

Phosphorylation of PEA-15 switches its binding specificity from ERK/MAPK to FADD

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Cell signalling pathways that regulate proliferation and those that regulate programmed cell death (apoptosis) are co-ordinated. The proteins and mechanisms that mediate the integration of these pathways are not yet fully described. The phosphoprotein PEA-15 (phosphoprotein enriched in astrocytes) can regulate both the ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway and the death receptor-initiated apoptosis pathway. This is the result of PEA-15 binding to the ERK/MAPK or the proapoptotic protein FADD (Fas-activated death domain protein) respectively. The mechanism by which binding of PEA-15 to these proteins is controlled has not been elucidated. PEA-15 is a phosphoprotein containing a Ser-104 phosphorylated by protein kinase C and a Ser-116 phosphorylated by CamKII (calcium/calmodulin-dependent protein kinase II) or AKT. Phosphorylation of Ser-104 is implicated in the regulation of glucose metabolism, while phosphorylation at Ser-116 is required for PEA-15 recruitment to the DISC (death-initiation signalling

complex). Moreover, PEA-15 must be phosphorylated at Ser-116 to inhibit apoptosis. In the present study, we report that phosphorylation at Ser-104 blocks ERK binding to PEA-15 *in vitro* and *in vivo*, whereas phosphorylation at Ser-116 promotes its binding to FADD. We further characterize phospho-epitope-binding antibodies to these sites. We report that phosphorylation does not influence the distribution of PEA-15 between the cytoplasm and nucleus of the cell since all phosphorylated states are found predominantly in the cytoplasm. We propose that phosphorylation of PEA-15 acts as the switch that controls whether PEA-15 influences proliferation or apoptosis.

Key words: calcium/calmodulin-dependent protein kinase II (CamKII), ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase), PEA-15, phosphorylation, protein kinase C, signal transduction.

INTRODUCTION

The regulation of cell proliferation and cell death is integrated as a result of cross-talk between the signalling pathways regulating them. The phosphoprotein PEA-15 (phosphoprotein enriched in astrocytes) is reported to regulate the proliferative ERK (extracellular-signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway by binding ERK and preventing its nuclear accumulation [1–3]. PEA-15 contains an N-terminal DED (death effector domain) and is also reported to block death receptor-activated apoptosis as a result of binding to the DED of FADD (Fas-activated death domain protein) protein and blocking caspase recruitment [4–7]. Moreover, the site on PEA-15 at which ERK binds also includes sequences in this DED (Figure 1A) [1]. How PEA-15 moves between these two pathways remains unknown.

In addition to the N-terminal DED, PEA-15 also contains two phosphorylated serine residues in its C-terminal tail. Ser-104 is phosphorylated by PKC (protein kinase C) [8]. Ser-116 is phosphorylated by both CamKII (calcium/calmodulin-dependent protein kinase II) [9] and AKT [10]. AKT is also reported to bind this sequence of PEA-15 [10]. Mutants in the C-terminal tail of PEA-15 can abrogate PEA-15 binding to ERK [11]. Phosphorylation at Ser-104 by PKC is required for PEA-15 to impair insulin-stimulated glucose transport and Glut4 translocation in skeletal muscle [12,13]. Phosphorylation by PKC is also reported to regulate the anti-apoptotic function of PEA-15 [14]. In particular, inhibition of PKC activity restored the sensitivity of human malignant glioma cells to TRAIL (tumour-necrosis-

factor-related apoptosis-inducing ligand)-induced apoptosis. This supports the hypothesis that phosphorylation at Ser-104 is required for PEA-15 to prevent apoptosis. Moreover, phosphorylation of Ser-116 was subsequently reported to be required for PEA-15 to translocate into the DISC (death-initiation signalling complex) and prevent TRAIL-activated apoptosis of glioma cells [15]. In these experiments, it is only the doubly phosphorylated form of PEA-15 that is anti-apoptotic. Phosphorylation at Ser-116 is also mediated by AKT [10]. Phosphorylation by AKT similarly enhanced the anti-apoptotic function of PEA-15. However, it is proposed in these studies that phosphorylation at Ser-116 altered PEA-15 function in part by stabilizing the protein. Taken together, these studies suggest that phosphorylation of PEA-15 is required for PEA-15 to prevent death receptor-initiated apoptosis.

PEA-15 is reported to bind directly to ERK/MAPK [1]. ERK does not phosphorylate PEA-15 and PEA-15 can bind both active and inactive ERK. PEA-15 binding to ERK prevents nuclear accumulation of ERK and blocks ERK-dependent transcription. It also slows down cell proliferation [1]. Finally, abrogation of ERK binding as a result of point mutations in PEA-15 restores normal ERK function [16,17]. Whether PEA-15 binding to ERK is regulated by phosphorylation has not been examined.

Here, we investigate whether PEA-15 binding to FADD and ERK is altered by phosphorylation at either Ser-104 or Ser-116. To facilitate these experiments, we made use of newly developed phospho-epitope antibodies specific for these sequences. We show that these antibodies are specific for the phosphorylated serine residues and that, in both Cos-7 cells and thymocytes, Ser-104 is

Abbreviations used: CamKII, calcium/calmodulin-dependent protein kinase II; DED, death effector domain; DISC, death-initiation signalling complex; DMEM, Dulbecco's modified Eagle's medium; FADD, Fas-associated death domain protein; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; NES, nuclear export sequence; NP40, Nonidet P40; PKC, protein kinase C.

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not phosphorylated unless the cells are treated with phorbol esters (PMA). Ser-116 is constitutively phosphorylated in these cells. Moreover, phosphorylation of PEA-15 at Ser-104 has no effect on the nuclear versus cytoplasmic subcellular localization of PEA-15. In all the phosphorylation states we examined, PEA-15 remains cytoplasmic. *In vitro* phosphorylation of PEA-15 by PKC substantially reduced ERK binding, while phosphorylation by CamKII had no effect on ERK binding. Conversely, phosphorylation by CamKII at Ser-116 promoted FADD binding. These results provide a mechanism by which phosphorylation of PEA-15 can determine whether it regulates ERK or FADD functions.

EXPERIMENTAL

Cell culture, plasmids, antibodies and reagents

Cos-7 cells and HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal bovine serum, antibiotics and non-essential amino acids. All transfections were carried out using Lipofectamine™ Plus transfection reagent (Invitrogen), according to the manufacturer's instructions. Constructs HA- and His-tagged PEA-15, the untagged PEA-15 in pCDNA3 vector and point mutants of PEA-15 His-S104A (Ser-104 → Ala) and His-S116A have been described previously [1,17,18]. FLAG-ERK2 was a gift from Dr M. Weber (University of Virginia) and pMT2-HA Rsk-2 was a gift from Dr Bjorbaek (Harvard Medical School). pcDNA3-AU1-FADD (human) was a gift from Dr V. Dixit (Genentech, South San Francisco, CA, U.S.A.). Antibody directed to PEA-15 was a gift from Dr H. Chneiweiss (College de France). Phospho-antibodies of PEA-15 against Ser-104 (pS104) and Ser-116 (pS116; rabbit, catalogue no. 44-836G) were generated in collaboration with Biosource International (Camarillo, CA, U.S.A.). Antibody directed to ERK1/2 was obtained from Santa Cruz Biotechnology, FLAG-tagged antibody from Stratagene and HA-tagged antibody from Cell Signaling Technology (Beverly, MA, U.S.A.). 12CA5 antibody was from Roche Applied Sciences (Indianapolis, IN, U.S.A.). Anti-FADD (Transduction Laboratories, Lexington, KY, U.S.A.) and anti-AU1 (Covance Research Products, Berkeley, CA, U.S.A.) were gifts from Dr E. White (Rutgers University). c-Myc antibody was a gift from Dr G. Xiao (Rutgers University) and laminB2 antibody was a gift from B. Firestein (Rutgers University). Protein A-Sepharose was obtained from Amersham Biosciences. Glutathione-agarose beads, PMA, protease inhibitor cocktail, calmodulin and thrombin were from Sigma. Purified PKC (catalytic subunit, rat brain) and CamKII (rat brain) were from Calbiochem.

Time course of phosphorylation

Cos-7 cells were cultured in 100 mm dishes and transfected with 2 µg of HA-PEA-15 and 2 µg of empty vector, and 30 h post-transfection they were serum-starved overnight. Cells were then either left unstimulated or were stimulated with 50 ng/ml PMA for 15 min followed by two washes with serum-free DMEM. Cells were then left in serum-free DMEM for different time periods of 30 min and 1, 2, 5, 16 and 25 h. Cells were lysed in sample buffer and phosphorylated PEA-15 was detected by Western blotting with pS104 and pS116 antibodies. Total PEA-15 was also detected with PEA-15 antibody.

In vitro phosphorylation

GST (glutathione S-transferase) and GST-PEA-15 fusion proteins were used for the *in vitro* phosphorylation reaction. Phos-

phorylation was performed according to Calbiochem's suggested method. The method is briefly described below. For *in vitro* phosphorylation at Ser-104, the reaction volume was 160 µl and the reaction mixture contained 20 µl of 400 mM MES [2-(*N*-morpholino)-ethanesulphonic acid; pH 6.0], 20 µl of 10 mM EGTA, 20 µl of 100 mM MgCl₂, 20 µl of 100 mM ATP, 15 µl of water and 65 µl of GST or GST-PEA-15 (50%) slurry. Reaction mixture was incubated at 30°C for 15 min. Reaction was terminated by washing three times with MLB buffer [25 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP40 (Nonidet P40), 0.25% sodium deoxycholate, 10%, v/v, glycerol, 10 mM MgCl₂ and 1 mM EDTA] supplemented with phosphatase and protease inhibitors. *In vitro* phosphorylation at Ser-116 was performed similarly using the following reaction conditions: 50 mM Hepes (pH 7.4), 5 mM MgCl₂, 0.3 mM CaCl₂, 30 µg/ml calmodulin, 0.6 µg/ml CamKII, 10 mM ATP and 65 µl of the substrate slurry (GST or GST-PEA-15) in a total reaction volume of 100 µl. An aliquot (one-third) of the phosphorylated GST or GST-PEA-15 slurry was used for detecting phosphorylation by Western blotting with phospho-PEA-15 Ser-104 and Ser-116 antibodies. Equal input of GST-PEA-15 in kinase-treated and untreated samples was verified by immunoblotting with total PEA-15 antibody. The remaining portion (two-thirds) of the phosphorylated fusion protein was used in the pull-down assay.

ERK pull-down assay

Cos-7 cells cultured in 100 mm dishes were lysed in MLB buffer supplemented with protease inhibitor, sodium fluoride and sodium orthovanadate. The *in vitro* phosphorylated GST and GST-PEA-15 protein beads were incubated with Cos-7 cell lysates for 2 h at 4°C, followed by three washes with MLB buffer. The pull-down complexes were resuspended in sample buffer and immunoblotted with ERK1/2 antibody.

FADD pull-down assay

Cos-7 cells cultured in 100 mm dishes were transfected with 2 µg of human FADD plasmid. The amount of DNA was brought up to 4 µg using pCDNA3 vector. Cells were lysed in complete MLB buffer 48 h after transfection. *In vitro* phosphorylation of GST and GST-PEA-15 was performed with CamKII as described in the *In vitro* phosphorylation subsection. One-third of the phosphorylated fusion protein was used for immunoblotting with pS116 antibody. Two-thirds of the phosphorylated protein was incubated with the Cos-7 cell lysate for 2 h at 4°C. Beads were washed three times with MLB buffer and once with thrombin wash buffer (50 mM Tris/HCl, pH 7.5 and 150 mM NaCl). Pull-down complexes were incubated with thrombin cleavage buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 2.5 mM CaCl₂) and thrombin (1 unit) for 30 min at room temperature (25°C). The eluate was immunoblotted with anti-FADD and anti-PEA-15 antibodies.

Biochemical fractionation

Cos-7 cells cultured in 100 mm dishes were transfected with 4 µg of HA-PEA-15 plasmid; 30 h post-transfection, they were serum-starved overnight. Cells were either left unstimulated or stimulated with 50 ng/ml PMA for a period of 15 min, 2 or 4 h, and subjected to biochemical fractionation. Cells were washed twice with cold wash buffer (125 mM KCl, 30 mM Tris/HCl, pH 7.5, 5 mM magnesium acetate, 5 mM EGTA and 45 mM 2-mercaptoethanol), scraped from the plates and kept in swelling buffer (10 mM KCl, 30 mM Tris/HCl, pH 7.5, 5 mM magnesium

acetate, 5 mM EGTA, 45 mM 2-mercaptoethanol and protease inhibitor cocktail) at 4°C for 30 min. Cells were lysed by repeated pipetting with a P 200 pipetman. The homogenate was then layered on an equal volume of swelling buffer containing 25% glycerol and centrifuged using Beckman Coulter Microfuge 22R (Rotor F241.5) at 370 *g* for 5 min at 4°C. The upper layer constituted the cytoplasmic fraction. The nuclear pellet was washed once with swelling buffer containing 25% glycerol and 0.1% Triton X-100 and the second time without Triton X-100. The nuclear pellet was resuspended in sonication buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 2 mM MgCl₂, 5 mM magnesium acetate, 5 mM EGTA and 45 mM 2-mercaptoethanol) and sonicated briefly to obtain the nuclear fraction. Samples were subjected to SDS/PAGE and immunoblotted with pS104, pS116, PEA-15 and c-Myc (nuclear control) antibodies.

In Figure 5, thymocytes were isolated from C57/B6 mice in serum-free RPMI 1640 and were either left untreated (0 min) or treated with 50 ng/ml PMA for 10 min, incubated for a period of 30 min, 2 or 4 h to allow nuclear translocation of ERK. Fractionation was performed as described for Cos-7 cells. Nuclear and cytoplasmic fractions were immunoblotted with ERK1/2, pS104, PEA-15 and murine nuclear lamin B2 (as control) antibodies respectively.

Immunoprecipitation

In Figure 4(A), Cos-7 cells were cultured in 100 mm plates and transfected with 1 μ g of FLAG-ERK2, 1 μ g of HA-Rsk-2 and increasing amounts of His-PEA-15 (0, 0.1, 0.5 or 1 μ g). The amount of DNA was brought up to 4 μ g with empty vector. Cells were lysed in NP40 lysis buffer (20 mM Tris, pH 8, 137 mM NaCl, 10% glycerol and 1% NP40) supplemented with protease inhibitors, sodium fluoride and sodium orthovanadate. Lysates were incubated at 4°C with 0.4 μ g of anti-FLAG or 12CA5 antibody. The immunocomplexes were precipitated with 50 μ l of Protein A-Sepharose beads for 1 h at 4°C. The precipitates were washed four times with NP40 lysis buffer supplemented with 1 M NaCl. Immunoprecipitated complexes were resuspended in 50 μ l of sample buffer and immunoblotted with FLAG, HA or PEA-15 antibody.

In Figure 6(B), HeLa cells were cultured in 100 mm plates and co-transfected with 2 μ g of pCDNA-AU1-FADD and 2 μ g of HA-PEA-15 or HA-S116A plasmids. Cells were lysed in NP40 lysis buffer 48 h post-transfection. Lysates were incubated with 5 μ l of anti-AU1 antibody overnight at 4°C. Immunocomplexes were precipitated with 50 μ l of Protein A-Sepharose for 1 h at 4°C. Immunoprecipitated complexes were washed four times, resuspended in 50 μ l of sample buffer and immunoblotted with FADD and HA antibodies.

In Figure 4(B), thymocytes from C57/B6 mice were harvested in serum-free RPMI 1640, treated with 50 ng/ml PMA(+) or DMSO(-) for 1 h at 37°C, washed once with PBS and pelleted by centrifuging for 10 min at 208 *g*. Cells were lysed in lysis buffer (0.5% NP40, 20 mM Tris, pH 7.6, 250 mM NaCl, 5 mM EDTA, 3 mM EGTA, 20 mM phosphate buffer and 3 mM β -glycerophosphate, protease and phosphatase inhibitors) followed by a brief sonication. Lysates were precleared with rabbit serum and 25 μ l of Protein A-Sepharose for 1 h at 4°C. Precleared lysates were incubated with 5 μ l of anti-PEA-15 (Figure 4B, lane IP) or 5 μ l of rabbit serum (lane C), overnight at 4°C. Immunocomplexes were precipitated with 50 μ l of Protein A-Sepharose for 1 h at 4°C. Immunoprecipitated complexes were washed four times in lysis buffer and resuspended in 50 μ l of sample buffer and immunoblotted with ERK2 or PEA-15 antibody.

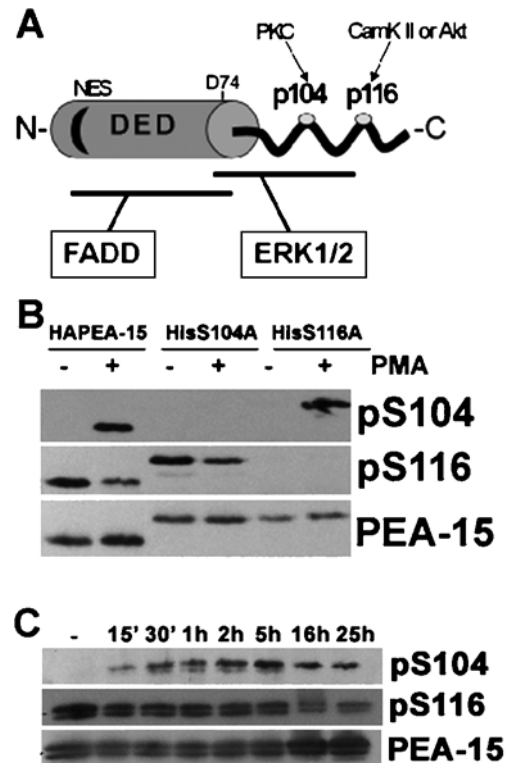


Figure 1 Characterization of phospho-PEA-15 antibodies

(A) A schematic representation of PEA-15. The protein is 130 amino acids in length and consists of a DED that constitutes amino acids 1–80. The DED is a homotypic binding domain found primarily in proteins that control extrinsic apoptosis. PEA-15 is phosphorylated at two serine residues. It is phosphorylated at Ser-104 by PKC and at Ser-116 by either CamKII or AKT. These phosphorylations are depicted by arrows. PEA-15 binding partners ERK and FADD are shown by connecting lines. (B) Cos-7 cells were transfected with the wild-type HA-PEA-15 or the mutant His-S104A and His-S116A constructs; 30 h post-transfection, cells were serum-starved for 18–20 h, and they were either left unstimulated (–) or stimulated (+) with 50 ng/ml PMA for 15 min and lysates were prepared. Lysates were subjected to SDS/PAGE and Western blotting. Phospho-epitope-specific antibodies were used to detect phosphorylation of PEA-15 at Ser-104 and Ser-116. PEA-15 expression was verified by immunoblotting. (C) Time course of phosphorylation of PEA-15 at Ser-104. Cos-7 cells were transfected with HA-PEA-15 construct, and 30 h post-transfection they were serum-starved overnight. Cells were then either left untreated (–) or treated with 50 ng/ml PMA for 15 min, washed twice with serum-free DMEM and left in serum-free DMEM for various time periods, namely 30 min and 1, 2, 5, 16 and 25 h. Cells were lysed and phospho-PEA-15 was detected by Western blotting with phospho-Ser-104 and phospho-Ser-116 antibodies. Total PEA-15 was also detected with PEA-15 antibody.

RESULTS AND DISCUSSION

PEA-15 is phosphorylated at Ser-104 in response to PMA

PEA-15 is phosphorylated at two distinct sites in the C-terminal tail of the protein (Figure 1A). PKC phosphorylates Ser-104 and CamKII phosphorylates Ser-116. The C-terminal tail of PEA-15 is flexible and lacks substantial structure, while the N-terminal region corresponds to a canonical DED [16]. To investigate whether phosphorylation of PEA-15 can alter PEA-15 function, we made use of two newly developed antibodies directed to these phospho-epitopes (pS104 and pS116, Biosource International). To verify that these antibodies were specific, we transfected Cos-7 cells with control wild-type PEA-15, PEA-15 with Ser-104 mutated to Ala (His-S104A) or PEA-15 with Ser-116 mutated to Ala (His-S116A). We found that the antibodies were specific for the phosphorylated serine residues (Figure 1B). Mutation of Ser-104 to Ala prevented pS104 binding and mutation of Ser-116 to Ala prevented pS116 binding by Western blotting. Moreover, in

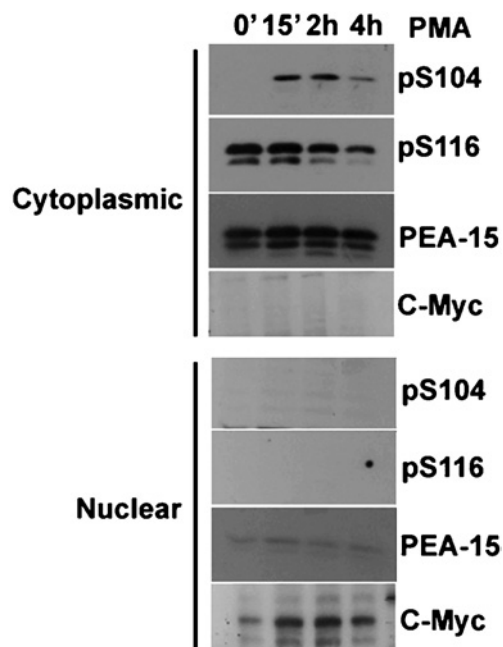


Figure 2 Localization of phospho-Ser-104 and phospho-Ser-116

Cos-7 cells transfected with HA-PEA-15 were serum-starved overnight and either left unstimulated (0 min) or stimulated with PMA at indicated time periods of 15 min, 2 or 4 h. Cells were subjected to biochemical fractionation and the localization of phospho-Ser-104 and phospho-Ser-116 was detected by immunoblotting. Total PEA-15 was detected with PEA-15 antibody. Blots were also probed with c-Myc antibody (nuclear control).

these cells, PEA-15 is not phosphorylated at Ser-104 under resting conditions. Only activation of the cells by PMA revealed specific binding of the pS104 antibody (Figure 1B). This phosphorylation persisted after the removal of PMA for up to 25 h (Figure 1C). This was probably due to PMA trapped in the cells. Conversely, Ser-116 was constitutively phosphorylated in these cells (Figures 1B and 1C).

PEA-15 has an NES (nuclear export sequence; see Figure 1A) and is present predominantly in the cytoplasm [1]. Its regulation of ERK and Rsk-2 has been attributed to its ability to prevent their accumulation in the nucleus and thus block phosphorylation of nuclear targets [1,18]. It is therefore important to know whether phosphorylation of PEA-15 can alter its subcellular localization. To test this hypothesis, we examined cytoplasmic and nuclear extracts using the phospho-epitope antibodies. We found that PEA-15 remained cytoplasmic regardless of whether or not it was phosphorylated at Ser-104 (Figure 2). Phosphorylation at Ser-116 also did not alter subcellular localization although we were unable to test PEA-15 with no phosphorylation at this site. Interestingly, we noticed that PMA treatment of cells, in addition to promoting phosphorylation at Ser-104, also promoted dephosphorylation of Ser-116 over the course of 4 h (Figure 2). We do not know what mechanism is responsible for this. Therefore phosphorylation of PEA-15 does not appear to regulate subcellular localization of the protein.

PKC phosphorylation of PEA-15 at Ser-104 prevents ERK binding

We previously reported that ERK does not phosphorylate PEA-15 and that PEA-15 can bind both active and inactive ERK [1]. Furthermore, PEA-15 binding to ERK is required for PEA-15 to regulate ERK localization [16], ERK-dependent transcription [1], integrin activation status [11,17] and cell proliferation [1].

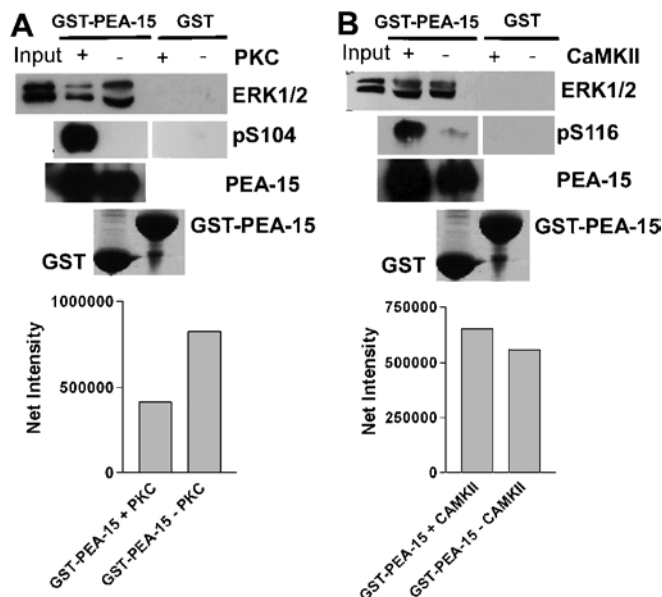


Figure 3 Phosphorylation of PEA-15 at Ser-104 impairs its association with ERK

Purified agarose-bound fusion proteins GST or GST-PEA-15 were *in vitro* phosphorylated using purified PKC (A) or CamKII (B) for 15 min, washed and incubated with Cos-7 cell lysates for 2 h. Amount of ERK1/2 associated with the fusion proteins was detected by Western blotting. An aliquot of GST or GST-PEA-15 (saved before pull down) was immunoblotted with phospho-Ser-104 or phospho-Ser-116 antibodies respectively. The section shown was blotted for phospho-Ser-104 and Ser-116 in the region corresponding to the 45 kDa band of GST-PEA-15 and the 27 kDa band of GST respectively. Blots were also probed with PEA-15 antibody to verify equal input fusion proteins. Bottom panel shows a Coomassie Blue-stained gel of the GST control and GST-PEA-15 fusion proteins. The amount of total ERK associated with PEA-15 was determined by densitometry and the net intensity in the presence or absence of PKC or CamKII treatment is plotted as a graph. Blots are representative of three independent experiments.

We examined whether phosphorylation of PEA-15 affected its ability to bind ERK. In particular, we *in vitro* phosphorylated PEA-15 with either PKC α or CamKII and then determined ERK binding in a pull-down assay. In these experiments, PEA-15 fusion protein (GST-PEA-15) was phosphorylated by both PKC α (Figure 3A, third panel) and CamKII (Figure 3B, third panel) as determined by the phospho-epitope antibodies. The specificity of these antibodies is striking, with nearly no cross-reaction with non-phosphorylated fusion protein (Figures 3A and 3B). Phosphorylation of the GST-PEA-15 fusion protein by both PKC and CamKII was incomplete. Phosphorylation at Ser-104 significantly and repeatedly abrogated PEA-15 binding to ERK (Figure 3A, top panel). It may be that significant unphosphorylated GST-PEA-15 is present and accounts for the remaining ERK binding. Phosphorylation at Ser-116 did not affect PEA-15 binding to ERK (Figure 3B, top panel).

To determine if phosphorylation of PEA-15 affects ERK binding to PEA-15 in cells, we examined Cos-7 cells transfected with increasing amounts of PEA-15 plasmid and FLAG-tagged ERK. FLAG-ERK was immunoprecipitated and the immunoprecipitate was blotted for PEA-15, pS104 or pS116. We found that ERK co-precipitated the PEA-15 phosphorylated at Ser-116 but not the PEA-15 phosphorylated at Ser-104 (Figure 4A). This confirms the *in vitro* result (Figure 3A). Therefore the ability of PEA-15 to bind and regulate ERK function is controlled by phosphorylation at Ser-104.

PEA-15 is also reported to bind the ERK substrate Rsk-2 [18]. PEA-15 is not a substrate for Rsk-2 and it binds both active and

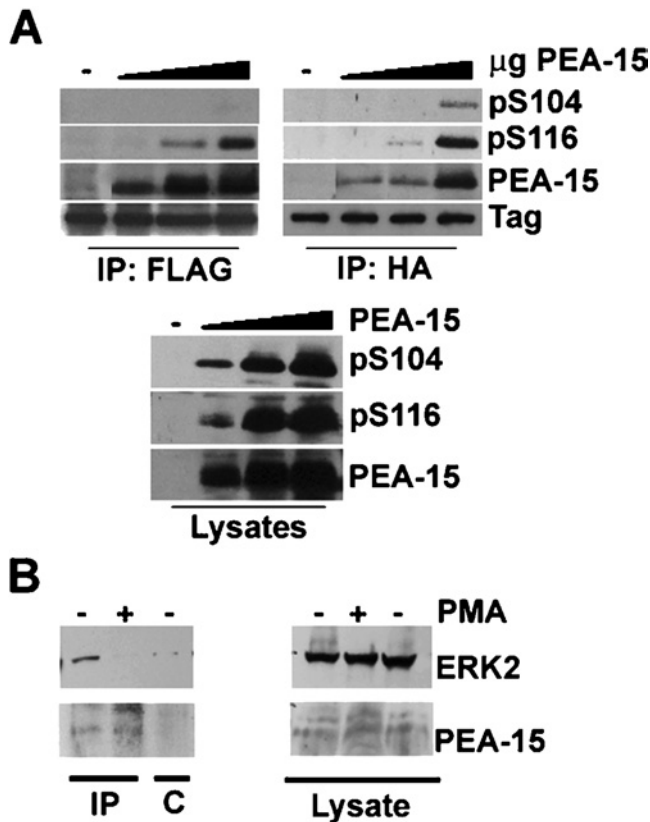


Figure 4 Phosphorylation of PEA-15 at Ser-104 impairs its interaction with ERK but not with Rsk-2

(A) Cos-7 cells were co-transfected with FLAG-ERK or HA-Rsk-2 and increasing amounts of His-PEA-15 constructs. Lysates were immunoprecipitated with FLAG (upper left panel) or HA (upper right panel)-tagged antibody and the amount of phosphorylated PEA-15 (at Ser-104 and Ser-116) and the total PEA-15 associated with ERK and Rsk-2 was detected by Western blotting. Blots were also probed with FLAG- or HA-tagged antibodies to verify the amount of immunoprecipitated protein. The input lysates were also immunoblotted with phospho-Ser-104, phospho-Ser-116 and total PEA-15 antibodies respectively (lower panel). (B) Thymocytes isolated from mice were either treated with PMA (+) or carrier DMSO (-) for 1 h and lysed. Lysates were immunoprecipitated with PEA-15 antibody (lane IP) or rabbit serum (lane C). The immunoprecipitated complex and input lysates were immunoblotted with ERK2 and PEA-15 antibodies respectively.

inactive Rsk-2. PEA-15 localizes Rsk-2 to the cytoplasm in the same way that it controls ERK localization. It is possible that phosphorylation of PEA-15 could control its ability to bind Rsk-2 in an analogous manner to that for ERK. To test this hypothesis, we immunoprecipitated HA-tagged Rsk-2, and determined whether pS104 or pS116 could be co-immunoprecipitated. PEA-15 phosphorylated at both serine residues bound Rsk-2 (Figure 4A). Therefore phosphorylation of PEA-15 does not regulate its binding to Rsk-2. This is one of the first differences regarding PEA-15 regulation of ERK and Rsk-2 so far reported. This difference further suggests that PEA-15 binding to these kinases can be independent.

These experiments were all done in overexpression systems. To determine whether the association of endogenous PEA-15 and ERK is affected by phosphorylation of PEA-15 on Ser-104, we immunoprecipitated PEA-15 from thymocytes treated with PMA and from control thymocytes not treated and then blotted for ERK. We found that PEA-15 did not co-immunoprecipitate ERK in thymocytes treated with PMA (Figure 4B). Therefore phosphorylation of PEA-15 at Ser-104 can prevent PEA-15 association with ERK in thymocytes.

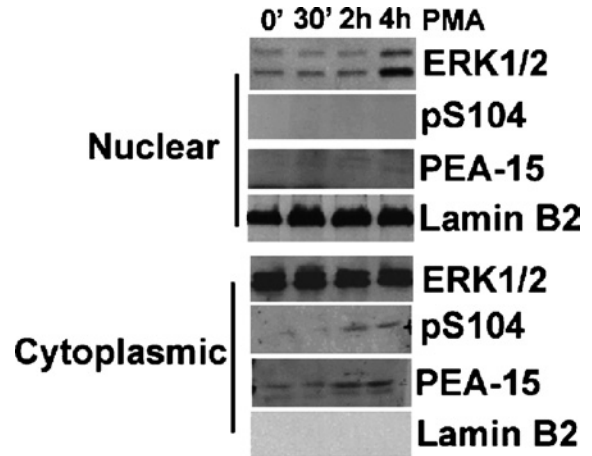


Figure 5 Effect of phosphorylation of PEA-15 at Ser-104 on ERK localization in thymocytes

Thymocytes were isolated from mice and treated with 50 ng/ml PMA for 10 min. Nuclear translocation was allowed to continue for 0, 0.5, 2 or 4 h post-stimulation. Cytoplasmic and nuclear fractions were immunoblotted for ERK1/2, pS104, PEA-15 and nuclear lamin B2 (as control) as indicated.

Phosphorylation at Ser-104 allows ERK translocation to the nucleus

PEA-15 binds ERK and prevents its accumulation in the nucleus by a mechanism dependent on the NES in PEA-15 [1]. We have now shown that phosphorylation of PEA-15 at Ser-104 impairs PEA-15 binding to ERK. We therefore tested whether the PEA-15 phosphorylated at Ser-104 permitted ERK translocation into the nucleus. We examined this in thymocytes, which express both PEA-15 and ERK, in order to extend our findings from Figure 4(B) at endogenous expression levels. We stimulated thymocytes with PMA as above and followed both ERK localization and PEA-15 phosphorylation in nuclear and cytoplasmic extracts. We found that, in these cells, at 4 h, PEA-15 is phosphorylated at Ser-104 and that ERK had translocated into the nucleus at this time point (Figure 5). Again, no nuclear PEA-15 was detected on these blots (Figure 5). This is the first observation of ERK translocation in the presence of PEA-15. Previously, PEA-15 expression was associated with cytoplasmic localization of ERK [1,11,19]. Based on our observations here, we propose that phosphorylation of PEA-15 at Ser-104 prevents PEA-15 binding to ERK and permits ERK translocation to the nucleus. Thus phosphorylation by PKC can regulate a central function of PEA-15 in controlling the ERK/MAPK pathway.

Phosphorylation at Ser-116 and Ser-104 promotes PEA-15 binding to FADD

PEA-15 is also reported to bind to FADD [4,6]. Moreover, phosphorylation at Ser-104 and Ser-116 has been reported to regulate the anti-apoptotic function of PEA-15 [10,15] in death receptor-initiated apoptosis and recruitment of PEA-15 into the DISC [15]. We therefore hypothesized that phosphorylation of PEA-15 might alter its interaction with FADD. We *in vitro* phosphorylated PEA-15 with CamKII and examined the ability of the phosphorylated PEA-15 to pull-down FADD from Cos-7 cells. We found that phosphorylation at Ser-116 promoted binding of PEA-15 to FADD (Figure 6A). We therefore propose that phosphorylation of PEA-15 at Ser-116 promotes its binding to FADD and, as a result, its recruitment to the DISC. To test this hypothesis further, we co-expressed tagged PEA-15 (wild-type) or PEA-15 containing an S116A mutation along with

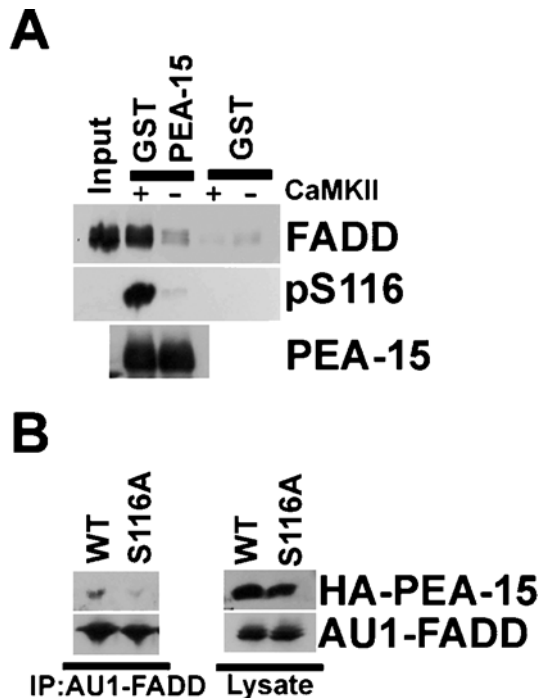


Figure 6 Phosphorylation of Ser-116 enhances its interaction with FADD

(A) Purified agarose-bound fusion proteins GST or GST-PEA-15 were *in vitro* phosphorylated using CamKII for 15 min, washed and incubated for 2 h with lysates made from Cos-7 cells transfected with human FADD plasmid. Pull-down complexes were treated with thrombin for 30 min. FADD associated with the fusion proteins was detected by Western blotting. Blots were also probed with PEA-15 antibody to verify equal input of fusion proteins. An aliquot of GST or GST-PEA-15 (saved before pull down) was immunoblotted with phospho-Ser-116 antibody. (B) HeLa cells were co-transfected with AU1-FADD and HA-PEA-15 or HA-S116A plasmids. Lysates were immunoprecipitated with AU1-tagged antibody and the amount of FADD associated with wild-type (WT) or mutant PEA-15 was detected by Western blotting. The input lysates were also immunoblotted with HA and FADD antibodies respectively.

FADD in Cos-7 cells. We then immunoprecipitated FADD and blotted for PEA-15. FADD did not immunoprecipitate the S116A mutant of PEA-15 (Figure 6B). This further establishes that phosphorylation at Ser-116 is required to promote FADD binding. In this way, phosphorylation of PEA-15 may control PEA-15 suppression of death receptor-initiated apoptosis (Figure 7). Moreover, phosphorylation at Ser-104 would prevent ERK binding and so the entire pool of dually phosphorylated PEA-15 would be free to move to the DISC.

We report in the present study that phosphorylation of PEA-15 does not regulate its nuclear versus cytoplasmic localization. We also report that in Cos-7 cells under standard tissue culture conditions, PEA-15 is primarily phosphorylated at Ser-116. Only after PMA stimulation do we see phosphorylation of Ser-104. Phosphorylation at Ser-104 blocks PEA-15 binding to ERK, while phosphorylation at Ser-116 promotes binding to FADD. Thus PEA-15 should bind and regulate predominantly ERK in tissue culture cells. These results may provide an explanation for the dual nature of the literature on PEA-15. There are many reports regarding PEA-15 binding to and regulation of ERK and there are also many concerning the binding to FADD and regulation of apoptosis. However, so far, there has been no explanation as to how PEA-15 could perform both functions. We propose that the different functions so far described result from studies examining differently phosphorylated PEA-15. Moreover, we suggest that phosphorylation of PEA-15 can serve to integrate life and death signalling in cells in which PEA-15 is expressed by

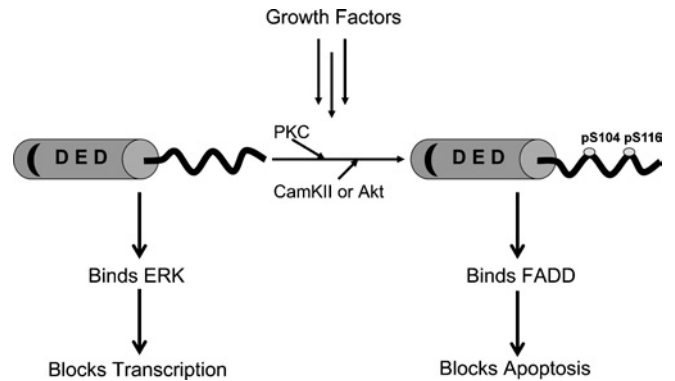


Figure 7 Phosphorylation state of PEA-15 modulates its function in blocking ERK-mediated transcription or DISC-mediated apoptosis

A model for control of PEA-15 function by phosphorylation is depicted. Unphosphorylated PEA-15 binds ERK and prevents accumulation of active ERK in the nucleus, thus blocking transcription and slowing proliferation. Upon phosphorylation at Ser-104, PEA-15 cannot bind ERK. Upon phosphorylation at Ser-116, PEA-15 binds FADD and is thus recruited to the DISC in response to death receptor ligation. In this way, PEA-15 can integrate ERK- and FADD-dependent signalling pathways.

switching it between regulation of ERK and regulation of FADD (Figure 7). We propose that mutants of PEA-15 at Ser-104 or Ser-116 will affect cell proliferation and apoptosis respectively. Further studies will be required to support this hypothesis.

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