Localization of Fas/CD95 into the Lipid Rafts on Down-Modulation of the Phosphatidylinositol 3-Kinase Signaling Pathway

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Abstract

Activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway is known to protect tumor cells from apoptosis and more specifically from the Fas-mediated apoptotic signal. The antitumoral agent edelfosine sensitizes leukemic cells to death by inducing the redistribution of the apoptotic receptor Fas into plasma membrane subdomains called lipid rafts. Herein, we show that inhibition of the PI3K signal by edelfosine triggers a Fas-mediated apoptotic signal independently of the Fas/FasL interaction. Furthermore, similarly to edelfosine, blockade of the PI3K activity, using specific inhibitors LY294002 and wortmannin, leads to the clustering of Fas whose supramolecular complex is colocalized within the lipid rafts. These findings indicate that the antitumoral agent edelfosine down-modulates the PI3K signal to sensitize tumor cells to death through the redistribution of Fas into large platform of membrane rafts. (Mol Cancer Res 2008;6(4):604–13)

Introduction

Fas (CD95/APO-1) belongs to the tumor necrosis factor receptor family and triggers an apoptotic signal, playing a pivotal role in the homeostasis of the immune system (1). Dysfunction of the Fas pathway leads to autoimmune diseases and lymphoma progression (2, 3). Fas contains an intracellular domain, termed death domain, which serves as a docking platform initiating the apoptotic signal. On binding to its cognate ligand, FasL, Fas recruits the adaptor molecule Fas-associating protein with death domain (FADD), which in turn aggregates the initiator caspase-8 and caspase-10. FADD carries a death domain and a death effector domain, which are essential to bind via homotypic interactions the receptor Fas and the proteases caspase-8 and caspase-10, respectively. This molecular complex (Fas/FADD/caspases) is termed death-inducing signaling complex (DISC; ref. 4). The concentration of these caspases in a condensed area allows their activation and the release in the cytoplasm of activated caspase-8 and caspase-10, thereby leading to the triggering of the apoptotic signal. According to the DISC formation, cells have been classified in two types: in type I cells, Fas engagement leads to the efficient formation of the DISC and a large amount of active caspases is released in the cytoplasm, and in contrast, in type II cells, DISC formation and caspase release are impaired (5).

Owing to the importance of Fas in homeostasis, this proapoptotic cascade is under the tight control of several factors, among which is the phosphatidylinositol 3-kinase (PI3K) signaling pathway. This signal is found constitutively activated in different solid tumors or leukemia cells and is involved in cell transformation as well as in tumor relapse after treatment with anticancer drugs (6). The function of class I PI3Ks is to convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the inner leaflet of the plasma membrane (see ref. 7 for review). The PIP3 production is tightly controlled and its amount is counterbalanced by the action of several PIP3 phosphatases (PTEN, SHIP1, and SHIP2). An increased production of PIP3 leads to the binding of intracellular enzymes containing pleckstrin homology domains, such as the kinase Akt (protein kinase B). Once Akt is recruited to the inner leaflet of the plasma membrane, this serine-threonine kinase undergoes activation through its phosphorylation on Thr^{308} and Ser^{473}. Activated Akt phosphorylates a variety of substrates involved in pleiotropic cell functions, such as proliferation,

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survival, glucose metabolism, and cytoskeletal changes (8). In T lymphocytes, activation of PI3K on CD3/CD28 cotriggering protects cells from a premature elimination by Fas-mediated cell death (9). This PI3K-mediated protection is achieved through induction of antiapoptotic genes, among which are *Bcl-xL* or *c-FLIP* (10, 11), through inhibition of the Bcl-2 antagonist of cell death apoptotic function (12), and through abrogation of the DISC formation by a yet unknown mechanism (9).

The classic fluid mosaic model of the cell membrane introduced by Singer and Nicolson in 1972 (13) has been extended in the last decade with the lipid raft theory (14, 15). Indeed, the extracellular leaflet is now depicted as a heterogeneous structure containing compacted proteolipidic assemblies (ordered phase) that float into the rest of a more fluid membrane (liquid phase). These subdomains are enriched in sphingolipids and cholesterol and they are resistant to cold lysis using nonionic detergents. These subdomains are currently designated as detergent-resistant microdomains, glycosphingolipid-enriched microdomains, lipid rafts, membrane rafts, or simply microdomains. We and others have reported that the Fas-mediated apoptotic signal is augmented when Fas is redistributed into these domains of the plasma membrane (16-18). Moreover, the canonical Fas-mediated apoptotic signal can be activated independently of the Fas/FasL interaction via the sole redistribution of Fas into the lipid rafts (18, 19). The ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3 or edelfosine) belongs to a novel class of promising antitumor agents (20, 21). When added to cell cultures, this lipid incorporates into the membrane of leukemic cell lines and induces the redistribution of Fas into the lipid rafts and the elimination of the tumor cell through a Fas-dependent but FasL-independent apoptotic signal (19). However, to date, the molecular mechanisms triggered by edelfosine and involved in the mobilization of Fas into lipid rafts remain unknown (22).

Because (*a*) the PI3K signaling pathway has been involved in controlling the lateral mobility of Fas in the plasma membrane (23) and (*b*) this antiapoptotic signal is well known to prevent the Fas-mediated apoptotic signal, we investigated whether inhibition of the PI3K activity could account for the redistribution of Fas into the lipid rafts observed on the edelfosine treatment (22).

Results

Edelfosine Modifies the Membrane Distribution of Fas and Is an Inhibitor of the PI3K Signaling Pathway

To confirm that the Fas signal is necessary for the edelfosine-mediated cell death signal, we tested the sensitivity of a Fas mutant-expressing Jurkat cell line on edelfosine treatment. We previously showed that hemizygous expression of a death domain–mutated Fas (FasQ257K) does not prevent the Fas-mediated apoptotic signal triggered on FasL incubation but significantly reduces it (24). Similarly, addition of edelfosine to the culture treatment killed less efficiently the FasQ257K-expressing Jurkat cell line than the parental cell (Fig. 1A).

Because it has been reported that edelfosine induces the Fas signal through the redistribution of Fas inside the detergent-resistant microdomains (19), we next determined whether the disruption of the lipid rafts was able to abrogate the edelfosine-induced apoptotic signal. Integrity of the lipid rafts depends on the concentration of cholesterol into the plasma membrane (15, 25), and preincubation of Jurkat cells with the methyl-β-cyclodextrin (MβCD), a chelator of cholesterol, dismantles these lipid rafts (26). Lipid rafts are considered resistant to cold lysis by nonionic detergent, and due to their lipid composition, they exhibit a lower density than the rest of the membrane. Owing to these properties, lipid rafts can be separated from the rest of the membrane after ultracentrifugation of a cell lysate into a discontinuous sucrose gradient (see Materials and Methods). We previously found that, in the T leukemia cell line Jurkat, the transmembrane protein CD28 is enriched into the microdomains (17); therefore, to confirm that MβCD treatment efficiently disrupts lipid rafts, we followed the submembrane localization of CD28 in Jurkat cells treated with

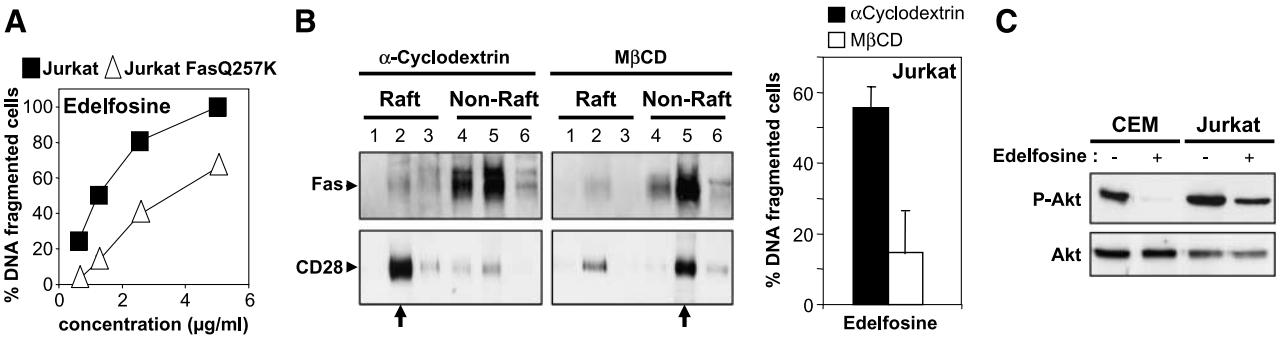

FIGURE 1. Edelfosine-mediated cell death. Apoptosis was quantified by propidium iodide staining and results were expressed as percentage of cells in the sub-G_1 phase. **A.** Parent and FasQ257K Jurkat cells were untreated or treated for 20 h with edelfosine before the sub-G_1 population was quantified using flow cytometry. **B.** Jurkat (10^8 cells per condition) was treated with 5 mmol/L of MβCD or α-cyclodextrin (control), lysed, and subjected to a sucrose gradient ultracentrifugation. For each fraction harvested (six fractions), 10 μg of proteins were loaded in a 12% SDS-PAGE and the presence of Fas and CD28 was detected by immunoblot using specific antibodies. Arrows, redistribution of CD28 from the lightest "raft" fractions to the "nonraft" fractions on MβCD treatment. Jurkat cells were pretreated with 5 mmol/L of MβCD or α-cyclodextrin (control) for 30 min, washed, and incubated for 8 h in serum-free medium in the presence of 5 μg/mL edelfosine. Columns, mean of three experiments; bars, SD. **C.** CEM and Jurkat cell lines were incubated for 2 h with 5 μg/mL edelfosine and phospho-Akt (*P-Akt*; *top*) and total Akt (*bottom*) was detected by immunoblotting. The data are representative of at least three independent experiments.

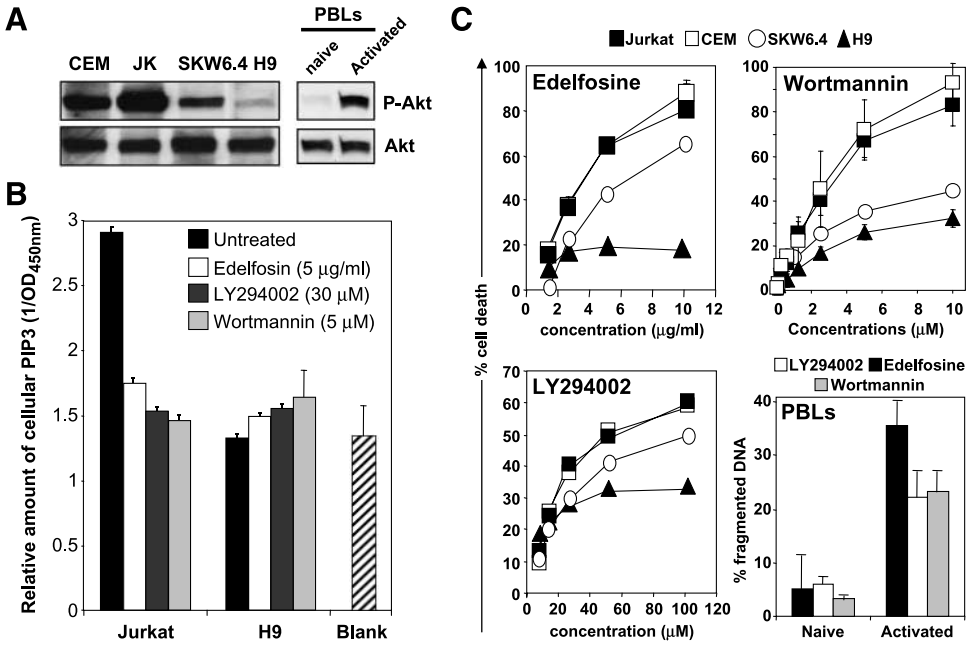

FIGURE 2. Edelfosine-induced cell death is preferentially transmitted in cells harboring a strong Akt activation. **A.** The T leukemia cell lines H9, CEM, and Jurkat or the B leukemia cell line SKW6.4 were lysed and the cellular Akt activity was determined by immunoblot using a phospho-Akt and a whole Akt protein antibodies. PBLs were harvested and activated as described in Materials and Methods. **B.** Using a competitive PIP3 ELISA (see Materials and Methods), we determined the amount of PIP3 in cells untreated or treated for 4 h with the indicated doses of PI3K inhibitors and edelfosine. Because the PIP3 ELISA is a competitive ELISA, the relative amount of PIP3 isolated from each cell was calculated using 1/absorbance at 450 nm ($1/OD_{450nm}$). A solution devoid of PIP3 was used to determine the blank. Columns, average of three independent experiments; bars, SD. **C.** The sensitivity of the indicated cells toward the PI3K inhibitors or the edelfosine incubation was assessed using a MTT assay. Cells were incubated for 16 h with the indicated agents. Columns, mean of three independent experiments; bars, SD.

the cholesterol chelator or with its nonactive counterpart (α-cyclodextrin; ref. 17). In α-cyclodextrin–treated cells, CD28 was concentrated into lipid rafts, whereas after treatment with a nontoxic dose of MβCD, CD28 was found enriched in the nonlipid raft membranes corresponding to the fractions harvested in the bottom of the ultracentrifuge tube (Fig. 1B). As it can be observed in Fig. 1B, a faint amount of Fas was localized into the lipid rafts (Fig. 1B) and the MβCD treatment slightly decreased the quantity of Fas present in these microdomains (Fig. 1B). We previously showed that, due to the weak amount of Fas distributed into the lipid rafts in the Jurkat cell line, the Fas-mediated apoptotic signal remained unchanged once lipid rafts are disturbed (data not shown; refs. 17, 27). On the other hand, the dismantling of detergent-resistant microdomains via the MβCD treatment efficiently prevented edelfosine-mediated apoptotic signal (Fig. 1B). These findings supported that the antitumoral effect mediated by edelfosine mainly relied on the redistribution of Fas into the lipid rafts (19).

Recently, edelfosine has been described as an inhibitor of the PI3K signal in different carcinoma cell lines through a still unknown mechanism (28). We analyzed whether edelfosine was also able to impair the PI3K activity in different leukemic cell lines. Untreated CEM and Jurkat cells constitutively exhibited an important level of Akt phosphorylation (Fig. 1C), which is a well known consequence of the PIP2 and PIP3 lipid generation by active PI3K. As observed in Fig. 1C, edelfosine dramatically decreased the amount of cellular phospho-Akt, whereas the amount of the whole protein remained unchanged (Fig. 1C). Taken together, these results indicate that edelfosine down-modulates the PI3K activity in leukemic cells, whereas its cytotoxic activity requires Fas and lipid raft integrities.

Edelfosine, Similarly to PI3K Inhibitors, Selectively Induces Cell Death in Cells Expressing High PI3K Activity

To study whether the apoptotic signal triggered by edelfosine effectively relies on PI3K inhibition, we graded various T and B leukemia cells according to their level of basal Akt activation. Three groups of cells were selected: one displaying a strong PI3K activation (T leukemia cells CEM, Jurkat, and activated T lymphocytes), one displaying an intermediate PI3K activation (B lymphoma cell line SKW6.4), and, finally, one displaying a weak PI3K activation (T leukemia cell line H9 and naive T lymphocytes; see Fig. 2A). Freshly isolated peripheral blood lymphocytes (PBL) did not harbor any phosphorylation of the kinase Akt, whereas in activated PBLs Akt is strongly phosphorylated and activated (Fig. 2A). Similarly to edelfosine, commercially available PI3K inhibitors, wortmannin and LY294002, efficiently abrogated the activation of the kinase Akt (Supplementary Fig. S1). It is noteworthy that the leukemic T-cell line Jurkat is deficient in protein expression of the lipid phosphatases Src homology 2 domain containing inositol polyphosphate phosphatase (SHIP) and the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which normally counterbalance the

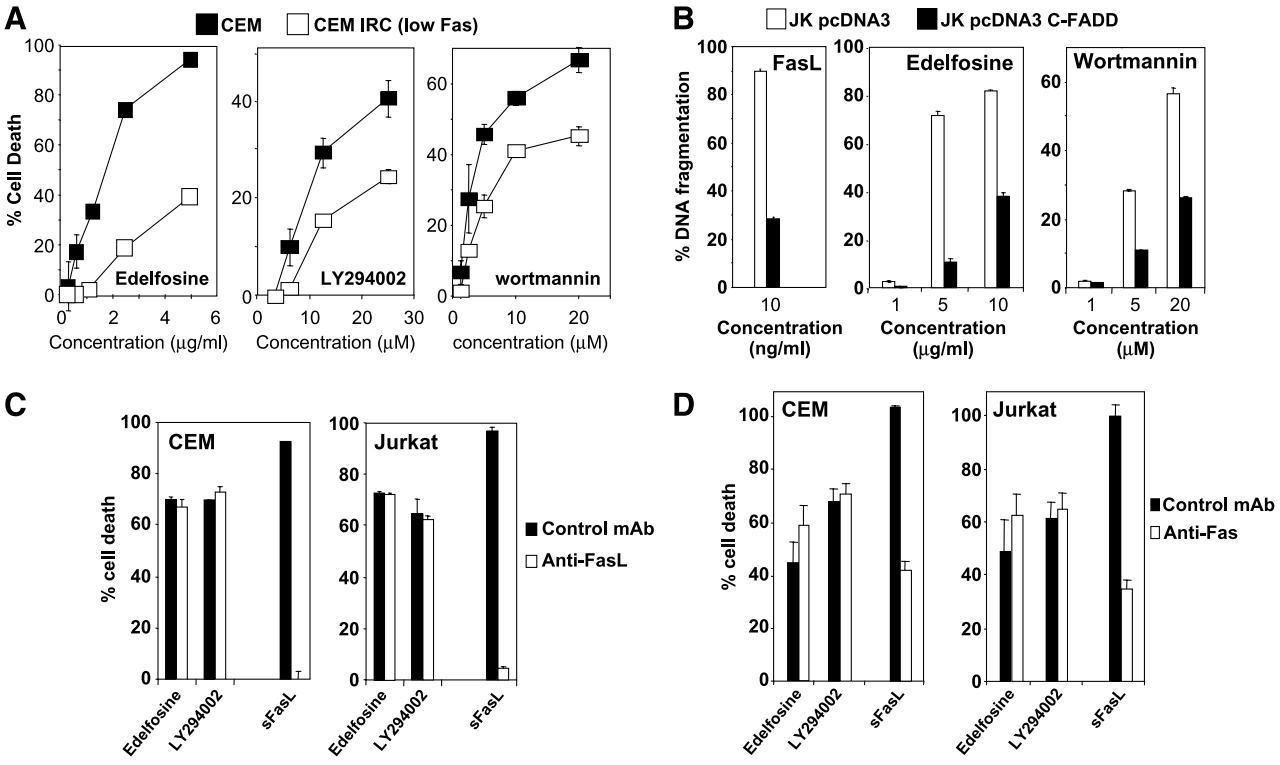

FIGURE 3. Edelfosine or PI3K inhibitors induce a Fas-mediated apoptotic signal independent of the Fas/FasL interaction. **A.** CEM (■) and CEM-IRC (□) were incubated 20 h with specific PI3K inhibitors or edelfosine and cell death was quantified using MTT assay. **B.** Stable Jurkat cells expressing empty vector or C-FADD were incubated for 20 h with the indicated agent and cell death was measured using a propidium iodide assay. **C.** CEM and Jurkat cells were preincubated for 30 min with 10 μg/mL of anti-FasL blocking antibody (clone 10F2) or isotype-matched control and treated with edelfosine (5 μg/mL), LY294002 (50 μmol/L), wortmannin (10 μmol/L), or soluble FasL (*sFasL*; 1 μg/mL) for 16 h. Viability was measured using a MTT assay. **D.** Cells were preincubated with 10 μg/mL of a blocking anti-Fas antibody (clone ZB4) or isotype-matched control and then treated as described above. Viability was measured using a MTT assay. In all four panels, data depict mean ± SD of three independent experiments.

PI3K-mediated *de novo* generation of PIP3 and hence the level of Akt phosphorylation (29). Thus, in the Jurkat cell line, the significant decrease of the Akt phosphorylation observed following edelfosine addition to cell culture could be associated to a direct down-modulation of the PI3K activity or to the abrogation of the PIP3 docking action.

The leukemic T-cell line Jurkat is devoid of SHIP and PTEN expressions (12), implying that in this cell line any modification of the PIP3 level will be associated to a direct effect on the PI3K activity. Because H9 cell line did not exhibit any activation of Akt (Fig. 2A), the cell was used to identify the baseline level of PIP3 present in a cell line. To show that edelfosine impaired the PI3K activity and so abrogated the production of PIP3, the cellular content of PIP3 was quantified using a PIP3-competitive ELISA. We confirmed that the amount of PIP3 in the H9 cell line was trivial because it was identical to the blank level (Fig. 2B). On the other hand, a high quantity of PIP3 was measured in the Jurkat cell line (Fig. 2B). Although edelfosine and the selective PI3K inhibitors LY294002 and wortmannin do not alter the PIP3 amount quantified in the H9 cell line, all these agents dramatically decreased the total amount of PIP3 in the Jurkat cell line to bring it down to the blank level (no PIP3).

Taken together, these findings strongly support the conclusion that edelfosine acts directly as a potent inhibitor of the PI3K activity, but to date, the molecular mechanism involved in this inhibition remains unknown.

Edelfosine, which was very efficient at killing CEM and Jurkat cells, was ineffective at eliminating both H9 cell line and naive PBLs (Fig. 2C). The SKW6.4 cell line possessed an intermediate sensitivity to this drug (Fig. 2C). To determine whether the inhibition of the PI3K signal behaves as edelfosine on these cell lines, we tested LY294002 and wortmannin on the different cell lines and T lymphocytes. Strikingly, these PI3K inhibitors displayed a similar apoptotic effect on the different cell lines and PBLs compared with edelfosine treatment. Indeed, edelfosine and the PI3K inhibitors exerted a trivial effect on cells harboring a weak PI3K activity (naive T lymphocytes or the H9 cell line), whereas, in contrast, cell death was efficiently transmitted in cells expressing a potent PI3K activity. Because we noticed a strong correlation between the activation status of the kinase Akt and the effect of edelfosine, we suggest that edelfosine mediates its Fas-dependent apoptotic signal (Fig. 1A; refs. 19, 22) through inhibition of the PI3K signal.

Inhibition of the PI3K Activity Triggers a Fas-Mediated Cell Death Independently of Fas/FasL Interaction

To formally prove that, similarly to edelfosine, PI3K inhibition induces a Fas-mediated apoptotic signal, we generated two cell lines carrying a defect in the Fas signaling

pathway. The Fas-deficient CEM cell line (CEM-IRC) has been obtained by a prolonged culture in the presence of a saturating concentration of soluble multimeric FasL. This cell line, which expressed a reduced amount of membrane Fas compared with the parental CEM, proved to be resistant to Fas-mediated cell death (Supplementary Fig. S2A). As well, the CEM-IRC was also resistant to the apoptotic signal triggered by incubation with the PI3K inhibitors and edelfosine (Fig. 3A). Expression of a truncated FADD molecule devoid of its death effector domain (C-FADD) has been shown to alter the DISC formation and to impair the Fas signaling pathway (30). Therefore, to show that the Fas signaling pathway was instrumental for the apoptosis mediated by PI3K inhibition, we generated a Jurkat cell line that stably overexpressed C-FADD. As can be seen in Fig. 3B, this cell line was resistant to both the Fas-mediated cell death and edelfosine or PI3K inhibitor treatments compared with the parental cell line (Fig. 3B). These data support that induction of apoptosis following PI3K inhibition requires an intact Fas signaling pathway.

Some antitumoral drugs have been described to eliminate tumor cells by triggering a Fas-dependent apoptotic signal via two distinct mechanisms. Whereas anticancer drugs such as doxorubicin and bleomycin promote *de novo* FasL expression and kill Fas-expressing tumor cells via suicide or fratricide (31, 32), edelfosine and cisplatin lead to the redistribution of Fas into the lipid rafts, which in turn allows the induction of the Fas pathway independently of the presence of its ligand (22, 33). In the first situation, the apoptotic signal can be abrogated by pretreating the cells with blocking anti-Fas or anti-FasL antibodies (31, 32), whereas in the second such antibodies are inefficient to prevent the Fas-mediated apoptotic signal. To distinguish between these two possibilities, we tested the efficiency of Fas or FasL blocking antibodies to inhibit cell death triggered on treatment with the different PI3K inhibitors. Although the blocking monoclonal antibodies (mAb) targeting Fas (clone ZB4) or FasL (clone 10F2) dramatically blocked the apoptotic signal induced by FasL in CEM or Jurkat, they did not influence cell death triggered by the PI3K inhibitors (Fig. 3C and D). Next, the expression of FasL was analyzed at the cell surface (flow cytometry) and in the whole cell (immunoblot on total lysates) after incubation with the different inhibitors of PI3K. CEM and Jurkat cells do not express any detectable amount of FasL (Supplementary Fig. S3). These results clearly showed that inhibition of the PI3K signal induces an apoptotic signal, which relies on the Fas pathway but is independent of the interaction of Fas with its natural ligand, FasL. Taken together, these findings suggested that the Fas-dependent apoptotic signal observed on PI3K inhibition may be dependent on the redistribution of the death receptor into the lipid rafts.

Inhibition of the PI3K Signaling Pathway Leads to the Redistribution of Fas into Low-Density Lipid Rafts

Because edelfosine triggers the membrane capping of Fas and its redistribution into lipid rafts by a still unknown mechanism (19, 22, 34), we investigated whether the PI3K inhibition could lead to the redistribution of Fas into the lipid rafts.

Lipid rafts were isolated from cells with high (Jurkat and CEM) or low (H9) PI3K activity using a discontinuous sucrose gradient ultracentrifugation. Fractions were harvested from the top to the bottom of the gradient tube and Fas was detected by Western blotting (Fig. 4A). As CD28, the lipid raft marker used in the Jurkat cell line, was not detected at the plasma membrane of the CEM, we decided to use another marker of the lipid rafts, the tyrosine kinase $p56^{Lck}$. This src kinase was found concentrated almost exclusively in the fractions 1 to 3 of the gradient. Strikingly, a significant amount of Fas was present in the lipid raft fractions of the H9 cell line (PI3K activity impaired), whereas it was barely detectable into the lipid raft fractions of the two leukemia cells CEM and Jurkat, which exhibit a strong Akt activation (Fig. 4A). To prove that PI3K activity controls the lipid environment of Fas in the cell membrane and that inhibition of this signaling pathway contributes to the redistribution of the apoptotic receptor into the lipid rafts, we incubated Jurkat and CEM cell lines with LY294002 or wortmannin and subsequently analyzed the

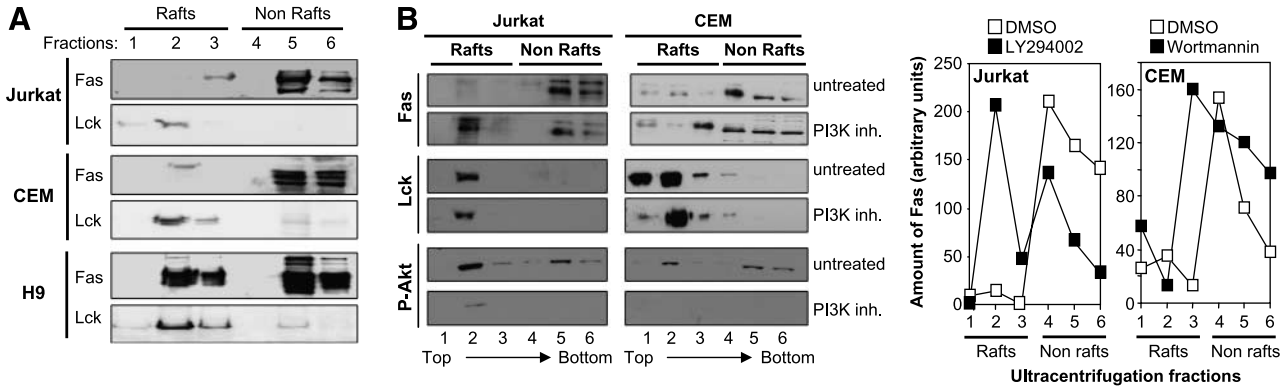

FIGURE 4. Inhibition of the PI3K signaling pathway induces the redistribution of Fas into the low-density microdomains. **A.** Lipid rafts were isolated using the sucrose gradient fractionation method as described in Materials and Methods. For each harvested fraction, 10 μg of proteins were loaded in a 12% SDS-PAGE. Lck (lipid raft marker) and Fas distribution were analyzed by immunoblotting. **B.** Jurkat or CEM cell lines (10^8 per condition) were treated or untreated for 4 h with LY294002 (20 μmol/L) or wortmannin (5 μmol/L), respectively, lysed, and subjected to a sucrose gradient ultracentrifugation. For each fraction harvested (six fractions), 10 μg of proteins were loaded in SDS-PAGE and the presence of Fas, Lck, and phospho-Akt was analyzed by immunoblot using specific antibodies. A densitometric analysis of the Fas amount present in each fraction was done using ImageJ software (W.S. Rasband, NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/).

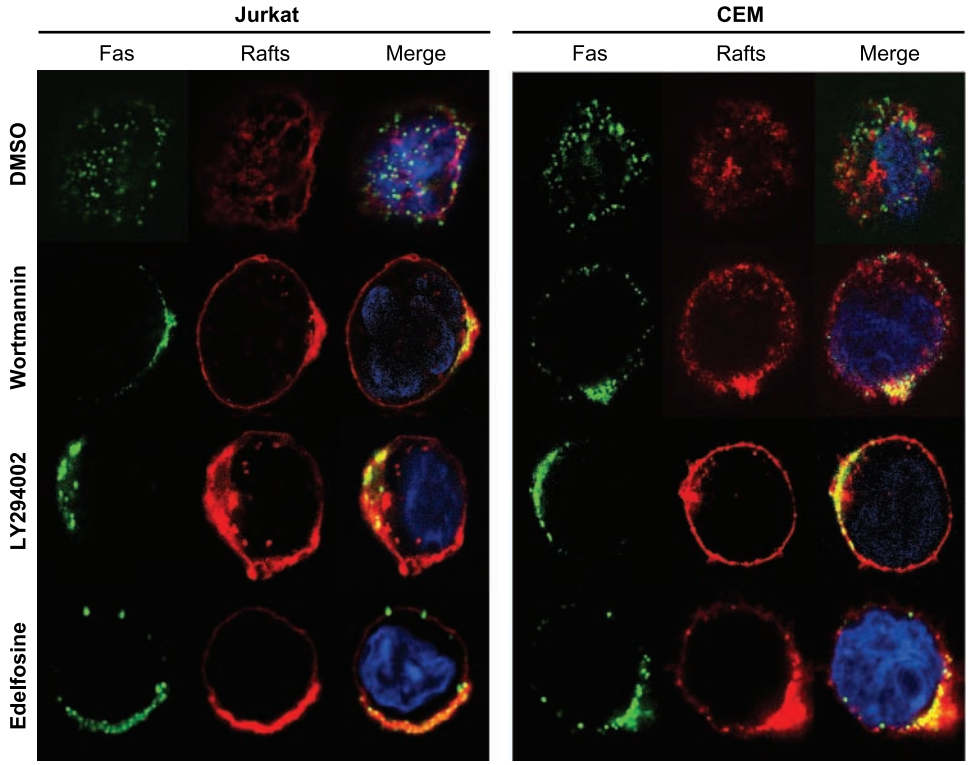

FIGURE 5. Capping of Fas is induced on PI3K inhibition and colocalizes with membrane lipid rafts. Jurkat and CEM cell lines were incubated for 4 h with LY294002 (20 μmol/L), wortmannin (5 μmol/L), and edelfosine (5 μg/mL) or left untreated. Green, Fas localization was followed using an anti-Fas mAb and a secondary Alexa Fluor 488–conjugated goat anti-mouse secondary antibody; red, microdomains were stained using Alexa Fluor 555–labeled cholera toxin subunit B; yellow, membrane area where Fas and lipid rafts were merged; blue, nuclei were stained using 4′,6-diamidino-2-phenylindole.

distribution pattern of Fas within the ultracentrifugation gradient. LY294002 or wortmannin treatment efficiently inhibited the PI3K activity because the phosphorylated form of Akt disappeared (Fig. 4B, *bottom*). Concomitantly to the inhibition of the PI3K activity, we observed that a significant amount of Fas was redistributed into the lipid raft–containing fractions in type II cell lines Jurkat and CEM (Fig. 4B, *top*). The enrichment of Fas into the lipid rafts was accurately quantified using ImageJ software (35) by scan analysis of the Fas bands (Fig. 4B). In conclusion, using a biochemical method for analyzing lipid rafts, we showed that the intracellular PI3K signal is mandatory to exclude Fas from the lipid rafts, whereas its down-modulation led to the enrichment of Fas into the microdomains.

To further confirm that Fas and lipid rafts colocalize on PI3K inhibition, we followed the plasma membrane distribution of Fas using confocal microscopy. PI3K inhibition potently triggered the clustering of Fas, which was estimated to reach a size around the micrometer range (green staining, Fig. 5). To determine whether these clusters of Fas were merged with the sphingolipid-enriched microdomains, we simultaneously stained Fas (green staining) and lipid rafts using the Alexa Fluor 555–labeled cholera toxin subunit B (red). This toxin is currently used to stain the lipid rafts because it possesses a high affinity for the monosialoganglioside GM1, which is concentrated into the microdomains (36). Confirming the results obtained using the sucrose gradient ultracentrifugation method, the untreated CEM and Jurkat cell lines did not exhibit colocalization of Fas with lipid rafts as visualized by confocal microscopy analysis (condition DMSO in Fig. 5). In contrast, PI3K inhibitors induced the formation of Fas clusters, which were colocalized with the cholera toxin B-FITC staining (Fig. 5). It is noteworthy that although the distribution of Fas into the lipid rafts was almost exclusive, an important part of the microdomains did not contain Fas. It is conceivable that, based on their molecular content, lipid rafts are not a uniform and homogenous pool constituted of identical domains but rather, as we previously showed, different types of lipid rafts may coexist and are consequently able to transmit distinct signals (27). Accordingly, we propose that, on PI3K inhibition, Fas is efficiently capped, whereas only a part of the cholesterol-enriched domains are mobilized.

To generalize the data obtained with the CEM cell line, we examined the status of the acute promyelocytic leukemia cell line HL-60, which displays a high PI3K activity (data not shown) and has been previously used extensively to characterize the redistribution of Fas into the lipid rafts on edelfosine treatment (19). Similarly to Jurkat and CEM cell lines, HL-60 cell line is highly sensitive to the inhibition of the PI3K signaling pathway (Fig. 6A, *left*). As depicted in Fig. 6A (*right*), the inhibitors of PI3K significantly increased the number of cells harboring Fas clusters colocalized with lipid rafts. Using confocal microscopy, we observed that Fas was excluded from the lipid rafts (Fig. 6B, *top*) in the untreated cells, whereas it was clustered and redistributed into these microdomains on PI3K inhibition or edelfosine treatment. To qualitatively show that Fas (green) and lipid rafts (red) were merged, a line was drawn around the cell perimeter (plasma membrane), and this region of interest was separately analyzed to the exclusion of the rest of the image for the intensity of each fluorescence (green and red; see Fig. 6B, *right* and *bottom*). Scan analysis of the cell perimeter clearly showed that the

intensity of green and red fluorochromes was accurately superimposed and, as a consequence, confirmed that the PI3K inhibition induced the redistribution of Fas inside a lipid environment enriched in GM1, the lipid rafts.

Discussion

Herein, we show that inhibition of the PI3K signaling pathway triggers the rapid formation of Fas cluster and the redistribution of Fas into the so-called lipid rafts. We also bring new evidences that the promising antitumoral agent edelfosine efficiently down-modulates the PI3K signal in T leukemia cell lines and in activated T lymphocytes, leading to (*a*) the induction of a Fas-mediated apoptotic signal and (*b*) the formation of micrometer range clusters of Fas. To our knowledge, this study is the first to link a molecular target of edelfosine to the previously well-described redistribution of Fas into the lipid rafts, leading to the elimination of leukemia cells (22, 34, 37). The class I PI3Ks are found constitutively activated in numerous leukemia cell lines and in cells isolated from various types of leukemia-suffering patients (38). The inhibition of the PI3K pathway promotes apoptosis in leukemia cells or activated PBLs and promotes the capping of Fas and its redistribution into the lipid rafts, which are essential processes in the efficient progression of the Fas-mediated apoptotic signal (16-18, 39).

As the T leukemic cell line Jurkat expresses neither the phosphatase SHIP nor the phosphatase PTEN, which normally counterbalances the PI3K-mediated *de novo* generation of PIP3 and hence the level of Akt phosphorylation (29), the significant decrease of the Akt phosphorylation observed following edelfosine treatment can reasonably be assigned to its sole

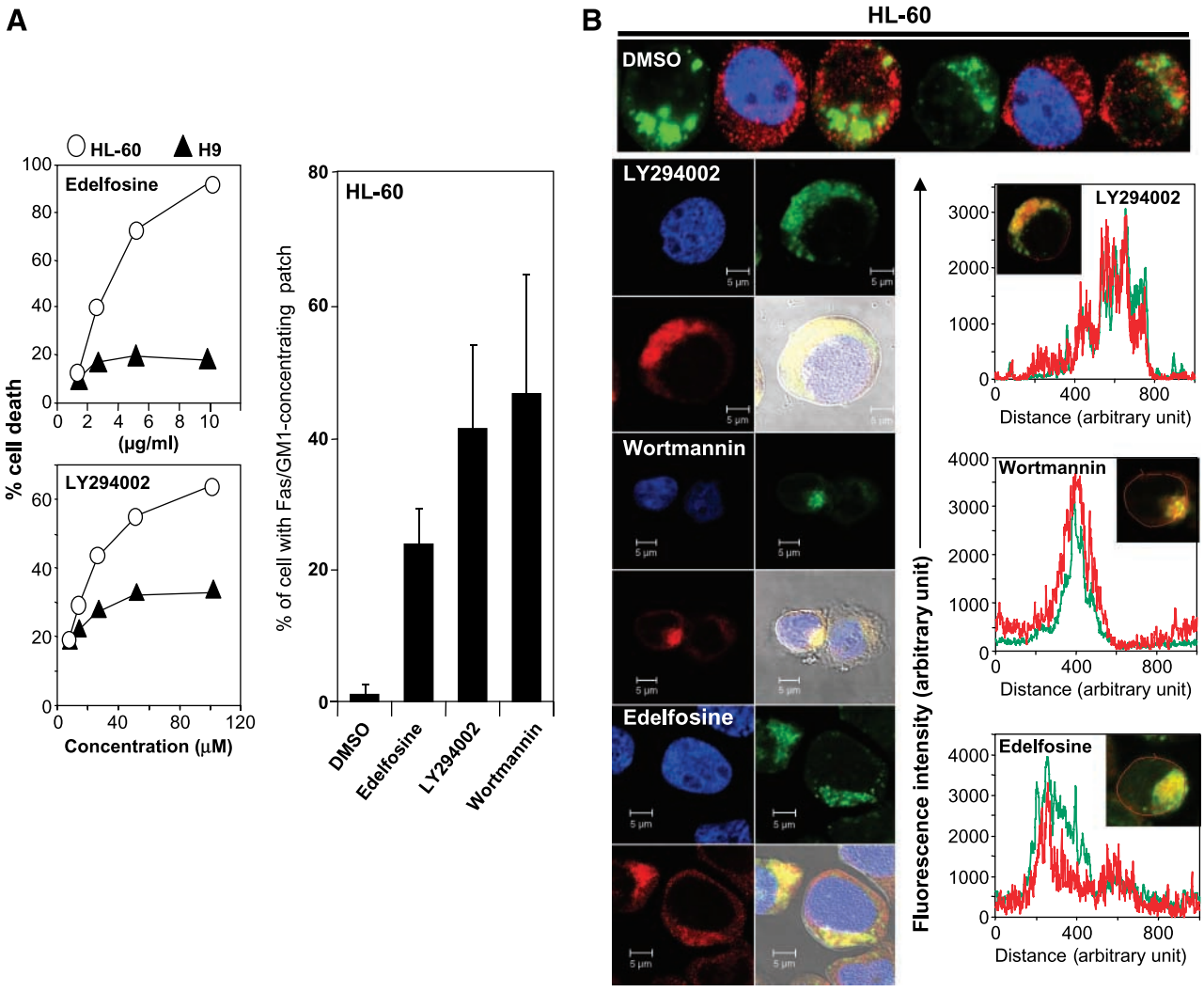

FIGURE 6. Fas and lipid rafts colocalize on PI3K inhibition. **A.** Left, H9 (▲) and HL-60 (○) cell lines were incubated for 20 h with edelfosine or LY294002 and viability was measured with a MTT assay. The HL-60 cell line was incubated for 2 h with the indicated agents. Cells were adhered on a poly-L-lysine slide, and then Fas and microdomains were stained. The percentage of cells exhibiting a clustering of Fas colocalizing with the lipid raft staining was quantified. At least 500 cells were counted for each condition. **B.** HL-60 cells were incubated with the indicated agents for 2 h, stained, and analyzed using confocal microscopy. Red and green fluorescence intensities at the plasma membrane were quantified around the perimeter of the cells and plotted in the corresponding graphs. *X* axis, distance around the periphery of the cell; *Y* axis, relative fluorescence intensity of the pixels.

inhibition of the PI3K. Moreover, recently, it has been reported that fluorescent analogue of edelfosine incorporates selectively into the plasma cell bilayer of leukemia cells and activated T lymphocytes, whereas it does not stain naive T cells (19). To date, we could envisage that (*a*) by virtue of its lipid nature edelfosine acts directly on the PI3K to down-modulate its activity or (*b*) its incorporation into the plasma membrane bilayer impinges on the PI3K substrate, PIP3, modifying its docking activity and thus preventing the downstream recruitment of Akt. The accurate characterization of edelfosine molecular target(s) will be essential as it would help to define new therapeutic agents and to understand the molecular ordering responsible for the Fas redistribution into the lipid rafts.

Herein, we showed that cell death following PI3K inhibition needs the integrity of the Fas signaling pathway. However, the overexpression of the dominant-negative mutant FADD, which impairs the formation of the DISC, was more efficient to prevent cell death triggered on PI3K abrogation than is the expression of a dominant-negative Fas mutant (FasQ257K) or the down-expression of membrane Fas (CEM-IRC). It is well established that, similarly to Fas, the tumor necrosis factor–related apoptosis-inducing ligand receptors death receptors 4 and 5 (DR4 and DR5) require the adaptor molecule FADD to transmit their apoptotic signal (40, 41), and a recent publication reported that DR4 and DR5 were also involved in the induction of the edelfosine-mediated apoptotic signal (37). Therefore, it is conceivable that, on PI3K inhibition, tumor necrosis factor–related apoptosis-inducing ligand receptors DR4 and DR5 may partially alleviate the default in cell death induction due to the down-expression of Fas or the hemizygous expression of a mutated Fas, whereas on the other hand the apoptotic signal is more dramatically abrogated when a dominant-negative FADD, which inhibits all the death receptor signals, is overexpressed. These findings imply that plasma membrane distribution of DR4 and DR5 on PI3K inhibition should also be studied in the future.

Formation of the supramolecular complexes of Fas has been previously observed as well as on soluble, membrane-bound FasL or agonistic anti-Fas stimulations, and these structures are essential for the transmission of the apoptotic signal (39, 42). All these extracellular ligands of Fas are well described to favor Fas capping and induction of cell death, whereas, herein, we point out that an intracellular stimuli (PI3K activation) is also able to control the aggregation level of the transmembrane receptor Fas.

The activated PI3K signaling pathway prevents the formation of large and oriented Fas-containing detergent-resistant microdomains reminiscent of what happens within the ''immune synapse'' observed on T lymphocyte activation (36). The formation of this synapse has been extensively studied and cumulative data favor the involvement of the actin cytoskeleton and actin-linked adaptors belonging to the ezrin/moesin/radixin family to the formation of this lipid raft–enriched supramolecular complex (43). Ezrin has been reported as essential to induce the Fas-mediated apoptotic signal (44) and to interact directly to the regulatory subunit p85 of the PI3K (45). Furthermore, recent works by Stickney et al. (46) showed that merlin, a protein related to the ezrin/moesin/radixin family, was able to link the actin cytoskeleton to the lipid rafts. Members of this family of membrane/filamentous actin linkers are therefore likely to play a role in the redistribution of Fas inside the lipid rafts under the control of the PI3K signal. However, the association between the actin cytoskeleton and the Fas-mediated signal of apoptosis is still a matter of debate because, rather to inhibit the Fas signal, the use of inhibitors of the actin filament elongation (e.g., cytochalasin D) has been reported to amplify or did not alter the apoptotic signal in epithelial cells (e.g., ovarian cancer cell lines or in airway epithelial cells) or in the T leukemia Jurkat cell line, respectively (47, 48).

Alternatively other molecular targets could play a role in the redistribution of Fas into the lipid rafts, as recently palmitoylation of Fas has been reported to promote the redistribution of Fas into microdomains (49). Therefore, it is conceivable that enzymes involved in the palmitoylation of proteins or other regulatory factors controlling the autoacylation process could be key components in the lipid raft redistribution of Fas and then could be considered as potential targets modulated by PI3K signaling pathway.

The antiapoptotic PI3K signal is found constitutively activated in numerous types of tumors, leading to the proposal that PI3K inhibitors can be used as therapeutic anticancer agents. However, because this pathway has pleiotropic effects in the cell, the currently available agents are likely to be too toxic, precluding their clinical use (6). We believe the exhaustive characterization of the molecular ordering between the blockade of the PI3K activity and the redistribution of Fas into the lipid rafts to be of interest to pinpoint more specific targets whose inhibition will be entailed with more limited side effects.

Materials and Methods

Cell Lines and PBLs

The leukemia cell lines Jurkat and CEM, the promyelocytic leukemia cell line HL-60, and the lymphoma cell line H9 were grown in RPMI 1640 supplemented with 8% heat-inactivated FCS and 2 mmol/L L-glutamine in a 5% CO_2 incubator at 37°C. Peripheral blood mononuclear cells from healthy donors were isolated by Ficoll centrifugation and washed twice in PBS. Monocytes were removed by a 2-h adherence step, and the naive PBLs were incubated overnight in medium supplemented with 1 μg/mL phytohemagglutinin (Sigma). Cells were washed extensively and incubated in the culture medium supplemented with 1,000 units/mL of recombinant interleukin-2.

The cell line CEM-IRC was selected as follows: parental CEM was cultured for a month in a medium supplemented with homemade soluble FasL (500 ng/mL; ref. 24). Then, soluble FasL–resistant cells were cloned by limiting dilutions and Fas expression at the surface of the clones was analyzed by flow cytometry using an anti-Fas mAb (clone 5D7). Jurkat cells (5×10^6 in 0.3 mL) were electroporated with 10 μg of C-FADD–containing pcDNA3 or empty vector at 300 V with one pulse using the BTM 830 electroporation generator (BTX Instrument Division). Twenty-four hours later, G418 (Life Technologies) was added to the cultures at a final concentration of 2 mg/mL. Stable clones were isolated by limiting dilution.

Antibodies and Other Reagents

The anti-human Fas mAb 5D7, the blocking anti-human FasL mAb 10F2, and the isotype-matched negative control 1F10 (IgG) mAb were all generated in the laboratory. The blocking anti-human Fas mAb ZB4 (IgG) was purchased from Immunotech. The anti-human Fas mAb used for confocal microscopy analysis is the clone DX2 from BD Biosciences. Anti-whole protein Akt and phospho-Akt–specific antisera were from Cell Signaling Technology, Inc. Anti-$p56^{Lck}$ mAb was from Transduction Laboratories (BD Biosciences). Edelfosine, LY294002, and wortmannin were purchased from Merck Biosciences. α-Cyclodextrin and MβCD were from Sigma.

Detergent Lysis Experiments and Western Blot Analysis

Cells were lysed for 30 min at 4°C in lysis buffer [25 mmol/L HEPES (pH 7.4), 1% Triton X-100, 150 mmol/L NaCl, supplemented with a mix of protease inhibitors (Sigma)]. Protein concentration was determined using the bicinchoninic acid method (Sigma) according to the manufacturer's protocol. Proteins were separated by SDS-PAGE on a 12% gel in reducing conditions and transferred to a nitrocellulose membrane (Amersham). The membrane was blocked 1 h with TBS-Tween 20 [50 mmol/L Tris, 160 mmol/L NaCl, 0.1% Tween 20 (pH 7.8)] containing 5% dried skimmed milk, and all subsequent steps were done in this buffer. Specific primary antibody was incubated overnight at 4°C. After intensive washes, the peroxidase-labeled anti-rabbit (CliniSciences) was added for 1 h and the proteins were visualized with the enhanced chemiluminescence substrate kit (Amersham).

Competitive PIP(3,4,5) ELISA

Using the competitive PIP_3 Mass ELISA kit (Echelon Biosciences, Inc.), we determined the relative amount of PIP3 present in cells treated or untreated by the different PI3K-specific inhibitors and edelfosine. Briefly, cells were treated or untreated with the indicated agents and lipids were extracted following the recommendation of the manufacturer. For each condition, the purified PIP3-containing suspension was treated exactly as recommended by the manufacturer and the colorimetric signal was measured at 450 nm using the Titertek Labsystems Multiskan reader.

Cell Death Assays

Quantification of fragmented DNA, also called sub-G_1 population, was done as previously described (5). Briefly, treated or untreated cells (0.2×10^6) were harvested and incubated 4 h in 300 μL of buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/mL of propidium iodide (Sigma). Cell fluorescence was analyzed using flow cytometry analysis. In experiments using MβCD, Jurkat cells were preincubated 30 min in serum-free medium with 5 mmol/L MβCD or α-cyclodextrin (negative control) at 37°C and then washed before the addition of the different inducers in medium containing no FCS.

Cell viability was also assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, exactly as previously described (50). In brief, 2×10^4 cells were cultured for 16 h in flat-bottomed, 96-well plates with the indicated concentrations of the apoptosis inducer in a final volume of 100 μL. MTT (15 μL, 5 mg/mL in PBS) solution was added, and after 4 h of incubation at 37°C, the absorbance was measured at 570 nm using the Titertek Labsystems Multiskan reader.

Flow Cytometry Staining of Cells

All the steps were done at 4°C. Cells were washed in PBS/1% bovine serum albumin and incubated 30 min with 10 μg/mL of anti-Fas mAb (clone 5D7) or isotypic mAb (1F10). Fas staining was revealed using a phycoerythrin-coupled goat anti-mouse antibody and analyzed with a three-color FACSCalibur flow cytometer (Becton Dickinson).

Sucrose Gradient Fractionation

Cells were treated or untreated with the indicated PI3K inhibitor for 4 h. Then, cells were harvested, washed twice in PBS, and lysed exactly as previously described (50). Briefly, indicated cells (10^8) were incubated with 1 mL of lysis buffer for 30 min at 4°C. The lysate was mixed with an equal volume of 85% (w/v) sucrose-containing lysis buffer, transferred to a centrifuge tube, and overlaid with 5 mL of 30% and 3 mL of 5% sucrose solutions. The sucrose gradient was centrifuged for 20 h at 4°C in a Kontron TST4114 rotor at 250,000 × *g*, and fractions were harvested from the top to the bottom of the centrifuge tube.

Immunofluorescence Staining

Cells were incubated with the indicated chemical for 4 h at 37°C and allowed to adhere for 5 min at room temperature to poly-L-lysine–coated slides (ESCO, VWR). Cells were then fixed in PBS containing 2% formaldehyde for 15 min, washed twice in PBS, and treated for 10 min with 50 mmol/L glycine in PBS to quench the aldehyde groups. Cells were incubated with Alexa Fluor 555–labeled cholera toxin B and anti-Fas mAbs (clone DX2) in PBS/1% bovine serum albumin for 30 min at 4°C, rinsed five times, and incubated for 30 min with the Alexa Fluor 488–conjugated goat anti-mouse antibody (Molecular Probes) in PBS/1% bovine serum albumin. Slides were washed with PBS, dried, and mounted with Fluoroprep (Biomerieux). Images were acquired and processed with a confocal microscope (Leica SP5) with a 63× objective.

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