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Paxillin phosphorylation and complexing with Erk and FAK are regulated by PLD activity in MDA-MB-231 cells

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Abstract

MDA-MB-231 cells are highly aggressive human breast adenocarcinoma cells that depend on PLD activity for survival. In response to the stress of serum withdrawal, there is increased motility and invasiveness of these cells that is associated with a rapid increase in PLD activity. In addition, PLD activity is elevated in response to most mitogenic signals. Similar to PLD, paxillin, a focal adhesion adaptor protein, and Erk, mitogen-activated protein kinase, play vital roles in cell motility through regulation of focal adhesion dynamics. Here, we addressed whether there is a functional correlation between paxillin and PLD that may influence cancer cell motility. We investigated the role of PLD activity on paxillin regulation, Erk activation and formation of a paxillin-Erk and paxillin-FAK association. Inhibition of PLD activity led to an increase in paxillin tyrosine phosphorylation, a decrease in Erk activation, as measured by phosphorylation, and enhanced association of paxillin with Erk. In addition, we found that paxillin tyrosine phosphorylation depends upon Erk activity and may be a consequence of an increased association with FAK. Taken together, these results suggest that Erk activity is governed by PLD activity and regulates the tyrosine phosphorylation of paxillin, potentially explaining its role in cell motility. This study indicated that PLD, Erk, paxillin and FAK participate in the same signaling pathway in this breast cancer cell line.

1. Introduction

The successful treatment of breast cancer requires greater understanding of the molecular and cellular basis of breast cancer phenotypes. Highly aggressive human breast cancer is often modeled using MDA-MB-231 cells. Invasiveness of these cells depends on phospholipase D (PLD) activity which is known to play an important role in cell proliferation and tumorigenesis [1,2]. In fact, PLD activity has been reported to be elevated in a majority of breast cancers examined [3]. Increased PLD activity has also been observed in cancer cell lines in response to the stress of serum removal, which is associated with increased cell motility and invasiveness [2]. PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and choline. PA is metabolically convertible to the mitogenic lipids, diacylglycerol and lysophosphatidic. There are two mammalian PLD genes: PLD1 and PLD2. Both are implicated in mitogenic signaling: PLD1 by functional association with Ral GTP-ase, a downstream target of Ras, and PLD2 by localization to light

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membrane fractions containing many signaling molecules, including EGFR [4–6]. However, the precise mechanism of the involvement of PLD activity in mitogenic signaling as well as associated proteins still remain to be revealed.

Previous studies have demonstrated that mitogen-activated protein kinases (MAPKs) are essential for cell migration. The MAPK family can be divided into three groups: extracellular-signal-regulated protein kinase (Erk/MAPK), p38 and Jun N-terminus kinase (JNK). It has been suggested that signaling through the Erk1/2 pathway is very important for breast cancer progression [7]. Erk has two isoforms; p44 (Erk-1) and p42 (Erk-2). Their activities are stimulated by various growth factors and mitogens which activate the Ras-Raf-1-MEK-1/2-Erk-1/2 signaling module [8]. Ras, activated by extracellular signals, recruits MAPKKK, Raf-1, to the membrane. Raf-1 phosphorylates and activates the MAPKKs, MEK-1 and MEK-2, which phosphorylate the Thr and Tyr residues of Erk1/2. Erk has been implicated in the migration of numerous cell types [9–14], leaving open the possibility that PLD affects motility through the regulation of Erk in tumor cells.

Migration and invasion of tumor cells is also highly dependent on adhesion molecules that regulate focal contacts with matrix components. The adaptor and scaffold protein paxillin is a component of focal adhesion complexes and mediates focal adhesion assembly and turnover and thus motility [15]. Dispersed throughout paxillin are many serine and threonine and tyrosine phosphorylation sites. It is known that various extracellular factors are able to induce tyrosine phosphorylation of paxillin [16–19]. One kinase known to phosphorylate paxillin on Tyr 31 and Tyr 118 is focal adhesion kinase (FAK) [20,21]. It is a paxillin binding partner in focal adhesions and is overexpressed in breast, ovary and colon cancers [15,22–24]. A major consequence of paxillin tyrosine phosphorylation is the generation of binding sites for SH2 domain-containing proteins. Therefore, it is not surprising that phosphorylation of paxillin leads to its recruitment of numerous signaling molecules, allowing it to regulate focal adhesion dynamics and cell migration. Interestingly, contradictions in the correlation between paxillin expression and cancer aggressiveness indicate that the roles of paxillin in various tumors are tissue-specific and context-specific [25–27]. Therefore, the investigation of the paxillin expression along with the co-expression of other paxillin binding partners may be important in determining the full biological impact of paxillin on tumor phenotype and thus on selecting appropriate therapies.

It has been suggested that Erk, by phosphorylation of FAK and paxillin, affects the formation of a complex between paxillin and FAK and thus plays a role in the regulation of focal adhesion dynamics [28,29]. However, the precise role of Erk and paxillin in controlling cell motility, along with their correlation with PLD remain unclear. In this report, we described the effect of PLD activity on paxillin phosphorylation, Erk activity, via its phosphorylation, and complex formation between paxillin and Erk in the MDA-MB-231 breast cancer cell line. Interestingly, our data show that Erk activity is governed by PLD activity and regulates the tyrosine phosphorylation of paxillin. Thus, Erk, PLD, and paxillin may act in concert to regulate cell motility in MDA-MB-231 cells.

2. Materials and methods

2.1. Cells, cell culture conditions, and transfection

MDA-MB-231 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified eagle’s medium (DMEM) with 10% bovine calf serum both purchased from Sigma (St. Louis, MO). Transfections were performed using Lipofectamine™ LTX and Plus reagent purchased from Invitrogen (Eugene, OR) according to the manufacturer’s instructions. Transfection efficiency was determined by transfection of pEGFP-C1 purchased from Clonetech (Mountain View, CA), which expresses green

fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 70%.

2.2. Materials

1-butanol and tertiary-butanol were from Sigma (St. Lous, MO). [^{3}H] myristate was obtained from New England Nuclear (Newton, MA). U0126 was obtained from Promega (Fitchburg WI). Anti-paxillin mouse antibody was obtained from Calbiochem (La Jolla, CA). Anti-phosphotyrosine antibody, anti-phospho-ERK1/2 antibody, anti-FAK antibody and anti-HA-tag antibody were obtained from Cell Signaling Technology (Beverly, MA). ERK 1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was obtained from Sigma (St. Louis, MO). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA).

2.3. PLD assays

MDA-MB-231 cells were plated in 60 mm culture dish at 1×10^5 cells/dish. Two days later, cells were shifted into DMEM containg 0.5% bovine calf serum and kept overnight. Cells were then prelabeled for 4 h with [^{3}H] myristate (3 μCi, 40 Ci/mmol) in 3 ml of medium. The cells were then incubated for 20 min with of 1-butanol or tertiary-butanol to achieve 0.8% final concentration. The extraction and characterization of lipids by thin layer chromatography were performed as described previously [30]. Radioactivity corresponding to the tertiary-butanol reaction (background) was subtracted from that of the butan-1-ol reaction to determine PLD activity.

2.4. Immunoprecipitation and immunoblotting

MDA-MB-231 cells were lysed in ice-cold RIPA buffer (65 mM Tris base, 154 mM NaCl, 1% IGEPAL, 0.25% Na-deoxycholate, 1 mM EDTA, pH 7.4) that contained 1 M Na-othovanadate, 100 mM NaF, 1X Roche Complete Protease Inhibitor Cocktail, Mini (Indianapolis, IN), 1X Phosphatase inhibitor cocktail set I obtained from Calbiochem (La Jolla, CA) and rotated for 20 min at 4° C. Lysates were clarified by centrifugation at 10,000×g for 10 min. Supernatants were normalized for total protein by Bio-Rad assay (Hercules, CA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Hybond-P membrane obtained from Amersham Bioscience (Piscataway, NJ) and immunoblotted with antibodies as described in the text. Depending upon the origin of the primary antibodies, either HRP-conjugated anti-mouse or anti-rabbit immunoglobulin G was used for visualization by enhanced chemiluminescent substrate for HRP detection obtained from Pierce (Rockford, IL).

For immuniprecipitations, precleared lysates were incubated with 1 μg antibody overnight on a rotating shaker at 4°C. Pure Proteome Protein A magnetic beads purchased from Millipore (Temecula, CA) were added to the sample and incubated on a rotatory shaker for 1 hour at 25°C. Beads were collected with a magnetic rack obtained from Millipore (Temecula, CA) and washed 3 times with PBS containing 0.1% Tween. Beads were resuspended in 50 μl PBS + 0.1% Tween and 10 μl 6X protein sample buffer, then boiled for 10 minutes at 90°C. Beads were then removed from suspension with the magnetic rack. The samples were electrophoretically separated on SDS-polyacrylamide gels and immunoblotted as described above.

2.5. siRNA

Cells were plated on 35 mm culture dish at 70% confluence in medium containing 10% serum without antibiotics. After one day, cells were transfected with siRNA using Lipofectamine Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer directions.

After 24 h the media were changed to fresh media containing 10% serum. The next day cells were lysed and analyzed for PLD activity and coimmunoprecipitations.

2.6. Statistical analysis

For quantitative analysis, data are presented as mean ± SEM from at least three independent experiments. The significance of differences was analyzed by the Student's t-test for pair-wise comparisons. A p value < 0.05 was considered statistically significant. Absence of a star means no significant difference from the control was found.

3. Results

3.1. Paxillin production does not depend upon PLD activity

It has been reported that serum withdrawal leads to increased PLD activity in MDA-MB-231 cells [2]. To confirm the elevated PLD activity in MDA-MB-231 cells upon serum removal, we performed PLD activity assay. As shown in Figure 1A, PLD activity was higher in MDA-MB-231 cells in 0.5% serum than in cells maintained in 10% serum, which is consistent with previous reports.

Given the confusion of whether paxillin expression correlates with cell motility and invasiveness, we examined whether serum removal and PLD activity, both of which stimulate motility and invasiveness in MDA-MB-231 cells [2], affects the level of paxillin production. The dependence of paxillin production on PLD activity was assessed using an "alcohol trap" assay. In this assay, primary alcohols are preferentially utilized over water by PLD to catalyze the formation of phosphatidylalcohol rather than phosphatidic acid, thus blocking signaling via phosphatidic acid. Western blot analysis of whole cell lysates revealed similar paxillin production in cells maintained in 10% serum, where PLD activity is low (Figure 1B, lane 1), as in cells exposed 0.5% serum, where PLD activity is high (Figure 1B, lane 2). In addition, paxillin production was not affected by treatment with 1-Butanol that inhibits PLD signaling (Figure 1B, lane 3) nor tertiary-Butanol, which is a poor substrate of PLD and is used as a negative control for 1-Butanol as it does not prevent phosphatidic acid formation (Figure 1B, lane 4). These data indicated that paxillin production is independent from serum levels and PLD activity.

To demonstrate that paxillin production is independent specifically from PLD activity, we suppressed PLD activity in MDA-MB-231 cells by introducing catalytically inactive PLD1(K898R) or PLD2(K758R), which have been used previously as dominant interfering mutants [31,32]. Expression of the dominant negative PLD proteins was determined by Western blot analysis using an antibody raised against HA tag (Figure 1C). The introduction of PLD1(K898R) or PLD2(K758R) via transient transfection suppressed the PLD activity in MDA-MB-231 cells to 50% of a vector control (Figure 1D). This is similar to what has been reported previously [2].

Paxillin production was further examined in cells expressing catalytically inactive PLD1(K898R) or PLD2(K758R) mutants. As expected, paxillin levels were not affected by expression of dominant negative PLD2 (Figure 1E, lane 2), nor of dominant negative PLD1 (Figure 1E, lane 3) demonstrating that paxillin production does not depend upon PLD activity.

3.2. Paxillin tyrosine phosphorylation parallels PLD activity in MDA-MB-231 cells

Given that serum removal affects the activity of PLD and increases cell motility [2], we were interested in observing the effect of serum withdrawal on the tyrosine phosphorylation of paxillin, which is known to regulate motility [33,34]. To examine the effect of PLD

activity on paxillin phosphorylation, antiphosphotyrosine blotting of paxillin immunoprecipitates from MDA-MB-231 cells was performed. As shown in Figure 2A, compared to 10% serum, where PLD activity is low (lane 1), paxillin phosphorylation was elevated in 0.5% serum, where PLD activity is high (lane 2). Addition of 1-Butanol which inhibits PLD signaling, repressed paxillin phosphorylation (lane 3), while tertiary-Butanol, which does not affect PLD, did not have an effect (lane 4). This suggested that paxillin tyrosine phosphorylation parallels PLD activity.

Paxillin tyrosine phosphorylation was further examined in cells expressing catalytically inactive PLD1(K898R) or PLD2(K758R) mutants. As seen in Figure 2B, paxillin tyrosine phosphorylation was suppressed in both dominant negative PLD2 (lane 2) and PLD1 (lane 3) mutants compared to the control cells (lane 1) suggesting that paxillin tyrosine phosphorylation is dependent upon PLD activity, and that the effect we observed with serum removal and alcohol addition were due to disrupted PLD signaling. Thus, increase in PLD activity led to an increase in tyrosine phopshorylated paxillin, while having no effect on total paxillin levels.

3.3. PLD activity inhibits Erk phosphorylation in MDA-MB-231 cells

PLD activity has been implicated in mitogenic signaling [6,35–38]. In addition, EGF-induced MAP kinase phosphoryalation is dependent upon PLD activity [31]. To determine whether there is a correlation between Erk activity and PLD activity in MDA-MB-231 cells, we examined the effect of serum removal and PLD inhibition (alcohol trap) on the level of Erk1/2 activation, as measured by its phosphorylation. Western blot analysis of whole cell lysates revealed that compared to the cells maintained in 10% serum, where PLD activity is low (Figure 3A, lane 1), there was a decrease in Erk1/2 phosphorylation in serum deprived cells, where PLD activity is high (Figure 3A, lane 2). Moreover, Erk1/2 phosphorylation in serum deprived cells is amplified by 1-Butanol, when PLD signaling is inhibited (Figure 3A, lane 3) but not by tertiary-Butanol, which does not inhibit PLD signaling (Figure 3A, lane 4). This suggested that PLD activity inhibits Erk phosphorylation and thus, activation.

We further examined Erk1/2 phosphorylation in cells expressing dominant negative PLD1 and PLD2 mutants. When compared to the control cells (Figure 3B, lane 1), the level of phosphorylated Erk1/2 was enhanced in cells expressing dominant negative PLD2 (Figure 3B, lane 2) and PLD1 (Figure 3B, lane 3) mutants. Along with the level of phosphorylated Erk1/2 seen in cells exposed to 1-Butanol, these results suggested that Erk activity, as measured by Erk phosphorylation, is inhibited by PLD activity.

3.4. PLD activity inhibits complex formation between Erk and paxillin in MDA-MB-231 cells

Finding that both paxillin tyrosine phosphorylation and Erk1/2 phosphorylation depend upon PLD activity prompted us to further explore a connection between paxillin and Erk. A physical association between paxillin and Erk has been described previously in renal medullary cells [28,39]. To elucidate whether there is a similar association between paxillin and Erk in MDA-MB-231 cells, we performed co-immunoprecipitation studies. Paxillin immunoprecipitates were examined for the presence of total and activated Erk. As indicated in Figure 4, compared to the cells maintained in 10% serum, where PLD activity is low (Figure 4A, lane 1), there is less Erk (either total or phosphorylated) associated with paxillin in serum deprived cells, where PLD activity is high (Figure 4A, lane 2). Furthermore, complex formation between Erk and paxillin was improved when PLD signaling was inhibited by addition of 1-Butanol to the serum deprived cells (Figure 4A, lane 3), while tertiary-Butanol did not have an effect (Figure 4A, lane 4). Thus, paxillin and Erk form a complex in MDA-MB-231 cells which depends upon PLD activity.

To demonstrate that complex formation between Erk and paxillin is dependent upon PLD activity, we used MDA-MB-231 cells transiently transfected with catalytically inactive mutants of either PLD1 or PLD2 (Figure 4B) or silenced by siRNA (Figure 4C). As shown in Figure 4B, when compared to the control cells (lane 1), the amount of total Erk and phosphorylated Erk associated with paxillin was enhanced in dominant negative PLD2 (lane 2) and PLD1 (lane 3) mutants. Similar results were seen when PLD activity was decreased to approximately 25% using siRNA to PLD1 or PLD2 (Figure 4C). Along with the results presented earlier, these results suggested that PLD activity inhibits complex formation between paxillin and Erk.

3.5. Supression of Erk activation allows paxillin tyrosine phosphorylation

Since our experiments showed an association between paxillin and Erk, we investigated the effect of Erk activity on paxillin phosphorylation. We measured paxillin tyrosine phosphorylation in the absence and presence of the selective MEK inhibitor U0126. This compound has been shown to inhibit MEK1 and MEK2 [40,41], thus preventing Erk phosphorylation and activation. As shown in Figure 5A, U0126 completely suppressed Erk phosphorylation in the cells in 10% serum. In addition, U0126 induced paxillin tyrosine phosphorylation (Figure 5B), indicating that paxillin tyrosine phosphoprylation is mediated through an Erk-dependent pathway.

3.6. FAK and paxillin complex formation depends upon PLD activity

To address the source of paxillin tyrosine phosphorylation, we examined the effect of serum on the association of paxillin with FAK, which is known to tyrosine-phosphorylate paxillin [20,21]. As shown in Figure 6A, paxillin is able to co-immunoprecipitate FAK in 10% serum, suggesting that these proteins form a complex. When the cells are serum deprived, the amount of FAK associating with paxillin is increased, arguing that serum removal favors the formation of a complex between FAK and paxillin.

To determine whether PLD activity was responsible for complex formation between FAK and paxillin, we examined paxillin immunoprecipitates for the presence of FAK in cells with decreased PLD activity due to dominant negative PLD1 or PLD2 mutants (Figure 6B), or siRNA for PLD1 or PLD2 (Figure 6C). When compared to the control cells (Figure 6B, lane 1), the level of FAK in paxillin immunoprecipitates was reduced in cells expressing dominant negative PLD2 (Figure 6B, lane 2) or PLD1 (Figure 6B, lane 3) mutants. A similar decrease in FAK association was seen when PLD2 (Figure 6C, lane 2) or PLD1 (Figure 6C, lane 3) was silenced with siRNA. These results suggest that formation of the complex between paxillin and FAK depends upon the activity of PLD.

4. Discussion

Metastasis occurs when malignant cells migrate to an organ distant from the primary tumor, adjust to the new microenvironment and expand secondary tumors. The metastatic process consists of tumor cell intravasation, survival in circulation, extravasation into a distant organ, angiogenesis and finally unconstrained tumor growth [42]. Specific gene expression patterns are correlated with various metastases and recent studies have focused on elucidating genetic factors critical for tumor metastasis [43,44].

In the center of a growing tumor, cells need to deal with the absence of serum growth factors. In order to survive under these conditions, cells need to bypass the immediate need for growth and survival factors and eventually obtain serum either by stimulating vascularization or by inducing migration to sites where nutrients are available. Hence, serum withdrawal in culture models the lack of vascularization in a developing tumor. In

accordance with this, survival and migration have been linked to the stress of serum removal in MDA-MB-231 cells [2], making them an excellent model for metastatic breast cancer.

When starved for serum, mimicking the conditions found in the center of a growing tumor, MDA-MB-231 cells are able to survive and avoid apoptosis by elevating PLD activity. As previously reported, PLD signaling is critical for prevention of apoptosis [2,45,46]. Elevated PLD activity leads to the elevated expression of Myc [47], and activation of mTOR, mammalian target of rapamycin [48,49]. In addition, it suppresses tumor suppressors such as p53 [50] and protein phosphatase 2A [51]. Thus, downstream targets of PLD have been identified in the suppression of apoptosis and the survival of cancer cells under serum stress.

While the role of PLD in survival of these cells has been examined, its role in the regulation of migration and invasion is less well understood. In other cell lines, it has been shown that PLD is involved in Erk signaling [31] and that Erk signaling is involved in cell migration through regulation of paxillin [28,29]. Consistent with this, our results suggest that PLD activity is involved in migration of MDA-MB-231 cells through controlling Erk and paxillin. Previous studies done in MDA-MB-231 showed that serum removal is associated with loss of phosphoryated Erk. In addition, RUNX2 (Runt-related transcription factor 2) which stimulates cell motility of MDA-MB-231 cells, is reciprocally linked to activity of the MEK-Erk signaling pathway [52]. In accordance with this, our data imply that Erk phosphorylation is inhibited upon serum removal, which is characterized by elevated PLD activity, motility and invasiveness of MDA-MB-231 cells [2].

In our study, disruption of either PLD1 or PLD2 activity both caused decreased paxillin tyrosine phosphorylation, increased Erk phosphorylation and increased paxillin-Erk association, implying some functional redundancy for the two PLD isoforms. Such redundancy is not unexpected, as it has been seen in EGF receptor endocytosis [31] and cell proliferation in cells overexpressing a tyrosine kinase [53]. PLD2 is localized primarily to light membrane fractions containing many signaling molecules including the EGFR, unlike PLD1 which while found in light membrane fractions, has much broader cellular distribution [54,55]. This would suggest that PLD2 is the major PLD isoform activated by mitogenic signals. However, PLD1 is known to be functionally associated with RalGTPase [4,56] a downstream target of Ras, implying a role for PLD1 in mitogenic signaling. Thus, genetic evidence links mitogenic signaling with PLD1 while circumstantial evidence implicates PLD2.

We showed that cells exposed to the stress of serum removal displayed a well characterized elevation of PLD activity. This led to a decrease in Erk phosphorylation, and presumably activity, and an increase in paxillin tyrosine phosphorylation. The increase in paxillin tyrosine phosphorylation is due to impaired Erk activity, as we were able to artificially reproduce this tyrosine phosphorylation by inhibiting Erk activity with U0126. Interestingly, Erk is known to phosphorylate paxillin on serine, not tyrosine [28]. Thus, the tyrosine phosphorylation of paxillin, a protein known to be involved in regulating motility, due to elevated PLD activity caused by serum removal, must be mediated by another kinase.

Paxillin regulates cell migration through controlling focal adhesion dynamics [57,58]. As an adapter protein, paxillin recruits a number of signal transducers to focal adhesions. One signaling protein known to complex and colocalize with paxillin is Erk [39]. Similar to what has been seen in other cells, we found that paxillin can bind Erk in MDA-MB-231 cells. In addition, we showed that formation of this complex is regulated by serum levels through PLD activity. When serum levels are low, PLD activity is high, which inhibits the binding of Erk to paxillin. There is a possibility that the physical association we see in immunoprecipitations is an artifact. However, given the fact that the associations are

responsive to serum, PLD activity and butanol and correspond with a physiological effect (migration), the chance of an artefactual association is low.

In addition to binding Erk, paxillin is known to bind the tyrosine kinase FAK. FAK is also localized at focal adhesions, with paxillin being its primary target [58,59]. Interestingly, Erk activity prevents paxillin from binding FAK as Erk phosphorylates FAK at Ser910 blocking the interaction of FAK with paxillin [29]. This is supported by our findings that the presence of serum or the inhibition of PLD activity, conditions which increase Erk activity, inhibits formation of a FAK - paxiliin complex. Thus, the observed decrease in paxillin tyrosine phosphorylation caused by increased Erk activity could be due to Erk disrupting the FAK-paxillin complex.

Previous studies have shown that adhesion-induced association of paxillin with FAK leads to paxillin phosphorylation of Tyr 31 and Tyr 118 [20,21]. A major consequence of paxillin tyrosine phosphorylation is the generation of binding sites for SH2 domain-containing proteins. This would, of course, lead to remodeling of focal adhesions and changes in motility. However, the role of paxillin phosphorylation in modulating cellular motility is not well established and appears to be dependent upon cell type and context [33,34]. Previous findings indicated that mTOR, mammalian target of rapamycin, may regulate phosphorylation of focal adhesion proteins, including paxillin [60,61], and cell motility and invasion [62]. In addition, phosphatidic acid, the metabolic product of PLD, is known to activate mTOR, [48,49]. Thus, it is also possible that increased PLD activity under serum deprived conditions activates tyrosine phosphorylation of paxillin and consequently, cell motility, in part through increased production of PA and mTOR activation.

5. Conclusion

For MDA-MB-231 cells, our data suggest that tyrosine phosphorylation of paxillin is associated with increased motility in response to serum withdrawal. Specifically, the absence of serum promotes PLD activity. This activity, either directly or indirectly, inhibits Erk activity. The decreased Erk activity allows the association of paxillin with FAK, leading to the tyrosine phosphorylation of paxillin. This causes changes in focal adhesions, leading to increased motility (Figure 7).

We have found that PLD, Erk, paxillin and FAK work coordinately to regulate cell motility in MDA-MB-231 cells, and therefore may play an important role in metastasis of breast cancer cells. In summary, our study suggests that elevated PLD activity is associated with low Erk activity and increased paxillin tyrosine phosphorylation, and constitutes a potentially novel target for inhibiting breast cancer cell motility and invasiveness.

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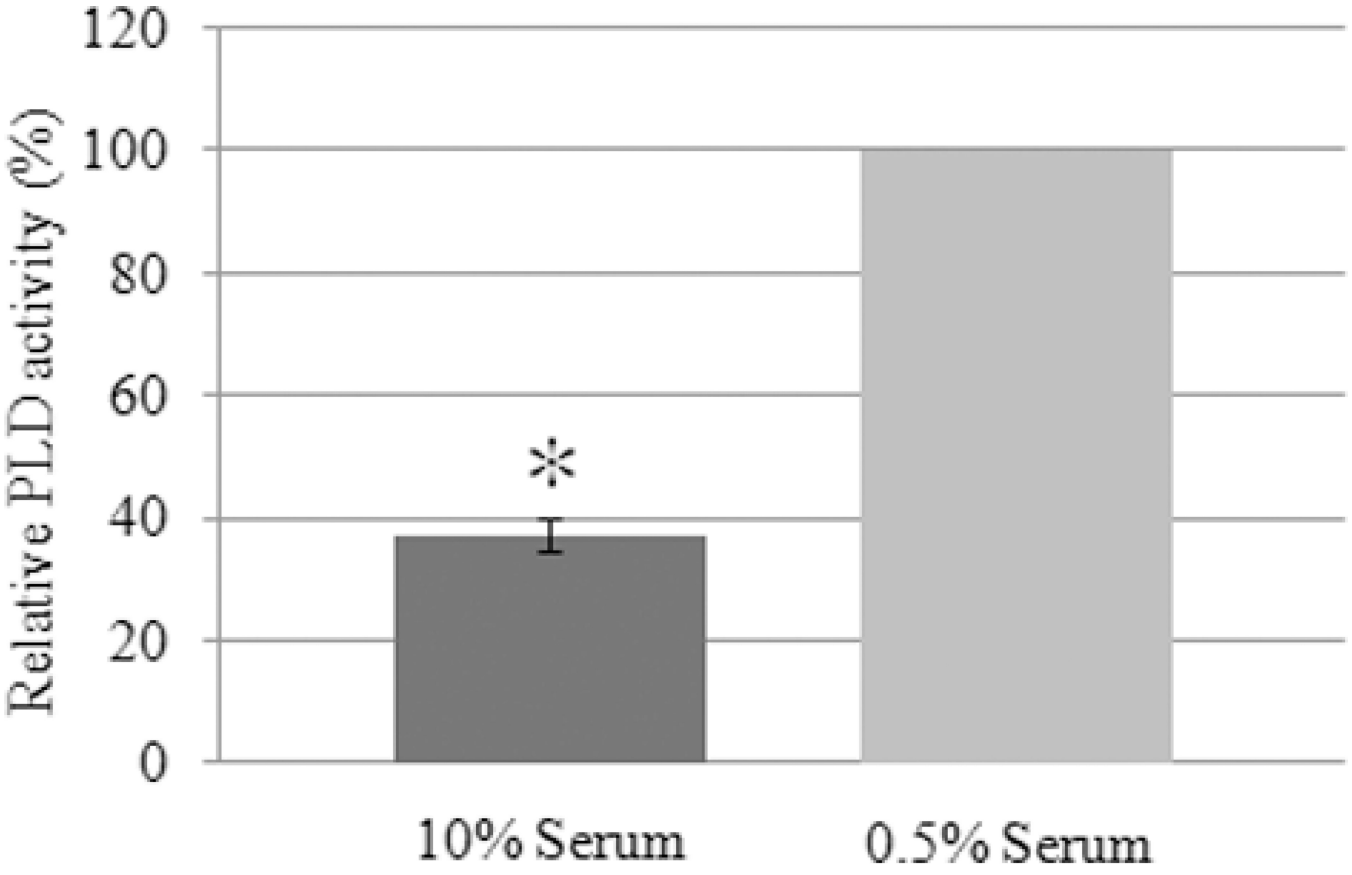
Highlights

- Paxillin production does not depend upon PLD activity
- Paxillin tyrosine phosphorylation parallels PLD activity in MDA-MB-231 cells
- PLD activity inhibits Erk phosphorylation in MDA-MB-231 cells
- PLD activity inhibits complex formation between Erk and paxillin in MDA-MB-231 cells
- Supression of Erk activation allows paxillin tyrosine phosphorylation
- FAK and paxillin complex formation depends upon PLD activity



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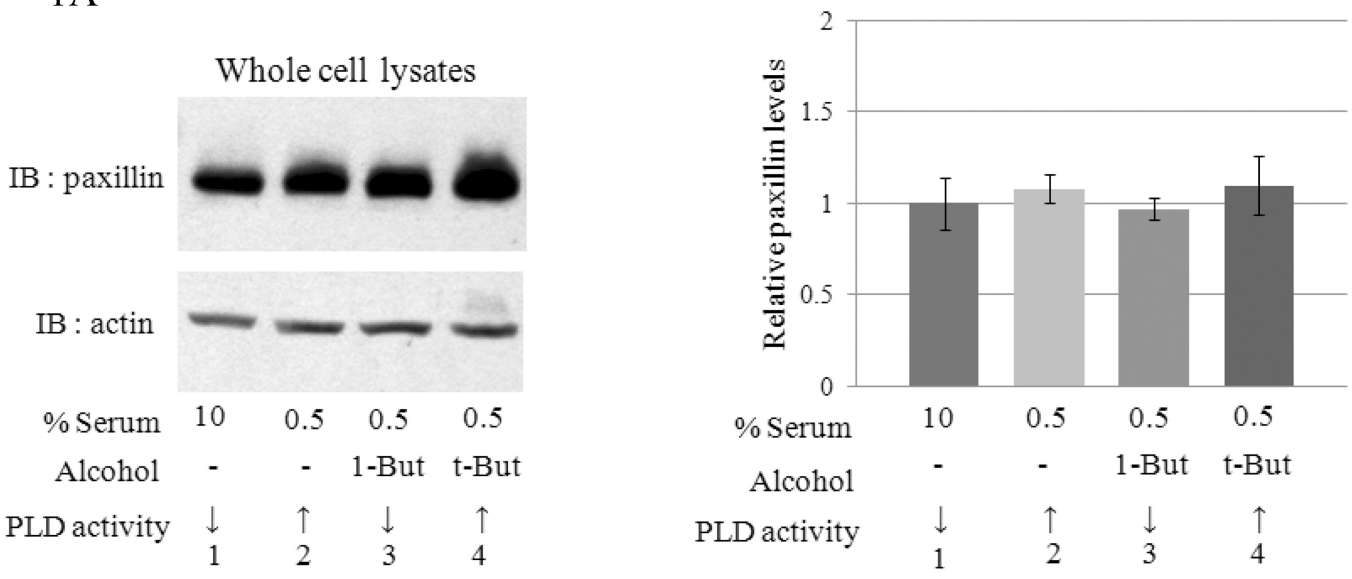

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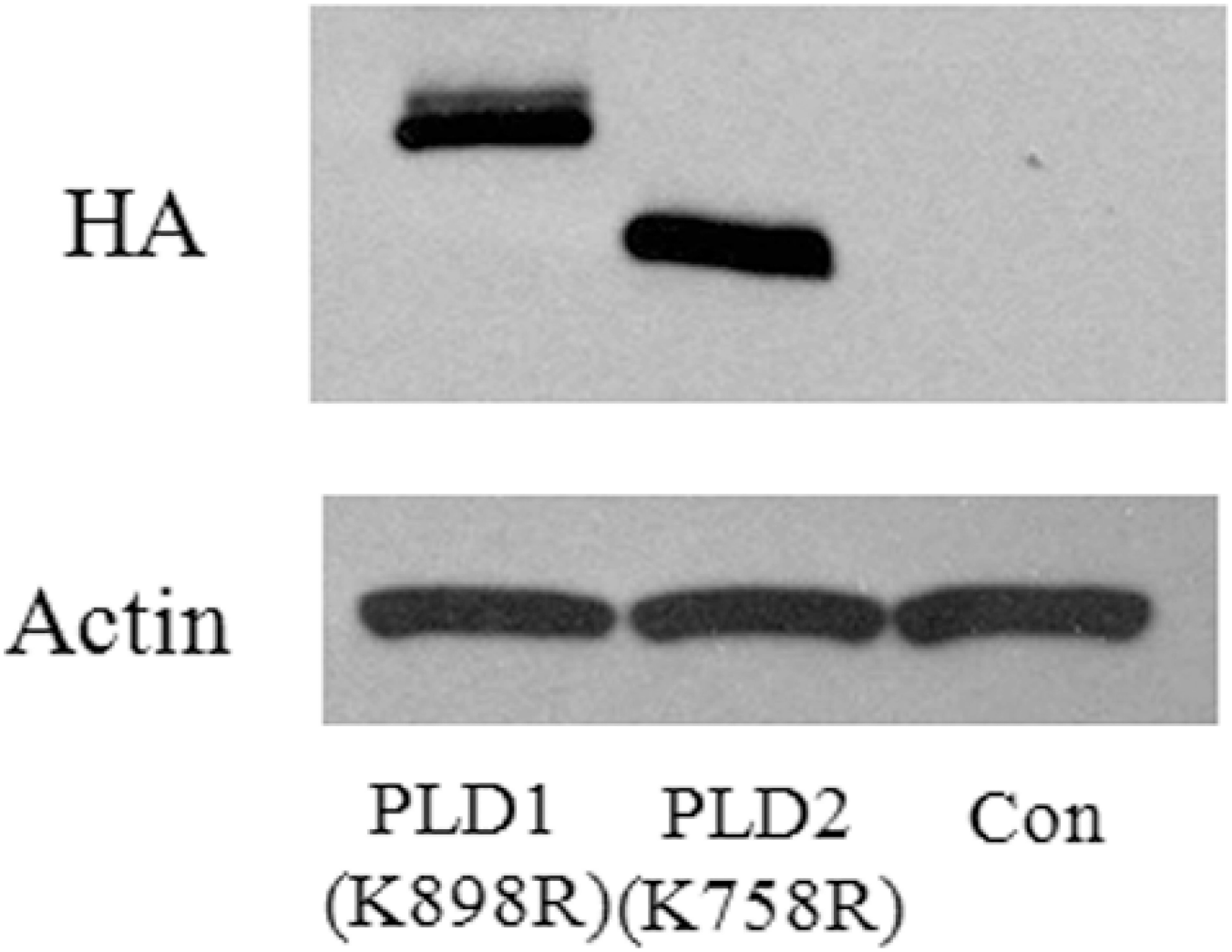

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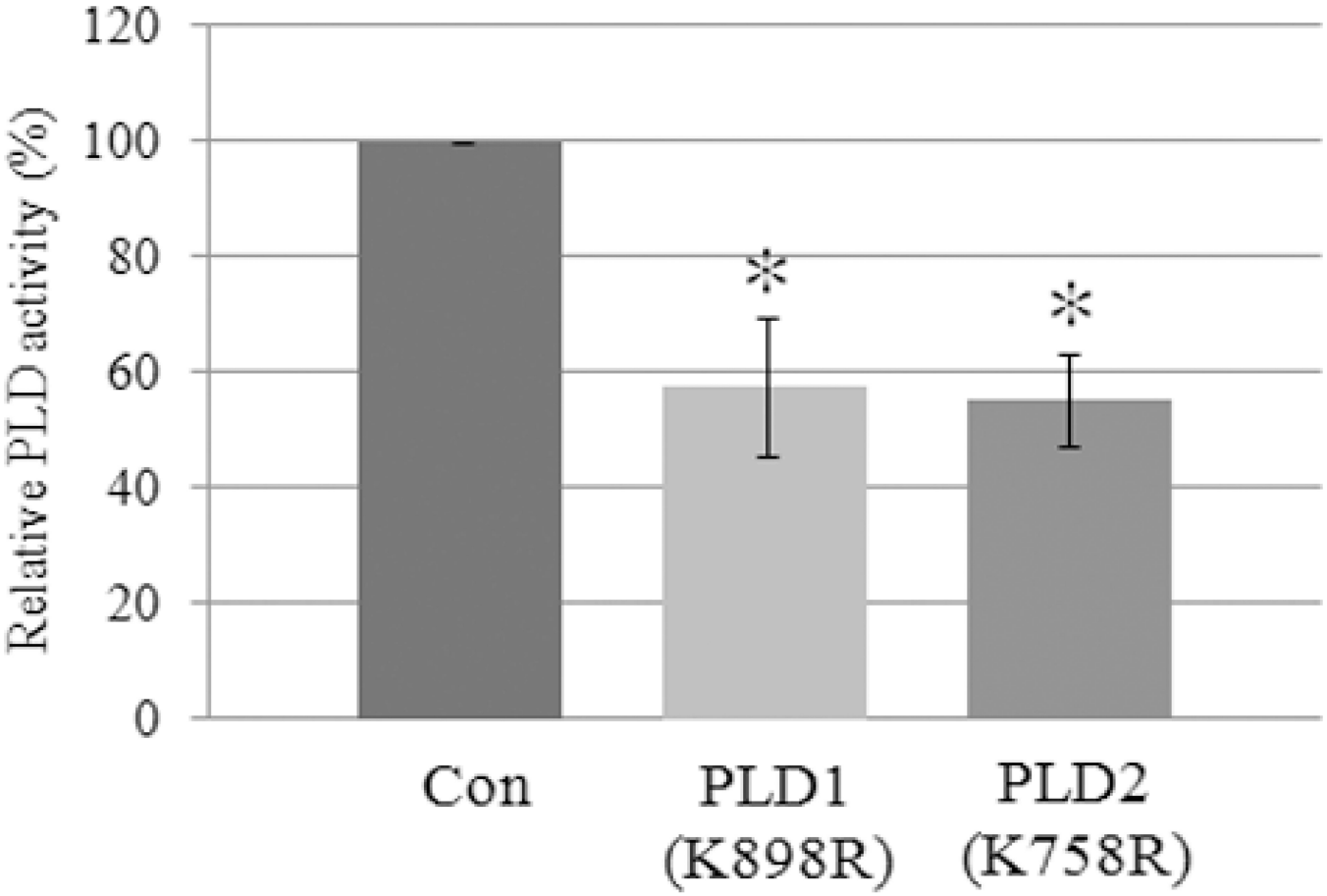

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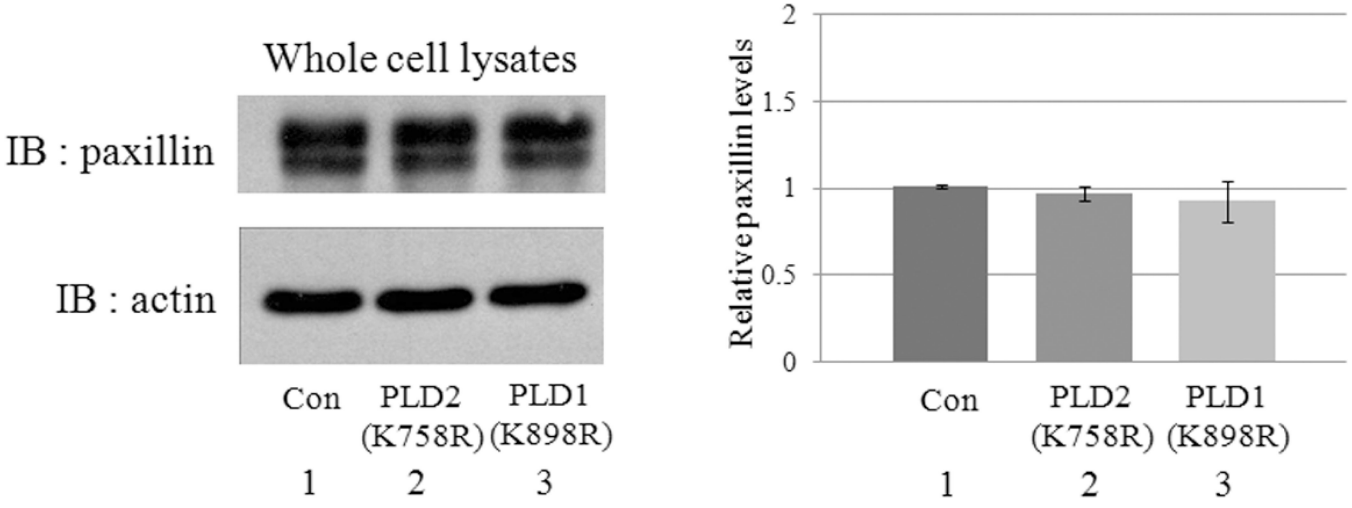
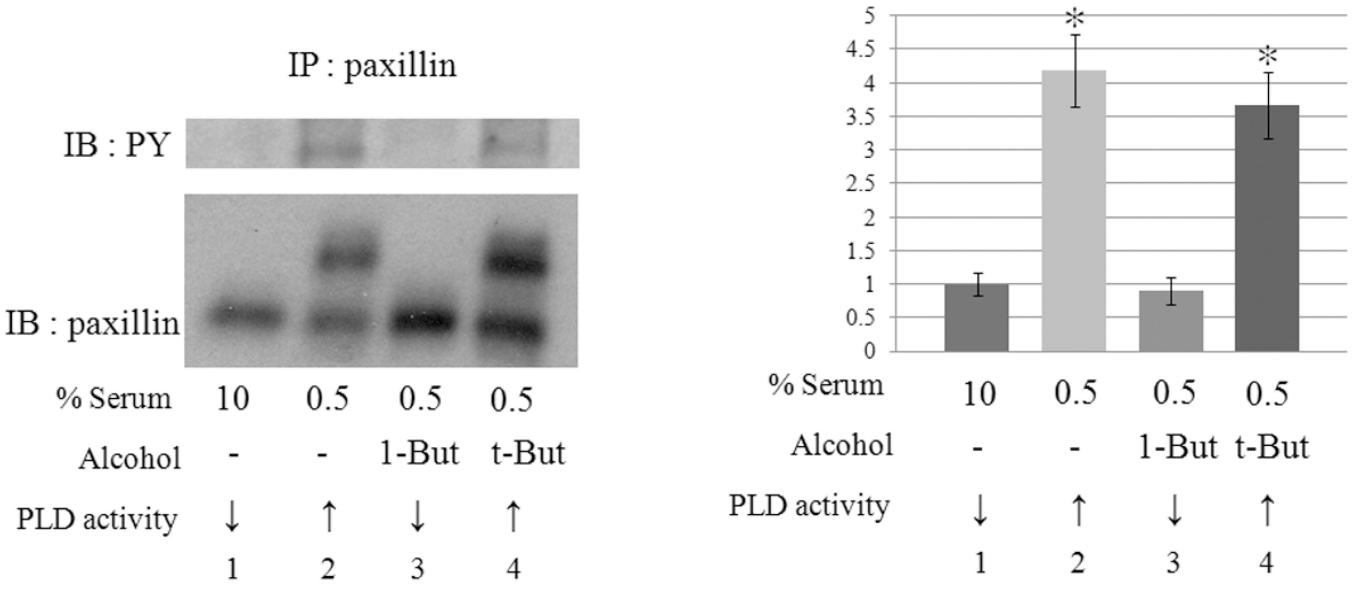


1E

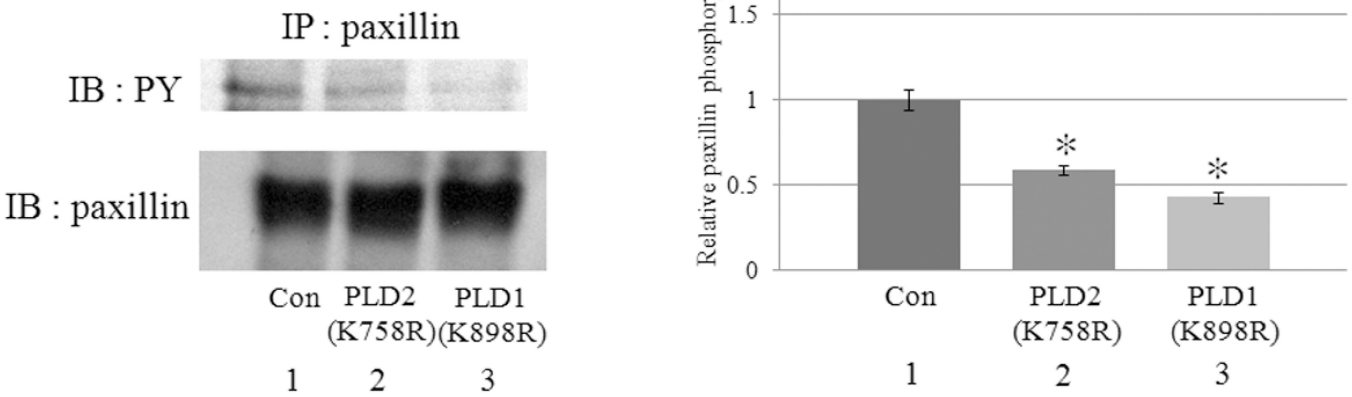
Figure 1.

Paxillin production does not depend upon PLD activity in MDA-MB-231 cells. A) MDA-MB-231 cells were placed in media containing 10% or 0.5% serum, and 24 h later, PLD

activity was determined using the transphosphatidylation reaction. B) Cell lysates were subjected to Western blot analysis with α-paxillin Ab followed by Western blotting using α-actin Ab as a control for equal loading. C) MDA-MB-231 cells were transiently transfected with PLD1 (K898R), PLD2 (K758R) or vector control (Con). Cell lysates were subjected to Western blot analysis with antibodies raised against the hemagglutinin (HA) tag on PLD1-K898R or PLD2-K758R and actin. D) MDA-MB-231 cells transiently transfected with either a vector control (Con) or with PCGN-PLD1-K898R or pCGN-PLD2-K758R were placed in media containing 0.5% serum, and 24 h later, PLD activity was determined using the transphosphatidylation reaction as described above. E) MDA-MB-231 cells transiently transfected with either a vector control (Con) or with PCGN-PLD1-K898R or pCGN-PLD2-K758R were placed in media containing 0.5% serum. 24 h later, cell were lysed and subjected to Western blot analysis with α-paxillin Ab followed by Western blotting using α-actin Ab as a control for equal loading. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 0.5% serum (A) or control (D) with $p < 0.05$. Representative blots are shown.



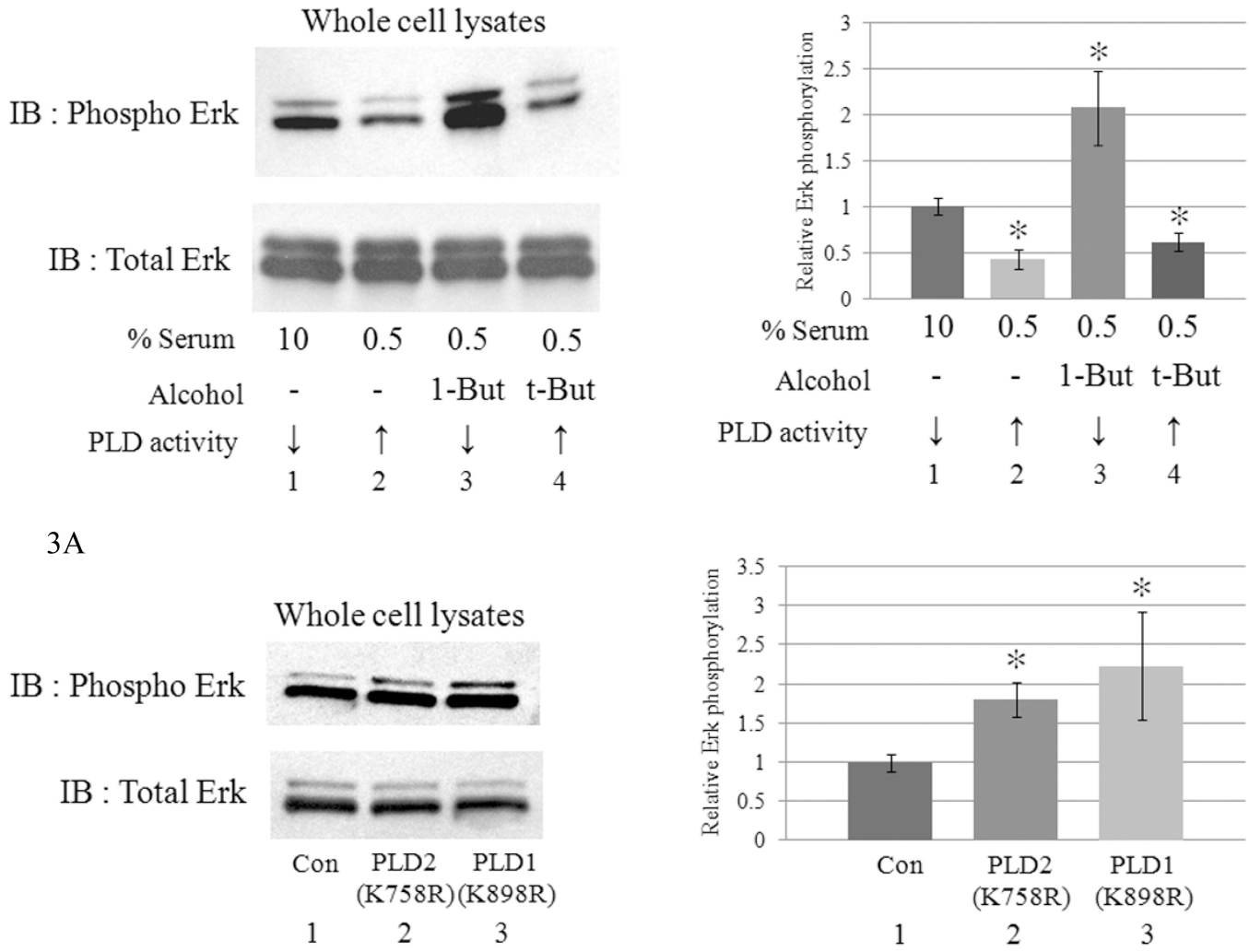
2A



2B

Figure 2.

Paxillin tyrosine phosphorylation parallels PLD activity in MDA-MB-231 cells. Cell lysates from MDA-MB-231 cells A) grown in 10% or 0.5% serum with or without Butanol or B) transiently transfected with either a vector control (Con) or with PCGN-PLD1-K898R or pCGN-PLD2-K758R were immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phosphotyrosine Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or control (B) with $p < 0.05$. Representative blots are shown.

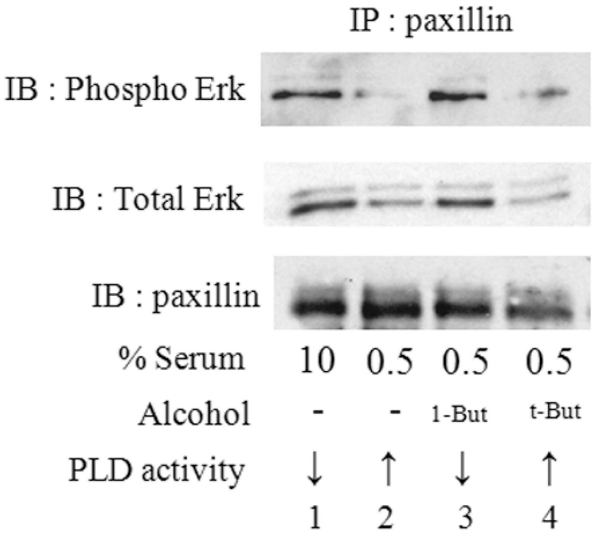


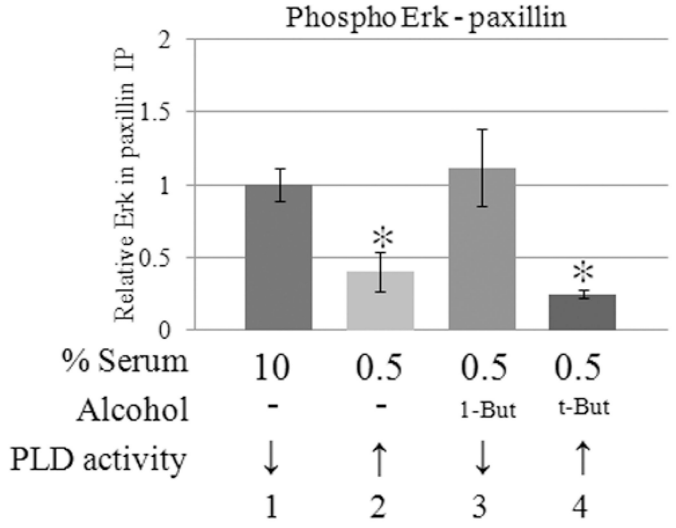
3A

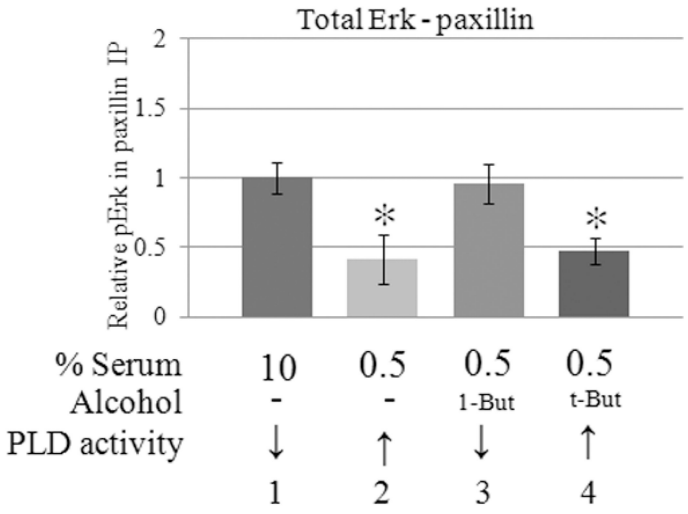
3B

Figure 3.

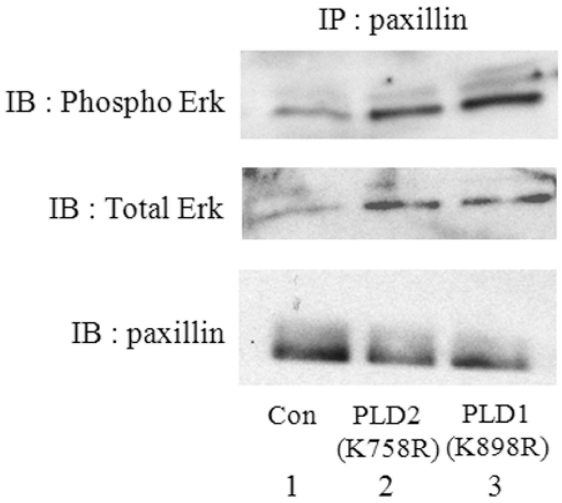
PLD activity inhibits Erk phosphorylation in MDA-MB-231 cells. Cell lysates from MDA-MB-231 cells A) grown in 10% or 0.5% serum with or without Butanol or B) transiently transfected with either a vector control (Con) or with PCGN-PLD1-K898R or pCGN-PLD2-K758R were subjected to Western blot analysis with α-phospho-Erk 1/2 Ab. The blots were stripped and reprobed with α-Erk 1/2 Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or control (B) with $p < 0.05$. Representative blots are shown.

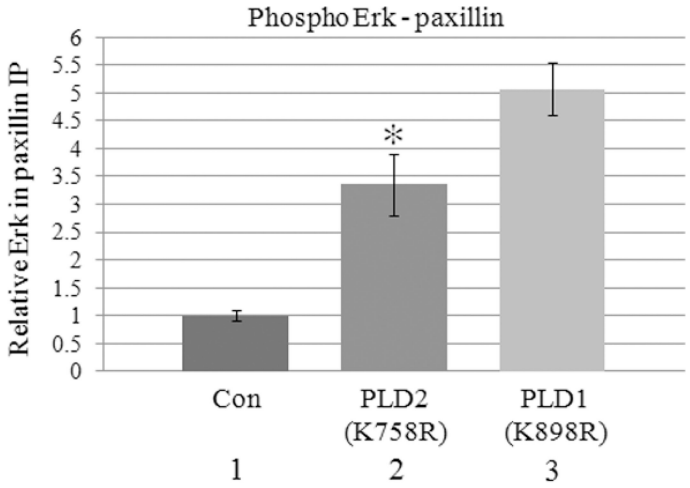


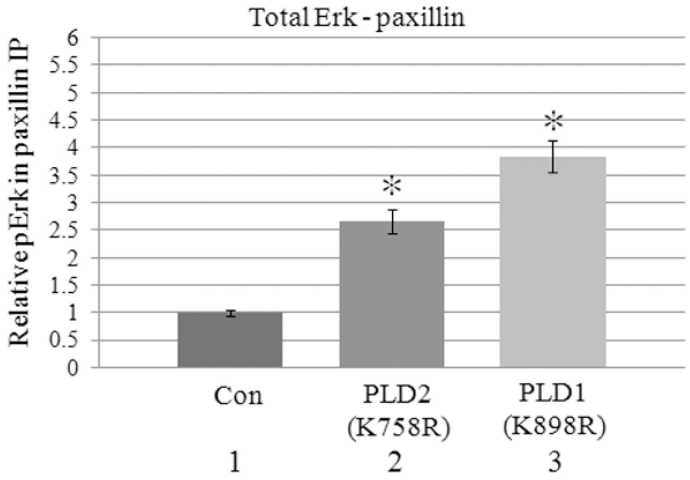




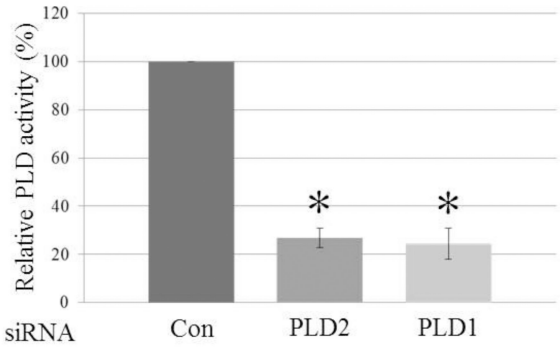
4A

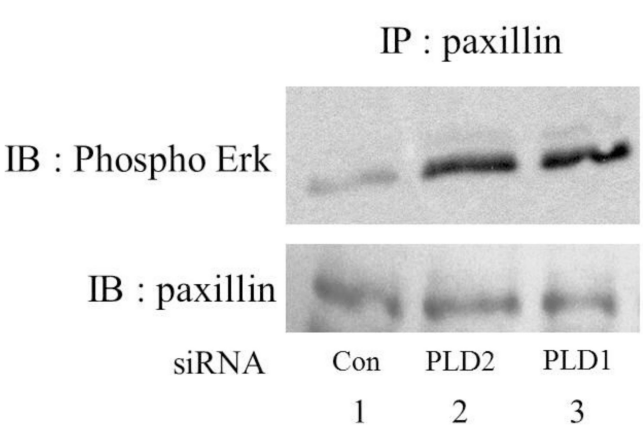


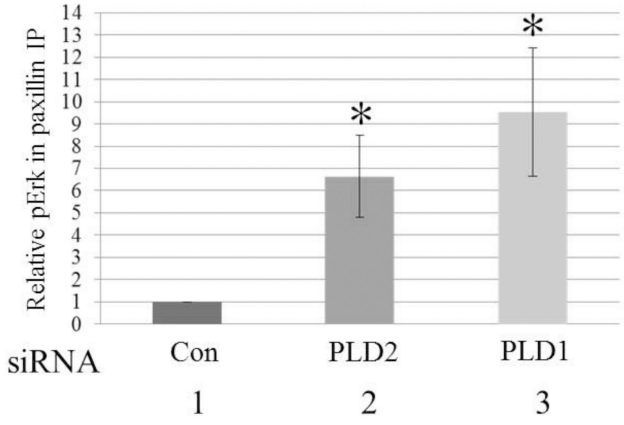




4B



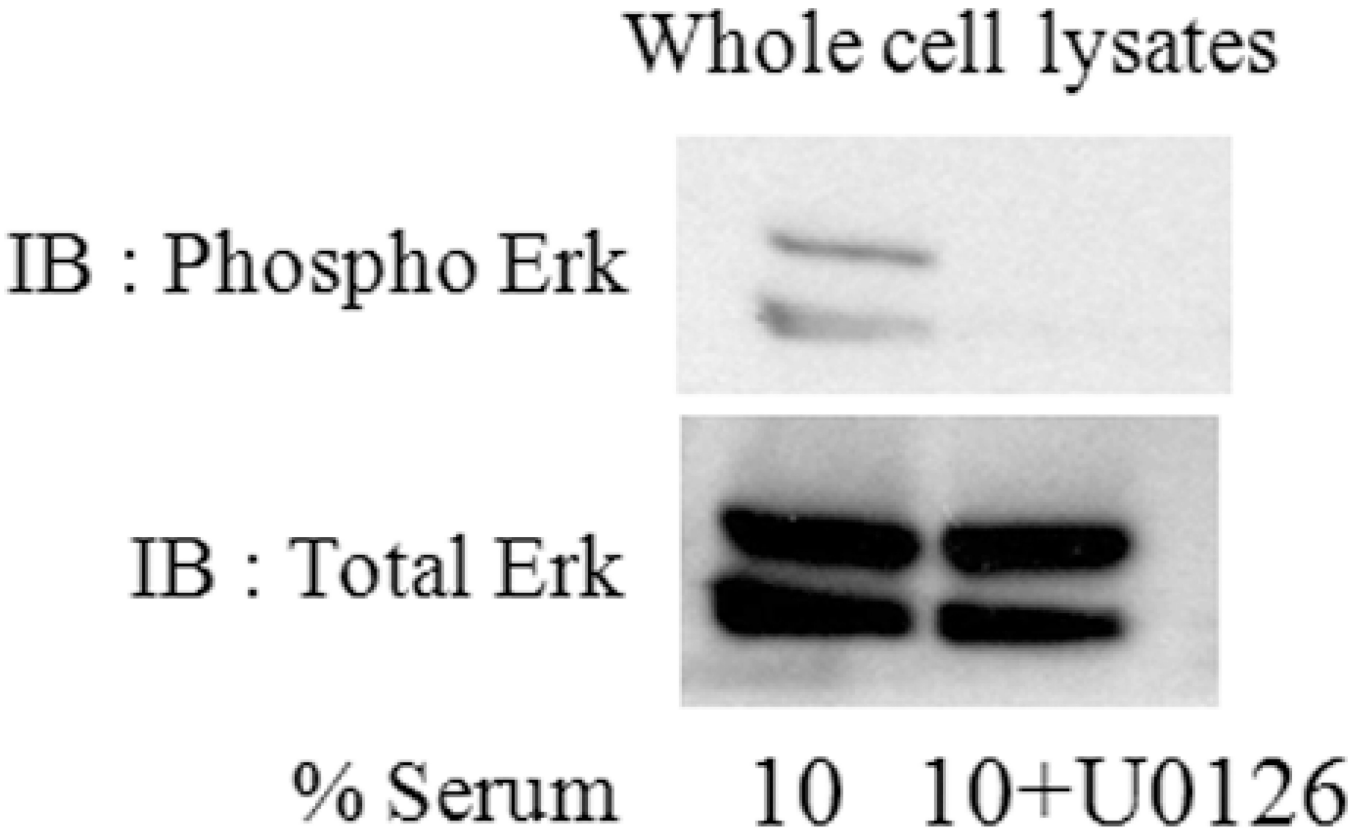




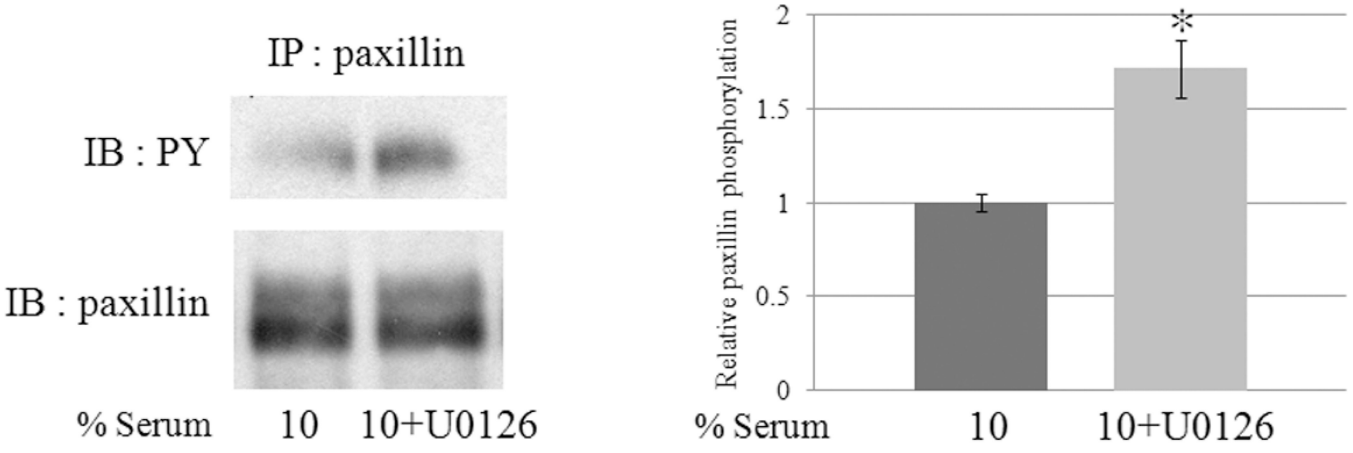
4C

Figure 4.

Erk - paxillin association is dependent upon PLD activity in MDA-MB-231 cells. Cell lysates from MDA-MB-231 cells A) grown in 10% or 0.5% serum with or without Butanol, B) transiently transfected with either a vector control (Con) or with pCGN-PLD1-K898R or pCGN-PLD2-K758R or C) transfected with either control (Con) scrambled siRNA or with siRNA specific for PLD2 or PLD1 were immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phospho-Erk1/2 Ab followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. PLD activity was determined using the transphosphatidylation reaction as described above. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to compared to 10% serum (A) or control (B, C) with $p < 0.05$. Representative blots are shown.



5A

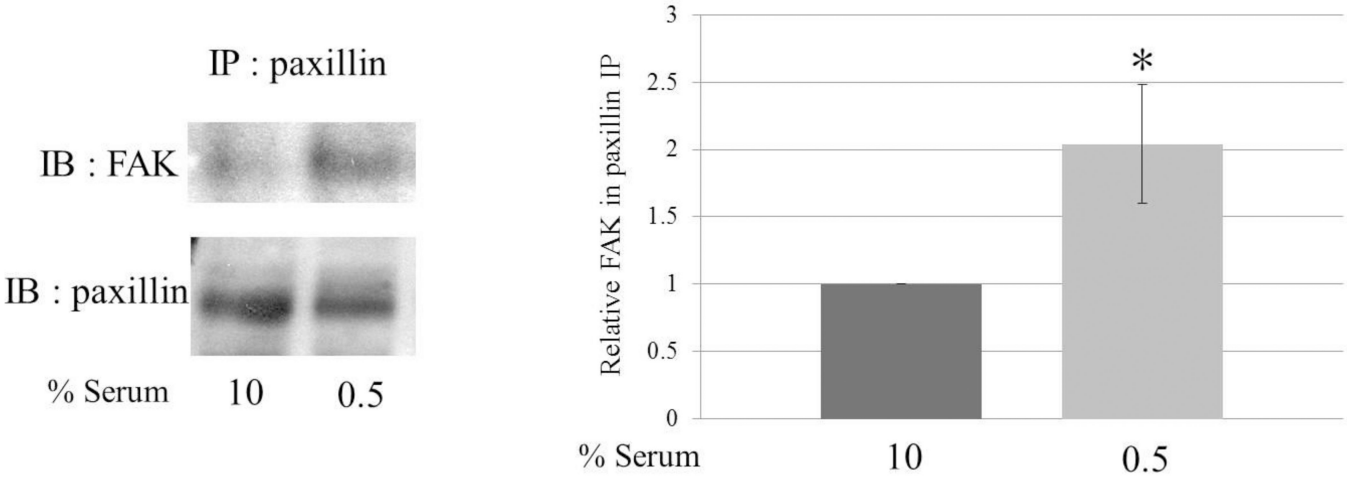


5B

Figure 5.

Erk activation inhibits paxillin tyrosine phosphorylation in MDA-MB-231 cells. A) MDA-MB-231 cells exposed to high serum (10%) were incubated overnight with U0126. Whole cell lysates were immunoblotted with α-phospho-Erk1/2 Ab. The blots were stripped and reprobed with α-Erk Ab. Intensity of the bands was analyzed by densitometry. B) MDA-MB-231 cells exposed to high serum (10%) were incubated overnight with U0126. Cells

were lysed, immnuoprecipitated with α-paxillin Ab and immunoblotted with α-phosphotyrosine Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to U0126 untreated cells with $p < 0.05$. Representative blots are shown.



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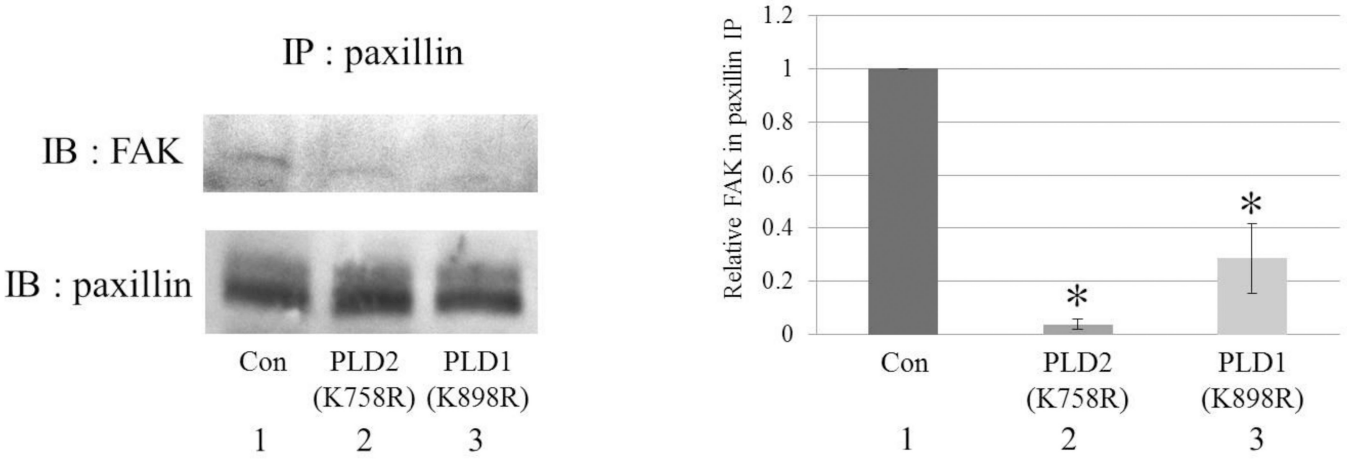

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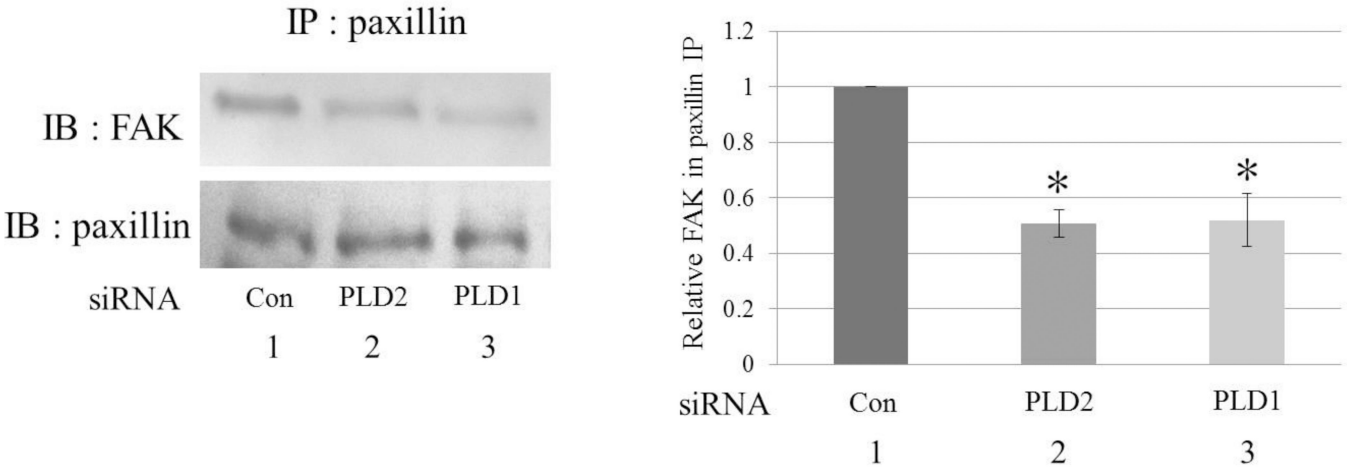

6C

Figure 6.

Association of paxillin with FAK is dependent upon PLD activity in MDA-MB-231 cells. Cell lysates from MDA-MB-231 cells A) grown in 10% or 0.5% serum with or without Butanol, B) transiently transfected with either a vector control (Con) or with pCGN-PLD1-K898R or pCGN-PLD2-K758R or C) transfected with either control (Con) scrambled siRNA or with siRNA specific for PLD2 or PLD1, were immnuoprecipitated with α-paxillin Ab and immunoblotted with α-FAK Ab, followed by stripping and reprobing with α-paxillin Ab. Intensity of the bands was analyzed by densitometry. Data presented are the means and SEM of at least three independent measurements. * indicates a significant difference compared to 10% serum (A) or control (B, C)) with $p < 0.05$. Representative blots are shown.

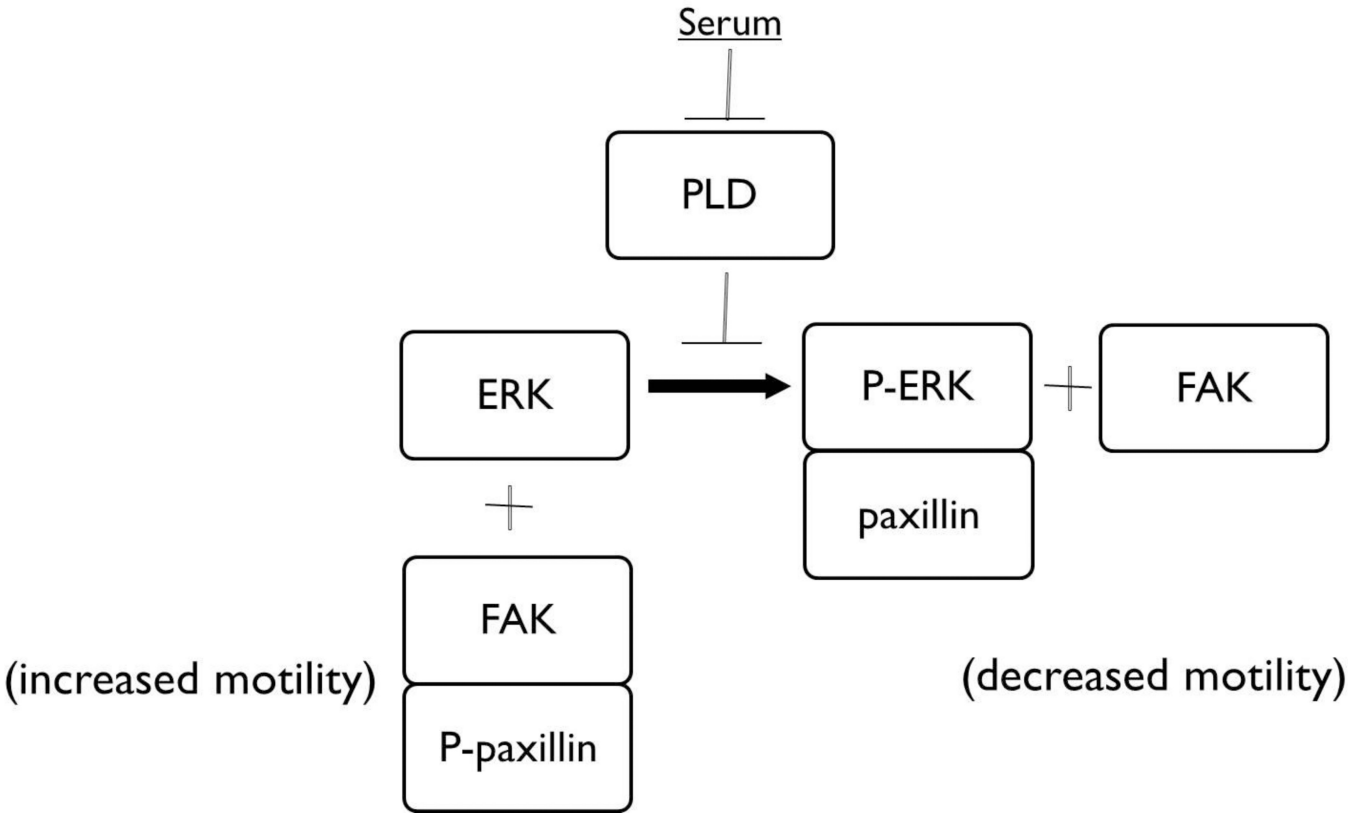

Figure 7.
Model. Increased motility of MDA-MB-231 cells after serum removal may be mediated by PLD, Erk, paxillin and FAK.