

Myc stabilization in response to estrogen and phospholipase D in MCF-7 breast cancer cells

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Abstract Estrogen, which has been strongly implicated in breast cancer, suppresses apoptosis in estrogen receptor (ER) positive MCF-7 breast cancer cells. Phospholipase D (PLD), which is commonly elevated in ER negative breast cancer cells, also suppresses apoptosis. Survival signals generated by both estrogen and PLD are dependent upon elevated Myc expression. We report here that estrogen- and PLD-induced increases in Myc expression are due to reduced turnover of Myc protein. Estrogen and PLD suppressed phosphorylation of Myc at Thr58 – a site that targets Myc for degradation by the proteasome. The data provide a mechanism for elevated Myc expression in hormone-dependent and hormone-independent breast cancer.

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1. Introduction

One of the most common defects in human cancer cells is deregulated expression of the Myc oncogene. Myc is a transcription factor that stimulates the transcription of factors that facilitate progression through critical cell cycle checkpoints and suppresses transcription of factors that suppress cell cycle progression [1]. Elevated Myc expression has been reported in a wide variety of human cancer [2,3]. Expression of Myc is regulated at many levels including: transcriptional, post-transcriptional (mRNA stability and translation), and post-translational (protein stability) [4–7]. Thus, there are many ways in which Myc expression can be altered in human cancers. In addition, there are mutations to the Myc gene that significantly enhance Myc stability and therefore expression [8,9]. Most significantly, mutations at Thr58 lead to a stabilized Myc protein [8,10]. Moreover, phosphorylation of Myc at Thr58 has been shown to regulate Myc stability by targeting Myc for degradation by the proteasome [11]. Thr58 is phosphorylated by glycogen synthase kinase-3 β (GSK-3 β), which phosphorylates many proteins fated for degradation by the proteasome [12].

We reported previously that treatment with 17- β -estradiol (E2) led to a bi-modal increase in Myc expression in the estrogen receptor (ER) positive MCF-7 human breast cancer cell line [13]. There was a short-term transient increase in Myc expression that peaked at about two hours. More striking however was a large sustained stable increase in Myc expression that peaked at five days of treatment. This long-term increase in Myc expression was critical for E2 to suppress apoptosis in MCF-7 cells subjected to the stress of serum withdrawal [13]. We also reported that elevated phospholipase D (PLD) activity could suppress apoptosis in MCF-7 cells, and that PLD similarly increased Myc expression [13,14]. PLD activity, like Myc expression, has been reported to be elevated in a substantial percentage of human breast cancers [15,16] and has been proposed to provide a survival signal in breast and other cancers [17–19]. Significantly, PLD activity is elevated in several breast cancer cell lines that are ER negative [14,20–22], suggesting the possibility that elevated PLD activity in breast cancer cells facilitates progression to hormone independence. In this report, we provide evidence that the induction of Myc expression by both E2 and PLD activity is due to stabilization.

2. Materials and methods

2.1. Cells, cell culture conditions and transfection

MCF-7 and 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. Phenol red free DMEM was employed for experiments as indicated. Cells were transfected with using Lipofectamine 2000 obtained by Invitrogen according to the vendor's instructions. The cells were then selected with G418 (Invitrogen) over a period of 15 days. Pools of clones were then collected and used for experiments.

2.2. Materials

Cycloheximide, lactacystin and okadaic acid were obtained from Calbiochem. Polyclonal antibodies to phosphorylated Myc (Thr58), GSK-3 β and phosphorylated GSK-3 β (Ser9) were obtained from Cell Signaling Technology. Antibody to c-Myc and Actin were obtained from Santa Cruz Biotechnology. The antibody to phosphorylated Myc (Ser62) was obtained from Anaspec, and a polyclonal rabbit anti-ubiquitin antibody was obtained from Dako.

2.3. Plasmids

The pcDNA3.1 control plasmid was obtained from Invitrogen. The plasmid expression vector for PLD2 (pCGN-PLD2) and a catalytically inactive PLD2 mutant (pCGN-PLD2-K758R) [23] were generous gifts of Dr. Michael Frohman (SUNY-Stony Brook). The pcDNA3.1-PLD2 constructs containing a neomycin resistance gene were constructed as described in [13].

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Abbreviations: CHX, cyclohexamide; E2, 17- β -estradiol; ER, estrogen receptor; GSK-3 β , glycogen synthase kinase-3 β ; PLD, phospholipase D; PP2A, protein phosphatase 2A

2.4. Immunoprecipitation

The cells were collected as previously described by Hui et al. [24]. The supernatant was precleared with protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences), and 500 μ g of the precleared proteins was adjusted to a volume of 500 μ l in modified radioimmune precipitation assay buffer and then incubated with the antibody for overnight. The immunocomplex was captured by incubation with 50 μ l of protein G-Sepharose 4 Fast Flow bead slurry and collected by centrifugation at 12000 \times g for 20 s at 4 $^{\circ}$ C. The beads were washed three times with modified radioimmune precipitation assay buffer and once with wash buffer (50 mM Tris, pH 7.6) and then subjected to Western blot analysis.

2.5. Western blot analysis

Extraction of proteins from cultured cells was performed as previously described [14,20].

3. Results

3.1. E2- and PLD-induced Myc expression is due to suppressed turnover

We previously reported that E2 enhanced proliferation and survival of MCF-7 cells subjected to serum withdrawal that became apparent after five days [13]. The ability of E2 to promote proliferation and survival was dependent upon a sustained increase in Myc expression that peaked after five days of E2 treatment [13]. The delayed increase of Myc expression occurred without induction of Myc mRNA, indicating that the increase in Myc expression is not due to an increase in transcription [13]. Since Myc expression is also regulated at the

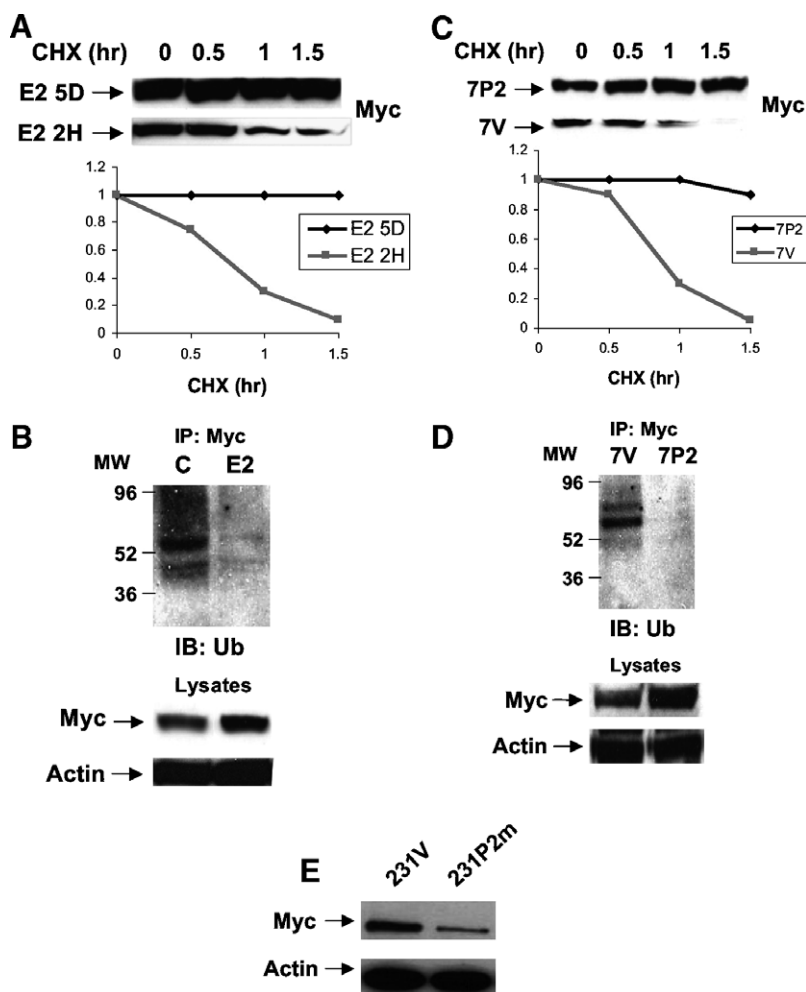


Fig. 1. E2- and PLD-induced Myc expression is due to suppressed turnover. (A) MCF-7 cells were plated at 2×10^4 cells/60 mm culture dish in DMEM with 10% bovine calf serum. After 24 h, the medium was changed to phenol red free DMEM with 0% serum. E2 (2 nM) was then added for either 2 h or five days as indicated. CHX (80 μ g/ μ L) was then added for the indicated times. The cells were then collected and lysates were analyzed for the level of Myc protein using Western blot analysis using an anti-Myc antibody. The relative Myc protein levels normalized to their respective levels in the cells not treated with CHX (zero time point) were determined by using densitometer quantification of the data shown in the upper panel. (B) MCF-7 cells were plated as in (A). 24 h later the medium was changed to phenol red-free DMEM without serum. E2 (2 nM) was then added for five days where indicated. At day five, cells were treated with 10 μ M lactacystin for 6 h at which time cells were collected and lysates were immunoprecipitated with an anti-Myc antibody. Immunoprecipitates were then subjected to Western blot analysis using an anti-ubiquitin antibody (top panel). The lysates were also analyzed for the levels of total Myc by Western Blot analysis. This blot was reprobed with an antibody to actin to control for loading. (C) MCF-7V vector control and MCF-7P2 cells, expressing elevated levels of PLD2, were generated as described previously [13]. Cells were treated and analyzed for the level of Myc protein as in (A). (D) The levels of ubiquitinated Myc in MCF-7V and MCF-7P2 cells was determined as in (B). (E) MDA-MB-231 cells stably transfected with either pCDNA3 vector control (231V) or with pCDNA3-PLD2-K758R (231P2m) were plated at 2×10^4 cells/60 mm culture dish in DMEM with 10% bovine calf serum. After 24 h, the medium was changed to phenol red free DMEM with 0% serum. Myc levels were determined by Western blot analysis 24 h later. The data shown are representative of at least three independent experiments.

level of translation [25], we examined whether E2-induced increases in Myc expression were dependent upon protein synthesis. MCF-7 cells were treated with E2 for five days. The protein synthesis inhibitor cycloheximide (CHX) was added to inhibit newly synthesized protein for increasing times out to 1.5 h. Since E2 also induces a short-term transient increase in Myc expression that is dependent on an increase in Myc translation [25], we also examined the effect of CHX on Myc expression in MCF-7 cells treated with E2 for 2 h. As shown in Fig. 1A, Myc protein levels induced by E2 for five days were very stable in the presence of CHX, whereas the Myc levels induced by E2 at 2 h dropped substantially in the presence of CHX. These data indicate that the high levels of Myc seen after five days of E2 treatment are not affected by inhibition of protein synthesis, indicating that increased Myc expression is not due to increased translation.

Since Myc can be degraded via the ubiquitin–proteasome pathway [8,9], we examined whether long-term E2 treatment inhibited the ubiquitination of Myc. MCF-7 cells were treated with E2 for five days at which time Myc was recovered by immunoprecipitation. The Myc immunoprecipitates were then analyzed by Western blot analysis with an anti-ubiquitin antibody. As shown in Fig. 1B, E2 reduced the amount of ubiquitinated Myc protein in cell lysates in spite of the fact that there were highly elevated levels of Myc protein after E2 treatment. The proteasome inhibitor lactacystin was included to prevent degradation of ubiquitinated Myc. These data provide evidence that E2 inhibits the ubiquitination of Myc protein, and consequently, degradation via the ubiquitin/proteasome pathway.

As observed with E2, elevated PLD activity in MCF-7 cells also provides a survival signal [14,20] that is dependent upon elevated expression of Myc [13]. And although E2 did not stimulate PLD activity, E2-induced Myc expression was dependent on PLD activity [13]. We therefore extended our study to MCF-7 cells with elevated expression of PLD2 [13]. As demonstrated previously [13], Myc protein levels are elevated in MCF-7 cells with over-expressed PLD2 (MCF-7P2 cells) relative to MCF-7 vector control cells (MCF-7V cells) without a corresponding induction of Myc mRNA levels [13], indicating that increased Myc expression in the MCF-7P2 cells is not due to increased transcription. We therefore examined the effect of suppressing protein synthesis on Myc expression in the MCF-7V and MCF-7 P2 cells. MCF-7V and MCF-7P2 cells were put in media lacking serum as was done with the E2 experiments and as reported earlier [13]. CHX was added to the MCF-7V and MCF-7P2 cells and Myc protein levels were examined at 30 min intervals for 1.5 h. As shown in Fig. 1C, Myc protein levels in the MCF-7P2 were very stable over this period, whereas Myc levels in MCF-7V cells dropped substantially during the 1.5 h incubation period. While the data were obtained using conditions with prolonged exposure to the serum free media, similar results were seen at all times of exposure to either low or no serum (not shown). We next examined whether ubiquitination of Myc was suppressed in the MCF-7P2 cells relative to the MCF-7V cells. MCF-7V and MCF-7P2 cell lysates were immunoprecipitated with a Myc antibody and the immunoprecipitates were then analyzed by Western blot with an anti-ubiquitin antibody as in Fig. 1B. As shown in Fig. 1D, ubiquitinated Myc was substantially reduced in the MCF-7P2 cells relative to the MCF-7V cells. These data provide

evidence that overexpression of PLD2 in MCF-7 cells inhibits ubiquitination of Myc protein and thus its degradation via the proteasome pathway.

We previously reported that Myc expression is elevated in MDA-MB-231 cells [20], which have highly elevated PLD activity that is stimulated by serum withdrawal [21]. We examined the effect of a catalytically inactive PLD2 mutant that functions as a dominant negative PLD2 mutant on Myc expression in these cells and as shown in Fig. 1E, the PLD2 mutant suppressed Myc expression in these cells – further establishing a link between PLD activity and Myc stability in breast cancer cells.

3.2. E2 and PLD increase phosphorylation of Myc at Ser62 and suppress phosphorylation at Thr58

The ubiquitination and stability of Myc is regulated by phosphorylation of Myc at two key sites – Ser62 and Thr58. Phosphorylation at Ser62 by MAP kinase suppresses ubiquitination and degradation; whereas the phosphorylation at of Myc at Thr58 by GSK-3 β targets Myc for ubiquitination and degradation [26,27]. Importantly, Ser62 must be phosphorylated before GSK-3 β can phosphorylate Thr58, and phosphorylated Ser62 is dephosphorylated by phosphatase-2A (PP2A) [11]. Dephosphorylation at Ser62 allows degradation to proceed by removing the stabilizing phosphate at Ser62. We examined the effect of E2 on the phosphorylation status of Myc at Ser62 and Thr58. MCF-7 cells were treated with E2 for five days and lysates were then analyzed by Western blot with antibodies specific for phosphorylated Myc. As shown in Fig. 2A, E2 increased phosphorylation Myc at Ser62 after five days of treatment compared to the control. However, total Myc protein was also elevated after E2 treatment. The increased phosphorylation of Ser62 was compared with increased Myc levels using densitometry scans from several experiments and as shown in Fig. 2C, there were higher increases in phosphorylation at Ser62 than observed for total Myc protein, indicating that there was a net increase in Myc phosphorylated at Ser62.

We also examined the phosphorylation state of Myc at Thr58, and as shown in Fig. 2B, E2 strongly reduced the level of phosphorylated Myc at Thr58 compared to the control. The reduced levels of Myc phosphorylated at Thr58 were seen in spite of increased levels of Myc protein. Densitometric analysis revealed that the suppression of phosphorylation at Thr58 in response to E2 was more than 20-fold (Fig. 2C). These data reveal that E2 suppresses phosphorylation at Thr58 – a site that when phosphorylated, targets Myc for ubiquitination and degradation [26,27]. In addition, the data also reveal that E2 also increases phosphorylation at Ser62 – a site that suppresses ubiquitination and degradation [11]. While the effect of E2 on the phosphorylation at Ser62 was reproducible, the effect on phosphorylation at Thr58 was far more dramatic. These data support the hypothesis that the elevated Myc expression in response to E2 is due to stabilization through elevated phosphorylation of Ser62 and reduced phosphorylation at Thr58.

The effect of elevated PLD2 expression on the phosphorylation of Myc at Ser62 and Thr58 in MCF-7 cells was also examined. As shown in Fig. 2D, PLD2 over-expression also resulted in increased phosphorylation of Myc at Ser62. As observed with E2 treatment, total Myc was elevated in the MCF-7P2 cells relative to the MCF-7V cells. However, as observed with

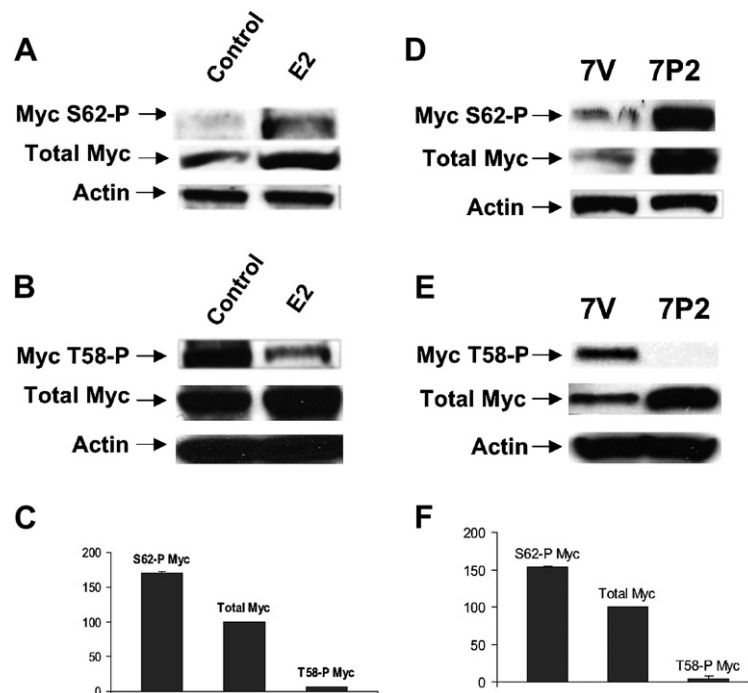


Fig. 2. E2 and PLD increase phosphorylation of Myc at Ser62 and suppress phosphorylation at Thr58. (A) MCF-7 cells were prepared and collected as described in Fig. 1 and placed in serum-free, phenol red-free DMEM with or without E2 (2 nM) as indicated. Lysates were prepared and analyzed for the levels of phosphorylated Myc protein at Ser62 by Western blot analysis with an antibody against Myc phosphorylated at this site (Anaspec). The blot was stripped and re probed with an antibody to total Myc protein. The blot was also re probed with an antibody to actin to control for loading. (B) MCF-7 cells were treated and collected as in (A) and the lysates were analyzed for the levels of phosphorylated Myc protein at Thr58 using an antibody that recognizes Myc phosphorylated at this site. The blot was stripped and re probed with antibodies to Myc and actin as in (A). (C) The levels of phosphorylated Myc in presence of E2 were normalized to their respective levels in the untreated control was determined using densitometer quantification. The relative changes in phosphorylated Myc for both Sre62 and Thr58 were compared to level of total Myc protein. (D) MCF-7V and MCF-7P2 cells were prepared and collected as described in (A) and placed in phenol red-free DMEM with 0.5% serum. Lysates were prepared five days later and analyzed by Western blot for the level of Myc phosphorylated at Ser62 (D) and Thr58 (E) as in (A) and (B) respectively. (F) The levels of phosphorylated Myc in the MCF-7P2 cells were normalized to the level in the MCF-7V control cells was determined using densitometer quantification. The relative changes in phosphorylated Myc for both Ser62 and Thr58 were compared to level of total Myc protein. Experiments shown in (A), (B), (D) and (E) are representative of three independent experiments. The data in (C) and (F) are the averages of three independent experiments normalized to the levels of Myc protein. Error bars represent the standard deviation.

E2 treatment, there were still larger increases in Myc phosphorylated at Ser62 than observed for total Myc protein in the MCF-7P2 cells. Densitometric analysis indicated a 50% increase in Myc phosphorylated at Ser62 (Fig. 2F). The phosphorylation state of Myc at Thr58 in MCF-7V and MCF-7P2 cells was also examined, and as shown in Fig. 2E, the level of phosphorylated Myc at Thr58 in MCF-7P2 cells was dramatically reduced relative to the MCF-7V cells. Densitometric analysis revealed that the suppression of phosphorylation at Thr58 in response to PLD over-expression was more than 50-fold (Fig. 2F). These data reveal that PLD, like E2, strongly suppresses phosphorylation at Thr58 and increases phosphorylation at Ser62 – supporting the hypothesis that the increase in Myc levels is due to stabilization.

3.3. E2 and PLD induce phosphorylation of GSK-3 β at the negative regulatory site Ser9

Since the effect of E2 on phosphorylation at Thr58 was so dramatic, we examined the effect of E2 on GSK-3 β . GSK-3 β is a ubiquitous protein kinase that is inhibited by phosphorylation at Ser9 [28–30]. E2 was previously reported to inhibit GSK-3 β in the hippocampus [31]. We therefore examined the effect of E2 on the phosphorylation of GSK-3 β at Ser9. MCF-7 cells were treated with E2 for five days and lysates

were then analyzed by Western blot using an antibody that recognizes GSK-3 β phosphorylated at Ser9. As shown in Fig. 3A, the five-day treatment with E2 led to a large increase in the phosphorylation of GSK-3 β at Ser9. The total GSK-3 β protein level was not affected by E2 treatment. We then examined whether PLD, like E2, induced an increase in the phosphorylation of the negative regulatory site Ser9. MCF-7V control or MCF-7P2 cells lysates were analyzed by Western blot analysis with the anti-Ser9 GSK-3 β antibody. As shown in Fig. 3B, PLD2 over-expression substantially increased phosphorylation of GSK-3 β at Ser9 relative to the MCF-7V control cells. Total GSK-3 β protein levels were not affected by PLD2. These data indicate that both E2 and elevated PLD expression can result in the inhibition of GSK-3 β by increasing phosphorylation of GSK-3 β at Ser9.

4. Discussion

Suppression of apoptosis is critical in tumor progression [32]. Myc has been implicated in the suppression of apoptosis and progression past cell cycle checkpoints where apoptosis is regulated [3]. The regulation of Myc expression is complex and is controlled at the level of transcription, translation and

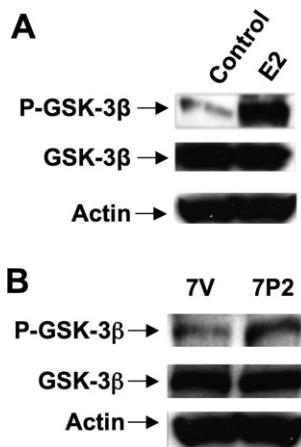


Fig. 3. E2 induces phosphorylation of GSK-3 β at the negative regulatory site Ser9. (A) MCF-7 cells were prepared as described in Fig. 1 and placed in serum-free phenol red-free DMEM in the absence or presence of E2 (2 nM) for five days as indicated. Lysates were prepared and analyzed for the levels of phospho-GSK-3 β (Ser9) by Western blot analysis using an antibody that recognizes GSK-3 β phosphorylated at this site (Cell Signaling Technology). The blot was stripped and reprobed for the levels of total GSK-3 β protein. The blot was then reprobed with an antibody to actin to control for loading. (B) MCF-7V and MCF-7P2 cells were prepared as in (A) and placed in phenol red-free DMEM with 0.5% serum. Lysates were prepared five days later and analyzed for the levels of phospho-GSK-3 β (Ser9) protein as in (A). The data shown are representative of three independent experiments.

stabilization [33]. We reported previously that prolonged exposure to E2 leads to very high levels of Myc expression that peaked at five days and that elevated Myc expression was required for the suppression of apoptosis in ER positive MCF-7 cells subjected to the stress of serum withdrawal [13]. E2 could be replaced with elevated PLD expression, which similarly increased Myc expression and suppressed apoptosis in MCF-7 cells subjected to serum withdrawal. In this report, we have characterized the mechanism for the sustained induction of Myc. We found that the increased Myc expression induced by both E2 and PLD was due to stabilization. Both E2 and PLD suppressed phosphorylation of Myc at Thr58 – a site that targets Myc for degradation by the proteasome [8,34]. Myc is phosphorylated at Thr58 by GSK-3 β [26,27], and consistent with the reduced phosphorylation of Myc at Thr58, both E2 and PLD stimulated a substantial increase in the phosphorylation of GSK-3 β at Ser9, which suppresses GSK-3 β activity [28–30]. A model for the E2 and PLD stabilization of Myc through suppression of GSK-3 β is shown schematically in Fig. 4.

The phosphorylation of Myc at Ser62 was elevated in response to both E2 and PLD. The effect was not as dramatic as observed for Thr58, but the increased phosphorylation at Ser62 is also consistent with increased stability of Myc. Myc must be first phosphorylated at Ser62 before it can be phosphorylated at Thr58 [11], and then it must subsequently be dephosphorylated by PP2A before it can be ubiquitinated. Consistent with this model we previously reported that PLD activity suppresses PP2A activity in MCF-7 cells [24]. Thus, the increased phosphorylation of Myc at Ser62 in response to PLD likely reflects the suppression of PP2A activity by PLD. We did not see a significant effect of E2 on PP2A activity

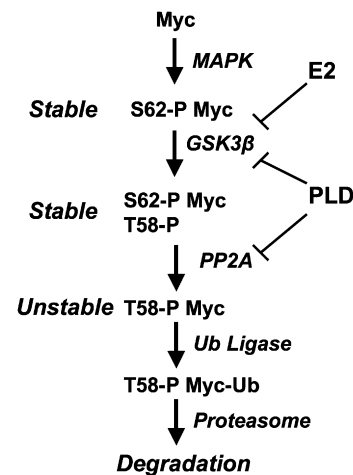


Fig. 4. Model for Myc stabilization in response to E2 and PLD. MAP kinase (MAPK) phosphorylates Myc at Ser62 (S62-P), which stabilizes Myc. Myc, phosphorylated at Ser62, can then be phosphorylated at Thr58 (T58-P) by GSK-3 β . The doubly phosphorylated Myc can then be dephosphorylated at Ser62 by PP2A. Myc phosphorylated only at Thr58 is recognized by a ubiquitin (Ub) ligase that leads to the ubiquitination of Myc and degradation by the proteasome [33]. Both E2 and PLD can suppress ubiquitination and degradation by suppressing the GSK-3 β phosphorylation of Myc at Thr58. In addition, PLD can also suppress the PP2A [24]. The effect of E2 and PLD on Myc is the stabilization of Myc, which is required for the survival signals generated by both E2 and PLD [13].

in the MCF-7 cells. Thus, at this time we are not sure as to how E2 increases phosphorylation of Myc at Ser62.

We have proposed that elevated PLD activity in human breast cancer provides a means for progression to hormone independence [17]. This was largely based on the observation that breast cancer cell lines with highly elevated PLD activity were all ER negative and most breast cancer cell lines with low PLD activity were ER negative [13,14,20–22]. While these observations only provide correlative evidence for the involvement PLD in progression to hormone independence in breast cancer, there is increasing evidence that PLD generates survival signals that would compensate for the loss of hormone responsiveness and dependence. As we reported previously, and observed by others, E2 provides a survival signal in ER positive breast cancer cells [13,34–36]. PLD similarly provides a survival signal in breast cancer cells subjected to the stress of serum withdrawal [14,22], conditions that occur in an emerging solid tumor prior to vascularization. The lack of serum would also restrict access to E2 and select for cells that could survive these conditions. Cells with elevated PLD activity would have the ability to survive these conditions. The finding here that PLD, like E2 can promote the stabilization of Myc further supports a critical role for the elevated PLD activity commonly seen in ER negative breast cancer cells for survival as tumors progress to hormone independence. The study suggests that in hormone resistant breast cancers, targeting PLD survival signals could be an effective therapeutic strategy for inducing apoptosis in breast cancer cells dependent on PLD activity for survival.

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