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CDC50A plays a key role in the uptake of the anticancer drug perifosine in human carcinoma cells

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ABSTRACT

Functional aminophospholipid translocases are composed of at least two proteins: an alpha subunit from the P4 subfamily of P-type ATPases and a beta subunit from the CDC50-Lem3p family. Over-expression and knockdown of the human beta subunit CDC50A in KB cells enhanced and decreased, respectively, the uptake of both fluorescent aminophospholipid analogues and the anticancer alkyl-phospholipid perifosine. Confocal microscopy showed that CDC50A-V5 was localized at the endoplasmic reticulum and the Golgi complex of both KB (perifosine-sensitive) and KB PER-R (perifosine-resistant, alkyl-phospholipid uptake deficient) cells, but was only widely distributed in the early and late endosomes in KB cells. Biotinylation of cell surface proteins allowed CDC50A-V5 to be detected in the plasma membrane of KB cells but not in KB PER-R cells, thereby suggesting a defect in CDC50A trafficking that could explain the inability of KB PER-R to uptake perifosine. Over-expression of CDC50A in HeLa and HEK293T cells did not increase uptake, since the protein was retained at the endoplasmic reticulum and Golgi. However, when CDC50A was co-expressed with the P4-ATPase Atp8b1, the two proteins co-localized at the plasma membrane and the uptake of aminophospholipids and perifosine increased strikingly in both cell lines. These findings suggest that CDC50A plays a key role in perifosine uptake in human cells, presumably by forming a functional plasma membrane translocator in combination with a P4-ATPase.

1. Introduction

Perifosine is an oral alkyl-phospholipid (ALP) Akt inhibitor that is currently being tested in phase II clinical trials. Unlike most kinase inhibitors, which target the adenosine triphosphate-binding region, perifosine targets the pleckstrin homology domain of Akt, thereby preventing its translocation to the plasma membrane [1]. Single-agent activity with perifosine has been observed in sarcoma and Waldenström macroglobulinemia patients [2]. However, the disappointing response rates of common solid tumors to perifosine as a single agent [3–6] have diminished expectations and prompted further investigation into its mechanism of action.

The ability to identify patients likely to respond to targeted therapeutics would decrease the size, duration, and cost of clinical trials, thus resulting in more efficient translation to improved patient outcomes. In this sense, Hennessy et al. [7] reported a strong correlation between proportional modulation of PI3K pathway biomarkers and quantified perifosine efficacy, thereby suggesting that the early measurements of such biomarkers will optimize the selection of responsive patients and guide perifosine dosage during cancer treatment. Furthermore, the identification of other potential biomarkers for perifosine efficacy in cancer patients would allow those patients most likely to respond to perifosine, either alone or in combination with other therapies, to be selected.

ALP uptake is a prerequisite for triggering the intracellular events that finally cause cell death in different cellular systems, including yeasts [8,9], the protozoan parasite *Leishmania donovani* [10] and cancer cells [11]. Indeed, a clear correlation between ALP uptake and apoptosis induction has been reported previously [12]. Two pathways for the entry of ALP into cancer cells, namely raft-mediated endocytosis in lymphoma cells [13] and translocator-mediated uptake in carcinoma cells [14–16], have been described. A clearer insight into the molecular determinants of perifosine entry into cancer cells would therefore be of enormous importance for the identification of additional biomarkers for the efficacy of perifosine as an anticancer drug.

Data from our previous work [14] strongly supported the hypothesis that a putative aminophospholipid translocase (APLT; P-type ATPase P4 subfamily) could be responsible for the uptake of

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ALPs in human KB carcinoma cells. Indeed, the plasma membrane P4-ATPase homologues of yeast and *Leishmania* have been shown to play a key role in the uptake of miltefosine and edelfosine [9,17] in combination with a second protein from the Cdc50p-Lem3p family that functions as a beta subunit [8,10,18,19]. As fourteen putative APLTs [20] and only three beta subunits (CDC50A, B and C) [21] have been identified in humans, we decided to try to elucidate the role of these human beta subunits as functional elements of the putative plasma membrane perifosine translocator (and other related ALPs) in cancer cells, and found that CDC50A plays a key role in the uptake of perifosine in human cancer cells, presumably by forming a functional plasma membrane translocator in combination with a P4-ATPase.

2. Materials and methods

2.1. Chemicals

Perifosine (octadecyl-[*N*,*N*-dimethyl-piperidinio-4-yl]-phosphate), and [^{14}C]-perifosine (30.9 mCi/mmol) were provided by Zentaris GmbH (Frankfurt, Germany). Lipofectamine 2000, Anti-V5 monoclonal antibody, Alexa Fluor 633-conjugated anti-mouse IgG, pEF6-V5/His and pcDNA3.1-HYGRO vectors, FITC-concanavalin A, BODIPY-lactosylceramide, and Alexa Fluor 488-labelled dextran 10,000 were purchased from Invitrogen (Prat de Llobregat, Barcelona, Spain). Pre-designed ON-TARGETplus siRNA for human *CDC50A* and *CDC50B* were purchased from Dharmacon (Lafayette, CO). Fluorescent (NBD-labelled) phospholipid analogues 1-palmitoyl-2-{6-[(7-nitro-2-(1,3-benzoxadiazol)-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoethanolamine (NBD-PE), -phosphoserine (NBD-PS) and -phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-rabbit IgG monoclonal antibody, monoclonal anti-α-tubulin antibody, and the rabbit polyclonal antibodies anti-*myc*, anti-calnexin, anti-GM130, anti-TGN46, anti-Rab5 and anti-Rab7, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma–Aldrich (Madrid, Spain). Fugene HD, High Pure RNA isolation kit and Expand High Fidelity PCR System were purchased from Roche (San Cugat del Vallés, Barcelona, Spain), and the High-capacity cDNA Reverse transcription kit plus RNAse inhibitor from Applied Biosystems (Alcobendas, Madrid, Spain). The Perfectprep® plasmid isolation kit from Eppendorf Ibérica (San Sebastian de los Reyes, Madrid, Spain) was used to isolate purified plasmids. QuickChange II XL Site-directed Mutagenesis kit was purchased from Stratagene-Cultek SLU (Madrid, Spain), and the pGEM-T Easy vector from Promega Biotech Ibérica (Alcobendas, Madrid, Spain). All other chemicals were purchased from Sigma–Aldrich (Madrid, Spain).

2.2. Cell lines and cultures

The mammalian cell lines described in the present work were grown at 37 °C in a humidified atmosphere and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamax I, 1:50 Micokill AB solution 50× (PAA Laboratories GmbH) and 250 U/mL penicillin G plus 250 μg/mL streptomycin sulfate (Invitrogen), unless indicated otherwise. The following cells were used: murine fibroblast embryo NIH 3T3 cells (ATCC number CCL-1658); chinese hamster ovary CHO-K1 cells (ATCC number CCL-61; cultured in F-12K medium); canine kidney MDCKII cells [22]; monkey epithelial kidney Vero and COS-7 cells (ATCC CRL-1586 and CRL-1651); human epidermal carcinoma KB cells (wild-type) (ATCC number CCL-17) and its perifosine-resistant counterparts KB PER-R clone 4, 8 and 10 [14]; human breast carcinoma MCF-7 and MDA-MB-23 cells [23,24]; human cervix epithelial adenocarcinoma HeLa cells (ATCC CCL-2); human epithelial embryonic kidney HEK-293T cells (ATCC CRL-11268); human pancreas carcinoma AsPC1 and MiaPaca2 cells (ATCC CRL-1682 and CRL-1420); human 2008 ovary cells [25], kindly provided by Prof. Piet Borst (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands); and human osteosarcoma HOS cells (ATCC CRL-1543).

2.3. MTT survival assays, [^{14}C]-perifosine uptake, accumulation of fluorescent phospholipid analogues and measurement of endocytosis rates

Unless otherwise indicated, these four different assays were performed as described in our previous work [14], with the only modification that, in the MTT-based colorimetric assay to determine perifosine sensitivity, the cell lines were incubated with the anticancer drug for only 2 h instead of 24 h before adding MTT to the culture medium. This modification allowed us to better link the observed cytotoxicity with the amount of perifosine incorporated in a short time by each cell line, and therefore to establish whether cell death actually correlated with intracellular accumulation of perifosine.

2.4. Isolation of CDC50A, CDC50B and Atp8b1, and DNA constructs

The human cDNAs of *CDC50A* (GenBank accession no. NM_018247, longer transcription variant 1) and *CDC50B* (GenBank accession no. NM_001017970) genes, which code for the putative beta subunits of the human APLT(s) (P-type ATPase P4 subfamily), were isolated by RT-PCR with the High Capacity cDNA Reverse transcription kit plus RNAse inhibitor (Applied Biosystems), using total RNA obtained from KB cells with the High Pure RNA isolation kit (Roche) as template; [primers: CDC50A-F (5′-ACCATGGCGATGAACTATAACG), and CDC50A-R (5′-TTAAATGGTAATGTCAGCTGTATTAC); CDC50B-F (5′-ACCATGACCTGGAGCGCCACGGC) and CDC50B-R (5′-TCACTCCTCGTCGTCGTCCTGG)]. The resulting PCR products were cloned into the pEF6-V5/His TOPO TA expression vector from Invitrogen, in frame with the C-terminal V5 epitope and the polyhistidine (6 × His) tag. The cDNA of *Atp8b1* (GenBank accession no. BC117946), the murine orthologue of the human gene that encodes for the putative APLT ATP8B1 (95% identity), was purchased from Source BioScience Geneservice (Cambridge, UK) as a full open-reading frame (ORF) cloned into pCR-BluntII-TOPO vector. The construct was isolated and purified using the Perfectprep® plasmid isolation kit (Eppendorf), and the ORF subsequently amplified using the Expand High Fidelity PCR System with the primers: 8b1-F (5′-ACCATGGGCACAGAAAGAGACTCG); 8b1-R (5′-TCACAGATCCTCTTCAGAGATGAGTTTCTGCTCGCTCTCCACTGTGCGCCGGTA), (*myc*-tag coding sequence underlined). The resulting PCR product was cloned into pGEMT-Easy from Promega. The nucleotide sequences of all cloned cDNAs were determined automatically, as described previously [26], and compared against their respective records annotated in GenBank. Unexpectedly, after repeating PCR and isolating new clones, we found that the supplier had provided us with a mutant variant of the *Atp8b1* gene (A1954G, which resulted in the amino acid mutation R652G). Since we were concerned about the possible undesired effects that this mutation could cause in the phenotypic function of the resulting protein, we decided to fix it by using the QuickChange II XL Site-directed Mutagenesis kit from Stratagene using the primers (fixing point-mutation is underlined): FAtp8b1-mut (5′-AAGTGAGACTCTCAGGACCCTGTGCCTGTG); RAtp8b1-mut (5′-CACAGGCACAGGGTCCTGAGAGTCTCACTTG). Once this annoying problem had been resolved, we re-amplified the *Atp8b1* ORF with the primers FAtp8b1-NheI (5′-TTATTGCTAGCACCATGGGCACAGAAAGAGACTCG); RAtp8b1-BamHI (5′-TTATTGGATCCTCAA-

GATCTCTTCAGAGATGAG), and cloned the resulting PCR product as an NheI/BamHI fragment into the pcDNA3.1-HYGRO expression vector.

2.5. Cell transfection

KB Wt, KB PER-R, HeLa and HEK-293T cells were stably transfected with pEF6-*CDC50A-V5* and pEF6-*CDC50B-V5* using Lipofectamine 2000 following the manufacturer's protocol. The stable transfectants were selected with 10 μg/mL blasticidin in only one week, and the expression levels of both proteins readily visualized by Western blotting or indirect immunofluorescence (IFI) under a microscope using monoclonal anti-V5 1:5000 from Invitrogen. Untransfected and *CDC50A-V5*-transfected KB Wt and KB PER-R cells were refractory to both stable and transient transfection with the pcDNA3.1-HYGRO-*Atp8b1-myc* construct using either Lipofectamine 2000 or Fugene HD, as judged by the fact that no *Atp8b1-myc* expression was observed, either by Western blot or by IFI using several anti-myc antibodies. In contrast, HeLa and HEK-293T cells could be transiently transfected with *CDC50A-V5* alone or in combination with *Atp8b1-myc*, with the best results being obtained using Fugene HD.

2.6. siRNA-based CDC50A and CDC50B knockdown

Human *CDC50A* and *CDC50B* were targeted using SMARTpool siRNA oligonucleotides (Dharmacon), following a previously described procedure [14], at concentrations of 1, 10 and 100 nM of siRNA oligonucleotides in order to monitor the concentration dependency of the gene silencing.

2.7. Biotinylation of cell surface proteins

KB Wt and PER-R cells were washed with PBS and incubated on ice with 1 mM EZ-Link Sulfo-NHS-SS-biotin (Thermo Scientific Pierce, Madrid, Spain) in 1 mL PBS for 2 h at 4 °C. The biotinylation reaction was quenched by washing the cells three times with 50 mM Tris–HCl, pH 7.4, then cells were incubated on ice for 30 min in 100 μL lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40) plus a protease inhibitor cocktail (Sigma–Aldrich). The cell lysate was clarified by centrifugation at 21,000 × *g* for 10 min at 4 °C, and the supernatant incubated for 2 h at 4 °C in continuous agitation with 100 μL of packed streptavidin-agarose beads (Thermo Scientific Pierce) pre-washed with lysis buffer. The agarose beads were then washed three times with 1 mL of PBS + 1 M NaCl and two times with 1 mL of PBS, and the biotinylated proteins eluted in 30 μL of 2 × Laemmli buffer.

2.8. Immunoblotting

Protein samples were fractionated by SDS-PAGE under standard conditions and electrotransferred onto Immobilon P membranes (Millipore, Bedford, MA). Immunodetection of the V5-tagged beta subunits and Atp8b1-myc was performed with 1:5000 monoclonal anti-V5 and 1:1000 anti-myc polyclonal antibody in buffer A (PBS containing 0.01% Tween 20 and 0.1% BSA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary rabbit anti-mouse or goat anti-rabbit (1:5000) immunoglobulin G (Dako, Barcelona, Spain) in buffer A. Immunodetection of α-tubulin for sample normalization was performed with monoclonal anti-α-tubulin antibody (Sigma–Aldrich) at 1:5000. Signals were detected using either the ECL or the ECL Plus chemiluminescent substrate (Thermo Scientific Pierce).

2.9. Immunofluorescence and confocal microscopy

Cells were seeded onto cover slip circles (diameter: 12 mm; thickness: 0.13–0.16 mm, from Menzzel-Glässer) in 24-well plates (Thermo Scientific Nunc, Madrid, Spain) and cultured under standard conditions. On the day of the experiment, the cells were washed three times with PBS and fixed with 2% para-formaldehyde in PBS for 20 min at room temperature. After quenching with 10 mM Tris–HCl pH 7.4 and washing twice with the same buffer, the cells were permeabilized with a 0.1% solution of Triton X-100 detergent (Sigma–Aldrich) in PBS for 10 min at room temperature. They were then washed with PBS and incubated for 30 min with PBS 1% BSA to block the non-specific binding of antibodies. The primary antibodies anti-V5 (1:5000), anti-myc (1:1000), anti-calnexin (1:200), anti-GM130 (1:2500), anti-TGN46 (1:200), anti-Rab5 (1:200) and anti-Rab7 (1:200) were incubated at 37 °C in a humidified atmosphere for 1–2 h in PBS 1% BSA. The cells were then rinsed three times with PBS and treated with the secondary antibodies FITC-conjugated anti-rabbit IgG antibody (1:1000–1:2000) from Sigma–Aldrich, and AlexaFluor 633-conjugated anti-mouse IgG antibody (1:500) from Invitrogen in PBS 1% BSA. After the labelling procedure was complete, the cells were washed three times with PBS and mounted onto glass slides using Prolong Gold mounting medium with DAPI (Invitrogen).

Confocal images were acquired using a Leica TCS-SP5 confocal scanning laser microscope equipped with Ar and He-Ne lasers and connected to a Leica DMIRBE inverted epi-fluorescence microscope. The following filter settings were used: DAPI (blue): excitation at 405 nm (405 diode laser, UV), emission at 409–469 nm; FITC (green): excitation at 458 nm (argon laser, 20% power), emission at 494–603 nm; Alexa Fluor 633 (red): excitation at 633 nm (633 HeNe laser, 20% power), emission at 648–692 nm. Serial fluorescence images were obtained using the 63 × objective and processed and assembled with LAS AF Leica confocal software. Image analysis and subcellular co-localization fluorograms were generated and analyzed using the Leica confocal software package.

2.10. Semi-quantitative RT-PCRs

Semi-quantitative RT-PCRs were performed using the total cDNAs synthesized above as templates to establish the endogenous expression of *CDC50A* and *ATP8B1* genes in KB, HeLa and HEK293T cells. Standard PCRs were performed using serial dilutions of total cDNAs as templates and highly specific primers [ATP8B1 forward (5′-CAG ACT GCA TAC GAG GAT TGG); ATP8B1 reverse (5′-CCT TCA ACC GCT TGC GAT G); CDC50A forward (5′-CTA TAA CGC GAA GGA TGA AGT G); CDC50A reverse (5′- TCC ATA ATA CAT AAA CAC GTT GCC); β-actin forward (5′-CCT CAT GAA GAT CCT CAC CG); β-actin reverse (5′-CAG TGA TCT CCT TCT GCA TCC)] to amplify internal 631-, 357- and 660-bp sequences from the *ATP8B1*, *CDC50A* and *β-actin* cDNAs, respectively. The PCR conditions for each step were as follows: Initial denaturation (95 °C, 5 min); 35 cycles (denaturation: 92 °C, 30 s; annealing: 52 °C, 45 s; extension: 72 °C, 45 s); final extension: 72 °C, 3 min. The resulting PCR products were run in 0.8% agarose gels stained with ethidium bromide and visualized in a UV transiluminator.

3. Results

3.1. Perifosine and NBD-phosphatidylethanolamine (NBD-PE) uptake are linked processes in KB cell lines

In our previous work we generated a perifosine-resistant line (designated as KB PER-R clone 10) from the perifosine-hypersensitive KB Wt cells (with an EC_{50} of less than 1 μM for 24 h perifosine exposure). Along with a high resistance to perifosine

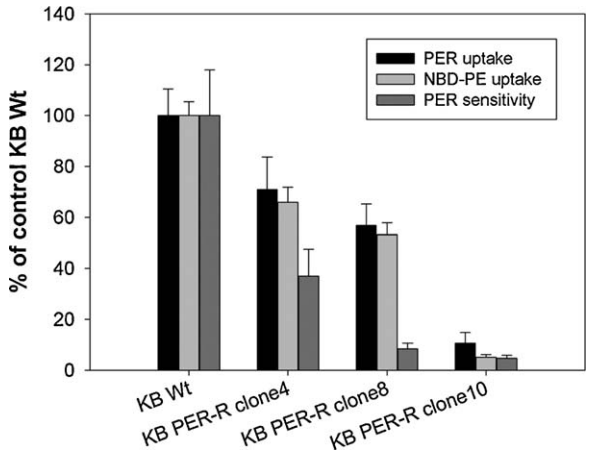

Fig. 1. Correlation between perifosine sensitivity and ^{14}C-perifosine and NBD-PE uptake in KB Wt cells and different KB PER-R clones. Perifosine sensitivity (EC_{50} values) was determined by MTT assay after incubation for 2 h with different drug concentrations. Uptake of 1 μM [^{14}C]-perifosine (0.03 μCi/mL) and 2 μM NBD-PE was performed at 37 °C for 1 h and 10 °C for 20 min, respectively, as described previously [14]. Data were normalized to those measured in KB Wt, which were considered as 100%. The values shown are the average ± S.D. of three to six different experiments performed in triplicate.

(grows suitably at 20 μM perifosine), this cell line showed significantly decreased perifosine uptake, a low rate of NBD-aminophospholipid translocation through the plasma membrane, a relatively high exposure of endogenous phosphatidylserine in the cell surface, and lacked NEM- and orthovanadate-sensitive, plasma-membrane-associated ATPase activity [14]. Similar perifosine-resistant clones generated in our laboratory from KB Wt cells (clones 4 and 8) showed intermediate levels of perifosine uptake with respect to KB cells and KB PER-R clone10 cells, along with intermediate rates of NBD-PE translocation through the plasma membrane, and were only moderately resistant to perifosine compared with clone 10 (Fig. 1). This correlation between perifosine uptake and NBD-PE translocation therefore suggested that, as proposed previously [14], a link could exist between both processes in KB cells. We also tested the uptake rates of BODIPY-lactosylceramide (raft-dependent endocytosis marker) and Alexa Fluor 488-dextran 10,000 (fluid phase endocytosis marker) in a subset of fourteen cell lines, and found no significant correlation upon comparing their rates of perifosine uptake (Fig. 2).

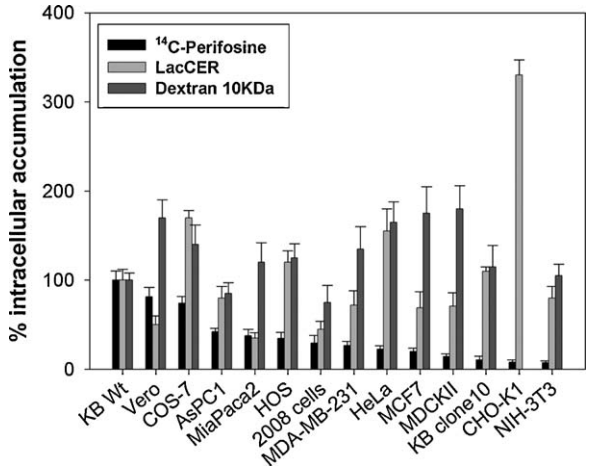

Fig. 2. Comparative analysis of perifosine and endocytosis markers in different representative cell lines. Perifosine-uptake experiments were performed as described in the legend to Fig. 1. Uptake of the endocytic markers BODIPY-Lactosyl-ceramide (LacCER, 0.5 μM) and Alexa488-labelled dextran 10 kDa (Dextran 10 kDa, 0.25 mg/mL) was performed for 7 min at 37 °C and analyzed by flow cytometry as described previously [14]. Bars represent the average ± S.D. of three different experiments performed in duplicate. Data were normalized to those measured in KB Wt cells, which were considered as 100%.

3.2. CDC50A is implicated in both perifosine and fluorescent aminophospholipid analogues uptake in KB cells

In our previous work we proposed that a putative APLT could be responsible for the internalization of both ALPs and aminophospholipids through the plasma membrane in human KB carcinoma cells. APLTs seems to be a functional complex of at least two proteins (alpha and beta subunits) [10,18,19], and since fourteen different putative alpha but only three presumed beta subunits (CDC50A, B and C) have been described in humans, we decided to start our study of the potential implication of the latter proteins on perifosine uptake in mammalian cells. Since *CDC50C* transcripts have been found to terminate aberrantly in humans [27], only CDC50A and B were likely to play a putative role in perifosine uptake. We therefore generated KB cells expressing CDC50A-V5 or CDC50B-V5 (Fig. 3A) and found that,

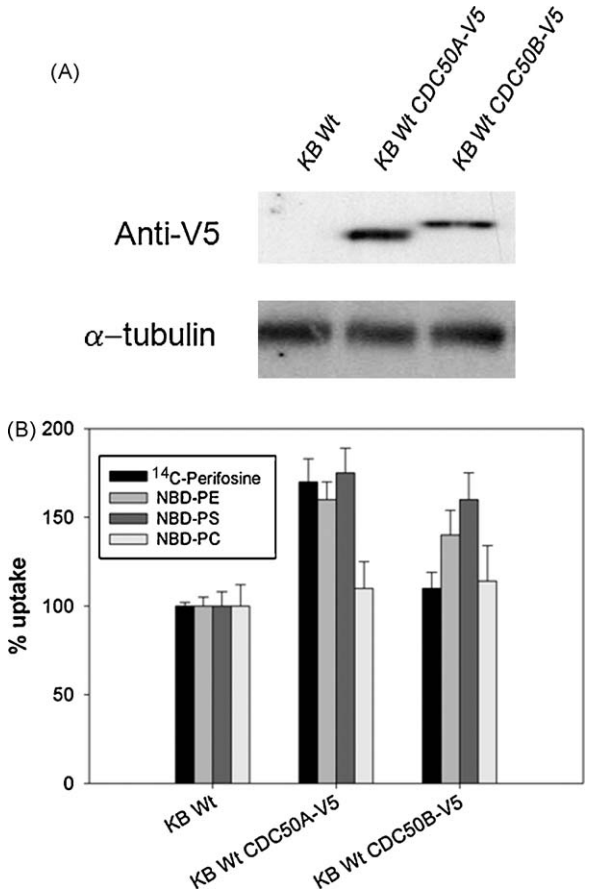

Fig. 3. Uptake of perifosine and NBD-phospholipids in KB Wt cells over-expressing CDC50A or CDC50B. (A) Western blot analysis. KB Wt cells were transfected with pEF6-V5 vector containing the corresponding gene in phase with a C-terminal V5-tag. Expression levels were determined using anti-V5 monoclonal antibody. A blot using anti-α-tubulin was performed in parallel as control of protein loading. (B) Uptake experiments. Uptake of perifosine and NBD-PE, -PS and -PC was performed as described in the legend to Fig. 1. This is a representative experiment performed twice in triplicate ± S.D. from two different transfections. Data were normalized to those measured in KB Wt cells (perifosine uptake: 3082 ± 151 pmol/mg protein; NBD-phospholipids uptake expressed in flow cytometry mean channel values after substracting the cell autofluorescence: NBD-PE: 558 ± 57; NBD-PS: 497 ± 63; NBD-PC: 463 ± 84), which were considered as 100%.

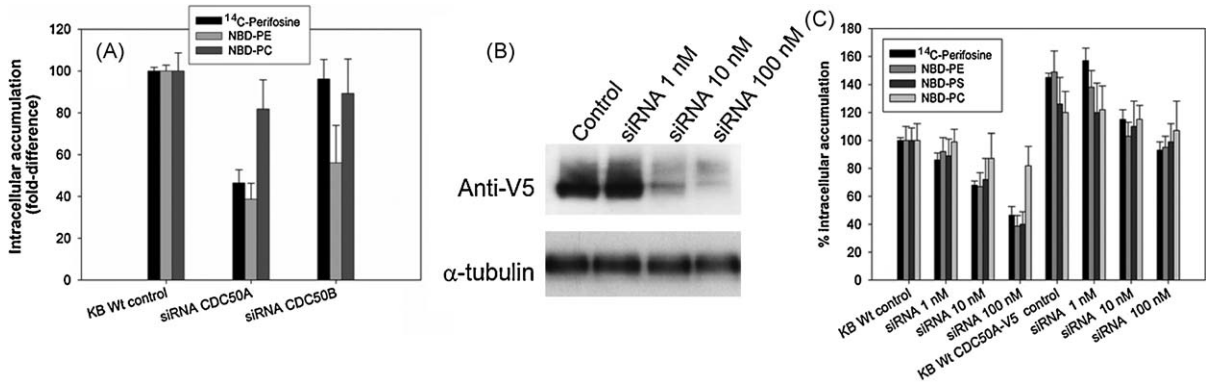

Fig. 4. Silencing of CDC50A and CDC50B in KB Wt cells. (A) Uptake of NBD-phospholipids and perifosine. KB Wt was transfected with 100 nM specific SMARTpool CDC50A or CDC50B siRNA oligonucleotides and the uptake of [^{14}C]-perifosine and NBD-PE and -PC determined as described in the legend to Figure 1. The figure shows a representative experiment performed in triplicate ± S.D. of four independent experiments. Data were normalized to that measured in DharmaFECT 1 treated (transfectant agent without siRNAs) KB Wt cells (perifosine uptake: 3450 ± 96 pmol/mg protein; NBD-phospholipids uptake expressed in flow cytometry mean channel values after substracting the cell autofluorescence: NBD-PE: 597 ± 28; NBD-PC: 513 ± 49), which was considered as 100%. (B) Western blot showing the increasing reduction of CDC50A-V5 expression in CDC50A-V5-transfected KB Wt cells at increasing concentrations of the specific siRNA. A blot using anti-α-tubulin was performed in parallel as control of protein loading. (C) The same as in (A) but targeting only CDC50A-V5 transfected cells with 1, 10 or 100 nM specific CDC50A siRNA oligonucleotides and determination of [^{14}C]-perifosine and NBD-PE, -PS and -PC uptake. The figure shows a representative experiment performed in triplicate ± S.D. of five independent experiments.
The absolute uptake values measured in KB Wt cells with Dharmafect 1 in this set of experiments were: perifosine: 3657 ± 162 pmol/mg protein; NBD-phospholipids uptake expressed in flow cytometry mean channel values after substracting the cell autofluorescence: NBD-PE: 609 ± 71; NBD-PS: 505 ± 63; NBD-PC: 521 ± 66.

whereas NBD-aminophospholipid uptake was increased by about 50% when either of these proteins was over-expressed, perifosine uptake was only boosted when CDC50A was over-expressed (Fig. 3B). No significant increase of NBD-PC uptake was detected in KB cells over-expressing CDC50A-V5 or CDC50B-V5. Interestingly, over-expression of CDC50A-V5 in KB PER-R clone10 cells did not increase the rate of perifosine or aminophospholipid uptake (data not shown). Moreover, knockdown of CDC50A and CDC50B reduced perifosine and NBD-aminophospholipid uptake to around 40% of the normal rate when CDC50A expression was lowered, whereas CDC50B silencing only affected NBD-aminophospholipid uptake (Fig. 4A). No significant reduction of NBD-PC was detected after the knockdown experiments. However, since we had no suitable antibody for monitoring the expression levels of endogenous CDC50A in KB cells, we also performed the same experiments in *CDC50A-V5*-transfected KB cells, thereby allowing us to monitor CDC50A-V5 expression levels by Western blot (Fig. 4B). This study showed that lower CDC50A-V5 levels correlated with lower rates of perifosine and NBD-aminophospholipid uptake (Fig. 4C).

3.3. CDC50A subcellular localization in KB Wt vs. KB PER-R clone 10 cells

Cdc50-Lem3p proteins play a role as both molecular chaperones needed for the correct folding and export of the alpha subunit P4-ATPase from the endoplasmic reticulum (ER) [28,29], and also as catalytically relevant, integral components of the phospholipid translocation machinery [30]. Since we have found previously that human CDC50A appears to play a role in perifosine uptake in KB cells, we decided to establish the cellular localization of this protein. Thus, we performed indirect immunofluorescence of V5-tagged CDC50A expressed in KB cells and then visualized the samples by confocal microscopy. It is apparent from Fig. 5, which shows the scattered intracellular distribution and co-localization with several markers of different intracellular membrane compartments, that CDC50A-V5 is a membrane protein that is distributed via the endocytic and exocytic pathways in KB cells. However, we cannot conclude from this figure whether CDC50A-V5 reaches the plasma membrane or not. A comparison of KB and KB PER-R clone10 cells, both of which express similar levels of

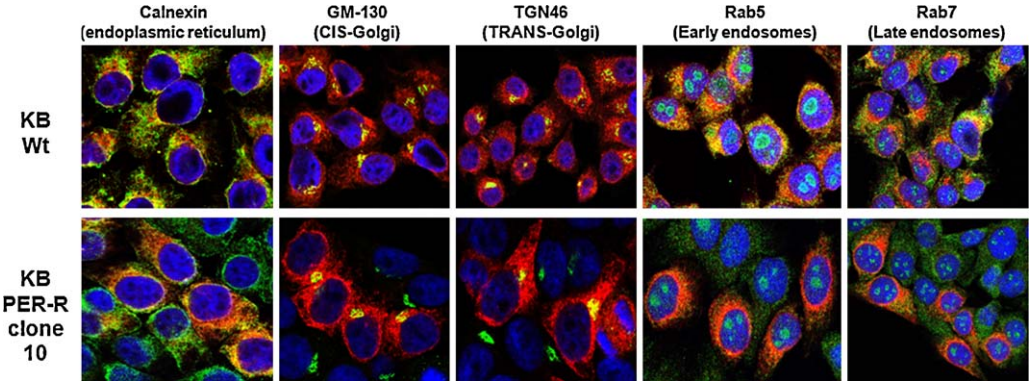

Fig. 5. CDC50A subcellular localization by confocal microscopy analysis. KB Wt and KB PER-R clone 10 cells transfected with CDC50A-V5 were fixed and immunostained as described in Section 2.9. Different protein markers specific for each subcellular compartment (green) were used to track the presence of CDC50A-V5 (red) in each of them (yellow pixels denote the co-localization of CDC50A-V5). The pictures are representative of at least three different microscopic fields per sample of fixed and immunostained cells. Three different cell samples from different days and different transfection batches were processed for each cell line and visualized under the microscope, in order to confirm that the intracellular distribution of CDC50A-V5 was not an artefact of the transfection procedure, cell batch or culture conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

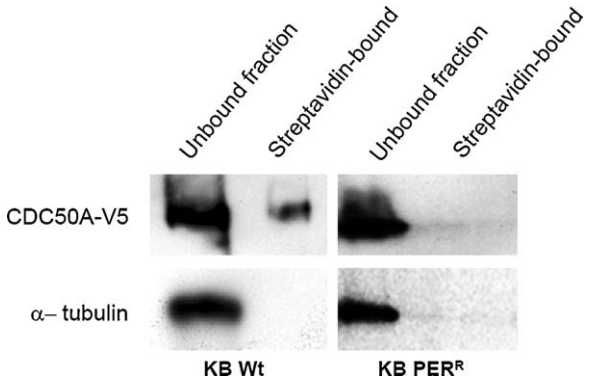

Fig. 6. Cell-surface expression of CDC50A. KB Wt and KB PER-R clone10 cells transfected with CDC50A-V5 were biotinylated as described in Section 2.7. Samples were subjected to SDS-PAGE and immunoblotted with anti-V5 antibodies. Immunoblot of α-tubulin, a typical cytoplasmic protein, was used as control of plasma membrane integrity. The unbound fraction represents proteins that could not be biotinylated with the EZ-Link NHS-Biotin reagent, since the labelling was performed in whole cells with intact plasma membrane, and only surface proteins could be labelled (avidin-bound fraction).

CDC50A-V5, as monitored by Western blot (not shown), highlighted a striking difference in the intracellular localization of CDC50A-V5. Thus, this protein was expressed well at the ER level, and was also exported to the CIS-Golgi and TRANS-Golgi network (TGN) in both cell lines, whereas its presence in early and late endosomes was significantly lower in KB PER-R cells than in KB cells, thereby suggesting that CDC50A-V5 distribution via the pathways connecting TGN, early endosomes, the plasma membrane and late endosomes is hindered in KB PER-R cells.

As we were unable to ascertain the presence of CDC50A at the plasma membrane of KB cells by confocal microscopy, we biotinylated the cell surface proteins in *CDC50A-V5*-transfected KB and KB PER-R cells in parallel. Fig. 6 shows that most of the CDC50A-V5 is intracellular (unbound fraction to streptavidin-agarose beads) in KB cells, as also determined by confocal microscopy, but that a detectable amount of CDC50A-V5 reaches the plasma membrane as well. In contrast, no CDC50A-V5 was observable in the fraction of biotinylated cell surface proteins in KB PER-R clone10 cells, although its intracellular levels were comparable to those for KB cells. We used α-tubulin as a reference to compare the amount of CDC50A-V5 expressed in both cell types and as a control of cell membrane integrity throughout the entire biotinylation process. Only cell surface proteins were biotinylated, since α-tubulin is only present in the unbound fraction. The inability of CDC50A-V5 to reach the plasma membrane in KB PER-R cells is in agreement with its defective membrane trafficking, as suggested previously by confocal microscopy, and further supports our hypothesis that perifosine uptake is very low in this cell line due to the lack of a P4-ATPase APLT at the plasma membrane.

3.4. CDC50A and Atp8b1 associate to exit the ER and traffic to the plasma membrane in HeLa and HEK293T cells, where they form a functional perifosine and aminophospholipid translocator

In light of the functional involvement of CDC50A in perifosine uptake in KB cells, we decided to investigate whether CDC50A was also involved in perifosine uptake in other cell lines. Thus, we expressed CDC50A-V5 in HeLa and HEK-293T cells, although with disappointing results: no increase was observed in perifosine or NBD-phospholipid uptake in either of these cell lines (see Fig. 8B). However, this finding does not allow us to state that CDC50A plays no role in perifosine uptake in this case, as CDC50A may lack the appropriate alpha subunit partner in these cell lines. To test this hypothesis, we performed semi-quantitative RT-PCR to

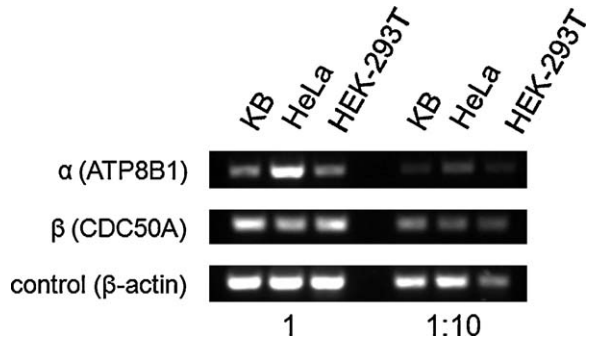

Fig. 7. *CDC50A* and *ATP8B1* mRNA levels in different cell lines. Semi-quantitative RT-PCRs showing the relative mRNA levels of *CDC50A*, *ATP8B1* and *β-actin* (control) in KB, HeLa and HEK-293T human cell lines were performed as described in Section 2.10. Aliquots of the undiluted (1) and 10-fold diluted (1:10) cDNAs were used as template in the PCR reactions.

compare the mRNA expression levels of *CDC50A* and *ATP8B1* (the best studied P4-ATPase [31], which was likely to be responsible for aminophospholipid translocation in these cell lines). This study showed (Fig. 7) that the endogenous expression

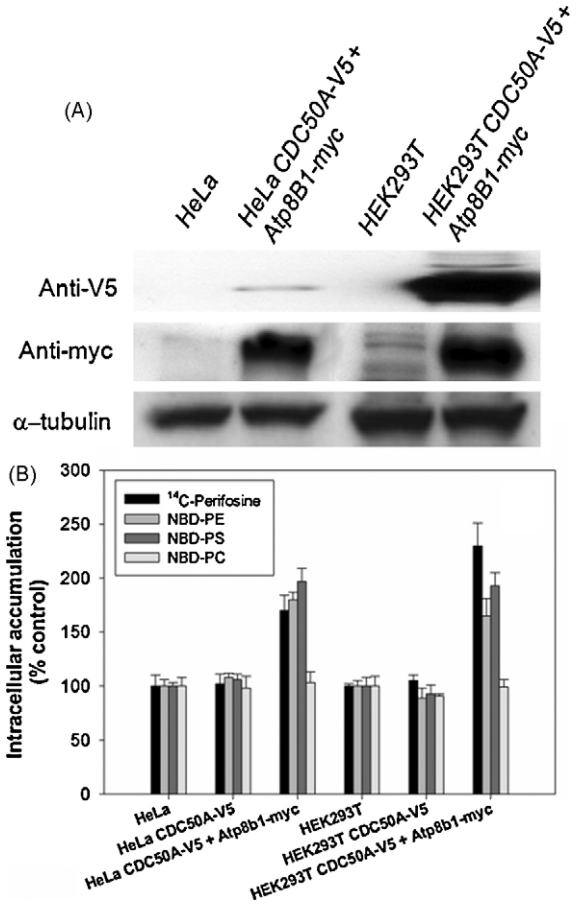

Fig. 8. Uptake of NBD-phospholipids and perifosine in cells over-expressing both CDC50A and Atp8b1. (A) Western blot showing the over-expression of CDC50A-V5 and Atp8b1-myc in HeLa and HEK-293T cells. A blot using anti-α-tubulin was performed in parallel as control of protein loading. (B) Effect of the co-expression of the two proteins on the accumulation of NBD-PC, -PE, -PS and ^{14}C-perifosine. Uptake experiments were performed as described in the legend to Fig. 1. The histograms represent the average ± S.D. of two different experiments performed in triplicate. The absolute uptake values measured in HeLa cells were: perifosine: 726 ± 123 pmol/mg protein; NBD-PE: 313 ± 29; NBD-PS: 272 ± 17; NBD-PC: 313 ± 45. In HEK293T cells were: perifosine: 877 ± 27 pmol/mg protein; NBD-PE: 446 ± 48; NBD-PS: 340 ± 53; NBD-PC: 403 ± 81. NBD-phospholipids uptake expressed in flow cytometry mean channel values after substracting the cell autofluorescence.

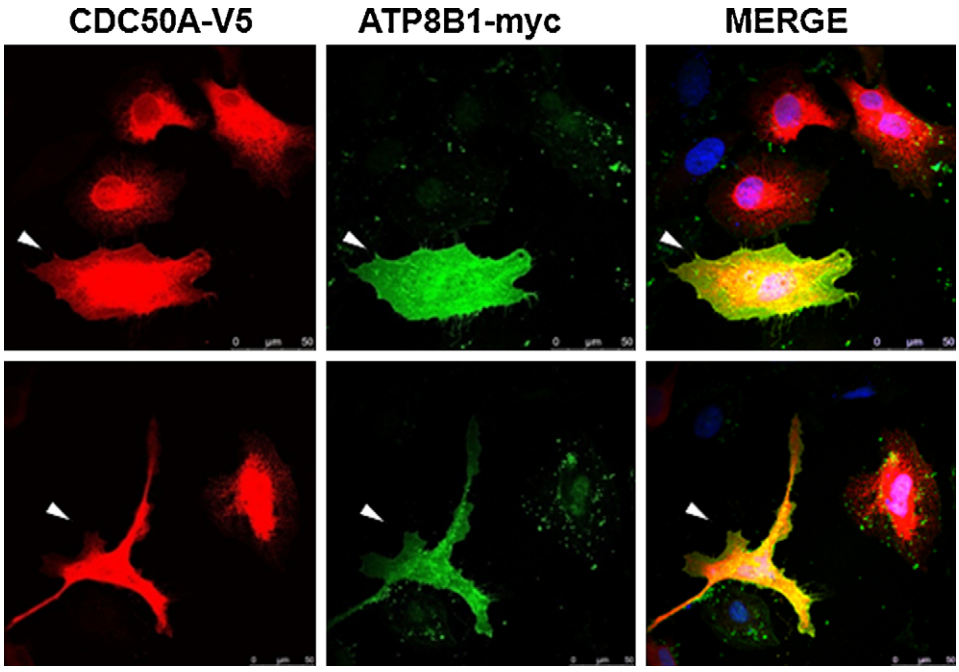

Fig. 9. Confocal microscopy analysis of the co-expression of CDC50A and Atp8b1 in human cancer cells. HeLa cells were co-transfected with CDC50A-V5 and Atp8b1-myc, fixed and immunostained with anti-V5 (red) and anti-myc (green) antibodies as described in Section 2.9. Arrowheads indicate cells co-expressing both proteins, which also co-localize at the cell surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

levels of *CDC50A* were similar in all three cell lines, and that *ATP8B1* was expressed at similar levels in KB and HEK-293T cells, and at even higher levels in HeLa cells (Fig. 7). This finding suggests that a P4-ATPase other than ATP8B1 is likely to be the alpha subunit responsible for the APLT activity observed in KB, HeLa and HEK293T cells. At this point, instead of simply determining the expression levels of the remaining thirteen putative human alpha subunits, we adopted a more straightforward approach involving the simultaneous transfection of *CDC50A-V5* and murine *Atp8b1* (a well-studied P4-ATPase, 95% homologous to its human counterpart [34]) as a C-terminal myc-tagged protein in HeLa and HEK-293T cells (Fig. 8A). Interestingly, we found that the rates of perifosine uptake in HeLa and HEK-293T cells increased by 60% and 130%, respectively (Fig. 8B). The uptake of NBD-aminophospholipids (but not NBD-PC) was also similarly boosted in both cell lines (Fig. 8B). Confocal microscopy studies revealed that CDC50A-V5 was retained in the intracellular membrane compartments in HeLa cells when over-expressed alone, whereas when it was co-expressed with Atp8b1 in the same cells (Fig. 9), both proteins co-localized at the plasma membrane, in a similar manner to that reported for the pair ATP8B1 + CDC50A [29]. Co-localization of these two proteins was also observed when co-expressed in HEK-293T cells (data not shown).

4. Discussion

A detailed knowledge of the molecular mechanism of action of anticancer drugs will help to identify those patients who are most likely to respond to a given treatment based on such drugs, to overcome drug resistance phenomena and to improve the final treatment outcome. Indeed, there seems to be a general consensus amongst researchers regarding the manner in which perifosine causes cell death in several different types of cancer cells. However, some discrepancies have arisen concerning how perifosine enters cancer cells, and what previous steps are required for the drug to reach its intracellular targets and trigger cell death. Thus, it has been proposed that ALP enters cells either by raft-dependent endocytosis [13,32,33] or by transporter-mediated translocation of perifosine through the plasma membrane [14–16].

We firmly support the latter hypothesis, and in this present work, which is a continuation of a previous study [14], we present strong evidence to suggest that perifosine enters cancer cells via APLT(s) at the plasma membrane, and that CDC50A plays a key role in this process. Thus, we have found a positive correlation between perifosine uptake and NBD-aminophospholipid translocation (a well-established marker for aminophospholipid translocase activity) in several KB clones obtained in our laboratory. Furthermore, we found no correlation whatsoever between perifosine uptake and endocytosis rates in a subset of fourteen cell lines, which is in stark contrast with previous works where the role of raft-dependent endocytosis in perifosine uptake has been proposed [13,32,33]. This discrepancy may, however, only be apparent if we consider that only adherent cell lines, but no leukemia or lymphoma cells, were used in the present study, whereas the authors who proposed the raft-dependent hypothesis for perifosine uptake have recently published a further study [15] where they discriminate between lymphoma and carcinoma cells on the basis of their prevalent perifosine-uptake mechanism (raft-dependent endocytosis in the former, and a plasma membrane translocator in the latter). It is also possible that both mechanisms of perifosine uptake could co-exist in the same cell, with their relative importance varying with cell type. Thus, for example, KB cells accumulate perifosine very rapidly, and independently of rafts endocytosis, due to their high APLT activity [14,15], whereas HeLa cells accumulate perifosine five times slower as they exhibit lower APLT activity. In this case, the slow drug uptake would depend mainly on endocytosis, as suggested previously [33].

We also found that modulating the CDC50A expression levels in KB cells markedly affected the rate of perifosine and aminophospholipid uptake, thereby suggesting that a putative APLT is responsible for translocation of both types of molecules through the plasma membrane. It also appears that the CDC50A beta subunit should be the limiting factor for the APLT complex in KB cells, where it is distributed throughout the endo-membranous compartments, trafficking from the ER to the plasma membrane of

KB cells. In contrast, CDC50A exports from the ER only to the TGN in KB PER-R clone10 cells and does not reach the cell surface, thereby suggesting that these perifosine-resistant cells harbor a defect that results in the incorrect trafficking of CDC50A at this point of the exocytic pathway. Since CDC50A must couple with a P4-ATPase for correct trafficking of the full APLT complex to the plasma membrane [29], the observed traffic defect of CDC50A in KB PER-R cells could explain the inability of this cell line to translocate perifosine. In contrast to KB cells, over-expression of CDC50A in the human cell lines HeLa and HEK-293T did not modulate perifosine uptake, thus suggesting that, in this case, the alpha subunit rather than CDC50A may be the limiting component of the putative APLT. Human ATP8B1 is a P4-ATPase with APLT activity when complexed with CDC50A, although the expression levels of both *CDC50A* and *ATP8B1* in HeLa and HEK-293T cells were found to be very similar to those in KB. Therefore, if we assume that no differential regulation of the APLT complex occurs in each cell line, and that the overall APLT activity depends mainly on the relative expression levels of its two components, it seems likely that ATP8B1 is not the alpha subunit responsible for the different APLT activities measured in these cell lines. However, we found that the uptake of perifosine and NBD-aminophospholipids (but not NBD-PC) increased remarkably when both HeLa and HEK-293T cells were co-transfected with *Atp8b1-myc* and *CDC50A-V5*. Moreover, CDC50A-V5 remained intracellular when over-expressed alone, but when co-expressed with Atp8b1 both proteins exported from the ER and co-localized at the plasma membrane, as is the case with human ATP8B1 + CDC50A [29]. CDC50A may be coupled with a P4 ATPase to drive perifosine and aminophospholipids uptake and could contribute to the transport specificity of the complex.

Taken together, the results of this work strongly support the role of an APLT as the means of entry of the anticancer drug perifosine through the plasma membrane in adherent carcinoma cells. Such an APLT in humans is most likely formed by the beta subunit CDC50A plus a P4-ATPase alpha subunit(s) whose identity remains to be established.

Acknowledgements

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