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Phospholipase D-mTOR Requirement for the Warburg Effect in Human Cancer Cells

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Abstract

A characteristic of cancer cells is the generation of lactate from glucose in spite of adequate oxygen for oxidative phosphorylation. This property – known as the "Warburg effect" or aerobic glycolysis – contrasts with anaerobic glycolysis, which is triggered in hypoxic normal cells. The Warburg effect is thought to provide a means for cancer cells to survive under conditions where oxygen is limited and to generate metabolites necessary for cell growth. The shift from oxidative phosphorylation to glycolysis in response to hypoxia is mediated by the production of hypoxia-inducible factor (HIF) – a transcription factor family that stimulates the expression of proteins involved in glucose uptake and glycolysis. We reported previously that elevated phospholipase D (PLD) activity in renal and breast cancer cells is required for the expression of the α subunits of HIF1 and HIF2. We report here that the aerobic glycolysis observed in human breast and renal cancer cells is dependent on the elevated PLD activity. Intriguingly, the effect of PLD on the Warburg phenotype was dependent on the mammalian target of rapamycin complex 1 (mTORC1) in the breast cancer cells and on mTORC2 in the renal cancer cells. These data indicate that elevated PLD-mTOR signaling, which is common in human cancer cells, is critical for the metabolic shift to aerobic glycolysis.

Keywords

Phospholipase D; Warburg effect; glycolysis; metabolic transformation; hypoxia-inducible factor

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Conflict of interest
There are no conflicts of interest regarding this paper.

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1. Introduction

A hallmark of cancer cells is aerobic glycolysis whereby there is an increased utilization of glucose and glycolysis for energy and the raw materials needed for cell growth [1]. This effect is commonly referred to as the Warburg effect after its discoverer [2,3]. Glycolysis generates the precursors needed for the synthesis of lipids and nucleotides for generating membranes and nucleic acids [4]. A shift away from mitochondrial respiration also occurs as a response to the stress of hypoxia where oxidative phosphorylation is not an option [5]. Much of the response to hypoxia is due to elevated expression of hypoxia inducible factor-α (HIFα)[2] – a family of transcription factors that stimulate the expression glycolytic and angiogenic genes [5]. HIFα expression is elevated in a significant percentage of human cancers [6].

The expression of the α subunits for both HIF1 and HIF2 is dependent upon phospholipase D (PLD) in human kidney and breast cancer cells [7,8]. Elevated PLD activity in human cancer cells provides both survival and migration signals [8,9]. The primary metabolite of PLD is phosphatidic acid (PA) and it is required for the activation of the mammalian target of rapamycin (mTOR) [10-12], which has also been implicated in survival signals and HIFα expression [13-15]. mTOR has been implicated as a sensor of nutritional sufficiency and elevated mTOR promotes cell cycle progression when there is sufficient nutrition for cells to double their mass and divide [16,17]. Thus, there is a connection between PLD-mTOR survival signals and the Warburg effect in cancer cells. We have investigated whether the Warburg effect is dependent on PLD-mTOR signaling in human cancer cells.

2. Materials and Methods

2.1. Cells, Cell Culture Conditions and Transfection

The 786-O, MDA-MB-231, MCF-7, and HEK293 cells used in this study were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Transfections were performed using Lipofectamine LTX (Invitrogen) according to the vendor's instructions.

2.2. Materials

Antibodies against mTOR, Rictor, Raptor, HIF2α, GLUT1, Actin, and hemagglutinin (HA) were obtained from Santa Cruz Biotechnology; antibodies against Akt1, Akt2, GLUT3 and GLUT4 were obtained from Cell Signaling. The antibody to HIF1α was obtained from BD Biosciences. siRNAs targeting Akt1, Akt2, Raptor, Rictor, and mTOR were obtained from Sigma Aldrich. Rotenone was purchased from EMD biosciences.

2.3. Plasmids

The pcDNA3.1 control plasmid was obtained from Invitrogen. The plasmid expression vectors for HA-tagged catalytically inactive PLD1 and PLD2 (pCGN-PLD1- K898R and pCGN-PLD2-K758R) [18,19] were generous gifts of Dr. Michael Frohman (SUNY-Stony Brook, NY).

2.3. Western Blot Analysis and PLD assays

Extraction of proteins and Western blot analysis of extracted proteins was performed using the ECL system (Amersham) as described previously [20]. PLD activity was determined using the transphosphatidylation reaction as described previously [21].

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2.4. siRNA

Cells were plated on 12-well plates at 30% confluence in medium containing 10% serum without antibiotics. After one day, cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer directions. After 24 hr, the media was changed to fresh media containing 10% serum and two days later cells were lysed and analyzed by Western blot.

2.5. Measurement of glucose uptake

Cells were incubated in DMEM containing 0.5% fetal bovine serum in the presence of 200μM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Molecular Probes) for 2 hours. 2-NDBG uptake by live cells was captured using a fluorescent inverted microscope and was quantified using a spectrofluorimeter by using 470 nm as the excitation wavelength and 545 nm as the emission wavelength. Basal fluorescence was subtracted from all measurements.

2.6. Lactate measurement

24 hours before the assay, cells were counted and 5.0×10^5 cells were incubated in 3 ml of DMEM containing 0.5% fetal bovine serum. Lactate concentration in the same media samples was determined using an EnzyChrom Lactate Assay colorimetric Kit (Bioassays Systems) according to manufacturer's instructions. Optical Density was measured with a spectrophotometer at 565 nm.

2.7. Intracellular ATP level measurements

Intracellular ATP levels were quantified using an ATPLite assay kit (Perkin Elmer) according to the manufacturer's instructions. Luminescence from cell lysate was measured with a microplate luminometer (Luminoskan Ascent, Thermo) and normalized to the protein concentration.

2.8. Reactive oxygen species and H_2O_2 measurements

Intracellular ROS production was measured by staining with dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes). Cells were loaded with 5 μM H_2DCFDA for 1 hr, washed in phosphate-buffered saline, and incubated with fresh media without H_2DCFDA for 30 min. The oxidation product of H_2DCFDA – 2’,7’-dichlorofluorescein (DCF) fluorescence was visualized using inverted fluorescence microscope and quantified with a spectrofluorimeter by using 507 nm as the excitation wavelength and 530 nm as the emission wavelength. Basal fluorescence was subtracted from all measurements.

Intracellular hydrogen peroxide levels were measured using a QuantiChrom Peroxide Assay colorimetric Kit (Bioassay Systems) according to the manufacturer's instructions. Optical Density was measured with a spectrophotometer at 585 nm and normalized to the protein concentration.

3. Results

3.1. Elevated glucose uptake in human cancer cells is dependent on PLD activity

Glucose uptake was examined in four human cell lines – two breast cancer cell lines (MCF-7 and MDA-MB-231), a kidney cancer cell line (786-O), and HEK293 human embryonic kidney cells. These cells have been analyzed previously for their PLD activity with MDA-MB-231 cells having high levels of PLD activity relative to the MCF-7 cells, and 786-O cells having high levels of PLD activity relative to the HEK293 cells [7,22,23]. This is shown graphically in Fig. 1A. The level of glucose uptake in these cells was investigated

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by examining the uptake of a fluorescent-tagged glucose 2-NBDG. As shown in Fig. 1B, the level of glucose uptake was strongly correlated to the level of PLD activity in these cell lines. We next examined whether the elevated glucose uptake observed in the MDA-MB-231 and 786-O cells was dependent on the elevated PLD activity in these cells. The MDAMB-231 and 786-O cells were transiently transfected with catalytically inactive PLD1 and PLD2 mutants, which function as dominant negative PLD mutants [7,20,21]. As shown in Fig. 1C, the dominant negative mutants for both PLD1 and PLD2 suppressed the uptake of glucose into the cytoplasm of both MDA-MB-231 and 786-O cells. The expression of both PLD1 and PLD2 mutants together was even more effective in suppressing glucose uptake – especially in the 786-O cells (Fig. 1C). These data indicated that the elevated glucose uptake in these cancer cells is dependent on both PLD1 and PLD2.

3.2. Glucose transporter expression is dependent on PLD activity

A key aspect of increased uptake of glucose is the expression level of glucose transporters (GLUT) – membrane proteins that facilitate uptake of glucose. We therefore examined the effect of the dominant-negative PLD mutants on the levels of GLUT1, GLUT3 and GLUT4 in 786-O and in MDA-MB-231 cells. As shown in Fig. 2, the levels of all three glucose transporters was substantially reduced in both the 786-O and MDA-MB-231 cells in the presence of the dominant negative PLD1 and PLD2 mutants. The expression of HIF2α was also suppressed by the dominant negative PLD mutants in the 786-O cells as reported previously [7]. These data indicate that PLD activity is required for the expression of GLUTs and is consistent with the PLD requirement for increased glucose uptake.

3.3. Suppression of PLD activity reverses phenotypes correlated with the Warburg effect

A hallmark of the Warburg effect is the reduction of pyruvate, the end product of glycolysis, to lactic acid, which is accompanied by the oxidation of NADH to NAD^+. This reaction takes place instead of the conversion of pyruvate to acetyl-CoA, which can enter the tricarboxylic acid (TCA) cycle [29]. We therefore examined the levels of lactic acid in the media of 786-O and MDA-MB-231 cells with and without the dominant negative PLD mutants. As shown in Fig. 3A, the levels of secreted lactic acid were substantially reduced in the cells that were transfected with the PLD mutants. These data reveal that PLD activity is required for increased lactate production – a characteristic of the Warburg effect.

Another hallmark of the Warburg effect is the shut down of oxidative phosphorylation in the mitochondria. Oxidative reactions in the mitochondria result in the production of reactive oxygen species (ROS), which are converted to H_2O_2 by superoxide dismutase. We therefore examined the effect of suppressing PLD activity on the production of ROS and H_2O_2 in both MDA-MB-231 and 786-O cells. We examined the effect of the dominant negative PLD mutants on the level intracellular oxidants by staining cells with H_2DCFDA, which is oxidized by ROS to the highly fluorescent DCF. As shown in Fig. 3B, introduction of the dominant negative PLD1 and PLD2 mutants significantly increased the level of DCF. The effect was reversed by the mitochondrial complex 1 inhibitor rotenone, indicating that increased level of ROS was due to mitochondrial activity. We also examined the level of H_2O_2 in the MDA-MB-231 and 786-O cells. As indicated in Fig. 3C, the level of H_2O_2 in both cell lines was dramatically increased when the dominant negative PLD mutants were introduced. These data support the hypothesis that PLD activity is required for suppression of mitochondrial oxidative phosphorylation.

Although mitochondrial respiration generates ATP more efficiently, ATP is generated more rapidly with elevated glucose uptake and glycolysis [29]. Shutting down the TCA cycle has been shown to increase cellular ATP levels [28]. We therefore examined the impact of the PLD dominant negative mutants on cellular ATP levels in the MDA-MB-231 and 786-O

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cells, and as shown in Fig. 3D, the PLD mutants reduced cellular ATP levels. Collectively, the data in Fig. 3 reveal that the elevated PLD activity in both MDA-MB-231 and 786-O cancer cell lines is required for the production of lactate and the suppression of mitochondrial respiration – both hallmarks of the Warburg effect in cancer cells.

3.4. The effect of PLD on glucose uptake is mediated by mTOR

A critical downstream target of PLD is mTOR – the mammalian target of rapamycin [12,14]. We have reported previously that PLD activity is required for the activation of both mTORC1 and mTORC2 in both the 786-O and MDA-MB-231 used in this study [21,22]. We therefore examined whether the dependence of glucose uptake was also dependent on mTOR. mTOR exists as two complexes – mTORC1 and mTORC2, which have differential sensitivities to rapamycin. The mTOR complexes have different components that are required for their activity – mTORC1 is dependent upon a protein known as Raptor and mTORC2 is dependent on Rictor [30]. We examined the effect of depleting cells of mTOR, Raptor, and Rictor on glucose uptake in the MDA-MB-231 and 786-O cells. In the 786-O cells, we found that siRNA for mTOR strongly suppressed glucose uptake (Fig. 4A). Rictor siRNA also strongly suppressed glucose uptake, whereas the siRNA for Raptor did not strongly suppress glucose uptake. These data indicate that the elevated glucose uptake in the renal cancer cell line 786-O is dependent on mTORC2. We also examined the effect of suppressing mTOR, Raptor, and Rictor on glucose uptake in the MDA-MB-231 cells where glucose uptake was also strongly suppressed by mTOR siRNA [Fig. 4A]. However, we saw the reverse effect with Raptor and Rictor siRNA in the MDA-MB-231 cells whereby the Raptor siRNA suppressed glucose more strongly than the Rictor siRNA. Thus, in the MDA-MB-231 cells, the elevated glucose uptake is apparently dependent upon mTORC1.

A target of mTORC2 is Akt, which gets phosphorylated by mTORC2 at Ser473 [31]. Akt has also been shown to stimulate aerobic glycolysis [32]. There are three Akt isoforms, of which Akt1 and Akt2 are expressed ubiquitously [33]. Akt1-deficient mice have developmental defects and Akt2-deficient mice have defects in glucose homeostasis. The MDA-MB-231 and 786-O cells were treated with siRNAs for Akt1 and Akt2 and the levels of glucose uptake was evaluated. As shown in Fig. 4B, depleting cells of Akt2, but not Akt1 abolished glucose uptake in the 786-O, but not in the MDA-MB-231 cells. Depleting cells of Akt1 did not have any significant impact on glucose uptake in either the MDA-MB-231 or 786-O cells. These data indicate that Akt2, a critical downstream target of mTORC2, is critical for the elevated glucose uptake in 786-O cells and are consistent with the dependence of glucose uptake in these cells on mTORC2 observed in Fig. 4A. They are also consistent with the dependence of glucose uptake on mTORC1 in MDA-MB-231 cells – in that Akt is not a target of mTORC1 [30]. This finding is consistent with the observation that mice with defective Akt have defects in glucose homeostasis [34].

4. Discussion

The “metabolic transformation” [1] that takes place in most cancer cells has attracted renewed attention as it has become apparent that the altered metabolism is closely integrated with oncogenic transformation. The metabolic changes that occur in cancer cells confer several advantages that allow cells to survive in an emerging tumor mass where there is inconsistent vascularization. We reported previously that elevated PLD activity in renal cancer cells is required for the expression of both HIF1α and HIF2α [7]. In this report, we have provided evidence that the elevated PLD activity in both breast and renal cancer cells is required for the metabolic changes that take place in most cancer cells. A critical target of the PA generated by PLD is mTOR [12], and consistent with a role for mTOR in the PLD-dependent increase in glucose uptake, suppression of mTOR expression reduced glucose uptake in both the 786-O and MDA-MB-231 cells. Interestingly, glucose uptake in the

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MDA-MB-231 cells was dependent mTORC1, whereas in the 786-O cells, glucose uptake was dependent on mTORC2. There is substantial data supporting a role for PA in the regulation of mTORC1 [12]. However, the mTORC2 requirement for glucose uptake demonstrated here suggests that PA also regulates mTORC2. Consistent with this hypothesis, we recently reported that PA is required for the assembly of both mTORC1 and mTORC2 complexes [21]. The lack of an mTORC2 requirement in the MDA-MB-231 cells is consistent with the observation that there are very low levels of Akt phosphorylation at the mTORC2 site at Ser473 [9]. The observation here that Akt2 is required for the effect in 786-O, but not in the MDA-MB-231 cells, is also consistent the differential dependence on mTORC1 and mTORC2.

We demonstrated previously that HIF1α levels are elevated in response to PLD activity in the MDA-MB-231 cells [8]. The 786-O cells used here express only HIF2α, which is also dependent on PLD activity [7]. Since both HIF1α and HIF2α have been implicated in the Warburg effect and the metabolic shift to aerobic glycolysis in cancer cells, the data provided here suggest two independent routes to metabolic transformation – one that goes through mTORC1 and HIF1α and another that goes through mTORC2, Akt2 and HIF2α. PLD is apparently required for both pathways.

While the Warburg effect has been revisited lately, it has become apparent that the metabolic changes that occur in cancer cells is due to a need for the raw materials needed for cell growth [35-37]. Thus, the altered metabolism of cancer cells may provide new opportunities for therapeutic intervention since this property distinguishes the cancer cells from normal cells. Consistent with this hypothesis, tumorigenicity was severely diminished by knockdown of the lactate dehydrogenase gene [38]. Suppression of PLD activity in the absence of serum resulted in apoptotic cell death in both 786-O and MDA-MB-231 cells [7-9]. Moreover, suppression of HIF2α expression in renal cancer cells suppressed tumorigenesis [39]. Thus, targeting both the metabolic changes and the signals that bring about these metabolic changes has already been shown to have therapeutic potential. In fact, it was recently proposed that tumor cell metabolism might be the Achilles' heel for cancer cells [40,41]. Thus, learning more about the metabolic shift that occurs in cancer cells will likely reveal many new possibilities for therapeutic intervention.

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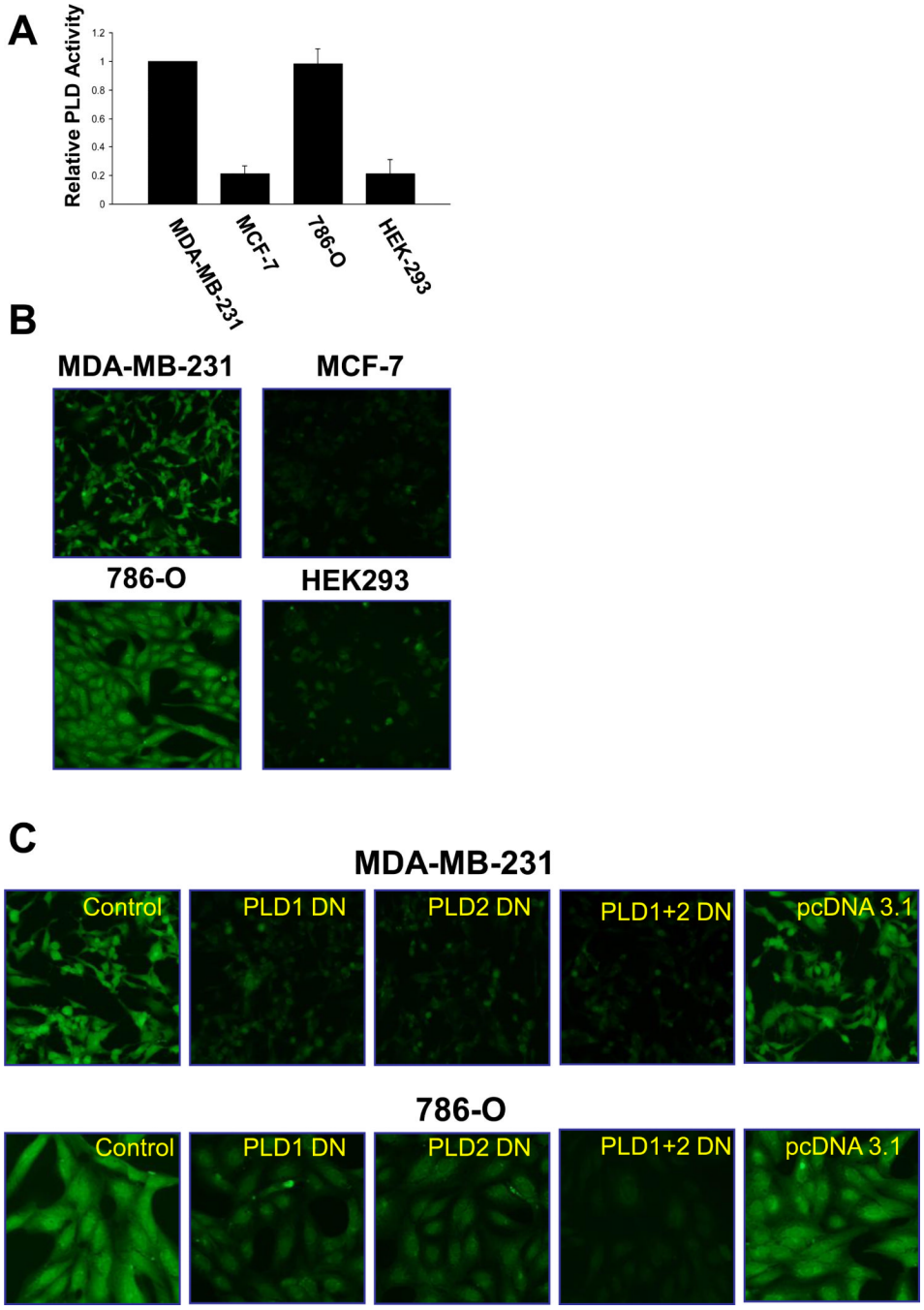

Fig. 1.

Glucose uptake in human cancer cells correlates with PLD activity. (A) MDA-MB-231, MCF-7, 786-O, and HEK293 cells were plated at 80% confluence. 24 hr later, the cells were shifted to media containing 0.5% serum overnight. PLD activity was determined as described previously [21]. The PLD activity was normalized to that observed in the MDA-MB-231 cells. Error bars represent the standard error for three independent experiments. (B) MDA-MB-231, MCF-7, 786-O, and HEK293 cells, prepared as in (A) were treated with 2-NDBG (200μM) and incubated for 2 hr. The fluorescence in the cells was then visualized by inverted fluorescent microscopy. All images were taken at the same exposure. (C) MDA-MB-231 and 786-O cells were plated at 80% confluence. 24 hr later, the cells were

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transfected with plasmid vectors expressing either PLD1 or PLD2 as indicated. The parental vector was pcDNA 3.1, which was used as an empty vector control. Control cells shown were treated with transfection reagent, but without DNA. 24 hr later, the cells were given fresh media with 0.5% serum and incubated for an additional 24 hr. The following day 2-NDBG uptake was evaluated as in (B). All experiments shown are representative of at least three independent experiments.

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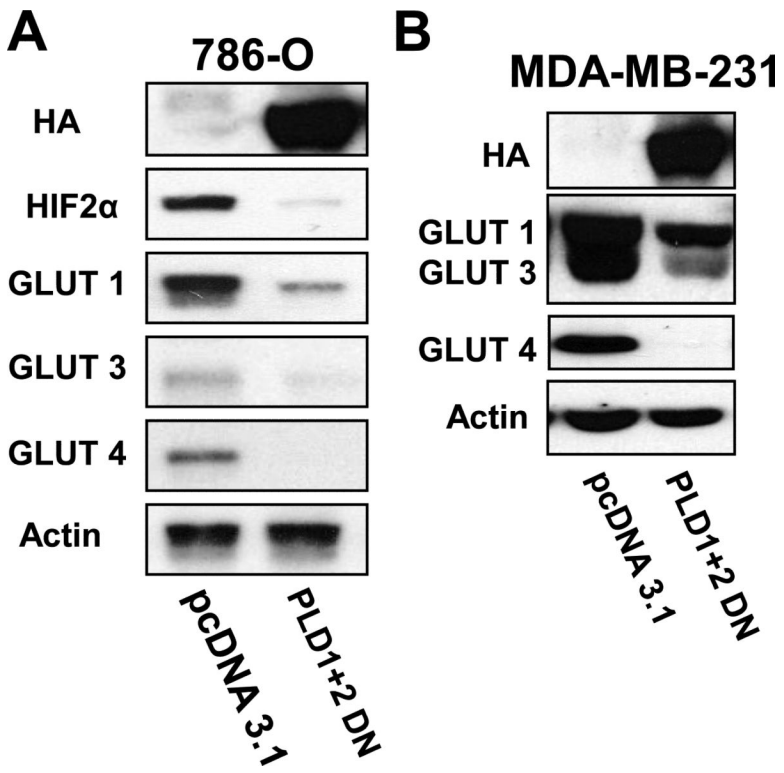

Fig. 2.

Glucose transporter expression is dependent on PLD activity. 786-O and MDA-MB-231 cells were plated and 24 hr later were transfected with the indicated plasmid vectors as in Fig. 2. 24 hr later the cells were shifted to DMEM containing 0.5% serum. The following day the cells were evaluated for the expression of GLUT1, GLUT3, and GLUT4 by Western blot analysis. The expression of HIF2α in 786-0 cells was also evaluated. Expression of the PLD mutants was evaluated by probing the blots for the HA tags on the PLD mutants [18,19]. Blots were also probed for actin as loading controls. Experiments shown are representative of at least two independent experiments.

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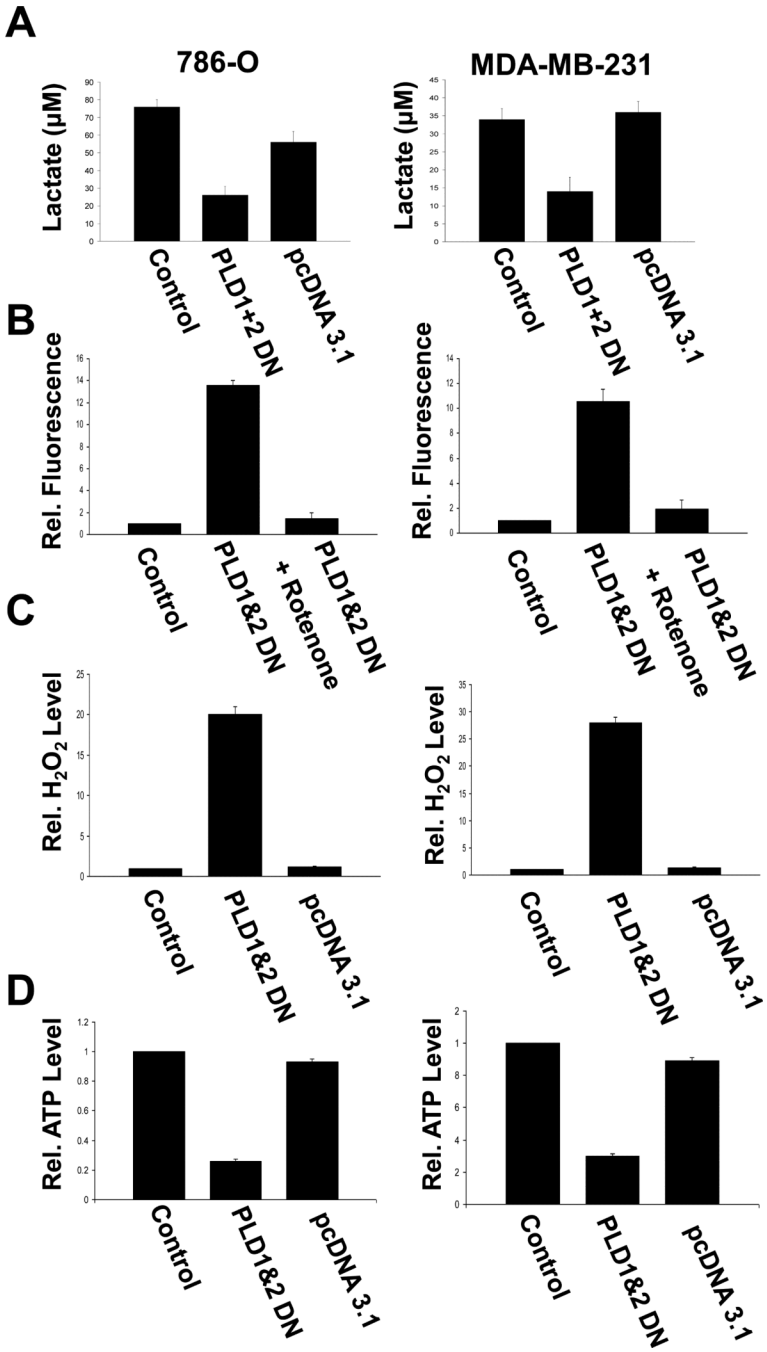

Fig. 3.

Lactate production is dependent on PLD activity. (A) MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in Fig. 1. 24 hr after transfection, the cells were counted and replated in 60mm plates at 8.0×10^5 cells/plate, in media containing 0.5% serum. (A) The level of lactic acid (μM) was determined 24 hr later as described in Materials and Methods. Error bars represent the standard deviation for triplicate samples from a representative experiment that was repeated twice. (B) Cells were prepared and transfected with the indicated vectors as in (A). 24 hr later, the media was replaced with fresh media containing 0.5% serum and the cells were incubated for an additional 24 hr in the absence or presence of rotenone (0.1 μM). H_2DCFDA (5 μM) was then added for 1 hr,

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after which the cells were washed and incubated in fresh media without H_2DCFDA for an additional 30min. The fluorescent oxidized DCF was evaluated as described in Materials and Methods. (C - D) MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in (A) and the levels of H_2O_2 (C) and intracellular ATP (D) were determined as described in Materials and Methods. The data in B-C were normalized to the controls which were given a value of one. Error bars represent the standard deviation for at least two independent experiments.

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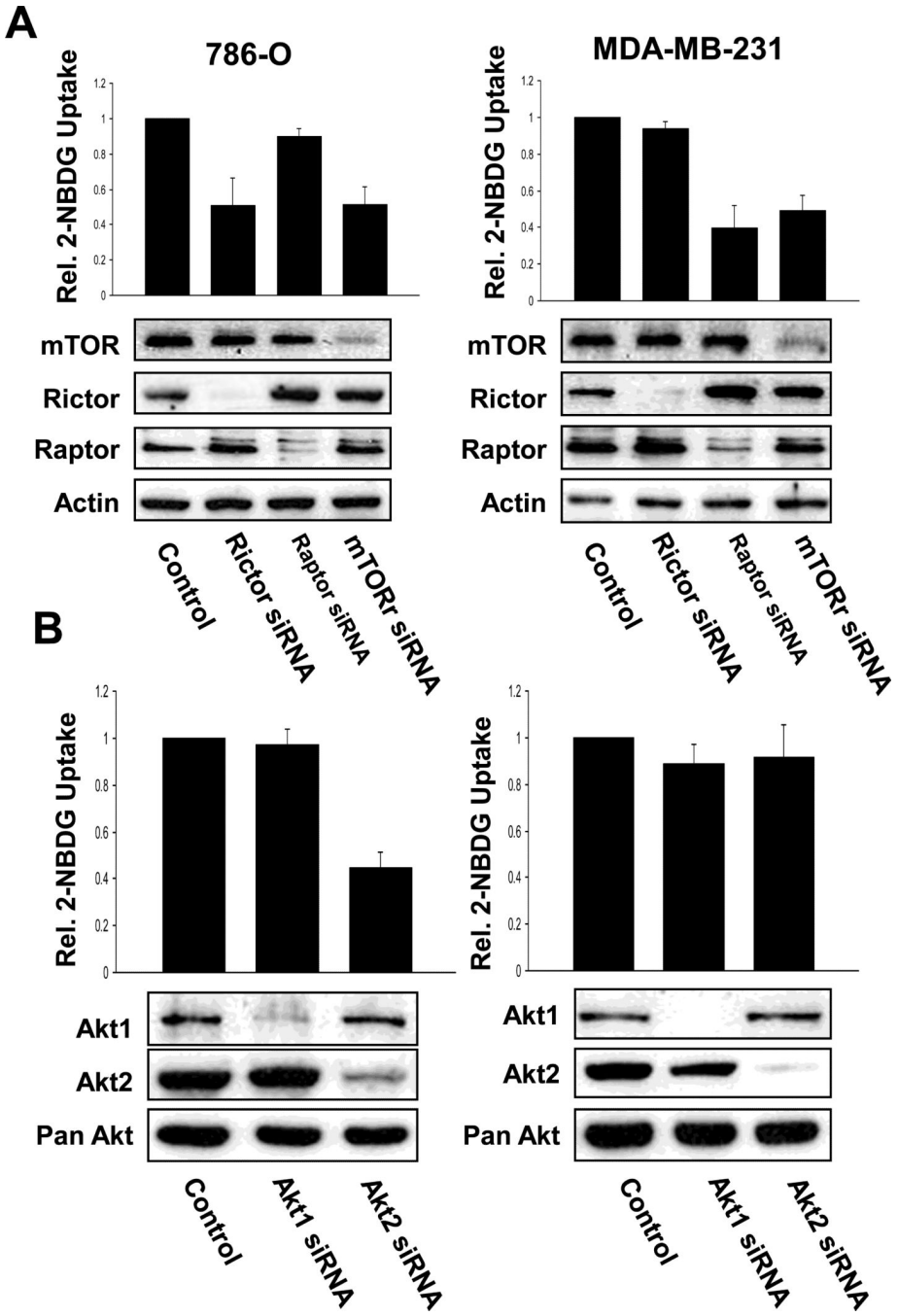

Fig. 4.

The effect of PLD on glucose uptake mediated by mTOR and Akt signals. (A) 786-O and MDA-MB-231 cells were plated at 30% confluence. 24 hr later the cells were transfected with siRNAs for mTOR, Raptor and Rictor. 24 hr later, the cells were shifted to media containing 0.5% serum and 2-NDBG uptake was evaluated 24 hr later as described in Materials and Methods. The expression of mTOR, Raptor, and Rictor was evaluated by Western blot. (B) 786-O and MDA-MB-231 cells were transfected with siRNAs for Akt1 and Akt2 as described in (A). 24 hr later, the cells were shifted to media containing 0.5% serum and 2-NDBG uptake was evaluated 24 hr later. The data were normalized to the controls which were given a value of one. Error bars represent the standard deviation for at

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least two independent experiments. A Western blot is shown that reveals that the siRNAs successfully suppressed the expression of Akt1 and Akt2. Experiments shown are representative of at least two independent experiments.