Published in final edited form as:
J Mol Biol. 2011 May 20; 408(5): 850–862. doi:10.1016/j.jmb.2011.03.017.

Cell Invasion of highly metastatic MTLn3 cancer cells is dependent on Phospholipase D2 (PLD2) and Janus Kinase 3 (JAK3)

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Abstract

MTLn3 cells are highly invasive breast adenoacarcinoma cells. The relative level of EGF-stimulated invasion of this cell line is greater than 2 other breast cancer cell lines, MDA-MB-231 and MCF-7, and one non-small cell lung cancer, H1299. We have determined that the mechanism of cancer cell invasion involves the presence of an enzymatically active phospholipase D (PLD), with the PLD2 isoform more relevant than PLD1. Silencing PLD2 abrogated invasion whereas ectopic expression of PLD2 augmented cell invasion in all four cell lines with an efficacy (MTLn3≥MDA-MB-231>H1299≥MCF-7) that correlated well with their abilities to invade matrigel *in vitro*. We also report that PLD2 is under control of Janus Kinase-3 (JAK3) with the kinase phosphorylating PLD2 at the Y415 residue, thus enabling its activation. Y415 is located downstream a PH domain and upstream the catalycic HKD-1 domain of PLD2. JAK3 knockdown abrogated lipase activity and EGF-stimulated cell invasion directly. For the purposes of activating PLD2 for cell invasion, JAK3 operates via an alternative pathway that is independent of STAT, at least in the MTLn3 cells. We also consistently found that JAK3 and PLD2 pathways are utilized at maximum efficiency (phosphorylation and activity) in highly invasive MTLn3 cells *vs.* a relatively low utilization in the less-invasive MCF-7 cell line. In summary, a high level of cell invasiveness of cancer cells can be explained for the first time by a combined high JAK3/PLD2 phosphorylation and activity, involving PLD2's Y415 residue, which might constitute a novel target to inhibit cancer cell invasion.

Keywords

Phospholipase; JAK3; MTLn3 cells; MCF-7 cells; cell invasion

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Introduction

Invasion through basement membranes is the first step leading to cancer metastasis. When carcinoma cells reach the stromal compartment, they begin to intravasate in capillaries and lymphatic vessels and transfer to other sites where they can colonize new tissues [1; 2]. Invasion is a form of cell migration that enables malignant cells to penetrate into neighboring tissues and degrade the extracellular matrix with proteases MMP-1, -2 and -9. Cancer cell invasion is thus critical in the overall multi-step phenomena of tumor metastasis, but in spite of this, a clear understanding of the signal pathways relevant to invasiveness is still lacking. Our laboratory and others have demonstrated that phospholipase D (PLD) is a key element needed for cell migration of leukocytes [3; 4; 5; 6; 7;8]. For the present study, we hypothesize that in a similar fashion, cancer cell invasiveness could be dependent on the presence of PLD at the time of metastatic initiation. For this we have utilized a panel of breast cancer cells that are responsive to EGF as chemoattractant, including MTLn3, a highly metastatic rat mammary adenocarcinoma cell line [4], and human MDA-MB-231, H1299 and MCF-7 cells. MDA-MB-231 cells possess the largest number of EGF receptors per cell calculated to be ~700,000 followed in descending order by MTLn3 cells, H1299 cells and MCF-7 cells have the fewest EGF receptors per cell estimated at ~3000–6000 per cell [9; 10; 11; 12].

PLD is an enzyme that breaks down phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. These second messengers, PA and choline, are involved in many cellular functions ranging from cytoskeletal rearrangement, phagocytosis, vesicle trafficking, exocytosis [13; 14] and, importantly, cell migration [15]. PLD has been implicated in cell proliferation and cancer [16; 17; 18]. PLD has two common isoforms, PLD1 and PLD2. PLD1 is a 1072 amino acid 120 kDa protein localized to the long arm (q) of chromosome 3 (3q26) and is found in perinuclear, Golgi and heavy membrane fractions [16; 19]. The PLD1 location, 3q26, is a very common chromosomal alteration that is found in many cancers, including that of ovarian lung, prostate, esophageal, nasopharyngeal and breast cancer [20; 21; 22; 23; 24]. PLD2 is a 933 amino acid 106kDa protein, located on the short arm (p) of chromosome 17(17p13) and predominately found in the plasma membrane [25]. Additionally, the PLD2 location, 17p13, is situated in an area of chromosome 17 that besides being involved in brain and eye development and in somatic growth is also found to be frequently altered in breast cancers and contributes to high S-phase, a higher aggressive tumor phenotype and possibly metastasis [26; 27; 28].

In the present paper, we have investigated cell invasion and defined a new mechanism that explains the highly invasive potential of adenocarcinoma cells. It is shown for the first time that JAK3-specific lipase phosphorylation of PLD2 directly mediates MTLn3 cell invasion, which implicates a role for JAK3 in EGF-mediated processes and cancer.

Results

Cell invasion of MTLn3 and MCF-7 cells is negatively affected by silencing PLD2

MTLn3 and MDA-MB-231 cancer cells are two highly invasive breast adenocarcinoma cell lines. Figure 1(a) represents hematoxylin-stained MTLn3 cells that invaded through the matrigel medium in response to chemoattractant and are shown as an example of the morphology of the invading cell. In the matrigel setting, we have determined the relative levels of EGF-mediated cell invasion of 3 breast adenocarcinoma cell lines and 1 non-small cell lung cancer cell line in response to 3 nM EGF (Figure 1(b), black bars). The hierarchical level of cell invasion for these 4 cancer cell lines was as follows: MTLn3 > MDA-MB-231 > H1299 > MCF-7. Overall, the highly invasive MTLn3 cells were ~7-fold more invasive than the MCF-7 cells. We found that all four cancer cell lines were negatively affected by

silencing PLD2 with siRNA (Figure 1(a), grey bars) which indicates a dependency of cell invasion on PLD. Perhaps as expected, the effectiveness of PLD2 silencing varied by cell line (Figure 1(c)), as did the endogenous amount of PLD2 in each cell line. At any rate, silencing of PLD and a concomitant robust inhibition of cell invasion indicates how crucial PLD for invasion is.

Overexpression of wild-type PLD1 or PLD2 can augment cell invasion

We next overexpressed PLD1 and PLD2 constructs (wild-type and lipase-dead mutants) in the four cell lines and analyzed western-blotting (Figure 2(a)), cell invasion (Figure 2(b–e)) and immunofluorescence (Figure 3). Figure 2(a) shows a robust protein expression of all constructs in MTLn3 cells as an example of transfection. Overexpression of PLD1 and PLD2 led to significant increases in cell invasion in non-stimulated and EGF-stimulated MTLn3 cells (Figure 2(b), black bars). This was observed only with catalytically active PLD constructs (wild-type) but not with lipase dead mutants (KR) (Figure 2(b), grey bars). A similar pattern of cell invasion responses were seen in MDA-MB-231 (Figure 2(c)) and MCF-7 (Figure 2(d)) cells, with PLD2 having a greater effect than PLD1, whereas H1299 (Figure 2(e)) cells showed a robust cell invasion only in the presence of EGF, yet again with PLD2 showing a greater response than PLD1. All these overexpression results reinforce the silencing data of Figure 1 and both together serve to indicate the dependency of cancer cell invasion on PLD2 and its enzymatic activity.

Immunofluorescence microscopy of non-transfected (mock) MTLn3 cells indicates a slightly increased punctate staining of endogenous PLD2 in vesicles following EGF stimulation when compared to the negative control (Figure 3(a)). This effect was significantly increased when MTLn3 cells were transfected with myc-tagged PLD2 (yellow arrowheads) (Figure 3(b)) when compared to that of the endogenous control sample. Additionally, we determined the effect of silencing PLD2 on the immufluorescence localization of PLD2 in MTLn3 cells (Figure 3(c)), confirming that endogenous PLD2 staining as detected using an anti-PLD2 antibody is significantly reduced and virtually undetectable when compared to endogenous (Figure 3(a)) or overexpressed PLD2 (Figure 3(b)). When MTLn3 cells were first silenced for PLD2 and then subsequently transfected for PLD2 overexpression, we were able to document the partial rescue of PLD2 protein expression (Figure 3(d)).

Implicating Janus Kinase-3 (JAK3) and PLD2 activities in the four cancer cell lines

Recently, our lab has shown that JAK3 is involved in chemotaxis of IL-8-stimulated human neutrophils and differentiated HL-60 cells (dHL-60) and can modulate PLD2 activity [29; 30]. We decided to investigate if JAK3 would also play a role in regulating PLD2 activity in cancer cell invasion. For this, we first ascertained the relative levels of JAK3 in the four cell lines at hand. By western-blot, we proved the presence of JAK3 in MTLn3, MDA-MB-231, and H1299 cells (Figure 4(a)). We then hypothesized that the initial cell invasion results shown in Figure 1(a) could be also attributed to JAK3 kinase activity in these cell lines. We measured JAK3 kinase activity with a JAK3tide as an exclusive peptide substrate for JAK3, which has been utilized by other authors [31], as it is phosphorylated by JAK3 at Tyr-7 and not by any other JAK family kinase. Endogenous JAK3 activity (in the absence of EGF) was minimal for all four cell lines (Figure 4(b), open bars). Following EGF stimulation, endogenous JAK3 phosphorylation activity was then detectable in all cell lines (Figure 4(b), filled bars). MTLn3 cells had the highest EGF-mediated endogenous JAK3 activity, while the H1299 cells have the second highest level of endogenous JAK3 activity, followed by MDA-MB-231 and MCF-7 cells with the lowest levels of endogenous JAK3 activity. Similarly to JAK3 activity, endogenous PLD activity of unstimulated cells was relatively low for all 4 cell lines (Figure 4(c), open bars). Following EGF stimulation, MTLn3 cells

had the greatest level of endogenous PLD activity of all four cancer cell lines tested (Figure 4(c), filled bars) with considerably lower endogenous PLD activity levels present in the MDA-MB-231, H1299 and MCF-7 cells. Overall, these data are consistent with a role for PLD in EGF-mediated cell invasion, as was also found to be the case in MDA-MB-231 and MCF-7 cells used by [32].

In summary, all four cell lines have basal expression levels of both kinase and lipase activities which are robustly enhanced by EGF treatment. Out of the 4 cell lines investigated, we have found a trend where MTLn3 cells have the highest levels of cell invasion (Figure 1(a)), highest response to ectopic PLD2 overexpression (Figure 2(b)), highest endogenous PLD activity (Figure 4(b)) and highest JAK3 activity (Figure 4(a)), whereas MCF-7 cells showed the lowest levels of all four parameters considered. As such, we focused on MTLn3 and MCF-7 cells for all subsequent experiments and investigated the relationship between PLD2, JAK3 and cell invasiveness in these two cell models.

JAK3 activates PLD2 to a greater extent in highly invasive MTLn3 cells than in MCF-7 cells

We have observed that JAK3 can phosphorylate PLD2 *in vitro*. Figure 5(b) for MTLn3 cells and Figure 5(c) for MCF-7 cells (Figure 5(c)) show a phospho-PLD2 band as a result of treatment with recombinant, active JAK3 in a radioimmunokinase assay. Initial studies have indicated a role for Y-415 in regulating PLD2 activity[29]. As the PLD2 Y-415 residue is located within a JAK3 consensus site between the PH domain and the first of two HKD catalytic domains (Figure 5(a)), we prepared a PLD2 mutant (PLD2-Y415F) plasmid encoding for a protein incapable of being phosphorylated by JAK3. We determined the level of *in vitro* phosphorylation of PLD2-Y415F in MTLn3 (Figure 5(b)) and MCF-7 (Figure 5(c)) and it resulted in lower phosphorylation than that achieved by PLD2-WT. Figure 5(b)(c) depicts more JAK3 phosphorylation of PLD2-WT when compared to the mutant PLD2-Y415F, particularly in MTLn3 cells. Therefore, this data supports the notion that the PLD2-Y415 is a substrate for JAK3 kinase action.

Next, we determined the order of signaling events between JAK3 and PLD2, and to accomplish this we established the effect of JAK3 phosphorylation of PLD2 on its lipase activity in MTLn3 and MCF-7 cells. As shown in Figure 6(a–b), PLD2 activity increased by ~2-*fold* and ~1.3-*fold* (left black bars in graphs), respectively, when active, recombinant JAK3 protein was incubated *in vitro* with whole cell lysates from MTLn3 or MCF-7 cells overexpressing PLD2-WT (which phosphorylated the myc-tagged PLD2 protein) as compared to reactions devoid of the kinase (second empty bar from left in graphs). Importantly, when this phosphorylation site on PLD2 is removed in the PLD2-Y415F mutant, lipase activity of both cell lines (Figure 6(a)(b)) was reduced to basal levels. PLD2-Y415F activity did respond to JAK3 treatment in either cell line (two bars to the right). These data suggest that JAK3 can utilize PLD2 as an effective kinase substrate. However, JAK3 can phosphorylate PLD2 to a greater extent in MTLn3 cells compared to MCF-7 cells.

Site-specific phosphorylation of PLD2 by JAK3 is crucial to MTLn3 cell invasion

Next, we examined the phosphorylation site at play (Y415) on the PLD2 molecule during cell invasion to prove a direct JAK3 interaction with PLD2 by transfecting PLD2–WT or mutant PLD2-Y415F plasmid DNA into MTLn3 and MCF-7 cells. Both MTLn3 (Figure 6(c)) and MCF-7 (Figure 6(d)) cells overexpressing PLD2-WT in the presence of EGF are ~2-*fold* more invasive than unstimulated cells. MTLn3 cells that overexpress the PLD2-Y415F mutant resistant to JAK3 phosphorylation experience ~60% reduction in cell invasion in the presence of EGF when compared to wild-type (Figure 6(c)). However, the effect of PLD2-Y415F on MCF-7 cell invasion was much smaller ($p < 0.05$) than that of the MTLn3 cells (Figure 6(d)). This data implicates the greater importance of JAK3

phosphorylation of PLD2 for PLD2-mediated MTLn3 cell invasion compared to the less invasive MCF-7 cells.

JAK3 silencing is rescued by PLD2 overexpression

Data in the previous figure was of an *in vitro* nature and served to highlight the key role of JAK3 in enhancing PLD2 activity. Next, we tested suppressing JAK3 using small interfering RNA specific for JAK3 in MTLn3 (Figure 7(a)) or MCF-7 (Figure 7(b)) cells. Invasion of both cell lines that were silenced with siJAK3 was inhibited by ~50% or ~30%, respectively, when compared to the non-transfected controls. Overexpression of PLD2-WT following JAK3 silencing rescued cell invasion and increased motility by >200% in MTLn3 cells when compared to the siJAK3 only sample devoid of PLD2 overexpression (Figure 7(a)). However, the same rescue scenario utilized in MCF-7 cells only increased cell invasion by a mere 10% (Figure 7(b)). Regarding a possible participation of STAT3 in JAK3-PLD2 interaction, we found that in the absence of PLD2, invasion was inhibited by siJAK3 alone, siSTAT3 alone or both siJAK3 and siSTAT3 in combination in MTLn3 cells. Further, MTLn3 cell invasion was also rescued by PLD2 when both JAK3 and STAT3 were silenced but not that of MCF-7 cells. All this indicates that JAK3, for the purposes of activating PLD2 for cell invasion, operates via an alternative pathway that is independent of STAT3 at least in the MTLn3 cells.

Discussion

Our laboratory and others have reported that phospholipase D (PLD) is a key element needed for cell migration of leukocytes [3; 4; 5; 6; 7; 8]. In the present study, we have demonstrated that cancer cell invasiveness is dependent on the presence of PLD (particularly the PLD2 isoform) at the time of metastatic initiation. For this, we have utilized a panel of breast cancer cells, including MTLn3, a highly metastatic rat mammary adenocarcinoma cell line [4] and human MDA-MB-231 and MCF-7 cells, as well a non-small lung cancer cell line, H1299. Ectopic expression of PLD2 augmented cell invasion in all four cell lines with an efficacy (MTLn3≥MDA-MB-231>H1299≥MCF-7) that correlated well with their abilities to invade matrigel *in vitro* and that also correlated well with the relative levels of endogenous PLD2 in the cells. Previously, the greater invasive ability of MDA-MB-231 breast cancer cells has been demonstrated to be dependent on both mTOR and PLD2 and ties apoptotic suppression with metastasis progression [32]. This group also was able to document that the overexpression of PLD2 in MCF-7 cells resulted in increased cell migration and invasion when compared to negative controls.

Current literature indicates that JAK1, JAK2, JAK3 and Tyk2 are present to some extent in MCF-7 cancer cells, JAK1 and JAK2 are present in MDA-MB-231 cancer cells and JAK3 is present in MTLn3 cancer cells. We documented the presence of JAK3 protein in MTLn3, MDA-MB-231, MCF-7 and H1299 cancer cells using western-blot analysis. MTLn3 cells had the highest degree of EGF-mediated endogenous JAK3 activity, while the MDA-MB-231, MCF-7 and H1299 cells had lower levels of endogenous JAK3 activity following treatment with EGF. JAK3 catalytic activities of the 4 cancer cell lines did not necessarily correlate with relative JAK3 protein expression levels as detected using western-blot analysis as did that of PLD2. However, these data overall implicate a role for JAK3 in EGF-mediated cell migration processes and cancer. Additionally, we document that MTLn3 cells had the uppermost levels of EGF-mediated endogenous PLD lipase activity, while the other 3 cancer cell lines has the smallest levels of endogenous PLD lipase activity following EGF stimulation. We also report for the first time that PLD2 is under control of Janus Kinase-3 (JAK3) with the kinase phosphorylating PLD2 at the Y415 residue, which enables its activation. JAK3 knock-down abrogated lipase activity and EGF-stimulated cell invasion independently of STAT.

In the study at hand, EGF has been used as a motogen (i.e. an inducing agent of cell movement), a quality that it exhibits apart from its role as a growth factor. As is well known, EGF contributes to cell growth, proliferation and differentiation by binding to its receptor, EGF-R, a tyrosine kinase that initiates a signal transduction cascade that also promotes motility and invasiveness of cancer cells [33; 34; 35; 36]. EGF receptors activate PI3K/AKT, Ras/MAPK, and JAK/STAT signaling pathways that lead to cell survival [37]. We have shown the effect of EGF stimulation as a motogen on the invasiveness of 3 breast adenocarcinomas and one non-small cell lung cancer, whereby MTLn3 cells are highly invasive when compared to especially the MCF-7 cells.

Figure 8 is a model depicting JAK3 phosphorylation and activation following EGF-stimulation in both MTLn3 (Figure 8(a)) and MCF-7 (Figure 8(b)) cells, respectively, which ultimately leads to cell invasion. In general, phosphorylation of PLD2 by JAK3 mediates both its lipase activity and its role in cell invasion signaling. A strong positive interaction (bold lines) exists in MTLn3 cells between JAK3 and PLD2, whereby JAK3 phosphorylates and activates PLD2 at the tyrosine-415 residue. Both JAK3 and PLD are more catalytically active in the highly invasive MTLn3 cells when compared to the weakly invasive MCF-7 cells. PLD2 overexpression rescues MTLn3 cell invasion when JAK3 is silenced. As shown in Figure 8(a), upregulation of PLD2 or its product phosphatidic acid (PA) could control this positive effect on JAK3 expression. A considerably weaker positive interaction (dashed lines) exists between JAK3 and PLD2 in MCF-7 cells (Figure 8(b)). PLD2 overexpression has a much weaker positive affect on JAK3 silencing in MCF-7 cell invasion when compared to that of MTLn3 cells.

As shown for the first time in this study, we have found two proteins that are integral to cell invasion: PLD2 and JAK3. Activation of PLD represents a chief signaling pathway responsible for cancer cell sensitivity to chemotactic signals. EGF triggers the activation of PLD [38; 39], and its product of activation, phosphatidic acid (PA), is a critical mediator of EGF- dependent cell motility in cells [40; 41; 42]. Our use of the PLD1/2 lipase dead mutants has implicated a role for PA in PLD-mediated cell invasion, as was also found to be the case for breast cancer cells transfected with an HA-tagged PLD2-K758R mutant that ultimately resulted in reduced cell migration, spreading, invasion and metastasis and also decreased proliferation [32; 43]. A connection between MCF-7 cells and JAK3 parallels that of thymocytes and bone marrow progenitor cells from Jak $3^{-/-}$ mice, that have decreased chemotactic responses to the chemokines CXCL12 and CCL25 [44].

In summary, the current study links PLD2 and JAK3 to enhanced invasiveness potential. This knowledge suggests that a high JAK3/PLD2 activity combination might constitute a novel target to inhibit breast cancer cell invasion.

Materials and Methods

Reagents

DMEM was from Cellgro; α-MEM, Lipofectamine, Plus reagent and Lipofectamine 2000 were from Invitrogen; matrigels were from BD Biosciences; hematoxylin was from Ricca Chemical Co.; purified recombinant human JAK3 and JAK3tide synthetic peptide substrate were from Millipore; [^{3}H]-butanol was from American Radiolabeled Chemicals; [^{32}P]-γATP was from Perkin-Elmer; ECL reagent was from GE Healthcare; rabbit anti-myc and rabbit anti-HA antibodies were from Cell Signaling; rabbit polyclonal anti-myc-FITC IgG was from Santa Cruz Biotechnology; EGF was from Peprotech; and si-Neg, siJAK3 and siSTAT3 were from Applied Biosystems.

Cells and cell culture

MDA-MB-231 cells were obtained from ATCC. MCF-7 and H1299 cells were from Dr. Steven Berberich (Wright State University) and MTLn3 cells were from Dr. Jeffrey Segall (Albert Einstein College of Medicine). MCF-7, MDA-MB-231 and H1299 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), while MTLn3 cells were cultured in α-MEM supplemented with 5% (v/v) FBS.

Cell invasion assays

Cells that had been in either 10% FBS (MDA-MB-231, H1299 or MCF-7) or 5% FBS (MTLn3) were serum-starved for 2 hr in chemotaxis buffer that contained DMEM + 0.5 % bovine serum albumin for MDA-MB-231, H1299 and MCF-7 cells or HMEM + 0.5 % bovine serum albumin for MTLn3 cells and resuspended at a concentration 1.5×10^6 cells/ml in the appropriate chemotaxis buffer. 200 μl cells were applied to the upper chambers of 8 μm PET matrigels (24-well format) with a 6.5 mm diameter membrane. Final concentration of chemoattractant used was 0 or 3 nM EGF in 500 μl of chemotaxis buffer placed in the lower wells of 24-well plates. Cell invasion assays were incubated for 6 hr (MDA-MB-231 and H1299) or overnight (MTLn3 and MCF-7) at 37 °C in a humidified 5 % CO2 cell culture incubator. Cells were scraped from the matrigel insert and were then stained for 1 hr in hematoxylin at room temp to visualize cells that invaded the matrigel. The number of cells that migrated through the matrigel to the bottom surface of the insert was calculated by counting 3 fields of cells using a 20× objective and a 10× ocular.

Gene Silencing

The protocol for silencing involved transfection of 300 nM siPLD2, 300 nM siJAK3 or 300 nM siSTAT3 into MTLn3 or MCF-7 cells using Lipofectamine 2000 and α-MEM cell culture media containing 5 % (MTLn3) or Mirus siQuest and DMEM containing 10% % fetal calf serum (MCF-7). Silencing reactions were allowed to incubate at 37 °C overnight at which time cells were washed and re-fed with complete media minus antibiotics. After 48 hr, cells were transfected with 2 μg myc-PLD2 plasmid DNA using transfection reagents as described earlier for 3 hr at 37 °C in cell culture incubator. Cells were washed and re-fed with complete media and were allowed to be silenced for a total of 96 hr and simultaneously transfected with PLD for 48 hr at which time cell invasion was performed.

Immunofluorescence Microscopy

MTLn3 cells were seeded onto glass coverslips at a density of ~10–20% confluency and then were either mock-treated, transfected with recombinant myc-tagged PLD2, silenced using si-RNA specific for PLD2 or first silenced with si-PLD2 and then subsequently transfected with recombinant myc-tagged PLD2. Endogenous and silenced PLD2 proteins were detected using rabbit anti-PLD2 (H-133) monoclonal IgG antibody followed by donkey anti-rabbit IgG FITC secondary antibody. Overexpressed myc-tagged PLD2 was detected using rabbit anti-myc-FITC IgG conjugate. Nuclei were stained with 1:2000 DAPI in PBS. Coverslips were washed with PBS and then distilled water, dried and mounted onto glass slided using Vectashield mounting solution. Cells were imaged using a Nikon 50i eclipse epifluorescence microscope and a 100× PlanFluor OIL objective.

PLD2 lipase assay

Cells that had been in either 10% FBS (MDA-MB-231, H1299 or MCF-7) or 5% FBS (MTLn3) were serum-starved for 2 hr in chemotaxis buffer that contained DMEM + 0.5 % bovine serum albumin for MDA-MB-231, H1299 and MCF-7 cells or HMEM + 0.5 % bovine serum albumin for MTLn3 cells and resuspended at a concentration 1.5×10^6 cells/ml in the appropriate chemotaxis buffer. Samples containing 2×10^6 cells were either mock-

treated or treated with 3 nM EGF for 14 min in a 37 °C waterbath with vigorous shaking. After stimulation, cells were sedimented, washed and finally lysed via sonication in 100 μl Special Lysis Buffer (SLB) (5 mM HEPES, pH 7.8, 100 μM NaVO3, 0.4 % Triton X-100 and 5 μg/ml leupeptin and aprotinin) and split into 2 × 50 μl aliquots for duplicate determinations in the PLD2 assay, as performed in [29]. Results were quantified as DPM/μg.

JAK3 Kinase Assay

Cells that had been in either 10% FBS (MDA-MB-231, H1299 or MCF-7) or 5% FBS (MTLn3) were serum-starved for 2 hr in chemotaxis buffer that contained DMEM + 0.5 % bovine serum albumin for MDA-MB-231, H1299 and MCF-7 cells or HMEM + 0.5 % bovine serum albumin for MTLn3 cells and resuspen ded at a concentration 1.5×10^6 cells/ml in the appropriate chemotaxis buffer. Samples containing 2×10^6 cells were either mock-treated or treated with 3 nM EGF for 14 min in a 37 °C waterbath with vigorous shaking. After stimulation, cells were prepared as in [30]. After stimulation, cells were sedimented, washed and finally lysed via sonication in 20 μl SLB containing protease inhibitors. Lysates were incubated in the presence of the following final concentration of each: 8.25 mM HEPES, pH 7.5, 18.75 mM MgCl2, 1.25 mM EGTA, 18.75 μM Na Orthovanadate, 3.125 μM p-nitrophenylphosphate (PNPP), 0.625 μCi [^{32}Pγ]-ATP, 40 μM cold ATP and 42 μM JAK3tide substrate to yield a 40 μl total kinase reaction volume. Cell lysates were immunoprecipitated with an antibody at 1 μg/μl intended to be used for the kinase assay (i.e., with anti-JAK3 for JAK3 kinase assay). Each immunoprecipitate was washed and resuspended into 2 × 30 μl volumes of SLB and used in the kinase assays as listed above using no peptide substrate or the JAK3tide peptide substrate only. Reactions were incubated at 30 °C for 20 min and stopped by spotting 20 μl reactions onto 2 × 2.5 cm^2 pieces of P81 Whatman filter paper for duplicate determinations. After filter papers were dry, each was washed in cold running water for 5 min total. Filters were dried and individual filters placed into scintillation vials containing Scintiverse II (Fisher) liquid scintillation cocktail. All samples were counted in a Beckman LS 6000TA liquid scintillation counter using the [^{32}P] protocol for 1 min each. Results were quantified as DPMs and expressed in terms of *–fold* activation.

JAK3 Phosphorylation of PLD2

PLD2 was overexpressed in MTLn3 and MCF-7 cells for 48 hr and then lysates were prepared. The effect of JAK3 on PLD2 activity was measured using whole lysates containing overexpressed PLD2 were mixed with purified, recombinant JAK3 (that was first pre-activated for 10 min at 30 °C with 100 μM cold ATP and 1mM $MgCl_2$) and were incubated for 20 min at 30–37 °C in a rocking incubator. Phosphorylated PLD2 was then immunoprecipitated with anti-myc antibodies and the immunocomplex beads were used for PLD2 activity assays, as described earlier herein.

Statistical Analysis

Data are presented as mean ± SEM. The difference between means was assessed by the Single Factor Analysis of Variance (ANOVA) test. Probability of $p<0.05$ indicated a significant difference.

Acknowledgments

We thank Jennalee Post and Stephen Short for technical assistance and Erin Lintz for her expertise with the immunofluorescence microscopy of MTLn3 cells. This work was supported by the grant HL056653 (J.G.-C.) from the National Institutes of Health and the Research Challenge Grant 667131 from Wright State University.

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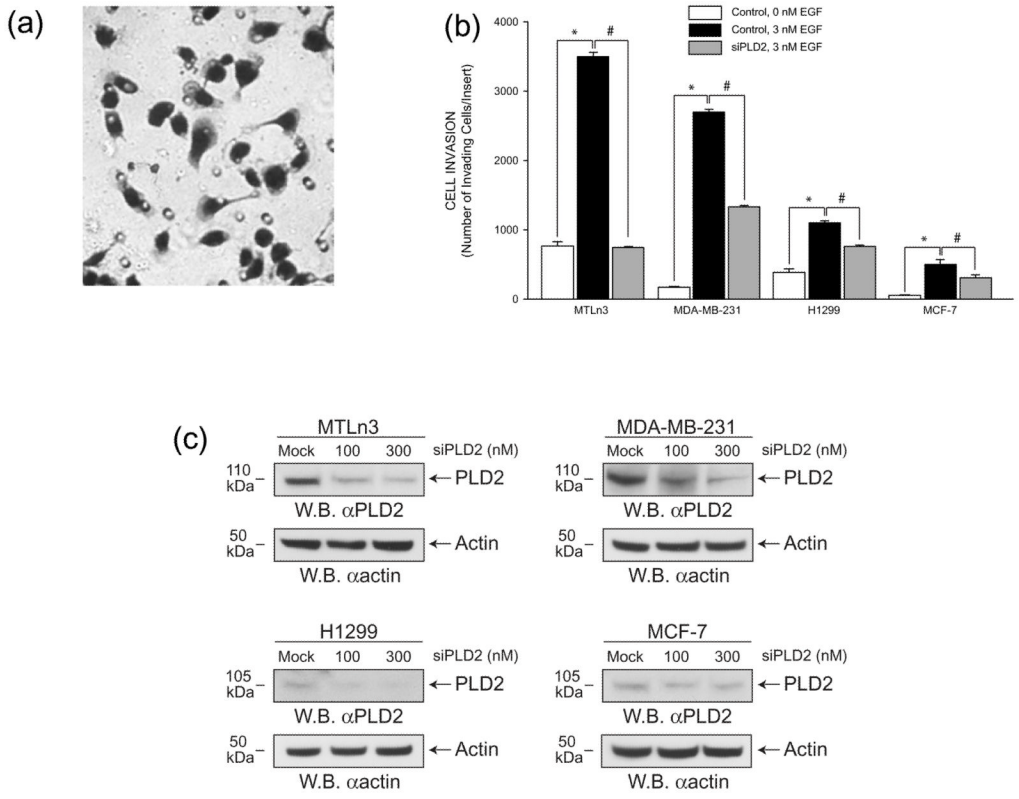

Figure 1.

PLD2 knock-down inhibits and PLD2 overexpression augments cancer cell invasion. (a) hematoxylin-stained MTLN3 cells on the underside of the matrigel filter after cell invasion. Pores in the filter (8 μm in diameter) are readily seen in the micrograph. (b) basal cell invasion of cancer cell lines and the effect of silencing PLD2 in response to 3 nM EGF as detailed in Materials and Methods. The (*) denotes a significant increase ($p < 0.05$ by ANOVA) in cell invasion with respect to the unstimulated control. The (#) denotes a significant decrease of the means ($p < 0.05$ by ANOVA) in cell invasion with respect to the EGF-stimulated control. Results represent mean ± SEM for four independent experiments. (c) western-blot analysis of endogenous PLD2 silencing of cancer cell lines using mouse anti-PLD2 monoclonal antibodies following by anti-mouse IgG HRP conjugated secondary antibodies and then ECL detection (top panels). Five hundred μg of each lysate was loaded per lane to achieve equal protein loading as indicated by the actin loading control (bottom panels).

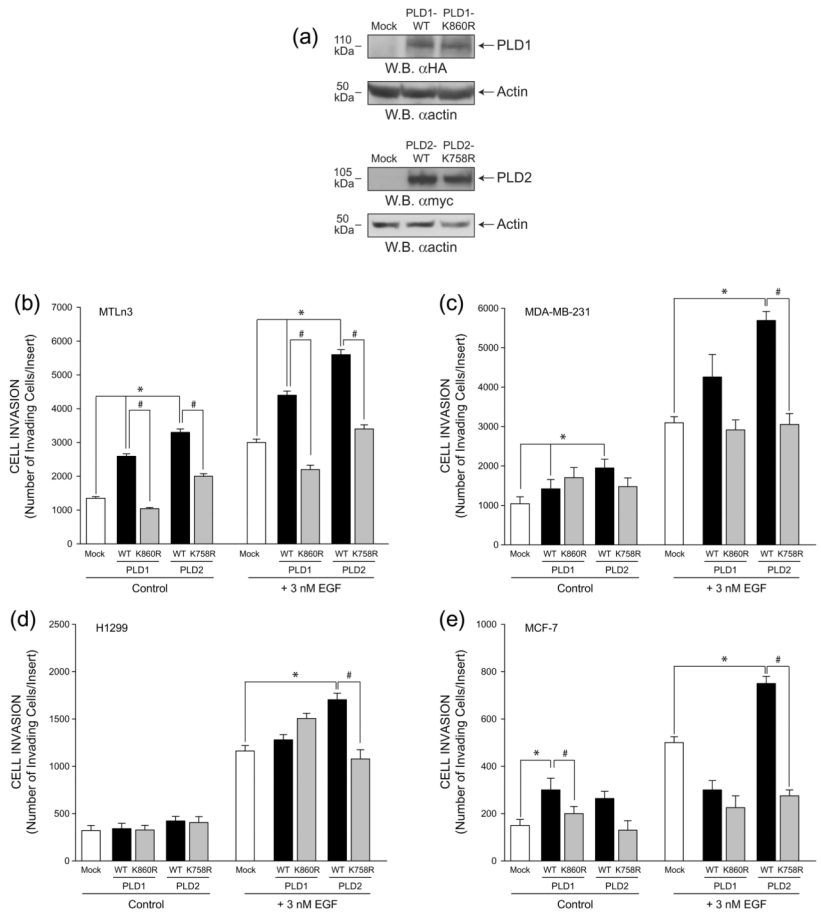

Figure 2.

Overexpression of wild-type PLD1 or PLD2 augments basal and chemoattractant-stimulated cell invasion of cancer cells. (a) overexpression of PLD1/2 wild-type or lipase-dead mutants in MTLn3 cells as detected using western-blots. (b–e) effect of PLD1/2 overexpression on cancer cell invasion of the MTLn3, MDA-MB-231, H1299 and MCF-7 cancer cell lines as detailed in Materials and Methods. The (*) denotes a significant increase in the means ($p < 0.05$ by ANOVA) in cell invasion with respect to the unstimulated control. The (#) denotes a significant decrease in the means ($p < 0.05$ by ANOVA) in cell invasion with respect to the EGF-stimulated control.

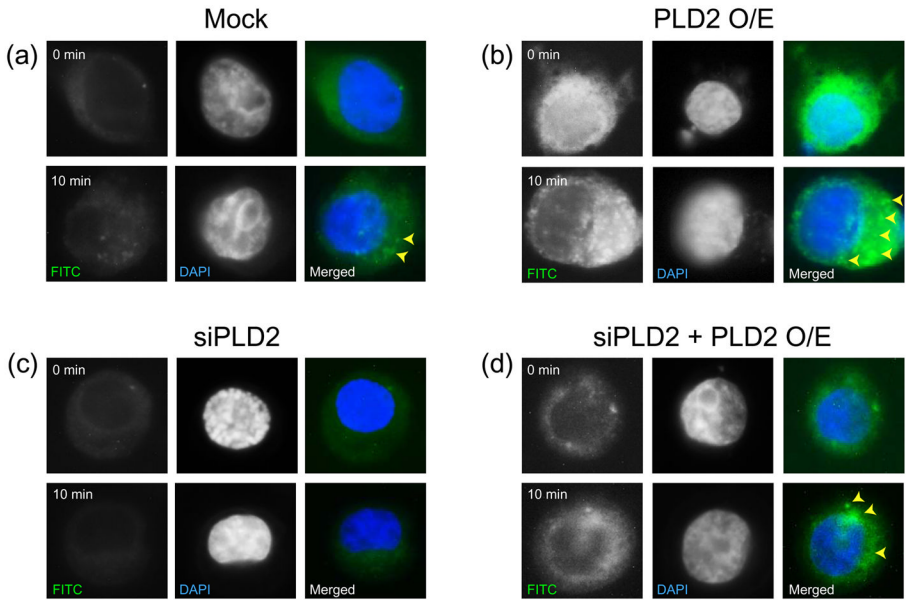

Figure 3.

Effect of PLD2 overexpression or PLD2 silencing in MTLn3 cancer cells before and after 10 min stimulation with 3 nM EGF as analyzed by immunofluorescence microscopy. (a) endogenous PLD2 protein in non-transfected MTLn3 cells. (b) MTLn3 cells overexpressing PLD2. (c) silenced PLD2 protein in MTLn3 cells. (d) PLD2 protein in non-transfected MTLn3 cells silenced for endogenous PLD2 and subsequently rescued with wild-type PLD2. Endogenous and silenced PLD2 proteins were detected using rabbit anti-PLD2 (H-133) monoclonal IgG antibody followed by donkey anti-rabbit IgG FITC secondary antibody. Overexpressed myc-tagged PLD2 was detected using rabbit anti-myc-FITC IgG conjugate. Yellow arrowheads indicate punctate staining after EGF stimulation.

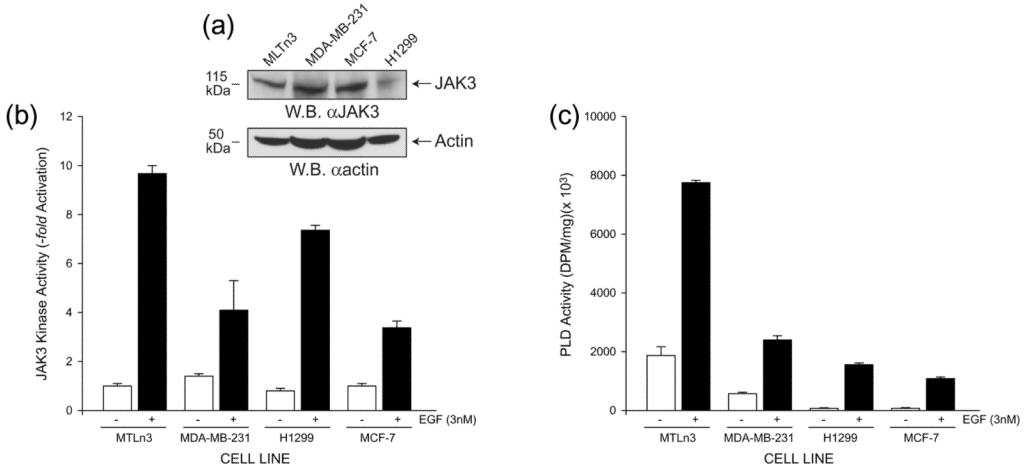

Figure 4.

Relative endogenous JAK3 kinase and PLD2 lipase activities of four cancer cell lines. (a) Western-blot analysis of endogenous JAK3 protein expression in MTLn3, MCF-7, MDA-MB-231 and H1299 cancer cells. PVDF membrane was probed with 1:1000 rabbit anti-JAK3 monoclonal antibodies, then probed with 1:3000 sheep anti-rabbit IgG HRP secondary antibodies and visualized using ECL reagents. (b) JAK3 kinase assay of MTLn3, MCF-7, MDA-MB-231 and H1299 cancer cells in the absence or presence of 3 nM EGF as detailed in Materials and Methods. The amount of endogenous JAK3 specific phosphorylation of the JAK3tide substrate was measured and quantified in terms of *–fold* activation ± SEM in regard to their respective negative control which is no peptide in the kinase assay. (c) Endogenous PLD assay of MTLn3, MCF-7, MDA-MB-231 and H1299 cancer cells in the absence or presence of 3 nM EGF as detailed in Materials and Methods. The amount of [^{3}H]-PBut that co-migrated with PBut standards was measured and quantified in terms of DPM/mg protein ± SEM.

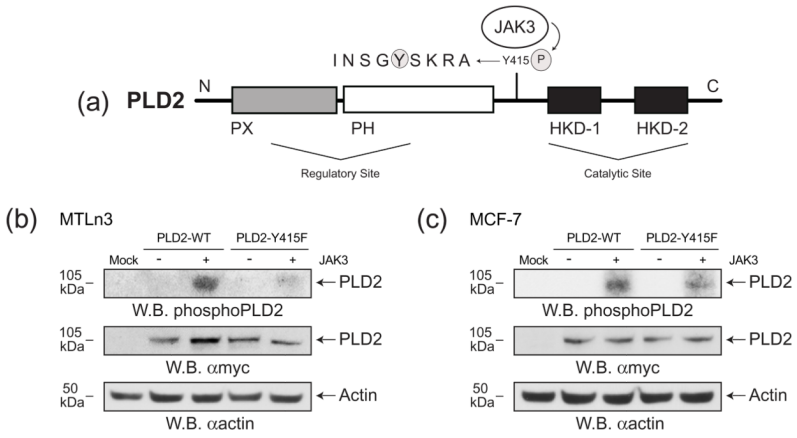

Figure 5.

In vitro phosphorylation of PLD2 by JAK3. (a) the Y-415 residue on PLD2 is within a JAK3 consensus site that is situated between the PH domain and the first of two HKD catalytic domains. (b)(c) Radioimmunokinase assay of western-blot of PLD2-WT or PLD2-Y415F transfected into MTLn3 (b) and MCF-7 (c) cells in the absence or presence of JAK3 phosphorylation. Top panel: PLD2 phosphorylation. Middle panel: western-blot analysis of the same MTLn3 and MCF-7 samples probed for the presence of the myc-tagged PLD2 using 1:1000 rabbit anti-myc IgG monoclonal antibodies followed by 1:3000 anti-rabbit IgG HRP conjugated secondary antibodies and then visualized using ECL reagents. Bottom panel: western-blot analysis of the same MTLn3 and MCF-7 samples probed for the presence of actin using 1:2000 rabbit anti-actin IgG monoclonal antibodies followed by 1:3000 anti-rabbit IgG HRP conjugated secondary antibodies and then visualized using ECL reagents.

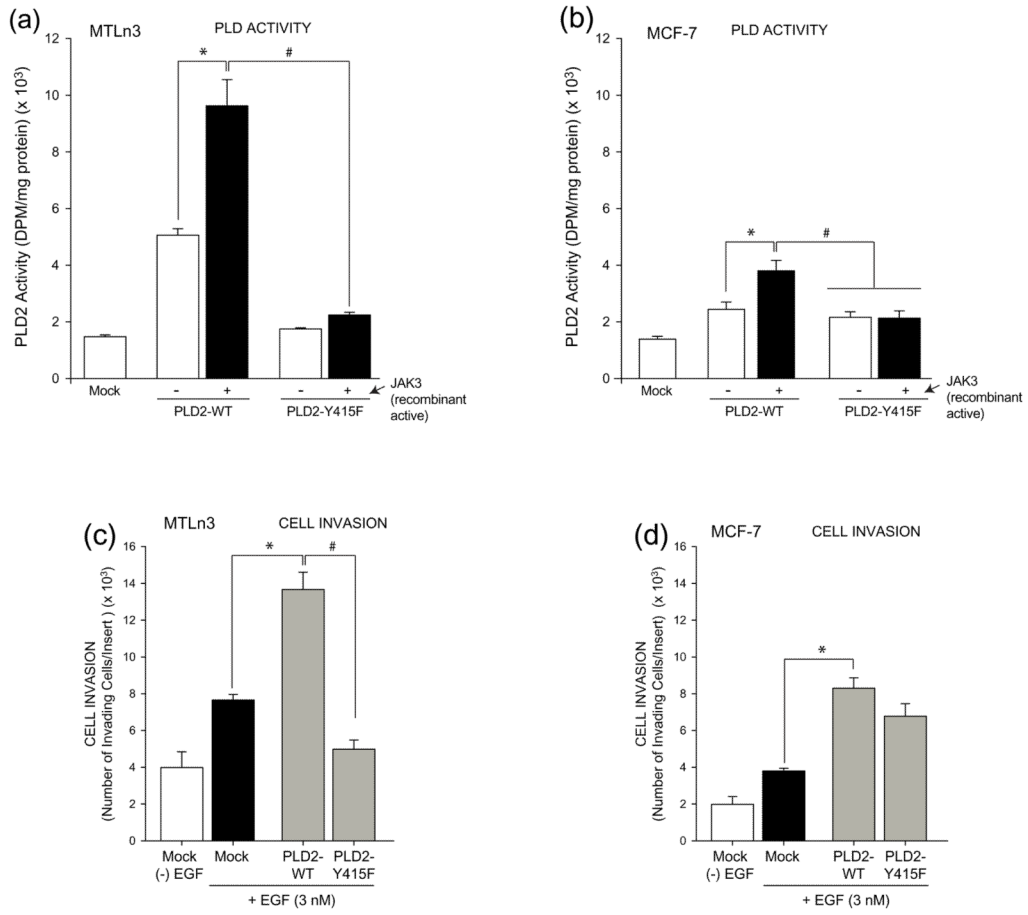

Figure 6.

JAK3 phosphorylation of PLD2 increases lipase activity in MTLn3 and MCF-7 cells, while dephosphorylation of PLD2 significantly decreases MTLn3 cell invasion. (a–b) JAK3 phosphorylation of overexpressed PLD2-WT or PLD2-Y415F in MTLn3 cells (a) and MCF-7 (b) cells. Results were quantified in terms of DPM/mg protein ± SEM. (c–d) effect of JAK3 phosphorylation on PLD2-mediated cell invasion of MTLn3 (c) or MCF-7 (d) cells. Results were quantified in triplicate as number of invading cells per insert ± SEM. (*) or (#) represent differences between means, as determined by ANOVA, above or below the negative control levels.

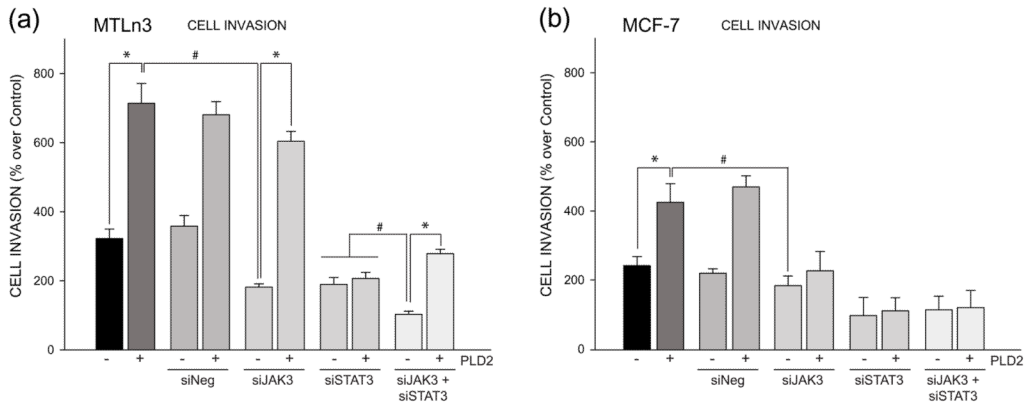

Figure 7.

PLD2 overexpression reverses and rescues the effect of JAK3 silencing in MTLn3 cell invasion but not in MCF-7 cell invasion as a result of a positive feedback loop. (a–b) effect of silencing JAK3 or STAT3 protein expression on PLD2-mediated cell invasion of MTLn3 (a) or MCF-7 (b) cells. Results were quantified in triplicate as number of invading cells per insert (% over control) ± SEM. (*) or (#) represent differences between means, as determined by ANOVA, above or below the negative control levels.

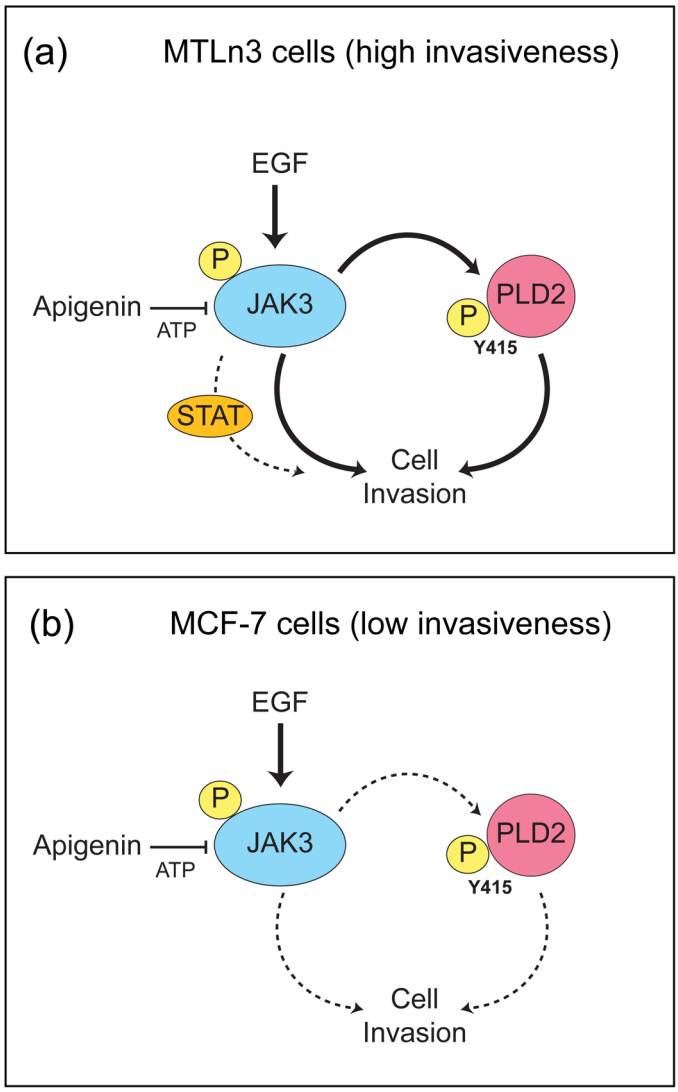

Figure 8.
Model representing a positive interaction between PLD2 and JAK3 during cancer cell invasion and the impact of apigenin on this interaction. (a–b) JAK3 phosphorylation and activation of PLD2-mediated cell invasion of MTLn3 (a) and MCF-7 (b) cells in response to EGF-stimulation and the effect of apigenin on this mechanism.