Alkyl-lysophospholipid Accumulates in Lipid Rafts and Induces Apoptosis via Raft-dependent Endocytosis and Inhibition of Phosphatidylcholine Synthesis*

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The synthetic alkyl-lysophospholipid (ALP), 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine, is an antitumor agent that acts on cell membranes and can induce apoptosis. We investigated how ALP is taken up by cells, how it affects de novo biosynthesis of phosphatidylcholine (PC), and how critical this is to initiate apoptosis. We compared an ALP-sensitive mouse lymphoma cell line, S49, with an ALP-resistant variant, S49AR. ALP inhibited PC synthesis at the CDP-phosphocholine-2-diylytransferase (CT) step in S49 cells, but not in S49AR cells. Exogenous phosphatidylcholine, providing cells with an alternative way (acylation) to generate PC, rescued cells from ALP-induced apoptosis, indicating that continuous rapid PC turnover is essential for cell survival. Apoptosis induced by other stimuli that do not target PC synthesis remained unaffected by lysophosphatidylcholine. Using monensin, low temperature and albumin back-extraction, we demonstrated that ALP is internalized by endocytosis, a process defective in S49AR cells. This defect neither involved clathrin-coated pit nor fluid-phase endocytosis, but depended on lipid rafts, because disruption of these microdomains with methyl-β-cyclodextrin or filipin (sequestering cholesterol) or bacterial sphingomyelinase reduced uptake of ALP. Furthermore, ALP was found accumulated in isolated rafts and disruption of rafts also prevented the induction of PC synthesis and apoptosis induction in S49 cells. In summary, ALP is internalized by raft-dependent endocytosis to inhibit PC synthesis, which triggers apoptosis.

The synthetic alkyl-lysophospholipid (ALP), 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (Et-18-OCH₃; Edelfosine), is a selective antitumor agent, known to induce apoptosis in various cell types (1, 2). Unlike most conventional chemotherapeutic drugs, ALP does not target the DNA but acts at the level of cell membranes. Because of its ether bonds ALP is resistant to phospholipases, and therefore accumulates in the plasma membrane as well as other subcellular membranes (3), where it inhibits mitogenic and survival signaling pathways and activates the stress-activated protein kinase/eJun NH²-terminal kinase stress pathway (2, 4). The mechanism by which ALP affects these pathways and induces apoptosis may relate to its disturbing effect on lipid metabolism and lipid signaling in cell membranes (5–7).

A major effect of ALP is the inhibition of de novo PC synthesis (7). Several studies have suggested that maintenance of PC biosynthesis is important for cell survival. A genetic defect in PC biosynthesis induced apoptosis in Chinese hamster ovary cells (8), whereas choline deficiency (via the culture medium) led to decreased PC levels and induced apoptosis in PC12 cells and in primary neural cultures (9, 10). PC biosynthesis occurs predominantly via the Kennedy pathway in which the conversion of phosphocholine to CDP-choline, catalyzed by CT, is the rate-limiting step, and the condensation of CDP-choline with diacylglycerol by choline phosphotransferase constitutes the final step. Inhibition of either of these enzymatic steps may lead to apoptosis (11, 12).

In the present paper, we determined the effect of ALP on PC synthesis in S49 mouse lymphoma cells, and found an inhibition at the CT step. This enzyme resides in the nucleus and the cytoplasm and translocates to the ER when activated (13). We questioned how ALP can reach this intracellular target from outside the cell and how this subsequently initiates apoptosis. There are reports on uptake of ALP correlating with apoptosis induction (1, 14). However, the mechanism by which ALP is internalized has remained uncertain. ALP is not taken up via a specific receptor (such as for the structurally related platelet-activating factor or lyso-PC) (1, 15–17). Some investigators argue that endocytosis is not a major pathway by which ALP is taken up (1, 14, 18), whereas others reach the opposite conclusion (15, 19). To address this issue in more detail, we investigated the uptake and action of ALP in S49 cells in comparison with an ALP-resistant variant cell line S49AR.

We report here that PC synthesis in S49AR cells remains undisturbed because these cells are unable to internalize ALP as efficiently as the parental cells. We provide evidence that ALP concentrates in cholesterol- and sphingomyelin-rich membrane microdomains, known as lipid rafts (20), that ALP is endocytosed via intact rafts, and that reduced internalization of ALP in the S49AR cells must be because of a defect in raft-dependent endocytosis. Furthermore, we demonstrate that the inhibition of PC biosynthesis in S49 cells depends on intact rafts and is the direct trigger for initiation of apoptosis. Maintenance of PC synthesis, for example, by providing the cell with...
an alternative route of PC synthesis, is a factor that contributes to cell survival.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methyl-[14C]choline chloride (58 mCi/mmol) was purchased from Amersham Biosciences. [3H]Choline-lysoPC (LPC) (55 mCi/mmole) was purchased from (American Radiolabeled Chemicals Inc., St. Louis, MO). ALP was purchased from BioMol (Plymouth Meeting, PA). [3H]Et-18-OCH3 ([3H]ALP; 58 Ci/mmol), was synthesized by Moravek Biochemicals (Brea, CA). Alexa-488 fluorescently labeled human transferrin and Sulfonhodamine 101 were purchased from Molecular Probes (Leiden, The Netherlands). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 × 20 cm) were purchased from Merck (Darmstadt, Germany). Cholera toxin B, rabbit anti-cholera toxin B, sphingomyelinase (Bacillus cereus), flippin, and MfCD were purchased from Sigma (Zwijndrecht, The Netherlands) and the peroxidase-conjugated swine anti-rabbit Ig was obtained from DAKO A/S (Glostrup, Denmark).

**Cells Cultures**—Mouse S49.1 lymphoma cells (S49) were grown in Dulbecco’s modified Eagle’s medium, supplemented with 8% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C and 5% CO2. ALP-resistant variants (S49AR) were isolated in two selection rounds of growth in 15 μl ALP (Et-18-OCH3) for 72 h, followed by plating in semisolid medium and isolation of colonies of surviving cells, as described by Smets et al. (21). The selective S49AR clone could be grown continuously in 15 μl ALP with a doubling time of 12 h, similar to that of the parent S49 cells. All experiments with S49AR cells were performed with cells grown without the selection agent for at least 1 week.

**Apoptosis Assay**—Cells were seeded at 1.5 × 104 cells/ml, cultured overnight, and incubated for the indicated time periods with various concentrations of Et-18-OCH3 (ALP). Cells were washed three times with phosphate-buffered saline (PBS) and lysed overnight at 4 °C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium iodide (22). Fluorescence intensity of propidium iodide-stained DNA was determined on a FACScan (BD Biosciences), and data were analyzed using Lysis software.

**ALP Uptake**—Cells were grown to a density of 2.5 × 105/ml and ALP was added in the apoptotic concentration of 15 μM, supplemented with 0.2 μCi/ml of [3H]ALP. At the given time points samples were taken followed by a 2-min incubation on ice and three washes with cold PBS. Samples were lysed in 0.1 N NaOH for scintillation counting. Back-extraction of ALP was performed by washing the cells with PBS containing 1% (w/v) fatty acid-free BSA.

**Endocytosis**—Cells were grown to a density of 2.5 × 105/ml and incubated for 30 min with Alexa-488 fluorescently labeled human transferrin (1 μg/ml) or with the fluorescent fluid-phase marker, Sulfonhodamine 101 (25 μg/ml). Endocytosis of these compounds was stopped on ice. Cells were washed thoroughly with cold PBS and the fluorescence measured by FACScan analysis (BD Biosciences). Data were analyzed using FCS express software.

**PC Biosynthesis Using [1-14C]Choline or [1-14C]LPC**—Cells at 2.5 × 105 cells/ml were incubated with [methyl-14C]choline chloride (1 μCi/ml) or [1-14C]LPC (0.1 μCi/ml). At the given time points, aliquots of cells were taken, washed, and resuspended in 200 μl of PBS. Lipids were extracted with chloroform/methanol (1:2, v/v), and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/H2O/chloroform (235:245:15, v/v/v), and separated by silica gel TLC using chloroform/methanol/acetic acid/water (60:30:8:5, v/v/v/v). Lipids were visualized and quantified using a Fuji BAS 2000 TR PhosphorImager and identified using internal standards, which were visualized by iodine staining.

**Isolation of Lipid Rafts**—A lipid raft fraction was prepared by detergent extraction of cells and sucrose gradient centrifugation, essentially as described (23). Cells were grown to a density of 2.0 × 106/ml, spun down, and washed with 2× 10 ml of ice-cold phosphate-buffered saline. Cells were solubilized into 2 ml of ice-cold MBST buffer (25 mM MES, 150 mM NaCl, 1% Triton X-100, 1 mM Pefabloc) and homogenized with a loose fitting Dounce homogenizer (10 strokes). The extract was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose in MBS (lacking Triton X-100) and put on the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was prepared by overlaying 5 ml of 40% sucrose and 3 ml of 5% sucrose (both in MBS), respectively. The tubes were centrifuged at 39,000 rpm in a SW41 rotor for 16–18 h at 4 °C and 12 × 1.0-ml fractions were collected manually from the top of the gradient. For incorporation of ALP in lipid rafts, cells were incubated with [3H]Et-18-OCH3 (25 μCi/ml; 15 μM) for 5 min to allow insertion into the outer leaflet of the plasma membrane lipid bilayer.

**Analysis of Raft Markers, Sphingomyelin and GM1**—Sphingomyelin levels in each fraction were determined after 24 h radiolabeling with 1 μCi/ml [methyl-14C]choline-HCl, after Triton X-100 solubilization and sucrose gradient centrifugation followed by TLC separation (system, see above). The ganglioside, GM1, level in each fraction was determined by a dot-blot technique using cholera toxin B binding. In short, 2.5 μl of each sucrose gradient fraction was spotted onto a nitrocellulose membrane, air-dried, and washed with PBS. Membranes were blocked with 3% (w/v) BSA in PBS, and incubated with 1 μg/ml cholera toxin B for 30 min at 4 °C. Blots were washed and incubated with rabbit anticholera toxin B in a 1:500 dilution and followed by an incubation at room temperature with horseradish peroxidase-conjugated swine anti-rabbit Ig (1:7500). Spots were visualized by chemiluminescence using the Amersham ECL kit according to the manufacturer’s instructions.

**RESULTS**

**ALP Induces Apoptosis in S49 Lymphoma Cells; S49AR Cells Are Resistant to ALP**—The synthetic ether lipid Et-18-OCH3 (Edelfosine; ALP) induces apoptosis in S49 cells in a dose-dependent fashion, with an EC50 of about 12 μM (Fig. 1, A and B). The onset of apoptosis in the S49 cell population was already apparent after 3 h. Apoptosis was maximal (80%) at 7 h, whereas half-maximal values were reached after 4 h (data not shown). The ALP-resistant variant S49AR did not undergo apoptosis during the time period of the experiment and at the ALP concentration range tested (Fig. 1A).

**ALP Inhibits PC Synthesis at the Level of Cytididylyltransferase in S49 Cells**—Not in S49AR Cells—To understand how
ALP induces apoptosis in S49 cells and why S49AR cells are resistant, we followed the suggestion that ALP would inhibit the *de novo* synthesis of PC (7). Using [14C]choline pulse-chase labeling, we found that ALP blocks [14C]choline incorporation into the PC and CDP-choline pools in S49 cells, but virtually not in S49AR cells (data not shown). ALP had virtually no effect on [14C]phosphocholine levels. These results are consistent with the notion that ALP inhibits the rate-determining enzyme in the Kennedy pathway of PC synthesis, CT (7). Contrary to S49 cells, this enzyme does not appear to be a target of ALP in the resistant S49AR variant.

**Lyso-PC Addition Prevents ALP-induced Apoptosis**—Because apoptosis induction by ALP correlated with inhibition of *de novo* PC synthesis, we questioned whether this lack of newly generated PC is the direct trigger that initiates the apoptotic machinery. To test this, we provided the cell with an alternative way to generate PC, that is by adding LPC via the culture medium. LPC, structurally related to ALP, readily incorporates into the plasma membrane and is very rapidly acylated to PC in S49 cells, while being structurally similar to ALP) is only slowly converted to PC in S49AR cells but not in S49 cells.

From these observations we conclude that inhibition of PC synthesis is sufficient to induce apoptosis, and that continual, unimpeded PC synthesis/turnover is important for cell survival. LPC, through conversion to cellular PC, alleviates ALP inhibition of PC synthesis and consequently rescues cells from ALP-induced apoptosis. LPC does not affect apoptosis induced by agents that have no direct effect on PC turnover.

**ALP Is Rapidly Internalized in S49 Cells, but Internalization in S49AR Cells Is Impaired**—CT, the target of ALP, is known to reside in the endoplasmic reticulum and the nucleus (13). We therefore addressed the questions how ALP is internalized in S49 cells and if the resistance of S49AR cells might be because of their inability to internalize the ether lipid. Fig. 3 shows that, in time, the uptake of radiolabeled ALP in the S49AR cells was considerably less than in S49 cells. Initially, the two cell types bound the same amount of label. However, after 30 min the uptake of ALP leveled off in S49AR cells but not in S49 cells.

Because the uptake of ALP in S49 cells does not reach saturation, it is unlikely that it would be mediated by a receptor (like that for PAF), which agrees with other reports (1, 15, 17). Instead, we envision that ALP, because of its single long alkyl chain, easily inserts into the outer leaflet of the plasma membrane lipid bilayer, prior to and/or during internalization. Employing the strong binding of ALP to albumin, we determined with albumin back-extraction (25) the fraction of [3H]ALP that remained located in this outer leaflet, and how much was internalized in time. Fig. 3 shows that, initially, most of the [3H]ALP associated to S49AR cells could be back-extracted by albumin, particularly during the first 30 min (this is also shown in an alternative way in the inset of Fig. 3). In contrast, in S49 cells, the majority of ALP remained cell-associated after back-extraction at all times, indicating that, in these cells, ALP is rapidly internalized. Interestingly in this respect, exogenous LPC is rapidly internalized (being structurally similar to ALP) is only slowly converted to PC in S49AR cells compared with S49 cells (Fig. 2), suggesting that S49AR cells, which show reduced ALP internalization, likewise show a reduced LPC internalization. Of note, ALP does not affect LPC uptake (conversion to PC; Fig. 2). The same holds for the reverse: addition of LPC does not affect the uptake of ALP by the cells (data not shown). We conclude that addition of ALP to S49 cells leads to its almost instantaneous internalization, in contrast to S49AR cells where ALP internalization is significantly delayed and occurs in lower amounts.

**Apoptosis Induction by ALP Requires Its Endocytosis**—We next addressed the question how this rapid internalization in
S49 cells occurs. Basically, two mechanisms could be envisioned: (a) passive diffusion involving plasma membrane transbilayer movement (flip-flop), which is unlikely because it is a relatively slow process (26), at least when it occurs spontaneously; (b) via formation, budding off, and intracellular release of membrane vesicles from the plasma membrane, i.e., endocytosis. We tested the latter, a more likely mechanism by two different ways to block endocytosis. First, cells were kept at 4 °C. Indeed, an ~80% reduced uptake of ALP was observed in S49 cells during a 2-h time period (data not shown). In the cold, the amount of ALP associated to S49 cells was the same as to the S49AR cells. Moreover, under these conditions the same amount (about 90%) of ALP could be back-extracted from these cells by albumin (data not shown), indicative of the same outer leaflet plasma membrane location of ALP in the two cell types in the cold. Therefore, the difference of (nonextractable) ALP prevents apoptosis (27). Also this treatment reduced ALP uptake in S49 cells (Fig. 4B), which further supports the notion that ALP, after insertion into the plasma membrane, reaches its intracellular target, CT, by an endocytic mechanism that does not involve clathrin-coated pits nor fluid-phase endocytosis. Therefore, we have concluded that ALP, after insertion into the plasma membrane, reaches its intracellular target, CT, by an endocytic mechanism that does not involve clathrin-coated pits nor fluid-phase endocytosis.

Cells can make use of clathrin-dependent or -independent routes of endocytosis, for signal transduction or uptake of nutrients. Fig. 5 (upper panel) shows that the ALP-resistant S49AR cells have normal receptor-mediated uptake of (fluorescent) transferrin, known to occur via clathrin-coated pits. Also the uptake of Sulforhodamine 101, a fluorescent marker for clathrin-independent fluid-phase endocytosis, does not differ between S49 and S49AR cells (Fig. 5, lower panel). Thus, the difference in ALP uptake between the two cell types must be because of a different mechanism of endocytosis. Therefore, we tested another clathrin-independent route of endocytosis, more recently discovered, that is via lipid rafts (28–30).

**Endocytosis of ALP, Inhibition of PC Synthesis, and Subsequent Apoptosis Depend on Intact Rafts**—Based on its structure, with an ether bond and a single saturated alkyl chain, ALP (Et-18-OCH3) is predicted to accommodate to the rigid, sphingolipid- and cholesterol-enriched membrane rafts (31, 32). We tested this directly by isolation of rafts using the well-established method of Triton X-100 solubilization and sucrose density gradient centrifugation (23) (Fig. 6). When cells were incubated with radiolabeled ALP for 5 min, most of the ether lipid was found accumulated in raft fractions 3 and 4 (Fig. 6C), and in equal amounts for S49 and S49AR cells. ALP co-distributed with the typical raft marker sphingomyelin (Fig. 6A). Another raft marker, the ganglioside GM1, visualized by cholera toxin B labeling using a dot-blot technique (Fig. 6B), accumulated in the same region of the gradient albeit consistently at a slightly higher density. Disruption of rafts by treatment of cells with MβCD, which extracts cholesterol, resulted in the redistribution of GM1 and ALP to non-raft fractions (Fig. 6, B and D).

**Fig. 5.** Transferrin receptor (TrR) and fluid phase-mediated endocytosis in S49AR cells do not differ from S49 cells. Cells were incubated for 30 min with Alexa-488 fluorescently labeled human transferrin (1 μg/ml; upper panel) or with the fluorescent fluid-phase marker, Sulforhodamine 101 (25 μg/ml; lower panel), and the uptake of fluorescence is shown by FACScan analysis (gray profile, relative to autofluorescence control).
Each treatment abrogated to different extents the capability of S49 cells to take up ALP (Fig. 7, at 120 min), without causing plasma membrane leakage (as determined by the lack of trypan blue uptake) or altering cell morphology (controls not shown). Fig. 7 also shows that the initial binding of ALP to the plasma membrane, measured at 10 min, was not affected by raft disruption. Although this disruption caused a partial displacement of ALP of the rafts (Fig. 6D), the overall initial binding of ALP to the plasma membrane remained the same. Only the subsequent cellular uptake through internalization was blocked by the raft disruption.

We next tested whether raft disruption abrogated the inhibitory action of ALP on the de novo synthesis of PC and the induction of apoptosis. Fig. 8A shows, in S49 cells, the time-dependent incorporation of \(^{14}\)C)choline into PC, which is 50% inhibited by ALP. This inhibition of PC synthesis is prevented by prior disruption of lipid rafts by bacterial sphingomyelinase or MβCD. These treatments alone did not affect PC synthesis (controls not shown). Fig. 8B shows that raft disruption by MβCD prevents ALP-induced apoptosis in a dose-dependent manner.

We thus conclude that intact rafts are required for the endocytic uptake of ALP by S49 cells, and the subsequent inhibition of PC synthesis that leads to the induction of apoptosis. Given that ALP partitions equally well in the raft fraction of S49\(^{AR}\) cells (Fig. 6C), it remains to be investigated why rafts in these cells do not mediate ALP internalization.

**DISCUSSION**

In this paper, we have shown that apoptosis induction by ALP requires its endocytosis, depending on intact lipid rafts, to inhibit de novo biosynthesis of PC at the level of CT inside the cell. Because an alternative pathway of PC synthesis, i.e. the acylation of exogenous LPC, alleviates shortage of newly produced PC and rescues cells from apoptosis induction, we conclude that impaired PC synthesis is the direct trigger of ALP-induced apoptosis. In other words, PC synthesis is a survival factor in this cell system. Consistent with this notion, apoptosis induced by other treatments, such as ionizing radiation, which acts predominantly by damaging DNA but does not affect PC synthesis, was found not counteracted by exogenous LPC (data not shown). Results by Jackowski and co-workers (7, 11) likewise point to PC synthesis as a survival factor in different cell systems. However, their results differ from our findings with respect to the cell cycle. In BAC1.2F5 macrophage-like cells,
ALP caused a cell cycle arrest (accumulation in the G2/M phase), which was overcome by LPC (24), whereas our FACS analysis in S49 cells did not show altered cell-cycle distribution by ALP or LPC in Fig. 1B. Apparently, these effects are cell type-dependent.

We have demonstrated, by albumin back-extraction of ALP and by low temperature and monensin treatment of S49 cells, that ALP is internalized by endocytosis, and that this internalization is impaired in the resistant S49AR cells. In this way, S49AR cells escape from the deleterious action of ALP. We have confirmed in a different cell system (HeLa) that internalization of ALP is dependent on the GTPase dynamin, a mediator of endocytosis (34–36). A dominant-negative mutant of dynamin blocked ALP internalization in these HeLa cells. Plasma membrane lipids can be endocytosed both in a clathrin-dependent and -independent fashion (29). The latter route occurs ubiquitously and constitutively, and plays an important role in rapid membrane recycling or remodeling of the plasma membrane and the formation of lipid second messengers in response to external stimuli (30). This route of endocytosis is, for example, also used by exogenous fluorescent membrane-permeable lipids (29, 37, 38). It is therefore not entirely unexpected, but was hitherto never shown that membrane-incorporated ALP is internalized in the same way.

One clathrin-independent way of endocytosis depends on lipid rafts/caveolae (28, 30, 39). Ligands as well as membrane lipid constituents (probably as entire rafts, clustered or not) can be internalized in this way. For example, the B subunit of cholera toxin, after binding to a typical raft lipid, GM1, is internalized in part directly via these microdomains in a dynamin-mediated fashion (35, 36, 40) (although the proportion that is internalized via this route may depend on the cell type) (41). Also, internalization of interleukin-2 by its receptor (42) and entry of pathogens (bacteria, viruses, etc.) (43, 44) occurs via lipid rafts.

Being enriched in sphingolipids and cholesterol, membrane rafts have a high degree of lipid structural order (high rigidity; low fluidity (32)). Based on its structure, with an ether bond and a single, long saturated alkyl chain, ALP will easily accommodate to these rigid microdomains (31, 32), as we have demonstrated here by isolating lipid rafts on a sucrose density gradient, using sphingomyelin and GM1 as genuine raft markers. ALP was found enriched in rafts, and in equal quantities in S49 and S49AR cells (Fig. 6C). In S49, but not in S49AR cells, ALP is rapidly internalized presumable as the molecular component of the constitutive raft-mediated endocytosis (which is impaired in S49AR cells). Disruption of rafts in three different ways, i.e. treatment with MβCD, filipin, or bacterial sphingomyelinase, prevented ALP internalization in the S49 cells. Furthermore, we demonstrated that ALP partitioning in rafts, a rapid event (within 5 min), was disturbed by MβCD pretreatment, resulting in a redistribution of ALP to nonraft fractions without altering the initial overall binding to the plasma membrane (Figs. 6D and 7).

The defect in S49AR cells to internalize ALP appeared unrelated to clathrin-coated pit-mediated or fluid-phase endocytosis, because the uptake of transferrin (via clathrin-coated pits) and a fluid phase marker were the same as in the S49 cells. These classical routes of endocytosis are usually directed toward late endosomes/lysosomes, whereas raft-mediated endocytosis does not target the lysosomes but is directed toward a rapid cycling pathway, via the Golgi or the ER back to the plasma membrane (28–30). This route may contribute to regulate protein/lipid sorting and trafficking (45). Accordingly, ALP is likely to follow such a pathway to inhibit CT in the ER. We have provided direct evidence for this notion; disruption of lipid rafts by MβCD or bacterial sphingomyelin prevented ALP inhibition of PC biosynthesis (Fig. 8).

Raft-mediated endocytosis and associated vesicular traffic is thus an active continuous cellular process (29, 38). We have demonstrated, by ALP uptake and albumin back-extraction data (Figs. 3, 4, and 7), that this process is disrupted in the ALP-resistant S49AR cells. However, the molecular details of this defect remain to be resolved. We showed that the amount of ALP initially (first 30 min) bound to S49 and S49AR cells is the same (Fig. 3) and that ALP partitions equally well in the raft fraction of the two cell types (Fig. 6C). Apparently, another property of the rafts, their molecular composition, and/or their functional dynamics is defective in the resistant cell. Preliminary data suggest a decreased sphingomyelin content in these cells (46) but this needs extensive further elaboration, and is subject of our current investigations.

The conclusion that new, unrestrained PC synthesis is important for cell survival raises the question, why? Not because PC is required for membrane expansion, which is only relevant for cell proliferation, but presumably because this PC synthesis is needed to replenish the continuous and rapid PC breakdown by phospholipases, types A2, C, and D, to generate the respective second messengers, arachidonate (and further metabolites), diacylglycerol, and phosphatidic acid, for different signaling purposes (47, 48). In addition, (new) PC is needed for Golgi-localized synthesis of sphingomyelin and diacylglycerol, both of which are involved in signal transduction as well (49, 50). At least some of these rapid conversions (turnover) of PC are believed to occur at/near the plasma membrane or, more likely, in (recycling) endosomes (51), which have recently been

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appreciated as sites of signal transduction (52). Moreover, there is intriguing evidence suggesting that PC biosynthesis may be regulated in response to the lipid requirements of vesicular trafficking (13, 50, 53). We have previously shown that exogenous LPC, which rescues cells from ALP-induced apoptosis, is first internalized before it is acylated to PC (54). This internalization most probably occurs via endocytosis, because LPC uptake and conversion to PC is severely hampered in the S49AR cells (Fig. 2) that, likewise, are impaired to internalize (by endocytosis) the structurally related ALP (Fig. 3). Thus, the compartment where PC is continuously degraded and where new PC is thus needed, is probably the same one where LPC is converted to PC, likely an endosomal compartment. We therefore speculate that the trigger that initiates apoptosis may actually be located in this endosomal compartment.

If de novo synthesis of PC exclusively occurs in the ER (which is not certain), it is conceivable that this newly made PC merges, through vesicular trafficking or a PC transfer protein, with this endosomal compartment (13, 50, 53).

We have argued (see "Results") that the very rapid internalization of ALP and LPC in S49 cells (Figs. 2 and 3) is not likely explained by transblayer movement (flip-flop) because this process is too slow. Yet, at some stage these lipids must adopt an altered orientation, because their respective "target" enzymes, CT and acyltransferase that would yield PC, are in the S49AR cells (Fig. 2) that, likewise, are impaired to inter-

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REFERENCES

1. Molinelo, F., Fernandez-Luna, J. I., Gajate, C., Martin-Martín, B., Benito, A., Martínez-Dávila, M., and Modéll, M. (1997) Cancer Res. 57, 1320–1328
2. Ruiter, G. A., Zerp, S. F., Bartelink, H., van Blitterswijk, W. J., and Verheij, M. (1999) Cancer Res. 59, 2457–2463
3. van Blitterswijk, W. J., Hilkman, H., and Storme, G. A. (1987) Lipids 22, 820–823
4. Ruiter, G. A., Verheij, M., Zerp, S. F., and van Blitterswijk, W. J. (2001) Int. J. Radiat. Oncol. Biol. Phys. 49, 415–419
5. Pavis, G., Seewald, M. J., Gratas, C., Melder, D., Rehbo, J., and Mender, E. J. (1992) Cancer Res. 52, 2835–2840
6. Zhou, X., and Arthur, G. (1995) Eur. J. Biochem. 232, 881–888
7. Boggs, K. P., Rock, C. O., and Jackowski, S. (1995) J. Biol. Chem. 270, 7757–7764
8. Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E., and Terre, F. (1996) J. Biol. Chem. 271, 14668–14671
9. Veen, C. L., Mar, M. H., and Zeisel, S. H. (1999) FASEB J. 13, 135–142
10. Yen, C. L., Mar, M. H., Meeke, R. B., Fernandes, A., and Zeisel, S. H. (2001) FASEB J. 15, 1704–1710
11. Babiru, I., and Jackowski, S. (1998) J. Biol. Chem. 273, 2169–2173
12. Miquel, K., Pradines, A., Verheij, M., and Van Rooij, H., and Salomons, G. S. (1990) Cancer Res. 50, 7505–7512
13. Kabarowski, J. H., Zhu, K., Le, L. Q., Witte, O. N., and Xu, Y. (2001) Science 293, 702–705
14. Zoeller, R. A., Laye, M. D., and Estolic, S. H. (1999) J. Biol. Chem. 274, 1866–1875
15. Bazill, G. W., and Dexter, T. M. (1990) Cancer Res. 50, 820–823
16. Kabarowski, J. H., Zhu, K., Le, L. Q., Witte, O. N., and Xu, Y. (2001) Science 293, 702–705
17. Ruiter, G. A., Zerp, S. F., Bartelink, H., van Blitterswijk, W. J., and Verheij, M. (2001) Int. J. Cancer 97, 811–819
18. Kelley, E. E., Moolenaar, W. H., and van Blitterswijk, W. J. (2002) Biochemistry 41, 2435–2439
19. Simall, G. W., Nurre, J. R., and Duggal, S. (1997) Lipids 32, 715–723
20. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
21. Smetts, I. A., Van Rooij, H., and Salomons, G. S. (1999) Apoptosis 4, 419–427
22. Nicotelli, L., Migrans, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) J. Immunol. Methods 139, 271–279
23. Sipani, M. F., Tang, Z., Scherer, P. E., and Sargiacomo, M. (1995) Methods Enzymol. 250, 655–668
24. Boggs, K. P., Rock, C. O., and Jackowski, S. (1995) J. Biol. Chem. 270, 16112–16118
25. van Meer, G., Stelzer, E. H., Wijnands-van Rees, L. W., and Simons, K. (1987) J. Cell Biol. 105, 1623–1635
26. Sprong, H., van der Ver, S. P., and van Meer, G. (2001) Nat. Rev. Mol. Cell. Biol. 2, 504–513
27. Tartakoff, A. M. (1983) Cell 32, 1026–1028
28. Hone, E. (2001) Curr. Opin. Cell Biol. 13, 470–477
29. Pur, W., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2001) J. Cell Biol. 154, 553–557
30. Naturels, B. J., and Lippincott-Schwarz, J. (2001) Trends Cell Biol. 11, 406–412
31. Mattiju, A. B., and Sotto, J. P. (1996) Langmuir 12, 1284–1290
32. van Blitterswijk, W. J., van der Meel, B. W., and Hilkman, H. (1987) Biochemistry 26, 1746–1756
33. Tepper, A. D., Ruurs, P., Wiedner, T. Sims, P. J., Strop, J., and van Blitterswijk, W. J. (2000) Cell 100, 155–164
34. van der Blik, A. M., and van der Bliek, A. M. (1991) Nature 351, 411–414
35. Oh, P., McIntosh, D. P., and Schnitzer, J. E. (1998) J. Cell Biol. 141, 101–114
36. Henley, J. R., Krueger, E. W., Oswald, B. J., and McVee, N. A. (1998) J. Cell Biol. 141, 85–99
37. Pagano, R. E., and Sletten, C. G. (1985) Science 229, 1051–1057
38. Hoo, M., and Maxfield, F. R. (2000) J. Biol. Chem. 275, 15279–15286
39. Porter, R. C. (1996) Curr. Opin. Cell Biol. 8, 542–548
40. Observa, P. A., and Fisher, J. P. H. (1998) J. Cell Biol. 141, 905–915
41. Torgerson, E. L., Skegg, C., de, D., and Sandvig, K. (2001) J. Cell Sci. 114, 3737–3747
42. Lamonza, C., Dojancoeur, A., Bata, T., Le, C. G., Benmerah, A., and Dautry-Varsat, A. (2001) Mol. Cell 7, 661–671
43. Rosenberger, C. M., Brunell, J. H., and Finlay, B. B. (2000) Curr. Biol. 10, R823–R825
44. Pellemans, L., Kartenbeck, J., and Helenius, A. (2001) Nat. Cell. Biol. 3, 473–483
45. Gagusc, G., Monameur, N., Parton, R. G., Huanzer, W., Huber, L. A., and Gruenhagen, J. (2000) Mol. Biol. Cell 11, 2775–2791
46. van Blitterswijk, W. J., Van der Luit, A. H., Hilkman, H., and Moolenaar, W. H. (1998) Eur. J. Biochem. 258, 386–392