Inhibition of phospholipase D2 induces autophagy in colorectal cancer cells

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Autophagy is a conserved lysosomal self-digestion process used for the breakdown of long-lived proteins and damaged organelles, and it is associated with a number of pathological processes, including cancer. Phospholipase D (PLD) isozymes are dysregulated in various cancers. Recently, we reported that PLD1 is a new regulator of autophagy and is a potential target for cancer therapy. Here, we investigated whether PLD2 is involved in the regulation of autophagy. A PLD2-specific inhibitor and siRNA directed against PLD2 were used to treat HT29 and HCT116 colorectal cancer cells, and both inhibition and genetic knockdown of PLD2 in these cells significantly induced autophagy, as demonstrated by the visualization of light chain 3 (LC3) puncta and autophagic vacuoles as well as by determining the LC3-II protein level. Furthermore, PLD2 inhibition promoted autophagic flux via the canonical Atg5-, Atg7- and AMPK-Ulk1-mediated pathways. Taken together, these results suggest that PLD2 might have a role in autophagy and that its inhibition might provide a new therapeutic basis for targeting autophagy.
Figure 1 Depletion of PLD2 induces autophagy. (a) HT29 and HCT116 cells were transfected with an siRNA directed against PLD2 and then immunostained with antibody specific for LC3. Endogenous LC3 punctate dots were observed by fluorescence microscopy, and the number of puncta per cell was quantified (7–10 cells were assessed). The data are representative of three independent experiments. (b) The cells were co-transfected with mRFP-GFP-LC3 and a PLD2-directed siRNA for 24 h, and the total number of RFP-positive/GFP-negative puncta per cell was counted. The data are representative of three independent experiments. (c) The cells were transfected with an siRNA directed against PLD2, and the lysates were immunoblotted with the indicated antibodies. The levels of LC3-II compared with that of α-tubulin were quantified using densitometer analysis. (d) HT29 cells were transfected with an siRNA directed against PLD2 and then cultured under amino acid and serum starvation conditions (HBSS media) for 6 h, after which the lysates were immunoblotted with the indicated antibodies. The levels of LC3-II compared with that of α-tubulin were quantified by densitometer analysis. (e) HT29 cells were transfected with the indicated siRNAs and then fixed and examined by transmission electron microscopy to detect autophagic vacuoles, which were counted in the field of view. Arrows indicate autophagic vacuoles. The values are the means ± s.d. of three independent experiments.
developing lung metastases more readily. In the present study, we demonstrate that PLD2 inhibition induces autophagy.

MATERIALS AND METHODS

Cell culture and reagents

HT29 and HCT116 human colorectal cancer cells were maintained in DMEM with 10% fetal bovine serum and were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Atg5+/+ and Atg5−− mice were provided by Dr G. Velasco (School of Biology, Complutense University); and ATG7 (Abcam, Cambridge, MA, USA). Rabbit polyclonal anti-phospho-AMPK (Thr172, Cell Signaling, Bray, Ireland), AMPK (Cell protein (GFP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), U2OS (Hank’s Buffered Salt Solution; amino acid-free) medium. The cells were then cultured under nutrient starvation conditions such as on HBSS (Hank’s Buffered Salt Solution; amino acid-free) medium. The cells were then transfected with red fluorescent protein (RFP)-LC3 and then cultured under nutrient starvation conditions such as on HBSS (Hank’s Buffered Salt Solution; amino acid-free) medium. The cells were then fixed with 4% paraformaldehyde for 20 min. The cells were then dehydrated with a series of graded ethyl alcohol and embedded in epoxy resin (Epon 812 mixture). Thick sections (1 μm) were stained with 1% toluidine blue for light microscopy. In addition, thin sections (50–60 nm) were prepared using an ultramicrotome (Leica, Reichert SuperNova, Heidelberg, Germany), double stained with uranyl acetate and lead citrate and then examined using a transmission electron microscope (JEM1200EX-II, JEOL, Tokyo, Japan).

Data analysis

The results are expressed as the means ± s.d. of the determinations. The statistical significance of the differences was determined by ANOVA, and significance was accepted when the P-value was lower than 0.05.

RESULTS

Knockdown of PLD2 induces autophagy

To investigate whether PLD2 is involved in the regulation of autophagy, we used RNA interference against PLD2. Autophagy mediates the bulk degradation of cytoplasmic components through the delivery of these components to lysosomes via autophagosomes. Microtubule-associated 1 LC3 localizes to autophagosomal membranes and is a specific marker for autophagosomes, and the development of autophagy is frequently assessed by the number and intensity of LC3 vesicles. We first assessed the effect of PLD2 depletion on autophagy. Depletion of PLD1 significantly induced autophagy in HT29 and HCT116 colorectal cancer cells, as indicated by punctate dots of endogenous LC3 (Figure 1a). Moreover, the maturation process through which autophagosomes are converted into autolysosomes can be monitored by the mRFP–GFP tandem fluorescence-tagged LC3 method. The GFP-LC3 punctate feature of the mRFP–GFP tandem autophagy flux reporter was not detectable in the acidic pH lysosomal environment by lysosomal hydrolysis, whereas the mRFP-LC3 punctate feature was still detectable in this compartment. Depletion of PLD2 significantly increased the transition of RFP–GFP/LC3-positive autophagosomes to RFP-positive, GFP-negative autolysosomes in RFP–GFP/LC3-transfected cells (Figure 1b), suggesting that knockdown of PLD1 promotes autophagic flux. The C-terminal fragment of LC3 is cleaved immediately following its synthesis to yield a cytosolic form called LC3-I (18 kDa), and a sub-population of LC3-I is further converted into an autophagosome-associating form, LC3-II (16 kDa), which localizes to autophagosomal membranes and delivers degraded cytoplasmic components to lysosomes. To examine whether PLD2 knockdown induces autophagy in HT29 and HCT116 cells, we investigated the conversion of LC3 from I to II, a known autophagosome marker. Depletion of PLD2 in HT29 and HCT116 cells increased the LC-II levels and reduced the expression of PLD2, as analyzed by immunoblotting (Figure 1c). In addition, depletion of PLD2 increased the level of LC3-II induced by amino acid and serum starvation conditions (HBSS media) for 6 h (Figure 1d), suggesting that silencing of PLD2 increases both basal and starvation-induced autophagy. Moreover, PLD2 depletion revealed a significant increase in the number of autophagic vacuoles, as analyzed by transmission electron microscopy (Figure 1e). Taken together, these results suggest that depletion of PLD2 induces autophagy.
Figure 2  Inhibition of PLD2 activity induces autophagy. (a) HT29 cells were pre-treated with or without PLD2 inhibitor (10 μM) for 1 h and then cultured in normal or HBSS medium for 6 h. The cells were then fixed, permeabilized and stained with a Texas Red-conjugated antibody specific for endogenous LC3, and the number of LC3 puncta per cell was counted (7–10 cells were assessed). The data are representative of three independent experiments. (b) HT29 cells were transfected with mRFP-GFP-LC3 and then pre-treated with or without PLD2 inhibitor (10 μM) for 1 h, after which the cells were cultured in normal or HBSS medium for 6 h. Autolysosomes were quantified by counting the RFP-positive/GFP-negative puncta per cell (7–10 cells were counted). (c) HT29 cells were co-transfected with GFP vector, GFP-WT-PLD2 or GFP-KRM-PLD2 and RFP-LC3 and then cultured in HBSS or normal medium for 6 h, after which the percentage of RFP punctate cells relative to the percentage of GFP-expressing cells was determined. The data are representative of three independent experiments. (d) HT29 and HCT116 cells were treated with the indicated concentrations of PLD2 inhibitor for 12 h or with 10 μM of PLD2 inhibitor for the indicated time, and the lysates were immunoblotted with the indicated antibody. The level of LC3-II compared with that of α-tubulin was quantified using densitometer analysis. The data are representative of three independent experiments. (e) HT29 cells were treated with PLD2 inhibitor (10 μM) for 12 h and then fixed and examined by transmission electron microscopy to visualize autophagic vacuoles. The quantification was based on counting the autophagic vacuoles in the field of view. Arrows indicate autophagic vacuoles. The values are the means ± s.d. of three independent experiments.
Inhibition of PLD2 activity induces autophagy

The PLD2 inhibitor VU0285655-1 is known to selectively inhibit PLD2. PLD2 inhibition significantly increased autophagy under basal conditions and starvation in HT29 cells, as indicated by punctate dots of endogenous LC3 (Figure 1a). We further examined the effect of VU0285655-1 on autophagy. HT29 cells were transfected with mRFP-GFP tandem LC3 and then treated with VU0285655-1. PLD2 inhibition significantly increased autophagy, as analyzed by mRFP-GFP tandem LC3 puncta (RFP-positive, GFP-negative puncta) formation (Figure 2b), suggesting that PLD2 inhibition promotes the on-rate of autophagy, at least in vitro, in cultured cells. To further examine whether PLD activity is involved in autophagy, we co-transfected RFP-LC3 with wild-type GFP-PLD2 or a catalytically inactive mutant form of PLD2, GFP-PLD2K758R (KRM-PLD2), in HT29 cells. Expression of PLD2 wt, but not of PLD2K758R, significantly inhibited GFP-positive RFP-LC3 puncta formation induced by starvation (Figure 2c), suggesting the involvement of PLD2 activity in the regulation of autophagy. Under basal conditions, PLD2 inhibition increased the level of the LC3-II protein in a dose- and time-dependent manner in HT29 and HCT116 cells (Figure 2d). Furthermore, PLD2 inhibition significantly increased the formation of autophagic vacuoles, as analyzed by transmission electron microscopy (Figure 2e), suggesting that PLD2 inhibition induces autophagy.

PLD2 inhibition induces autophagy via Atg5-, Atg7- and AMPK-Ulk1-mediated pathways

To investigate the regulation of autophagy pathways by PLD1, we examined whether PLD2 inhibition is dependent on Atg genes. A series of protein complexes composed of autophagy-related gene (Atg gene) products coordinate the formation of autophagosomes. In these experiments, we used isogenic MEFs, deficient in the essential autophagy genes, Atg5 or Atg7; these cells are completely defective for autophagy. PLD2 inhibition increased the LC3-II protein level and the number of LC3 puncta in Atg3 and Atg7 MEFs, but the LC3 level and number of LC3 puncta induced by PLD2 inhibition were dramatically suppressed in Atg5 and Atg7 null MEFs (Figures 3a and b). The ATG1/ULK1 complex (Atg1 in yeast and ULK1 in mammals) is an essential positive regulator of autophagosome formation, and PLD1 inhibition dramatically suppressed the level of LC3-II in Ulk1 null MEFs (Figure 3c). Autophagy is controlled

**Figure 3** PLD2 inhibition induces autophagy via Atg5-, Atg7- and Ulk1-mediated pathways. (a) Atg5 MEFs were treated with or without PLD2 inhibitor (10 μM) for 6 h, and the lysates were immunoblotted with the indicated antibodies. The level of LC3-II compared with that of α-tubulin was quantified by densitometer analysis. The data are representative of three independent experiments. (b) Atg7+/+ and Atg7−/− MEFs were transfected with RFP-LC3 for 24 h and then treated with PLD2 inhibitor (10 μM) for 6 h. RFP-LC3 punctate dot cells were observed by fluorescence microscopy, and the number of RFP punctate cells was quantified. The values are the means ± s.d. of three independent experiments. (c) Two isogenic MEF cell lines (Ulk1+/+ and Ulk1-null) were treated with PLD2 inhibitor (10 μM) for 6 h, and the lysates were immunoblotted with the indicated antibodies. The data are representative of three independent experiments. (d) HT29 and HCT116 cells were treated with PLD2 inhibitor (10 μM) for the indicated time, and the lysates were immunoblotted with the indicated antibodies. The data are representative of three independent experiments.
by several kinases, including mTOR, a negative regulator of autophagy, and monophosphate-activated protein kinase (AMPK), a positive regulator of autophagy. mTOR inhibits autophagy initiation by phosphorylating ULK1 Ser75, and the cellular energy-sensing pathway that is controlled by AMPK is upstream of mTOR. Under starvation conditions, activated AMPK inhibits mTOR to relieve the phosphorylation of ULK1 on Ser757, leading to the ULK1-AMPK interaction. AMPK then phosphorylates ULK1 on Ser555, Ser317 and Ser777, activates ULK1 kinase, and eventually leads to the induction of autophagy. PLD2 inhibition increased both the phosphorylation of ULK1 Ser757 by mTOR (Figure 3d). Collectively, these results suggest that PLD2 inhibition induces autophagy via canonical Atg5-, Atg7- and AMPK-Ulk1-mediated pathways.

**PLD2 inhibition increases the on-rate of autophagy**

The accumulation of autophagosomes and autolysosomes by inhibiting or depleting PLD2 could involve enhanced autophagic sequestration (on-rate) or reduced degradation of autophagic material by fusion with lysosomes (off-rate). To distinguish between these possibilities, we assessed PLD2 inhibitor-induced autophagic vacuolization by monitoring the colocalization of an autophagic marker, GFP-LC3, with a lysosomal marker, Lamp-1, in the presence or absence of PLD2 inhibition.
BafA1, which is known to inhibit autophagosome–lysosome fusion.28 The PLD2 inhibitor, but not BafA1, induced colocalization between Lamp-1 and LC3, suggesting that the autophagosome might encounter the lysosome in PLD2 inhibitor-treated HT29 cells (Figure 4a). Moreover, BafA1 further increased the PLD2 inhibitor-triggered induction of LC3-II in HT29 and HCT116 cells (Figure 4b). These results suggest that PLD2 inhibition promotes autophagic flux.

DISCUSSION
In the present study, we demonstrate that PLD2 modulates autophagy; specifically, overexpression of PLD2 suppresses autophagy, and inhibition of PLD2 induces autophagy. Overexpression of PLD1 or PLD2 has been reported to protect cancer cells from apoptosis,29 and aberrant expression of PLDs has been widely implicated in a variety of cancers.9 Furthermore, ablation and pharmacological inhibition of PLD suppresses tumor growth, invasion and metastasis.14,30,31 Autophagy is dysregulated in a wide spectrum of human cancers, and some of these abnormalities have been shown to correlate with clinicopathological parameters and disease outcomes such as overall survival in cancer patients. Thus, it is suggested that autophagy has a pivotal role in tumorigenesis. The role of autophagy in cancer and treatment responsiveness is complicated; the double-edged sword function of autophagy, both as a tumor suppressor and as a protector of cancer cell survival, likely impacts anticancer treatment efficacy in opposing ways. The exploitation of the functional autophagy status in tumor cells,32,33 and p53 has been reported to have a dual role in apoptosis. Thus, we have reported that PLD1 inhibition suppressed autophagic flux, and the treatment provides opportunities for cancer management. PLD2 inhibition increased the number of LC3 punctate dots and the treatment enhanced autophagic flux in colorectal cancer cells,32,33 and p53 has been reported to have a dual role in autophagy regulation.34 Thus, PLD1 may function as both a positive and a negative modulator of the autophagy pathway, depending on the cell type and the cellular context. Further studies will be required to understand the mechanisms underlying the dual role of PLD1 in autophagy. Collectively, the pharmacological and genetic inhibition of PLD2 provides a new therapeutic basis for targeting the autophagic process.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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