HeLa cells were induced to express the dominant-negative phenotype (K44A) of dynamin. Expression levels in time were measured by Western blot analysis. (B) Time-dependent induction of dominant-negative dynamin-K44A expression (white bars) decreases ALP-induced apoptosis, as determined by FACScan analysis. Time of induction (h) is indicated on the x-axis. Control cells (black bars) were untransfected. Data are means of three experiments $\pm$ S.D. (C) LysoPC (LPC, 50 μ M) rescue of HeLa cells from ALP (10 μ M)-induced apoptosis, visualized as nuclear fragmentation by FACScan analysis (as in Figure 1). HeLa cells were non-induced (control) or induced to express dominant-negative dynamin (K44A), as indicated. Horizontal bars indicate regions of subdiploid fragments; corresponding percentages of apoptosis are indicated above these bars. Data are representative of four independent experiments with similar results.

Figure 4 ALP-mediated inhibition of PC synthesis is alleviated by dominant-negative dynamin

[14 C]Choline incorporation into PC was measured in HeLa cells in the presence of 10 μ M ALP (present during the entire incubation period indicated). The [14 C]choline precursor was present during the last 60 min. Cells were either non-induced (black bars) or induced to express dominant-negative K44A dynamin (white bars). Data are expressed as percentages of [14 C]choline incorporation into PC in the absence of ALP, and are means $\pm$ S.D. for three experiments.

functional dynamin in order to induce apoptosis. Since ALP has previously been shown to inhibit the *de novo* biosynthesis of PC, expression of dynamin-K44A should therefore alleviate the inhibitory effect of ALP on PC biosynthesis. Indeed, when we measured the incorporation rate of [14 C]choline into the PC pool after treatment of cells with ALP, we observed that interference with functional dynamin partially alleviated ALP-mediated PC synthesis inhibition (Figure 4; the time-dependent decrease in PC values in the controls is due to the increasing exposure time to ALP).

Figure 5 Conversion of exogenous lysoPC into PC is unaffected by dynamin-K44A expression or the lipid raft-disrupting agents bSMase and MβCD

Lyso-[14 C]PC conversion into PC was determined as a function of time (indicated in min) in HeLa cells non-induced (upper panel) or induced (lower panel) to express dynamin-K44A. Cells were either not treated further (control), or were subjected to 30 min pre-incubation with bSMase (150 m-units/ml; from *B. cereus*) or MβCD (2.5 mg/ml). Choline phospholipid spots, lysoPC (LPC), PC and sphingomyelin (SM), as separated by TLC, are shown.

Conversion of exogenous lysoPC into PC does not depend on either dynamin or intact lipid rafts

From Figure 3(C), it is clear that, whereas ALP is internalized in a dynamin-dependent fashion, exogenous lysoPC remains able to rescue cells (residual) from ALP-induced apoptosis when dynamin function is impaired. We therefore tested the effect of dynamin on the conversion of lysoPC into PC. Figure 5 shows that this conversion is very fast, and is already detectable in the space of 15 min, in agreement with our previous data in lymphoma cells [2]. Furthermore, the rate of conversion of lysoPC into PC is not affected by induction of dynamin-K44A expression, indicating that functional dynamin is not involved.

Since lysoPC and ALP are both choline-‘lyso-phospholipids’ with a similar structure, and ALP internalization by S49 lymphoma cells depended on intact lipid rafts [2], we investigated the possibility that lysoPC internalization and subsequent acylation would likewise depend on intact rafts. Figure 5 shows that this is not the case. Disruption of lipid rafts by cholesterol extraction using MβCD, or by SM (sphingomyelin) breakdown with bSMase, did not affect the rate of conversion of lysoPC into PC. Figure 6 confirms that, in HeLa cells, raft disruption by MβCD abrogates ALP uptake, but leaves the uptake of lysoPC unaffected. Figure 6 also shows that the uptake of lysoPC, contrary to that of ALP, is not affected by expression of the dominant-negative dynamin mutant.

We conclude that lysoPC and ALP are internalized by different mechanisms: whereas ALP is taken up by endocytosis (depending on intact lipid rafts and functional dynamin), lysoPC must be internalized in a different way.

Exogenous lysoPC is acylated after transbilayer movement

From experimental results in a different cell system, we have previously suggested that acylation of exogenous lysoPC occurs inside the cell [12]. We set out to verify this hypothesis in the HeLa cell system used at present by employing the strong binding of lysoPC to fatty-acid-free BSA in ‘back-extraction’ experiments

Figure 6 Different effects of dynamin-K44A and/or MβCD on internalization of ALP compared with lysoPC

HeLa cells induced to express dynamin-K44A (black bars) or not (white bars) were pre-incubated for 30 min with MβCD (2.5 mg/ml) or not (control), and the amount of uptake of [³H]ALP (upper panel) or lyso-¹⁴C]PC (¹⁴C-LPC; lower panel) was determined at 120 min. Values are means ± S.D. for three experiments

Figure 7 Exogenous lysoPC traverses the plasma membrane lipid bilayer before acylation to PC, as demonstrated by BSA back-extraction

HeLa cells were incubated with lyso-¹⁴C]PC (LPC, 50 μM, 0.1 μCi/2 × 10⁵ cells) for 15 min at 4 °C, washed three times either with PBS (first panel) or in 5% fatty-acid-free BSA in PBS (second and third panels), and incubated for the times and temperatures indicated in culture medium, in either the absence (first and second panels) or presence (third panel) of 5% BSA in culture medium. Choline phospholipid spots dissolved by TLC are indicated. Data are representative of two experiments with similar results.

[19]. Lyso-¹⁴C]PC was allowed to insert into the plasma membrane of HeLa cells in the cold, and was then allowed to be converted into ¹⁴C]PC at 37 °C, after pre-washing of cells with PBS or 5% BSA to remove excess label. Figure 7 shows that, under control conditions ('pre-wash PBS'), most of the lysoPC was converted into PC within 15 min. A single BSA back-extraction of lyso-¹⁴C]PC (from the membrane outer leaflet) prevented ¹⁴C]PC formation by more than 60% (middle panel), whereas in the continued presence of BSA, PC formation was essentially eliminated and hardly exceeded the low background level (at 4 °C; right panel). These results indicate that conversion of lysoPC into PC does not take place at the outer membrane

Figure 8 Different partitioning of lysoPC and ALP in lipid rafts

(A) HeLa cells were labelled overnight with [¹⁴C]choline (0.5 μCi/2.5 × 10⁷ cells). Lipid rafts were isolated by a standard Triton X-100/sucrose-density-gradient centrifugation procedure. Distribution among the gradient fractions of the raft marker SM and of PC and lysoPC (LPC), separated by TLC, was visualized by phosphorimaging. (B) Cells were incubated with [¹⁴C]choline, as described above (at 37 °C) to label endogenous lysoPC (open squares), or were briefly (10 min) incubated with [³H]ALP (5 μM; 0.1 μCi/2.5 × 10⁷ cells; filled circles) at 4 °C to allow insertion into the outer leaflet of the plasma-membrane lipid bilayer. Distribution of radiolabelled ALP and lysoPC among the gradient fractions (numbered) is shown. Data are expressed as percentages of the total, and are representative of two experiments with similar results.

leaflet, but occurs after lysoPC traversal across the plasma-membrane lipid bilayer. Surprisingly, at 4 °C (the 15 min time point), some of the lysoPC has already been converted into PC, indicating that, under these conditions, lysoPC is able to traverse the lipid bilayer and that the acyltransferase has some activity in the cold.

ALP and lysoPC exhibit different lateral distributions in the plasma membrane

We have shown previously in lymphoma cells that ALP, after rapid insertion into the plasma-membrane outer leaflet, readily congregates at the rigid, sphingolipid- and cholesterol-enriched membrane rafts [2]. We verified this in HeLa cells by incubating the cells for 60 min with 5 μM ALP, with the subsequent isolation of lipid rafts using Triton X-100 solubilization and sucrose-density-gradient centrifugation. Figure 8(A) shows a typical distribution of (radiolabelled) choline-phospholipids over the gradient fractions. Lipid rafts equilibrated at fractions 3 and 4, characterized by the presence of SM and other raft markers (the ganglioside G_{M1} and caveolin; this pair are not shown in the Figure). As shown in Figure 8(B), as much as 30% of the ALP accumulated in the lipid-raft fractions. By contrast, endogenous lysoPC (labelled overnight with [¹⁴C]choline) was virtually excluded from the lipid-raft fractions (Figures 8A and 8B). Exogenous lysoPC, incubated over a short period of time (10 min) with cells on ice, resulted in a fraction of raft distribution of 15% (results not shown).

We conclude that ALP in HeLa cells is incorporated preferentially into lipid rafts, consistent with our previous results in lymphoma cells, whereas endogenous lysoPC is excluded from rafts. Exogenous lysoPC, by definition inserting into the outer leaflet, shows less affinity for lipid rafts than does ALP, even under forced conditions (a short period of incubation in the cold). This

different lateral membrane distribution of ALP compared with that of lysoPC may be causally related to their different modes of internalization by cells.

DISCUSSION

In the present paper, we have shown that exogenous lysoPC can rescue HeLa cells from ALP-induced apoptosis, which is in line with our previous findings in S49 lymphoma cells [2], and extends the work of Barburina and Jackowski [1], who reported HeLa cell rescue from ALP 'cytotoxicity'. We found that ALP is internalized via endocytosis, mediated by the GTPase dynamin and depending on intact lipid rafts, similar to the typical raft marker BODIPY-lactosylceramide. ALP causes the translocation of cytidylyltransferase from the soluble compartment to the particulate compartment, which is probably the ER [3,4]. By contrast, lysoPC conversion into PC, presumably a key step in the cell-rescue mechanism, does not depend on dynamin and lipid rafts. However, from BSA back-extraction experiments (Figure 8), it appears that lysoPC needs to be internalized before its acylation to PC. This conclusion was also drawn from previous work in our laboratory, in which exogenous phospholipase D was unable to hydrolyse the cellular PC pool labelled with exogenously added ³²P-labelled lysoPC [12]. Apparently, lysoPC is internalized in a different way from ALP, i.e. not by endocytosis, but by flipping over the plasma-membrane lipid bilayer. Our unexpected observation that some conversion of lysoPC into PC occurs even on ice, a condition that blocks endocytosis, provides additional evidence that lysoPC must have crossed the lipid bilayer by flipping. By the same token, acylation to PC occurs most likely at the inner leaflet of the plasma membrane, immediately after lysoPC has traversed the membrane without the involvement of vesicular trafficking (which is blocked on ice). In principle, lysoPC could transverse the plasma-membrane lipid bilayer spontaneously or with the help of a flippase. Evidence for the existence of a membrane flippase, mediating transbilayer movement of PC or lysoPC, has been found in the ER [20]. At the plasma membrane, no flippase for inward movement of lysoPC has yet been identified.

The synthetic ALP has a high structural similarity to lysoPC, being a 'lyso'-phospholipid with the same choline head-group. On this basis, we speculated previously that the two lipids might follow the same route in the cell [2], which we have now shown not to be the case. The structural difference must determine therefore their different mode of internalization. ALP contains one long alkyl chain and an additional ether-linked methyl group at the *sn*-2 position, whereas lysoPC contains one ester-linked acyl chain. We found that ALP, but not lysoPC, preferentially (but not exclusively) congregates to the liquid ordered phase of lipid rafts in the plasma membrane. This agrees with the results of Mattjus et al. [21], who showed that alkyl-phosphatidylcholines have a stronger interaction with cholesterol in laterally condensed microdomains (lipid rafts) than their acyl counterparts. Lipid rafts are subject to a continuous endocytosis vesicular trafficking pathway [18,22–24] that involves dynamin [25,26]. In addition, the internalization of interleukin-2 after receptor binding [27], and the entry of pathogens (bacteria, viruses, etc.) [28–30] have been shown to occur from lipid rafts mediated by dynamin. Figure 2(C) shows that internalization of the fluorescent BODIPY-lactosylceramide raft constituent also depends on dynamin. Whereas ALP, too, moves along this pathway [2], lysoPC apparently is excluded from it.

The effect of K44A-dynamin expression on the uptake of ALP is similar to that of other inhibitors of endocytosis (e.g. monensin and cold treatment), but is not as pronounced as is

observed usually for receptor proteins (e.g. for epidermal growth factor or transferrin). This can be explained by the nature of these molecules: the lipid ALP intercalates into, and will partly remain bound to, the plasma-membrane bilayer, while a minor part is even expected to follow the same internalization route as lysoPC (flipping). However, the part of ALP internalized by dynamin-mediated endocytosis both affects PC synthesis and induces apoptosis.

Our results show that exogenous and endogenous ([¹⁴C]choline-labelled) lysoPC are subject to different submembrane distributions. Whereas exogenous lysoPC in the outer leaflet of the plasma membrane showed some 15 % partitioning in lipid rafts, being less than that shown for ALP (30 %), endogenous lysoPC, labelled with [¹⁴C]choline, did not partition in rafts at all. Endogenous lysoPC is generated by deacylation (by phospholipase A₂) of PC, presumably at the plasma-membrane inner leaflet, but, at the least, not at the outer leaflet. Our raft fractionation data suggest that this inner-leaflet PC deacylation as well as the reverse reaction, the acylation of (exogenous) lysoPC, are both occurring outside rafts.

Our present and previous work confirms that continuous PC synthesis is essential for cell survival in actively proliferating (tumour) cells, and that exogenous lysoPC boosts an alternative pathway (acylation) of PC synthesis that apparently can substitute for ALP-inhibited *de novo* PC synthesis as a survival mechanism [2]. The question arises as to how the cell senses a deficiency in PC synthesis (via CT activity in the *de novo* or Kennedy pathway), believed to be located in the ER, and how it is capable of compensating for this by new PC formation (from lysoPC) at a topologically different site (the inner leaflet of the plasma membrane). One possibility is through direct ER contacts with the plasma membrane [31], similar to that observed between ER and the Golgi [32]. Another possibility is through a specific protein mediator that is capable of sensing PC levels in the cell. Evidence for the existence of such a regulatory protein for PC synthesis has been provided in yeast. In this system, PC levels are sensed by Sec14p, while PC-bound Sec14p down-regulates CT enzymic activity [33]. Replenishment of PC levels by addition of lysoPC at the plasma membrane might likewise be sensed by Sec14p, suppressing the need for CT in the *de novo* pathway for continuous PC formation. Verification of this speculative mechanism, however, first needs the identification of a mammalian homologue of Sec14p.

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REFERENCES

1. Baburina, I. and Jackowski, S. (1998) Apoptosis triggered by 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine is prevented by increased expression of CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **273**, 2169–2173.
2. Van der Luit, A. H., Budde, M., Ruurs, P., Verheij, M. and van Blitterswijk, W. J. (2002) Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. *J. Biol. Chem.* **277**, 39541–39547.
3. Clement, J. M. and Kent, C. (1999) CTP:phosphocholine cytidylyltransferase: insights into regulatory mechanisms and novel functions. *Biochem. Biophys. Res. Commun.* **257**, 643–650.
4. Boggs, K. P., Rock, C. O. and Jackowski, S. (1995) Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. *J. Biol. Chem.* **270**, 7757–7764.
5. Mollinedo, F., Fernandez-Luna, J. L., Gajate, C., Martin-Martin, B., Benito, A., Martinez-Dalmau, R. and Modolell, M. (1997) Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH₃ (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X(L). *Cancer Res.* **57**, 1320–1328.

- 6 Zoeller, R. A., Layne, M. D. and Modest, E. J. (1995) Animal cell mutants unable to take up biologically active glycerophospholipids. *J. Lipid Res.* **36**, 1866–1875
- 7 Kelley, E. E., Modest, E. J. and Burns, C. P. (1993) Unidirectional membrane uptake of the ether lipid antineoplastic agent edelfosine by L1210 cells. *Biochem. Pharmacol.* **45**, 2435–2439
- 8 Bazill, G. W. and Dexter, T. M. (1990) Role of endocytosis in the action of ether lipids on WEHI-3B, HL60, and FDCP-mix A4 cells. *Cancer Res.* **50**, 7505–7512
- 9 Small, G. W., Strum, J. C. and Daniel, L. W. (1997) Characterization of an HL-60 cell variant resistant to the antineoplastic ether lipid 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine. *Lipids* **32**, 715–723
- 10 Schmid, S. L., McNiven, M. A. and De Camilli, P. (1998) Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol.* **10**, 504–512
- 11 Damke, H., Baba, T., Warnock, D. E. and Schmid, S. L. (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127**, 915–934
- 12 van Dijk, M. C., Postma, F., Hilkmann, H., Jalink, K., van Blitterswijk, W. J. and Moolenaar, W. H. (1998) Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca²⁺ signaling pathways. *Curr. Biol.* **8**, 386–392
- 13 Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139**, 271–279
- 14 Lisanti, M. P., Tang, Z., Scherer, P. E. and Sargiacomo, M. (1995) Caveolae purification and glycosylphosphatidylinositol-linked protein sorting in polarized epithelia. *Methods Enzymol.* **250**, 655–668
- 15 Ruiter, G. A., Verheij, M., Zerp, S. F., Moolenaar, W. H. and van Blitterswijk, W. J. (2002) Submicromolar doses of alkyl-lysophospholipids induce rapid internalization, but not activation, of epidermal growth factor receptor and concomitant MAPK/ERK activation in A431 cells. *Int. J. Cancer* **102**, 343–350
- 16 Boggs, K. P., Rock, C. O. and Jackowski, S. (1995) Lysophosphatidylcholine attenuates the cytotoxic effects of the antineoplastic phospholipid 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine. *J. Biol. Chem.* **270**, 11612–11618
- 17 Tartakoff, A. M. (1983) Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell* **32**, 1026–1028
- 18 Puri, V., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L. and Pagano, R. E. (2001) Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* **154**, 535–547
- 19 Van Meer, G., Stelzer, E. H., Wijnaendts-van-Resandt, R. W. and Simons, K. (1987) Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* **105**, 1623–1635
- 20 Menon, A. K., Watkins, W. E. and Hrafnisdottir, S. (2000) Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum. *Curr. Biol.* **10**, 241–252
- 21 Mattjus, P., Bittman, R. and Slotte, J. P. (1996) Molecular interactions and lateral domain formation in monolayers containing cholesterol and phosphatidylcholines with acyl- or alkyl-linked C16 chains. *Langmuir* **12**, 1284–1290
- 22 Nichols, B. J. and Lippincott-Schwartz, J. (2001) Endocytosis without clathrin coats. *Trends Cell Biol.* **11**, 406–412
- 23 Hao, M. and Maxfield, F. R. (2000) Characterization of rapid membrane internalization and recycling. *J. Biol. Chem.* **275**, 15279–15286
- 24 Van Blitterswijk, W. J., van der Luit, A. H., Veldman, R. J., Verheij, M. and Borst, J. (2003) Ceramide: second messenger or modulator of membrane structure and dynamics. *Biochem. J.* **369**, 199–211
- 25 Oh, P., McIntosh, D. P. and Schnitzer, J. E. (1998) Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* **141**, 101–114
- 26 Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998) Dynamin-mediated internalization of caveolae. *J. Cell Biol.* **141**, 85–99
- 27 Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. and Dautry-Varsat, A. (2001) Interleukin-2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* **7**, 661–671
- 28 Rosenberger, C. M., Brumell, J. H. and Finlay, B. B. (2000) Microbial pathogenesis: lipid rafts as pathogen portals.
- 29 Pelkmans, L., Kartenbeck, J. and Helenius, A. (2001) Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* **3**, 473–483
- 30 Pelkmans, L., Puntener, D. and Helenius, A. (2002) Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* **296**, 535–539
- 31 Pichler, H., Gaigg, B., Hraštnik, C., Achleitner, G., Kohlwein, S. D., Zellnig, G., Perktold, A. and Daum, G. (2001) A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. *Eur. J. Biochem.* **268**, 2351–2361
- 32 Marsh, B. J., Mastronarde, D. N., Buttle, K. F., Howell, K. E. and McIntosh, J. R. (2001) Organellar relationships in the Golgi region of the pancreatic beta cell line, HIT-T15, visualized by high resolution electron tomography. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2399–2406
- 33 Skinner, H. B., McGee, T. P., McMaster, C. R., Fry, M. R., Bell, R. M. and Bankaitis, V. A. (1995) The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 112–116

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