Phosphoprotein Enriched in Astrocytes 15 kDa (PEA-15) Reprograms Growth Factor Signaling by Inhibiting Threonine Phosphorylation of Fibroblast Receptor Substrate 2α

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Changes in cellular expression of phosphoprotein enriched in astrocytes of 15 kDa (PEA-15) are linked to insulin resistance, tumor cell invasion, and cellular senescence; these changes alter the activation of the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein (MAP) kinase pathway. Here, we define the mechanism whereby increased PEA-15 expression promotes and sustains ERK1/2 activation. PEA-15 binding prevented ERK1/2 membrane recruitment and threonine phosphorylation of fibroblast receptor substrate 2α (FRS2α), a key link in fibroblast growth factor (FGF) receptor activation of ERK1/2. This reduced threonine phosphorylation led to increased FGF-induced tyrosine phosphorylation of FRS2α, thereby enhancing downstream signaling. Conversely, short hairpin RNA-mediated depletion of endogenous PEA-15 led to reduced FRS2α tyrosine phosphorylation. Thus, PEA-15 interrupts a negative feedback loop that terminates growth factor receptor signaling downstream of FRS2α. This is the dominant mechanism by which PEA-15 activates ERK1/2 because genetic deletion of FRS2α blocked the capacity of PEA-15 to activate the MAP kinase pathway. Thus, PEA-15 prevents ERK1/2 localization to the plasma membrane, thereby inhibiting ERK1/2-dependent threonine phosphorylation of FRS2α to promote activation of the ERK1/2 MAP kinase pathway.

INTRODUCTION

Growth factor receptors transmit signals that regulate cell proliferation and differentiation, promote cell migration and survival, and modulate cellular metabolism. The mitogen-activated protein (MAP) kinase pathway is an essential effector of growth factor receptor signaling. The terminal elements of this pathway are the extracellular signal-regulated kinase (ERK)1 and ERK2. ERK1/2 elicit biological outputs by phosphorylating nuclear targets such as the transcription factor Elk-1 (Gille et al., 1995), cytoplasmic substrates that include stathmin (Lovric et al., 1998) and ribosomal S6 kinase 2 (RSK2) (Jensen et al., 1999), and membrane targets such as fibroblast receptor substrate 2α (FRS2α; Lax et al., 2002). The localization of ERK1/2 dictates its access to substrates and therefore its biological activities. Proteins that regulate the localization of ERK1/2 include kinase suppressor of Ras (KSR), mitogen-activated protein kinase kinase (MEK)-partner 1, Sef, Paxillin, and phosphoprotein enriched in astro-

cytes of 15 kDa (PEA-15) (Jacobs et al., 1999, Formstecher et al., 2001; Zhou et al., 2002; Teis et al., 2002; Ishibe et al., 2003; Torii et al., 2004). PEA-15, in particular, functions as a potent inhibitor of ERK1/2-mediated transcription and cell proliferation by binding directly to ERK1/2 and preventing nuclear localization (Formstecher et al., 2001).

PEA-15 was originally discovered in astrocytes (Araujo et al., 1993) and subsequently found to be widely expressed in a variety of tissues and is conserved among mammals (Danziger et al., 1995). PEA-15 expression has been implicated in numerous pathologies, including glioma, breast cancer, ovarian cancer, astrogliosis, and diabetes (Bera et al., 1994; Hwang et al., 1997; Condorelli et al., 1998; Tsukamoto et al., 2000; Embury et al., 2001; Hao et al., 2001; Underhill et al., 2001; Sharif et al., 2004; Glading et al., 2007; Bartholomeusz et al., 2008). PEA-15 comprises of an N-terminal death effector domain (DED) and a largely unstructured C-terminal tail (Hill et al., 2002). PEA-15 binds directly to ERK1/2 and limits ERK1/2 entry into the nucleus by blocking nuclear import and promoting nuclear export (Formstecher et al., 2001; Whitehurst et al., 2004). NMR "footprinting" and site-directed mutagenesis show that residues in the DED and in the tail of PEA-15 are involved in ERK1/2 binding (Formstecher et al., 2001; Hill et al., 2002). Furthermore, protein kinase C and Ca^{2+}/calmodulin-dependent kinase II (CaMKII)/AKT phosphorylate PEA-15 at Ser104 and Ser116, respectively, thus inhibiting ERK1/2 binding (Krueger et al., 2005). Whereas PEA-15 inhibits ERK1/2 phosphorylation of the nuclear transcription factor Elk-1, it does not inhibit the phosphorylation of ERK1/2 cytosolic targets such as stathmin or RSK2 (Formstecher et al., 2001; Vaidyanathan and Ramos, 2003; Krueger et al., 2005). Therefore, PEA-15 func-

tions to redirect ERK1/2 signaling rather than to inhibit ERK1/2 intrinsic kinase activity. Paradoxically, the expression of PEA-15 enhances activation of Ras and thus MAP kinase kinase kinase (Raf-1) and MEK1/2 that lead to ERK1/2 phosphorylation and activation (Ramos *et al.*, 2000). The mechanism by which PEA-15 potentiates the ERK1/2 MAP kinase pathway is not known.

Here, we define the mechanism whereby PEA-15 increases activation of ERK1/2. Structure–function analysis revealed that PEA-15 binding to ERK1/2 is required for activation of MEK1/2. PEA-15 blocked the association of ERK1/2 with the plasma membrane, thereby preventing threonine phosphorylation of FRS2α, a signaling adapter that links several tyrosine kinase growth factor receptors to Ras and ERK1/2 activation. Increased PEA-15 lead to reduced ERK1/2-dependent threonine phosphorylation and therefore to prolonged tyrosine phosphorylation of FRS2α, resulting in sustained activation of MEK1/2 and thus ERK1/2. Furthermore, the capacity of PEA-15 to sustain signaling downstream of FRS2α is the primary mechanism by which it activates the MAP kinase pathway because genetic deletion of FRS2α abrogated the capacity of PEA-15 to activate MEK1/2. Thus, PEA-15 can prolong upstream activation of growth factor signaling and the MAP kinase pathway, while preventing cell proliferation by blocking the transcriptional activities of ERK1/2. Expression of this protein therefore reprograms the output and duration of growth factor signaling.

MATERIALS AND METHODS

Plasmids

PEA-15 cDNA expression constructs used in this work have been described previously (Chou *et al.*, 2003). The PEA-15 mutant L123R was described in Hill *et al.* (2002). Additional PEA-15 mutants S104D, S116D, and S104D S116D were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) by using wild-type pCDNA3-PEA-15 as the template. Human FRS2α cDNA was obtained from American Type Culture Collection (Manassas, VA) and subcloned into pCDNA3 via polymerase chain reaction (PCR). The FRS2α mutant T8V was generated with the QuickChange site-directed mutagenesis kit. All PEA-15 and FRS2α constructs in pCDNA3 included a C-terminal hemagglutinin (HA) tag and were verified by DNA sequencing. A plasmid-based short-hairpin RNA (shRNA) directed against PEA-15 and a control shRNA containing a scrambled sequence were constructed in pSilencer-U6 (Ambion, Austin, TX) and were gifts from Dr. Joseph Ramos (University of Hawai'i-Manoa, Honolulu, HI) and have been described previously (Glading *et al.*, 2007).

Cell Culture

Chinese hamster ovary (CHO)-K1 cells and glioblastoma A172 cells were obtained from the American Type Culture Collection. The generation of Raf-1:ER cells was described previously (Hughes *et al.*, 1997). Mouse embryonic fibroblast (MEF) cells carrying homozygous LoxP-flanked FRS2α alleles have been described previously (Lin *et al.*, 2007). All cells were cultured in DMEM (Lonza Walkersville, Walkersville, MD) containing 10% fetal calf serum, 1% nonessential amino acids, 2 mM glutamine (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin. Transient transfections of CHO, Raf-1:ER, and MEF cells were carried out with Lipofectamine Plus (Invitrogen, Carlsbad, CA) as described by the manufacturer. A172 cells were transfected using a nucleofection device (Lonza Walkerville, Walkersville) in combination with solution V and program U-029 (Lonza Walkersville).

Antibodies and Reagents

Anti-ERK2, -ERK1, -β1 integrin, -Rho GDI, and -FRS2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2, -MEK1/2, -phospho-MEK1/2, -phospho-FRS2α (Tyr196), and -phospho-threonine-proline were obtained from Cell Signaling Technology (Danvers, MA). Anti-HA monoclonal antibody was purchased from Covance Research Products (Princeton, NJ). Rabbit polyclonal anti-PEA-15 (4513) was raised against a glutathione transferase-PEA-15 fusion protein (GST-PEA-15) as described previously (Krueger *et al.*, 2005). Unless otherwise indicated, all antibodies were used at a 1:1000 dilution. 4-Hydroxy tamoxifen (4-OHT) was obtained from Sigma-Aldrich and used at a final concentration of 300 nM for the

induction of Raf-1. Calf intestinal phosphatase (CIP) was purchased from New England Biolabs (Ipswich, MA) and incubated with cell lysates for 2 h at room temperature.

Subcellular Fractionation

CHO cells were transfected with cDNA encoding wild-type PEA-15, PEA-15 mutant L123R, or empty vector. Cells were allowed to recover from transfection by allowing growth in complete media for 24 h. Next, cells were harvested in fractionation buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM NaF, 5 mM KCl, 1 mM NaVO₄, with Complete EDTA-free protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]). After incubation on ice for 20 min, cells were homogenized by shearing through a 27-gauge needle. Forty microliters of total cell lysate was saved, and the remaining sample was centrifuged at 2000 rpm to sediment the nuclei. The supernatant was then spun at 14,000 rpm for 30 min at 4°C to separate the sedimented membrane fraction from the soluble cytosolic fraction. The membrane pellet was washed with 1 ml of lysis buffer, centrifuged at 14,000 rpm for 20 min at 4°C, and resuspended in 180 μl of lysis buffer containing 1% NP-40. Total, cytosolic, and membrane fractions were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting.

RESULTS

PEA-15 Binding to ERK1/2 Is Necessary for PEA-15 Stimulation of the ERK MAP Kinase Pathway

Increased expression of PEA-15 activates Ras leading to activation of MEK1/2 and resulting activation of ERK1/2 (Ramos *et al.*, 2000). PEA-15 binds to ERK1/2 (Formstecher *et al.*, 2001); we therefore studied ERK1/2 binding-defective PEA-15 mutants to determine whether ERK1/2 binding was involved in activation of the MAP kinase pathway. PEA-15 binding to ERK1/2 is mediated by both the N-terminal DED and the C-terminal tail of PEA-15 (Figure 1A). In particular, mutation of PEA-15 Asp74 or Leu123 (Figure 1A) block ERK1/2 binding (Hill *et al.*, 2002). Cells transfected with wild type or either the D74A or L123R mutant PEA-15 were monitored for phosphorylated MEK1/2 and phosphorylated ERK1/2 to assess the activation of the MAP kinase pathway. Expression of wild type PEA-15 resulted in the expected increase of phosphorylated MEK1/2 and ERK1/2 (Ramos *et al.*, 2000), compared with cells transfected with an empty DNA vector. In contrast, neither cells transfected with the L123R nor D74A PEA-15 mutant increased MEK1/2 or ERK1/2 phosphorylation (Figure 1B) in spite of expressing similar levels of PEA-15. These data show that mutations in two distinct domains of PEA-15 that disrupt ERK1/2 binding abrogate the capacity of PEA-15 to activate MEK1/2 and their downstream targets.

Protein kinase C and CaMKII or AKT phosphorylate PEA-15 at Ser104 and Ser116, respectively, thus inhibiting ERK1/2 binding (Krueger *et al.*, 2005). As an additional test of the role of ERK1/2 binding in activation of the MAP kinase pathway, we examined the effect of aspartic acid substitutions that mimic the effect of these serine phosphorylations. PEA-15 mutants containing aspartic acid at either of the serine phosphorylation sites (S104D, S116D) or a mutant containing both mutations (S106D S116D) exhibited marked reduction in their capacity to stimulate phosphorylation of MEK1/2 and ERK1/2 (Figure 1C and Supplemental Figure S1). Thus, four different mutations that disrupt ERK1/2 binding block the ability of PEA-15 to activate the MAP kinase pathway. Notably, the L123R mutation lies outside the region required for the binding of phospholipase D (Zhang *et al.*, 2000; Viparelli *et al.*, 2008) and D74A, S104D, and S116D mutants do not affect binding to RSK2 (Vaidyanathan and Ramos, 2003). Thus, we conclude that PEA-15 binding to ERK1/2 is required for PEA-15–mediated activation of the ERK1/2 MAP kinase pathway.
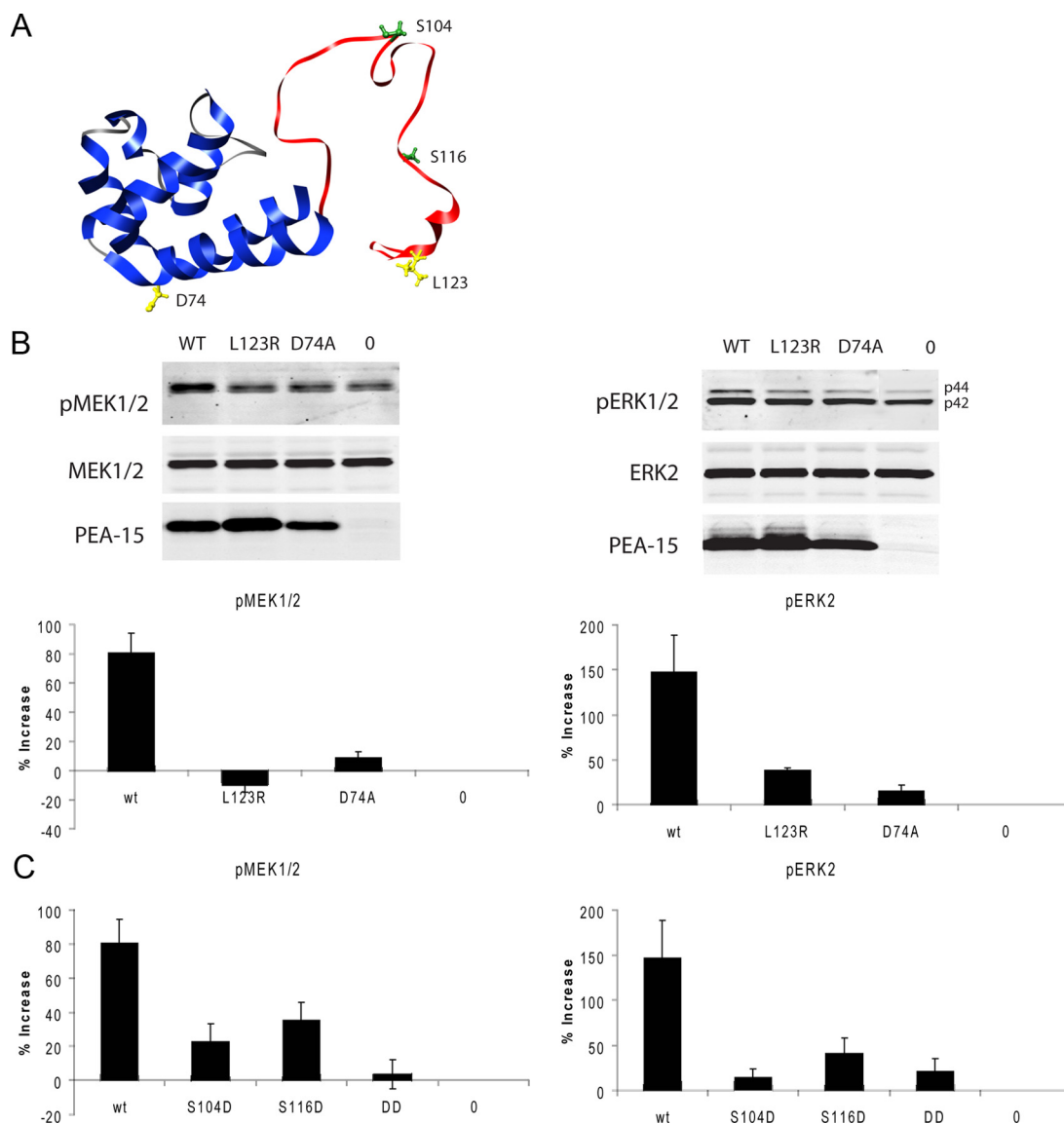
Figure 1. Binding of PEA-15 to ERK1/2 is required for activation of the ERK MAP kinase pathway. (A) Ribbon representation of the three-dimensional structure of PEA-15 (Protein Data Bank accession code 1N3K) with helices colored in blue and the loops of the C terminus in red. The side chains of selected residues are drawn as ball-and-stick models with Asp74 and Leu123 in yellow and Ser104 and Ser116 in green. (B) Wild-type PEA-15 increases levels of endogenous pMEK1/2 and pERK1/2, whereas mutations in PEA-15 that abrogate binding to ERK1/2 (L123R and D74A) do not. CHO cells were transfected with cDNAs encoding HA-tagged wild-type PEA-15 or the indicated PEA-15 mutants. After 24 h, the cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting. The blots were quantified by densitometric scanning and represented in bar graphs below ± SEM. (C) Phosphomimetic mutant PEA15 (S104D, S116D, or double mutant S104D S116D) with reduced binding to ERK1/2 do not increase pMEK1/2 and pERK1/2 to the same extent as wild-type PEA-15.

PEA-15 Blocks ERK1/2 Membrane Association

Binding of PEA-15 to ERK1/2 reroutes ERK1/2 from the nucleus to the cytosol (Formstecher *et al.*, 2001; Whitehurst *et al.*, 2004; Krueger *et al.*, 2005). Because the activation of Ras, the first step in the MAP kinase pathway, occurs at the cell membrane (Quilliam *et al.*, 1995; Downward 1996), we examined the capacity of PEA-15 to inhibit ERK localization to the plasma membrane. We transfected CHO cells with cDNA encoding PEA-15 or ERK1/2 binding-defective PEA-15(L123R) and examined the localization of endogenous ERK2 in the membrane and cytosol. Expression of PEA-15 reduced membrane-associated ERK2 compared with PEA-15 (L123R) or an empty vector

(Figure 2A). Similar results were observed for the localization of ERK1 (Supplemental Figure S2). Quantification revealed that PEA-15 caused a marked decrease in membrane-localized ERK2 and a corresponding increase in the cytosolic fraction (Figure 2B). In contrast, PEA-15 (L123R) had no effect on the distribution of ERK2 (Figure 2B). As expected, PEA-15 was confined to the cytosol (Danziger *et al.*, 1995) as was the marker RhoGDI (Shimizu *et al.*, 1991; Figure 2A). Similarly, the membrane marker, β1 integrin was detected only in the membrane fraction, thereby confirming the subcellular fractionation. Thus, PEA-15 sequesters ERK2 in the cytosol, limiting its association with the cell membrane (Figure 2C).
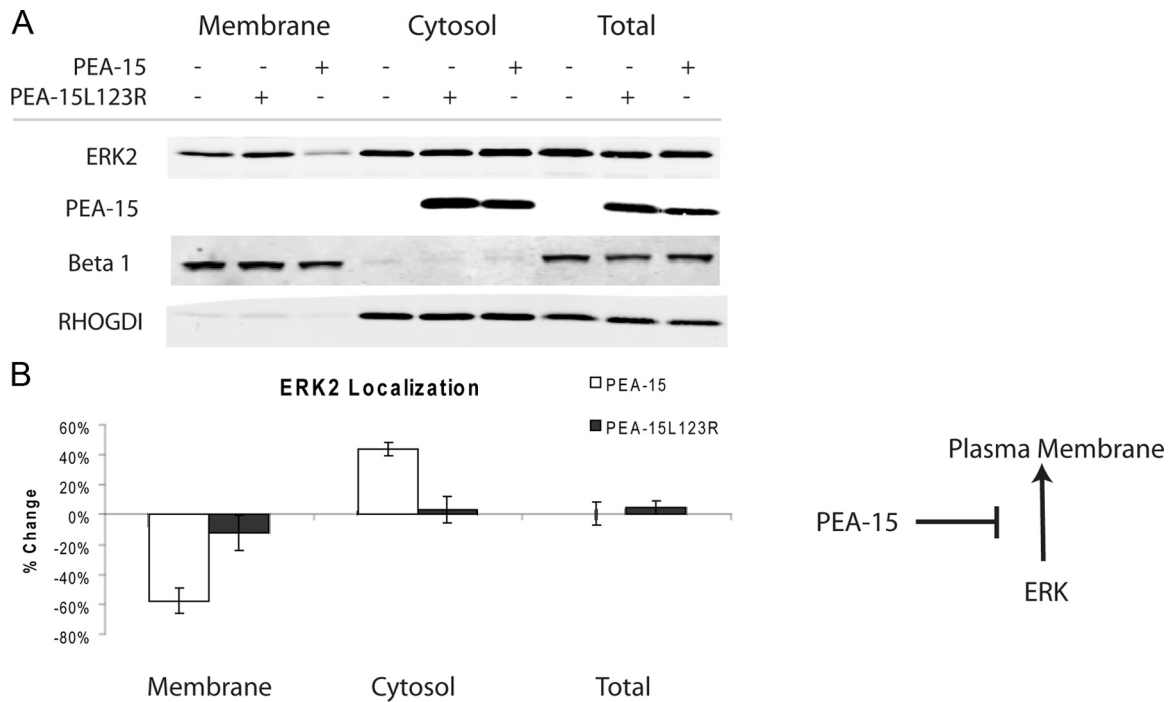
Figure 2. PEA-15 blocks the association of ERK1/2 with the plasma membrane. CHO cells were transfected with cDNAs encoding HA-tagged PEA-15, PEA-15 (L123R), or empty vector. After 24 h, the cells were disrupted by osmotic shock and fractionated into membrane and cytoplasmic fractions. Each fraction or the total lysate was separated by SDS-PAGE and analyzed by immunoblotting. Levels of ERK2 were quantified by densitometric scanning. (A) Wild-type PEA-15 reduced membrane association of ERK2, whereas the PEA-15 (L123R) did not. β1 integrin and RHO GDI serve as validation of subcellular fractionation for membrane and cytoplasmic fractions respectively. (B) Bar graph depicting the mean of data from three independent experiments ± SEM. Wild-type PEA-15 decreases levels of endogenous ERK2 in membrane fractions and increases levels of endogenous ERK2 in cytoplasmic fractions. The PEA-15 mutant L123R has little effect on ERK2 localization.
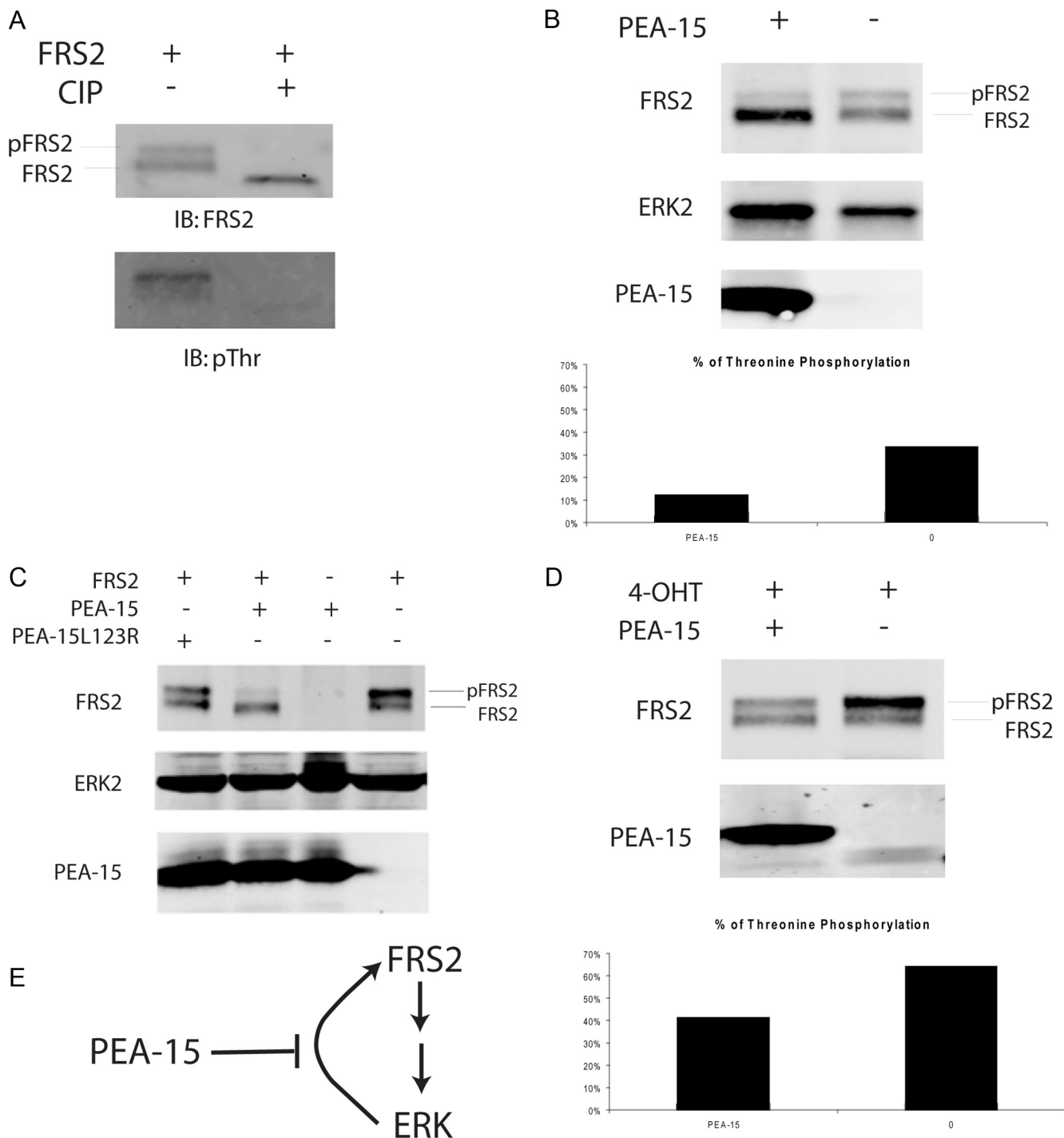
PEA-15 Blocks Threonine Phosphorylation of FRS2α

As noted above, PEA-15 blocked ERK1/2 localization to the plasma membrane leading us to seek potential membrane-associated ERK1/2 substrates. The docking protein FRS2α is myristoylated, resulting in membrane localization. ERK1/2 phosphorylates FRS2α, thereby inhibiting its tyrosine phosphorylation by growth factor receptors to reduce binding of growth factor receptor bound protein 2 (Grb2)–SOS complex, leading to reduced Ras activation and ultimately ERK1/2 activation (Lax *et al.*, 2002). We used a gel mobility shift to assay phosphorylation of FRS2α. Cells were transfected with cDNA encoding HA-tagged FRS2α, lysed, and anti-FRS2α immunoprecipitates were treated with an alkaline phosphatase or left untreated. Immunoblotting with an anti-HA or anti-phospho-threonine antibody revealed that the upper band detected by the HA antibody contained phosphorylated threonine and that this band was eliminated by dephosphorylation with alkaline phosphatase (Figure 3A). This result confirms that the large mobility shift of FRS2α (Lax *et al.*, 2002) can be used to monitor threonine phosphorylation. Overexpression of PEA-15 lead to a reduction in the upper band of FRS2α (Figure 3B and Supplemental Figure S3), whereas expression of PEA-15(L123R) did not reduce threonine phosphorylated FRS2α (Figure 3C). We used a CHO cell line containing tamoxifen-regulated Raf-1 to activate ERK1/2 (Hughes *et al.*, 1997) to increase threonine phosphorylation of FRS2α. When these cells were treated with tamoxifen, 64% of FRS2α was phosphorylated, whereas PEA-15–expressing cells exhibited a reduction in phosphorylation (41% phosphorylated; Figure 3D). Importantly, in the absence of tamoxifen, the PEA-15 effect was much stronger (Supplemental Figure S4), indicating that increased ERK activation can partially bypass the PEA-15 effect. Thus, PEA-15 blocks ERK1/2-mediated threonine phosphorylation of FRS2α (Figure 3E).

Depletion of Endogenous PEA-15 Decreases Tyrosine Phosphorylation of FRS2α

The foregoing experiments demonstrated that PEA-15 binding to ERK1/2 blocks their localization to the plasma membrane and reduces threonine phosphorylation of FRS2α. Such threonine phosphorylation is known to reduce FGF-induced tyrosine phosphorylation of FRS2α (Lax *et al.*, 2002). To examine the role of endogenous PEA-15 in regulating FRS2α signaling, we used glioblastoma A172 cells that express PEA-15 (Glading *et al.*, 2007). shRNA transfection induced a 90% reduction in PEA-15 expression (Figure 4A). When these cells were treated with basic FGF (FGFb), FRS2α tyrosine phosphorylation at residue 196 was markedly reduced in cells that had been depleted of PEA-15 (Figure 4A). Phosphorylation of tyrosine 196 functions as a docking site for Grb2–SOS complexes (Kouhara *et al.*, 1997) and is therefore important for downstream MAP kinase signaling. We quantified three such experiments and observed statistically significant ($p = 0.01$) five-fold reduction in FGF-induced FRS2α tyrosine phosphorylation in PEA-15–depleted cells (Figure 4B) at 20 min after FGFb addition. By 40 min after FGFb addition, no increase in FRS2α tyrosine phosphorylation was observed in PEA-15–depleted cells. Thus, endogenous PEA-15 increases and prolongs FRS2α phosphorylation in response to FGFb.

Figure 3. PEA-15 inhibits threonine phosphorylation of FRS2α. (A) The mobility shift of FRS2 is due to threonine phosphorylation. CHO cells were transfected with cDNAs encoding HA-tagged FRS2α. After 24 h, the cells were lysed and split into two fractions. One fraction was treated with CIP at room temperature for 2 h, whereas the other fraction was left untreated. Each fraction was immunoprecipitated with anti-FRS2α, separated by SDS-PAGE, and immunoblotted with anti-HA or anti-pThr antibodies. (B) PEA-15 reduces threonine phosphorylation of FRS2α. CHO cells were cotransfected with cDNAs encoding HA-tagged FRS2α and HA-tagged PEA-15 in indicated samples. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Immunoblot of ERK2 serves as a loading control. Threonine phosphorylation of FRS2α was quantified by densitometric scanning and represented in bar graph below. (C) A PEA-15 mutant that abrogates binding to ERK1/2 does not reduce threonine phosphorylation of FRS2α. CHO cells were cotransfected with cDNAs encoding HA-tagged FRS2α in combination with either HA-tagged wild-type PEA-15 or PEA-15(L123R). Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Note that PEA-15(L123R) does not reduce the upper band of FRS2α to the same extent as wild-type PEA-15. Immunoblot of ERK2 serves as a loading control. (D) Raf-ER CHO cells were cotransfected with cDNAs encoding HA-tagged FRS2α in combination with either HA-tagged PEA-15 or empty vector. After 24 h, the cells were treated with 4-OHT for 30 min at 37°C. Cells were lysed immediately and analyzed by SDS-PAGE followed by immunoblotting. Note that the upper band of FRS2α is reduced in the presence of PEA-15. Immunoblot of pERK1/2 serves as a control for Raf-MEK-ERK activation after the addition of 4-OHT. Threonine phosphorylation of FRS2α was quantified by densitometric scanning and represented in bar graph below. (E) PEA-15 inhibits ERK-mediated threonine phosphorylation of FRS2α.

A

FGFb Treatment:
(minutes)
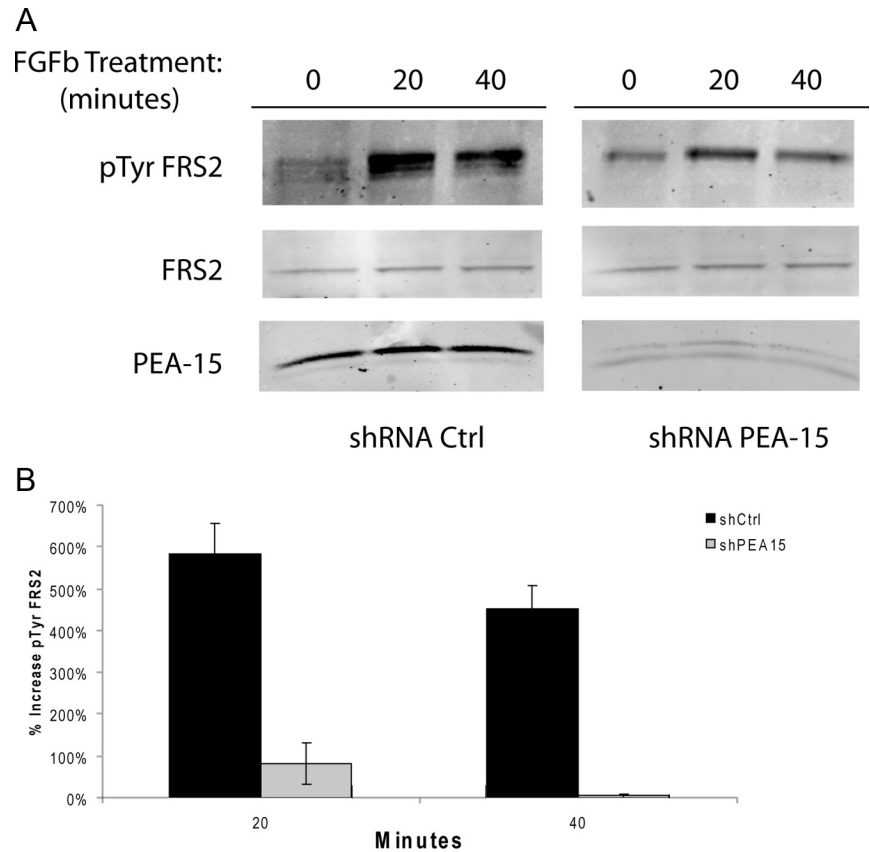
Figure 4. Knockdown of endogenous PEA-15 attenuates tyrosine phosphorylation of FRS2α. A172 cells were transfected with shRNA for PEA-15 (shPEA15) or a scrambled shRNA (shCtrl). All plates were serum starved for 16 h before stimulation with 100 ng/ml FGFb for the indicated amount of time. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting. Levels of FRS2α tyrosine phosphorylation (pTyr FRS2) were quantified by densitometric scanning. (A) Top, tyrosine phosphorylation of FRS2α (pTyr FRS2) is abundant at 20 and 40 min in shCtrl samples, whereas reduced pTyr FRS2 is observed at the same time points in shPEA15 samples. All samples were run on the same gel; irrelevant intervening bands were excised to save space. (B) The bar graph summarizes the mean percent increase of pTyr FRS2 relative to the 0 time point from three independent experiments. Note that the knock down of PEA-15 greatly diminishes pTyr FRS2 in response to FGFb.

PEA-15 Prolongs FGF-induced Activation of the ERK1/2 MAP Kinase Pathway

Endogenous levels of PEA-15 affected the degree and duration of FGF-induced tyrosine phosphorylation of FRS2α, suggesting that it might regulate the duration of FGF-induced activation of the MAP kinase pathway. To test this idea, we overexpressed either PEA-15 or PEA-15(L123R) and examined the effect on FGF-induced FRS2α tyrosine phosphorylation and ERK2 phosphorylation. Overexpression of wild type PEA-15 increased tyrosine phosphorylation of FRS2α relative to over expression of PEA-15(L123R) (Figure 5, second row); conversely, the PEA-15(L123R)–transfected cells exhibited a more threonine phosphorylation of FRS2α than PEA-15–transfected cells, as indicated by the appearance of the FRS2α of reduced mobility (Figure 5, top row). The increased tyrosine phosphorylation of FRS2α in wild-type PEA-15–transfected cells was associated with prolonged ERK2 activation (Figure 5, third row). In particular, PEA-15 overexpression led to a markedly prolonged response at 60 min, a time when the PEA-15(L123R) cells exhibited maximal threonine phosphorylation (Figure 5, first row). Furthermore, PEA-15 did not increase ERK1/2 phosphorylation in cells expressing FRS2α T8V, an ERK phosphorylation-resistant mutant (Supplemental Figure S5). Thus, PEA-15 extends FGF-induced activation of ERK1/2 by reducing threonine phosphorylation leading to increased tyrosine phosphorylation of FRS2α.

FRS2α Is Required for PEA-15 to Activate the MAP Kinase Pathway

The previous studies established that PEA-15 binding to ERK1/2 blocked threonine phosphorylation of FRS2α, lead-ing to prolonged tyrosine phosphorylation and activation of the MAP kinase pathway. We next asked whether the capacity of PEA-15 to increase activation of this pathway was dependent on FRS2α. We used immortalized MEFs derived from *Frs2α^flox* mice and infected them with an adenovirus encoding Cre-recombinase, resulting in the deletion of both *Frs2α* alleles (*Frs2α^-/-* cells). As a control, cells were infected with adenovirus encoding LacZ. The two resulting MEF cell lines were transfected with PEA-15, PEA-15(L123R), or empty vector. MEF cells lacking FRS2α did not exhibit activation of MEK1/2 (Figure 6A, left) when expressing wild-type PEA-15, whereas the control, LacZ adenovirus-infected cells did so (Figure 6A, right). Re-expression of FRS2α in *Frs2α^-/-* cells restored the phosphorylation of MEK1/2 in response to PEA-15 overexpression (Figure 6B, left). Thus, FRS2α is required for PEA-15 to induce activation of the MAP kinase pathway (Figure 6C).

DISCUSSION

The ERK1/2 MAP kinase pathway is a central regulator of cellular behaviors and PEA-15, an ERK1/2 binding protein, has profound effects on the activation and output of this pathway (Ramos *et al.*, 2000; Formstecher *et al.*, 2001). Here, we have defined the mechanism whereby PEA-15 increases activation of MEK1/2 and consequently, ERK1/2 (Figure 7). Mutational analysis revealed that PEA-15 binding to ERK1/2 is required for activation of MEK1/2 and ERK1/2. Because this pathway is initiated by activated Ras at the plasma membrane, we examined the effects of PEA-15 on ERK1/2 localization and function at the membrane. PEA-15 blocked the association of ERK1/2 with the membrane,

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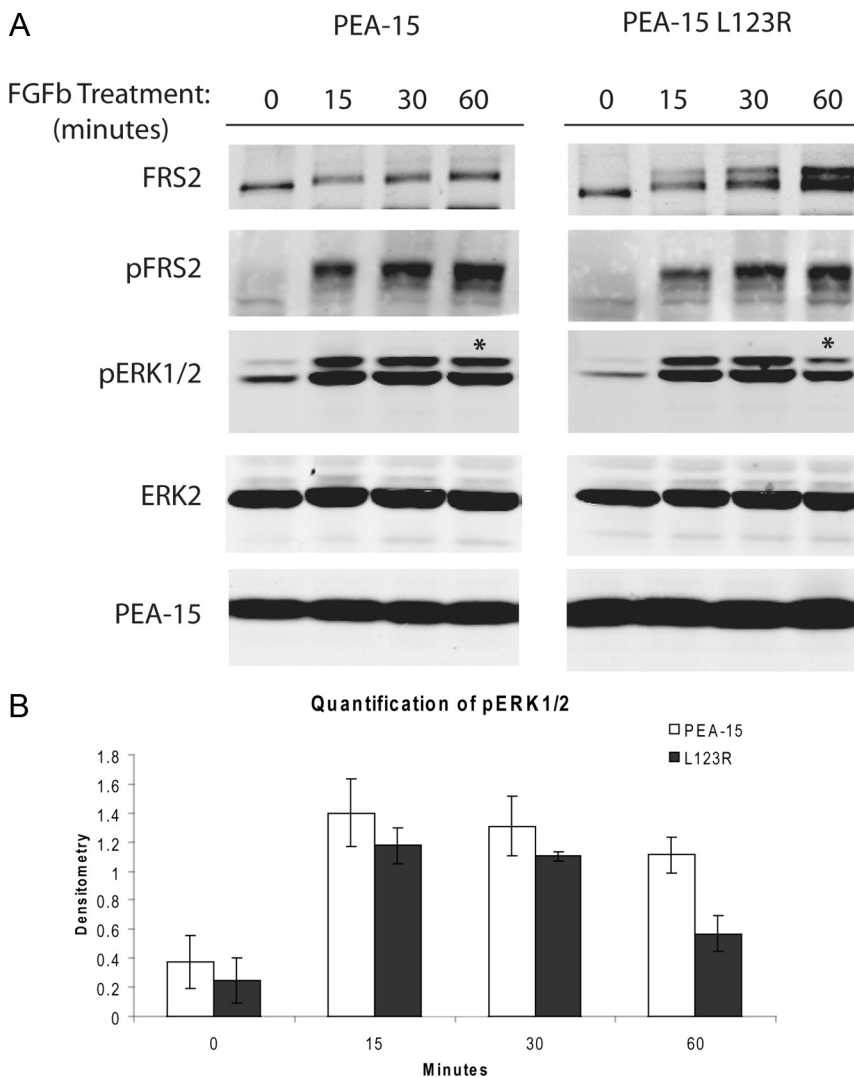
Figure 5. Overexpression of PEA-15 prolongs tyrosine phosphorylation of FRS2α and phosphorylation of ERK1/2. (A) CHO cells were cotransfected with cDNAs encoding HA-tagged FRS2α and either HA-tagged PEA-15 or PEA-15(L123R). All plates were serum starved for 16 h before stimulation of FGFb for the indicated length of time. Cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting with anti-HA (for the detection of FRS2α and PEA-15), anti-pY196 FRS2α, anti-pERK1/2, and anti-ERK2 antibodies. The kinetics of FGF-induced phosphorylation of FRS2α and ERK1/2 are altered in the presence of wild-type PEA-15 (left) relative to PEA-15(L123R) (right). Note that the upper band of FRS2α (previously established as threonine phosphorylated) is absent from cells transfected with wild-type PEA-15, whereas a robust upper band of FRS2α in cells cotransfected with PEA-15 L123R appears at 60 and 120 min. The asterisks in the pERK1/2 indicate the prolonged pERK1/2 phosphorylation in the presence of wild-type PEA-15. (B) Quantification of data (n = 4) from experimental design described in A. Depicted are the mean ± SEM the difference at 60 min was significant (p = 0.003, *t* test).

thereby preventing threonine phosphorylation of FRS2α, a membrane-tethered adapter that links several tyrosine kinase growth factor receptors to Ras and ERK1/2 activation. This threonine phosphorylation terminates FRS2α signaling by inhibiting tyrosine phosphorylation, thereby preventing the binding of downstream adapters such as Grb2 (Lax *et al.*, 2002). We now find that increased PEA-15 leads to prolonged tyrosine phosphorylation of FRS2α, which results in activation of MEK1/2 and thus ERK1/2. Furthermore, shRNA-mediated depletion of endogenous PEA-15 decreased FGF-induced tyrosine phosphorylation of endogenous FRS2α, thereby establishing the biological relevance of PEA-15 regulation of this pathway. Importantly, these studies used mouse embryo fibroblasts, human glioblastoma cells, and CHO cells, suggesting that these findings can be generalized to many cells types. Consequently, PEA-15 increases activation of the ERK 1/2 MAP kinase pathway by interrupting a negative feedback loop formed by ERK1/2 phosphorylation of FRS2α. Finally, genetic deletion of FRS2α abrogated the capacity of PEA-15 to activate MEK1/2, establishing that this is the major mechanism whereby PEA-15 activates the MAP kinase pathway. Thus, we describe a novel mechanism for prolonging upstream activation of growth factor signaling and the MAP kinase pathway; in particular, PEA-15 binds ERK1/2 preventing its

localization to the plasma membrane, thereby inhibiting threonine phosphorylation of FRS2α.
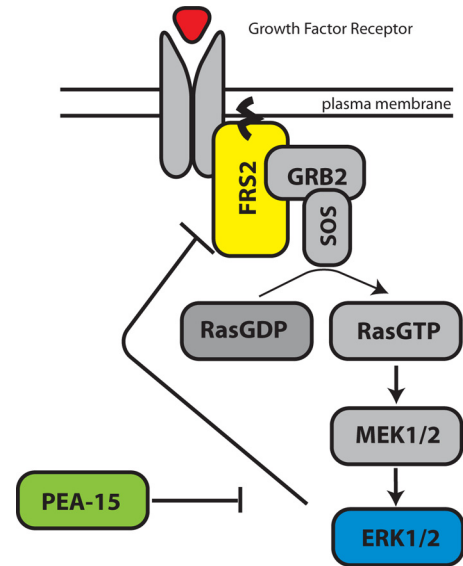
PEA-15 must bind to ERK1/2 to activate MEK1/2 and ERK1/2. Mutations in the folded PEA-15 DED (D74A) or its unstructured C terminus (L123R) and phosphorylation-mimicking Asp mutations at Ser104 and Ser116 block ERK1/2 binding. Each of these mutations abrogated the capacity of PEA-15 to activate MEK1/2 and ERK1/2. Importantly, these mutants were well expressed and several of them have only localized effects on the heteronuclear single quantum correlation nuclear magnetic resonance spectrum of PEA-15 (Hill *et al.*, 2002), indicating that these mutants do not disrupt the overall structure of PEA15. Furthermore, PEA-15 can bind to RSK2; however, the D74A mutation does not impair this interaction (Vaidyanathan and Ramos, 2003). Similarly, Leu123 lies outside the region of PEA-15 that binds to phospholipase D (Zhang *et al.*, 2000; Viparelli *et al.*, 2008). Yet, each of these mutants disrupted activation of the MAP kinase pathway, indicating that binding of RSK2 or phospholipase D is not required for the effect of PEA-15 on MEK1/2 and ERK1/2 activation. Importantly, phosphorylation of PEA-15 acts as a molecular switch that disengages PEA-15 from ERK1/2 (Krueger *et al.*, 2005; Renganathan *et al.*, 2005) and leads to PEA-15 recruitment to the death inducing signaling complex (Condorelli *et al.*, 1999; Kitsberg
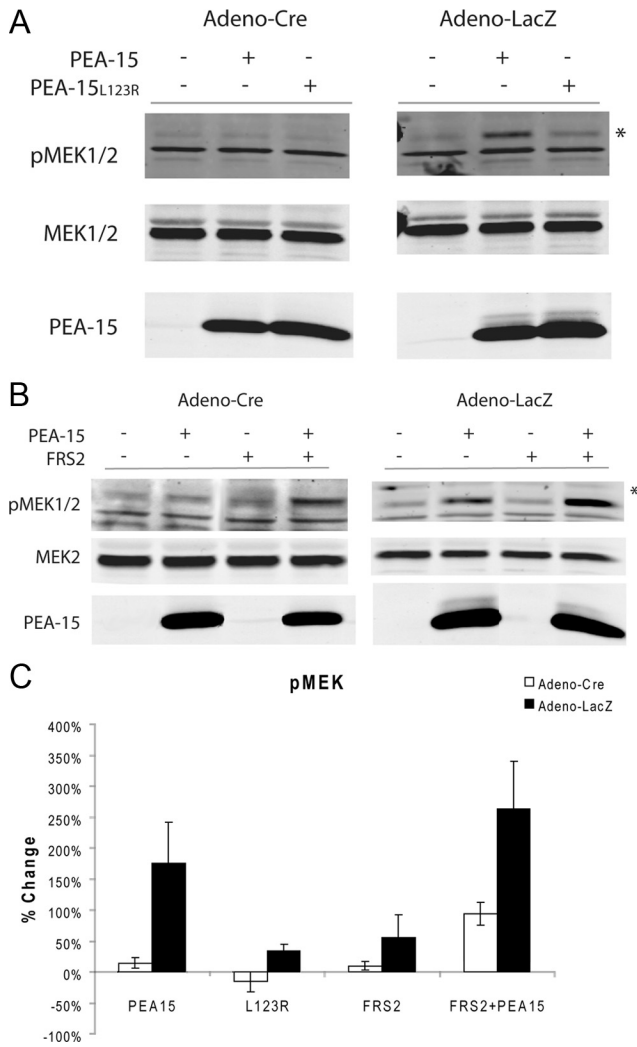
Figure 6. FRS2α is required for PEA-15 to the activate MEK1/2. FRS2α-floxed MEFs were transduced with an adenovirus encoding Cre-recombinase or LacZ. After 48 h, cells were transfected with cDNAs encoding HA-tagged wild-type PEA-15, PEA-15(L123R), and/or HA-tagged FRS2α. Twenty-four hours after transfection, cells were lysed, fractionated by SDS-PAGE, and analyzed by immunoblotting. (A) PEA-15 does not increase MEK1/2 phosphorylation in cells deficient in FRS2α. Asterisk denotes the band corresponding to pMEK1/2. (B) Re-expression of FRS2α restores the ability of PEA-15 to activate MEK1/2. Asterisk denotes the band corresponding to pMEK1/2. (C) Quantification of the data from four independent experiments described in A and B. Percentage of increase is calculated relative to cells transfected with empty vector ± SEM.

Figure 7. Schematic diagram of how PEA-15 inhibits a negative feedback loop between ERK1/2 and FRS2α. PEA-15 binds directly to ERK1/2, preventing its membrane localization. Consequently, this limits ERK-mediated threonine phosphorylation of the membrane tethered substrate FRS2α, resulting in increased and prolonged tyrosine phosphorylation and activation of downstream signaling pathways such as that leading to activation of ERK1/2 MAP kinase.

et al., 1999; Xiao et al., 2002). Our finding that PEA-15 phosphomimetic mutations block its ability to activate the MAP kinase pathway establish another regulatory role for PEA-15 phosphorylation.

We found a reduction of ERK2 in the membrane fraction of PEA-15–expressing cells and ascribe this effect to the interaction of PEA-15 with ERK1/2 because it was abolished by a L123R mutation that inhibits ERK binding. Previous studies showed that PEA-15 can reduce nuclear accumulation of ERK1/2 by both blocking nuclear entry (Whitehurst et al., 2004) and inducing nuclear export (Formstecher et al., 2001), thereby inhibiting the transcriptional effects of ERK 1/2. ERK1/2 are targeted to the plasma membrane by interacting with the scaffolding protein KSR that binds to membranes via its C1 domain (Muller et al., 2001; Zhou et al., 2002), leading to the formation of a Ras/MEK/ERK complex at the plasma membrane (Morrison and Davis, 2003). PEA-15 binds to ERK2 near the F-recruitment site (αG helix of ERK2), and ERK2 mutations at this site abrogate binding to PEA-15 (Chou et al., 2003); this same region is involved in binding to KSR (Cacace et al., 1999; Jacobs et al., 1999). Furthermore, PEA-15 blocks binding of ERK1/2 substrates (e.g., Elk-1) to the D-recruitment site (Callaway et al., 2007); an effect that may also contribute to blockade of membrane localization. In either case, PEA-15 blocks ERK1/2 signaling in the nucleus, and we now show that it blocks the membrane localization of ERK1/2 and the phosphorylation of a membrane-associated substrate.

PEA-15 blocks ERK-mediated threonine phosphorylation of FRS2α, a membrane-tethered (Kouhara et al., 1997) ERK1/2 substrate (Lax et al., 2002; Wu et al., 2003), thereby prolonging its tyrosine phosphorylation and downstream signaling. Another FRS2 family member, FRS3 or FRS2β, is 72% identical to FRS2α in amino acid sequence and is also able to activate ERK1/2 in a sustained manner (Gotoh et al., 2004). However, FRS2β does not contain the eight canonical ERK phosphorylation motifs—PXTP—and is therefore not involved in an ERK1/2-mediated negative feedback mechanism (Lax et al., 2002; Gotoh et al., 2004). Thus, our finding that PEA-15 blocks membrane-recruitment of ERK1/2 suggests that FRS2α is a preferred target of the effects of PEA-15, an idea supported by the observation that deletion of FRS2α blocked the ability of PEA-15 to activate MEK 1/2; hence, regulation FRS2α is a principal mechanism by which PEA-15 increases activation of the ERK1/2 MAP kinase pathway.

FRS2α is similar to other adaptor proteins such as DOK, Gab, and IRS; however, the FRS2 family is unusual in that it is myristoylated and therefore constitutively membrane associated. Other membrane-linked docking proteins contain a

pleckstrin homology domain, and their membrane localization is dependent on a variety of factors including the availability of specific phosphoinositides. For example, Gab1 is phosphorylated by ERK1/2 at two serine residues leading to Gab1 recruitment to the plasma membrane (Eulenfeld and Schaper, 2009). Because PEA-15 prevents ERK1/2 membrane association without inhibiting its kinase activity (Formstecher *et al.*, 2001), PEA-15 is unlikely to affect Gab1 recruitment. That said, it will be of interest to identify effects of PEA-15 on phosphorylation of other membrane localized ERK1/2 substrates.

Our study establishes a mechanism whereby PEA-15 promotes and sustains growth factor signaling: by binding to ERK1/2, PEA-15 blocks ERK1/2-mediated phosphorylation of FRS2α, thereby interrupting a major negative feedback loop that terminates growth factor signaling. These results have important implications for the biological effects of PEA-15 expression. In addition to increasing ERK1/2 activation (Ramos *et al.*, 2000), our data show that PEA-15 prolongs signaling downstream of FRS2α, suggesting much broader activating effects on signal transduction pathways. Unphosphorylated PEA-15 prevents nuclear translocation of ERK1/2 thereby inhibiting transcriptional effects of ERK1/2 that lead to cell cycle progression and tumor cell invasion (Formstecher *et al.*, 2001; Renault *et al.*, 2003; Glading *et al.*, 2007). Furthermore, PEA-15 can block tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)–induced apoptosis (Condorelli *et al.*, 1999; Kitsberg *et al.*, 1999; Xiao *et al.*, 2002), increase expression of phospholipase D (Zhang *et al.*, 2000) to promote glucose transport (Viparelli *et al.*, 2008), and act as a scaffold to promote activation of RSK2 (Vaidyanathan *et al.*, 2007). This combination of activities suggests that high level expression of PEA-15 may enable nonproliferative cells to survive for extended periods. Notably, PEA-15 is expressed in astrocytes (Araujo *et al.*, 1993) that can give rise to neural stem cells, a cell population that is long lived yet minimally proliferative (Emsley *et al.*, 2005). Similarly, it is highly expressed in mammary epithelium and in some mammary cancers (Glading *et al.*, 2007), a tumor type that is prone to prolonged survival of micrometastases (Demicheli *et al.*, 1996). Our study has elucidated a specific biochemical mechanism whereby PEA-15 prolongs growth factor signaling, thereby activating the MAP kinase pathway and thus contributing to its extensive armamentarium of biological effects.

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