

RSK2 Activity Is Regulated by Its Interaction with PEA-15*

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The ERK MAP (mitogen-activated protein) kinase cascade modulates many cellular processes including transcription, adhesion, growth, survival, and proliferation. One target substrate of ERK involved in regulating transcription is the p90 ribosomal S6 kinase (RSK) isozyme, RSK2. Here we demonstrate that a small death effector domain-containing protein called PEA-15 binds RSK2. RSK2 and PEA-15 (phosphoprotein enriched in astrocytes, 15 kDa) co-precipitated from cells and were co-localized in the cytoplasm. Furthermore, purified PEA-15 bound *in vitro* translated RSK2, suggesting that these proteins interact directly. PEA-15 does not bind to RSK1 and therefore exhibits some binding specificity. RSK2 binds the COOH terminus of PEA-15 and does not interact with its NH₂-terminal death effector domain. We show that this interaction has functional consequences including the inhibition of RSK2-dependent CREB transcription. PEA-15 expression also blocks histone H3 phosphorylation, an RSK2-dependent event that may contribute to effects on gene expression. These results can be attributed to two effects of PEA-15 on RSK2. First, PEA-15 blocks nuclear accumulation of RSK2 after epidermal growth factor stimulation. Second, PEA-15 inhibits RSK2 kinase activity by 50%. A mutant of PEA-15 that binds RSK2 but is localized to the nucleus had no effect on RSK2-dependent transcription. Interestingly, this mutant also did not affect RSK2 kinase activity. This may indicate that cytoplasmic retention of RSK2 is also required for PEA-15 to impair kinase activity. PEA-15 does not alter ERK phosphorylation of RSK2 and is not itself a substrate of RSK2. Hence the effects of PEA-15 on RSK2 represent a novel mechanism for the regulation of RSK2-mediated signaling.

The ERK¹ MAP kinase cascade can regulate many cellular processes including transcription, adhesion, growth, survival, and proliferation. The canonical ERK MAP kinase cascade is

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¹ The abbreviations used are: ERK, extracellular signal-regulated kinase; CREB, cAMP response element-binding protein; CTD, COOH-terminal catalytic domain; DED, death effector domain; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; EGF, epidermal growth factor; MBP, myelin basic protein; MEK, MAP/ERK kinase; NTD, NH₂-terminal domain; PDK1, phosphoinositide-dependent protein kinase 1; PEA-15, phosphoprotein enriched in astrocytes, 15 kDa; RSK, ribosomal S6 kinase; wt, wild type; MOPS, 4-morpholinepropanesulfonic acid.

stimulated upon the binding of extracellular growth factors such as EGF to their respective transmembrane tyrosine kinase receptors. The subsequent autophosphorylation of the cytoplasmic tails of the receptor on tyrosine leads to the recruitment of grb2, which binds the guanine exchange factor, SOS. Recruitment of SOS to the membrane promotes its interaction with the membrane localized H-Ras and results in GTP loading and activation of H-Ras (1). This is followed by the sequential recruitment and activation of Raf, MEK, and ERK. MEK binds and restricts inactive ERK to the cytosol (2). The MEK and ERK complex dissociates when MEK is activated and phosphorylates ERK. The ERK may then dimerize and translocate into the nucleus by an active transport mechanism (3–5). Alternatively, ERK may phosphorylate cytoplasmic targets. The specific outcome of activation of this ubiquitous pathway is directed by interactions with differentially expressed substrate, scaffold, and adapter proteins (6, 7).

One set of targets of ERK is the RSK family of proteins including RSK2 (8). RSK2 is a serine/threonine kinase activated by ERK1/2 MAP kinase and PDK1. Like other RSK family members, RSK2 consists of two kinase domains, an NH₂-terminal catalytic domain (NTD) that is similar to protein kinases B and C and a COOH-terminal catalytic domain (CTD) that is similar to calcium/calmodulin-dependent protein kinases I and II (9). Activation of RSK2 is complex. In response to growth factors like EGF, inactive RSK2 is phosphorylated by ERK at the catalytic loop of the CTD (10, 11). The CTD autophosphorylates RSK2 at serine 386 in the connecting domain (12). This enables further phosphorylation by the constitutively active kinase PDK1 at serine 227 in the NTD (10). The NTD is the kinase domain responsible for phosphorylation of substrate molecules such as histone H3, CREB, and Akt (13). Mutations in RSK2 are linked to Coffin Lowry syndrome in humans, an X-linked disease marked by cognitive disabilities and skeletal malformations (14). In cells derived from Coffin Lowry syndrome patients, EGF-induced CREB phosphorylation and *c-fos* transcription did not occur (15). Hence RSK2 appears to be required specifically for these functions. Finally, RSK2 is also required for EGF-induced phosphorylation of histone H3 (16) implicating RSK2 in the regulation of chromatin remodeling. The regulation of RSK activity presents a complex problem that is just beginning to be solved.

We have previously reported that the small death effector domain protein PEA-15 regulates the ERK MAP kinase pathway. PEA-15 is a 15-kDa protein that was originally identified as a major astrocytic phosphoprotein (17, 18). The first 80 amino acids of PEA-15 correspond to the canonical death effector domain (DED) sequence found in proteins that regulate apoptotic signaling pathways (19, 20). The remaining 51 amino acids contain a serine (Ser-104) that is phosphorylated by protein kinase C (17) and a serine (Ser-116) phosphorylated by calcium calmodulin kinase II (21). PEA-15 blocks an H-Ras to integrin signal (22). More recently we have shown that PEA-15

blocks ERK-dependent transcription and proliferation by binding ERKs and preventing their accumulation in the nucleus (23). PEA-15 contains a nuclear export sequence required for its capacity to maintain ERK in the cytoplasm. Genetic deletion of PEA-15 results in increased ERK nuclear localization with a consequent increase in *c-fos* transcription and cell proliferation. Thus, PEA-15 can redirect the biological outcome of MAP kinase signaling by regulating the subcellular localization of ERK MAP kinase (23).

We now show that PEA-15 binds RSK2 and alters its activity. PEA-15 does not bind to RSK1 and therefore appears to exhibit some binding specificity. PEA-15 expression impairs RSK2 kinase activity and prevents RSK2 accumulation in the nucleus. Moreover, PEA-15 blocks the ability of RSK2 to activate CREB mediated transcription and to phosphorylate histone H3. Hence we have identified a potentially crucial RSK2 binding partner that can regulate the outcome of RSK2 signaling.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Constructs, Reagents, and Antibodies—HeLa cells were obtained from ATCC (Manassas, VA), and COS-7 cells were a gift from Dr. B. Firestein (Rutgers University). Cells were cultured in Dulbecco's minimum essential medium (Invitrogen) containing 10% fetal bovine serum (Gemini), antibiotics, and non-essential amino acids. All transfections were carried out using either LipofectAMINE Plus (Invitrogen) or Polyfect (Qiagen) according to the manufacturer's protocols. pMT2-HA RSK2 was a gift from Dr. Bjorbaek (Harvard Medical School). pKH3-RSK2 wt and activated RSK2 (Y707A) were gifts from Dr. T. Sturgill (University of Virginia). HA-tagged or untagged PEA-15 in pcDNA3 vector and PEA-15 in the p-EGFP1 vector have been described previously (22, 23). Mutants of PEA-15 in the COOH terminus (L123R), the DED (D74A), and the nuclear export sequence (I15A) have been described previously (24). PEA-15 was subcloned from the pcDNA3 vector into the pcDNA3-HisC vector (Invitrogen) in frame with the His tag using *Bam*HI and *Eco*RI restriction sites. Protein expression was verified using both a PEA-15-specific antibody (from Dr. Hervé Chneiweiss) and a His-specific antibody (Novagen). RSK2 was cloned into pcDNA3 vector using forward primer 5'-GGATCCATGCCGCTGGCGCAGCTGGCGGAC-3', which contains a *Bam*HI restriction site, and a reverse primer 5'-CTCGAGTACAGGGCTGTTGAGGTGATTTT-3', which contains a *Xho*I restriction site. RSK2, ERK1, and ERK2 antibodies were from Santa Cruz Biotechnologies. A rabbit-RSK2 used for immunofluorescence and phospho-histone H3 antibody were from Upstate Biotechnology. Phospho-RSK2 antibody was from Cell Signaling Technologies, and the HA antibody 12CA5 was from Roche Applied Science. EGF was obtained from Sigma.

³⁵S]Methionine Labeling and GST Pull-downs—CHO-K1 cells were cultured to 90% confluency in a 100-mm tissue culture dish. Cells were then cultured for at least 4 h in the presence of ³⁵S-labeled methionine. Cells were lysed in MLB buffer (25 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, and protease inhibitors) and then subjected to a pull-down assay using equal amounts of purified GST or GST-PEA-15 immobilized on agarose beads. The cell lysates were incubated with the purified proteins for at least 1 h at 4 °C. The beads were washed extensively with MLB buffer and then once with buffer containing 50 mM Tris-Cl (pH 7.5) and 150 mM NaCl. The proteins were then cleaved with 1 unit of thrombin in a buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 2.5 mM of CaCl₂. The beads were discarded, and the supernatant was subjected to SDS-PAGE. The gels were dried and autoradiographed.

Immunoprecipitations and Kinase Assays—COS-7 cells were transfected with pMT2 HA-RSK2 in 100-mm dishes. The cells were serum-starved for 24 h prior to stimulation with 10 ng/ml EGF for 10 min. Cells were washed in phosphate-buffered saline, and lysates were obtained by scraping the cells in cold lysis buffer containing 20 mM Tris (pH 8), 137 mM NaCl, 10% glycerol and 1% Nonidet P-40. Cell debris was discarded after centrifugation at 14,000 rpm for 20 min at 4 °C. The supernatant was used to immunoprecipitate HA-RSK2 using the monoclonal HA-antibody 12CA5. Lysates were incubated with the antibody for 2–3 h on ice. Immune complexes were precipitated with 50 μl of protein A-Sepharose (Amersham Biosciences) beads for 1 h at 4 °C. The precipitates were washed extensively in lysis buffer supplemented with 1 M NaCl. For kinase assays, the precipitates were resuspended in 40 μl

of buffer containing 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM dithiothreitol and split into two equal aliquots. One aliquot was used in an S6 peptide kinase assay (Upstate Biotechnology), while the other aliquot was subjected to immunoblotting to verify equal RSK2 precipitation. The kinase assay was carried out according to the manufacturer's protocol using an S6 peptide substrate to determine radiolabeled phosphate incorporation by scintillation counting. Alternatively, the S6 peptide was substituted with 10 μg of myelin basic protein, GST-PEA-15, or GST, and the reaction was arrested with sample buffer after 10 min. Proteins were separated by SDS-PAGE, dried, and visualized by autoradiography.

His-tagged Protein Precipitations—His₆-tagged constructs of PEA-15 (wt and mutants) were transfected into HeLa cells using Polyfect (Qiagen) according to the manufacturer's protocol. The cells were cultured for 48 h prior to lysing with 1 ml of Nonidet P-40 lysis buffer/100-mm dish. Lysates were obtained after centrifugation at 14,000 rpm for 20 min at 4 °C to remove cell debris. For each sample, 50 μl of ProBond Ni²⁺ column (Invitrogen) was used after washing the column once with distilled water and lysis buffer. Lysates were incubated with the columns for 45–60 min at 4 °C. The pellets with the bound protein were washed extensively with lysis buffer and finally resuspended in 100 μl of sample buffer. Proteins were subjected to SDS-PAGE and immunoblotting for endogenous RSK2.

Immunofluorescence Assays—COS-7 cells were co-transfected with HA-RSK2 and GFP or PEA-15 fused to GFP. Cells were grown for 24 h and then serum-starved for 24 h. Cells were then treated with 50 ng/ml EGF for 0, 20, or 50 min before fixing with 4% paraformaldehyde for 10 min. Cells were then stained overnight at 4 °C with anti-RSK2 antibody (Upstate Biotechnology) in 3% bovine serum albumin and 0.2% Triton X-100 in phosphate-buffered saline. The antibody was detected using anti-rabbit Texas Red-conjugated antibody from Molecular Probes. The cells were imaged in a Zeiss Axiovert 100M fluorescent microscope using a 100X oil-immersion objective.

Transcription Assays—Transcription assays for RSK2 activity were carried out using the Stratagene path detect kit specific for CREB-mediated transcription. The strategy involves the use of a Gal4DBD-tagged CREB construct (pFA2-CREB) and a luciferase construct carrying the Gal4 promoter region (pFR-Luc). Using this approach, only the effect of transfected CREB on luciferase expression is measured. 250 ng of pcDNA3 or PEA-15 (wt and mutants) was co-transfected with 500 ng of pFR-Luc and 50 ng of pFA2-CREB constructs. Cells were left serum-starved for 16–20 h prior to stimulation with 20% serum for 1 h. Luciferase readings were obtained using the Promega assay kit according to the manufacturer's protocol. To verify the specificity of the effect for RSK2, activated RSK2 (Y707A) was co-transfected along with the other plasmids, and cells were left serum-starved prior to lysis. Luciferase readings were obtained in a luminometer for 5 μg of total cellular protein according to the manufacturer's protocol.

In Vitro Transcription/Translation—pcDNA3-RSK2 plasmid was used to transcribe RNA with a linked protein transcription/translation system (Ambion). The proteins were labeled using [³⁵S]methionine according to the manufacturer's protocol. One-tenth of the reaction was retained to load on the gel as input. The remaining 40 μl of the reaction was diluted to 500 μl in buffer containing 25 mM HEPES, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, and protease inhibitors. Purified GST-PEA-15 or GST immobilized on agarose beads was used as bait to carry out a protein pull-down. The translation mixture containing full-length proteins was first precleared with GST for one-half hour at 4 °C. Equal amounts of GST-PEA-15 or GST were used for a pull-down experiment with the precleared reaction mixtures. The proteins were incubated for 2–3 h at 4 °C before washing the pellet extensively with MLB buffer. The samples were resuspended in sample buffer and subjected to SDS-PAGE, drying, and autoradiography.

RESULTS

PEA-15 Binds RSK2—We reported previously that the small death effector domain-containing protein, PEA-15, binds ERK1/2 and redirects traffic in the ERK MAP kinase pathway (23). To identify other proteins that interact with PEA-15 and that might contribute to the effects on ERK signaling, we isolated PEA-15-interacting proteins from cell lysates by pull-down with GST-PEA-15 fusion proteins. We labeled CHO-K1 cells with [³⁵S]methionine, prepared lysates from these cells, and ran the lysates over a glutathione-agarose column coated with GST-PEA-15 fusion protein containing full-length PEA-15

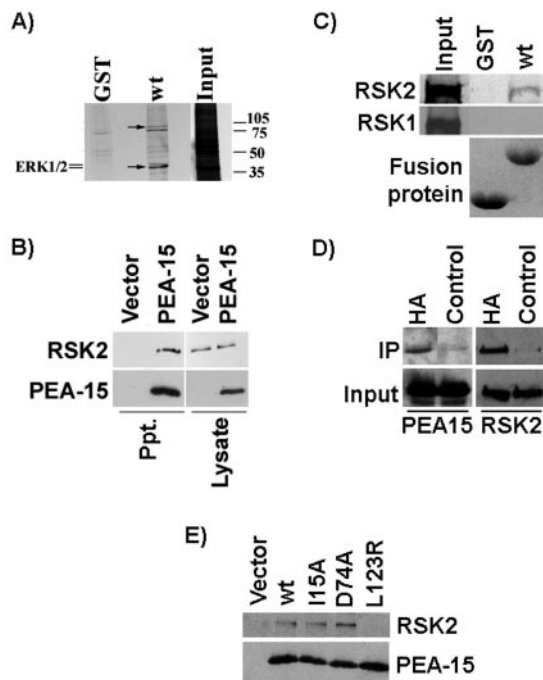


FIG. 1. *A*, a 90-kDa protein interacts with PEA-15. CHO-K1 cells were labeled with [³⁵S]methionine for 4 h. Cells were lysed, and the lysate was used in a pull-down with GST or GST-PEA-15. Proteins were subjected to SDS-PAGE and autoradiography to detect PEA-15-interacting proteins. *B*, RSK2 binds PEA-15. HeLa cells cultured in 10-cm dishes were transfected with His₆-tagged PEA-15 or vector control. The tagged proteins were precipitated using Probond Ni⁺² columns, washed extensively, and analyzed by SDS-PAGE and Western blotting to detect endogenous RSK2 associated with PEA-15. *Ppt.*, precipitate. *C*, RSK2 interacts directly with PEA-15. RSK2 and RSK1 were *in vitro* translated and labeled with ³⁵S. GST or GST-PEA-15 fusion proteins immobilized on agarose beads were used as bait to pull down the translated protein. The proteins were run on an 8% denaturing gel, dried, and autoradiographed. *D*, PEA-15 co-immunoprecipitated (IP) with RSK2. COS-7 cells were co-transfected with pMT2-HARSK2 and His PEA-15. RSK2 was immunoprecipitated using the 12CA5 monoclonal antibody to the HA tag on RSK2. Precipitates were subjected to SDS-PAGE and immunoblotted for PEA-15 as well as RSK2. Lysates were also run to verify equal protein used in the immunoprecipitation. *E*, the COOH terminus of PEA-15 may be required to bind RSK2. HeLa cells were transfected with His₆-tagged PEA-15 wt or mutants, and the precipitated protein complexes were subjected to Western blotting for RSK2. PEA-15 expression and precipitation were also verified.

fused to glutathione *S*-transferase. In addition to bands the size of ERK1 and -2, we also saw a band at 90 kDa (Fig. 1A). The p90RSK proteins are targets of ERK1/2 (8) and regulate transcription and cell survival events. We therefore tested whether the 90-kDa band was a member of the RSK family of proteins. Initial results showed that antibodies to RSK2 but not RSK1 reacted with a band in the GST-PEA-15 pull-down at 90 kDa (data not shown). To determine whether the PEA-15 could precipitate RSK2, we transfected HeLa cells with a His-tagged PEA-15 or the vector alone and isolated the over-expressed PEA-15 and any binding partners on a Ni⁺² column. We observed that endogenous RSK2 co-precipitated with PEA-15 (Fig. 1B). Protein expression and pull-down were verified by Western blotting as indicated.

To determine whether the interaction is direct, we used a linked *in vitro* transcription and translation system to produce [³⁵S]methionine-labeled RSK2 and RSK1 proteins. We then mixed the labeled RSKs with agarose beads coated with purified GST-PEA-15. As controls we included GST-coated agarose beads. PEA-15 binds RSK2 but not RSK1 or GST (Fig. 1C). Hence PEA-15 and RSK2 can interact directly as purified proteins.

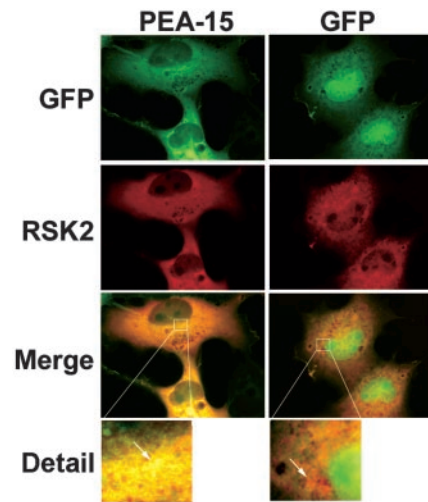


FIG. 2. **RSK2 and PEA-15 co-localize in the cell.** COS-7 cells were transfected with HA-RSK2 and GFP or PEA-15 fused to GFP. Cells were fixed in 4% paraformaldehyde, stained with anti-RSK2 antibody, and detected using Texas Red antibody. *Detail panels* show close-ups of regions with distinct co-localization of RSK2 and PEA-15. GFP controls do not show co-localization with RSK2.

To confirm the interaction we used an additional *in vivo* assay to isolate the protein complex. We co-transfected His tagged PEA-15 with HA-tagged RSK2 in COS-7 cells and allowed expression for 48 h. We then immunoprecipitated RSK2 using an anti-HA antibody (Cell Signaling Technology) and immunoblotted for PEA-15. We found a significant amount of PEA-15 co-immunoprecipitated with RSK2 (Fig. 1D). Additionally, we were able to observe endogenous PEA-15 that had also been precipitated in a complex with RSK2 (data not shown). Thus we have shown that PEA-15 and RSK2 interact with each other and that the interaction is direct.

To determine the regions of PEA-15 that may be necessary for this interaction, we used point mutants of PEA-15 in the DED as well as the COOH-terminal regions of the protein. These two regions have been shown to be important for the interaction of PEA-15 with ERK, and mutations in these regions affect the ability of PEA-15 to alter ERK function (24). The D74A mutation in the DED and the L123R mutation in the COOH-terminal tail abolish ERK binding to PEA-15 *in vitro*, and these mutants were therefore used as representatives. We also used a previously characterized point mutant in the nuclear export sequence of PEA-15 (I15A), which allows PEA-15 accumulation in the nucleus. We observed that whereas the D74A mutant and the I15A mutant bind RSK2 *in vivo*, the L123R tail mutant does not (Fig. 1E). Hence the interaction of PEA-15 with RSK2 involves the COOH-terminal domain of the protein. Furthermore, although the D74A mutation completely prevents ERK binding it has no effect on RSK2 binding. This supports a model in which PEA-15 can interact with ERK1/2 and RSK2 independently.

Because PEA-15 binds RSK2, we determined whether the two proteins co-localize in the cell. We found that whereas PEA-15 appeared to be strongly co-localized with RSK2, control cells transfected with GFP did not show similar co-localization patterns (Fig. 2). Neither PEA-15 nor RSK2 appeared to be confined to any particular compartment of the cytoplasm but instead, under these conditions, were diffusely distributed throughout. These co-localization experiments provided further *in vivo* evidence for an interaction between PEA-15 and RSK2. Thus we have shown that PEA-15 interacts directly with RSK2, co-localizing with it in the cell, and that the interaction involves the COOH-terminal domain of PEA-15.

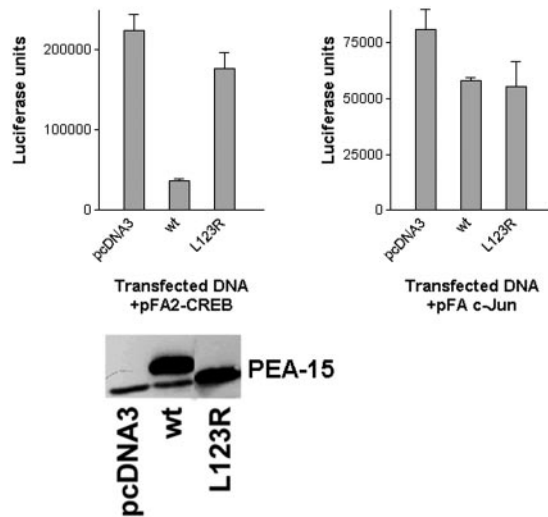


FIG. 3. PEA-15 inhibits CREB mediated transcription. COS-7 cells were co-transfected with PEA-15, a luciferase reporter construct, pFRLuc, and pFA2CREB or pFA-cJun. Cells were serum-starved for 16–20 h prior to stimulation with serum. 5 μ g of total protein was used for the luciferase assay. Lysates were blotted to verify protein expression.

PEA-15 Affects RSK2-dependent Transcription—Having found that PEA-15 binds to RSK2, we sought to determine whether PEA-15 expression affects RSK2 function. One known function of RSK2 is the activation of the transcription factor CREB (15, 25). To examine the functional consequences of PEA-15 binding to RSK2 we carried out *in vivo* transcription assays using a CREB-driven luciferase construct in the Pathdetect system. Cells were treated with serum or not and then lysed and assayed for luciferase activity as a measure of CREB-mediated activation of transcription. Cells transfected with PEA-15 showed impaired CREB activity (Fig. 3). For comparison, PEA-15 had little impact on the activation of the c-Jun transcription factor.

We then sought to exclude the possibility that PEA-15 blocks CREB activation by an RSK2-independent mechanism. We therefore examined whether PEA-15 expression could alter activation of CREB by an activated mutant of RSK2 (Fig. 4, Y707A). PEA-15 was co-transfected with the activated RSK2 construct. Cells transfected with wt PEA-15 strongly inhibited RSK2-dependent CREB-mediated transcription (Fig. 4). Furthermore a non-ERK-binding tail mutant (L123R) and the nuclear export sequence mutant, I15A, did not inhibit transcription (Fig. 4). Activated RSK2 expression was verified by blotting for RSK2 in the lysates (Fig. 4). Hence, PEA-15 is able to impair RSK2 activation of CREB.

Over-expression of PEA-15 Decreases Phosphorylation of Histone H3—RSK2 is reportedly required for the phosphorylation of Histone H3 (16). To further determine the functional consequences of PEA-15 expression on RSK2 kinase, we examined the effect of PEA-15 on the RSK2-specific nuclear substrate histone H3. We co-transfected COS-7 cells with RSK2 and PEA-15 or vector control. The MEK inhibitor was used to remove the high background phosphorylation of histone H3 at Ser-10, which was observable even upon serum starvation (data not shown and previous reports). Cells were then stimulated with EGF. We observed that PEA-15 expression blocked H3 phosphorylation compared with the vector control (Fig. 5) in response to EGF stimulation.

PEA-15 Affects RSK2 Kinase Activity—One mechanism by which PEA-15 may block RSK2 activation of CREB and histone H3 phosphorylation is by blocking the kinase activity of RSK2. To address this hypothesis, we looked at the effects of PEA-15

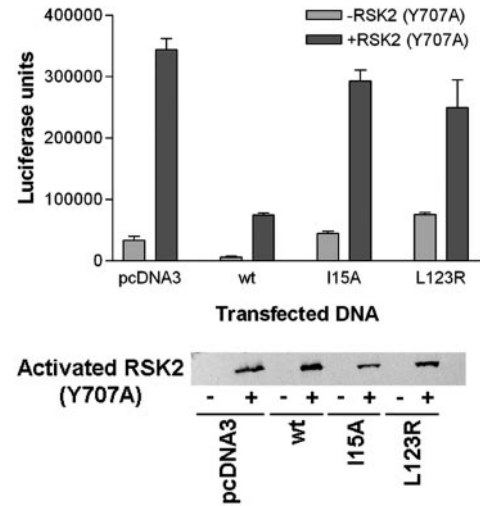


FIG. 4. PEA-15 inhibits RSK2-specific CREB transcription. COS-7 cells were co-transfected with activated RSK2 (Y707A) and PEA-15, mutants, or vector control. The cells were left serum-starved for 16–20 h prior to lysis. 5 μ g of total protein was used for the luciferase assay, and lysates were blotted to verify protein expression.

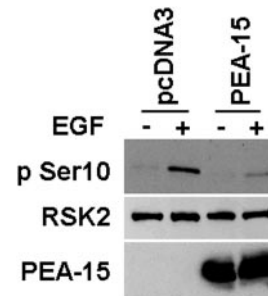


FIG. 5. PEA-15 inhibits phosphorylation of histone H3 by RSK2. COS-7 cells were transfected with pMT2-RSK2 and PEA-15 or vector control. Cells were serum-starved and treated with the MEK inhibitor UO126 for 16 h. Cells were then stimulated with 30 ng/ml EGF for 30 min. Cells were lysed in sample buffer and immunoblotted for pSer10-histone H3, RSK2, and PEA-15.

on RSK2 kinase activity using an S6 peptide-based kinase assay. In this assay PEA-15 or control vector alone were co-transfected with HA-tagged RSK2 into cells followed by immunoprecipitation of the RSK2 and determination of its ability to phosphorylate S6 peptide. We found that PEA-15 inhibits RSK2 kinase activity by 50% compared with a vector control (Fig. 6A). Surprisingly, the nuclear localized PEA-15 mutant (I15A) did not appear to inhibit RSK2 kinase activity (Fig. 6A) suggesting that the inhibition of RSK2 kinase activity by PEA-15 occurs in the cytoplasm and is not simply a function of PEA-15 binding itself.

Alternatively, PEA-15 may impair RSK2 phosphorylation of S6 by acting as a competitive substrate for RSK2. To determine whether PEA-15 is a substrate of RSK2, we immunoprecipitated RSK2 after stimulation with EGF and carried out a kinase assay using purified GST-PEA-15, myelin basic protein (a good substrate for RSK2 activity), or GST as substrate. After the kinase reaction, proteins were separated by SDS-PAGE and subjected to autoradiography. Although myelin basic protein was efficiently phosphorylated by RSK2, neither PEA-15 nor GST was phosphorylated (Fig. 6B). Hence, PEA-15 is not a substrate of RSK2 and does not act as a competitive substrate in blocking S6 kinase activity.

PEA-15 may inhibit RSK2 kinase activity by preventing full activation of RSK2 in cells. Complete activation of RSK2 requires phosphorylation of Ser-386 by the COOH-terminal ki-

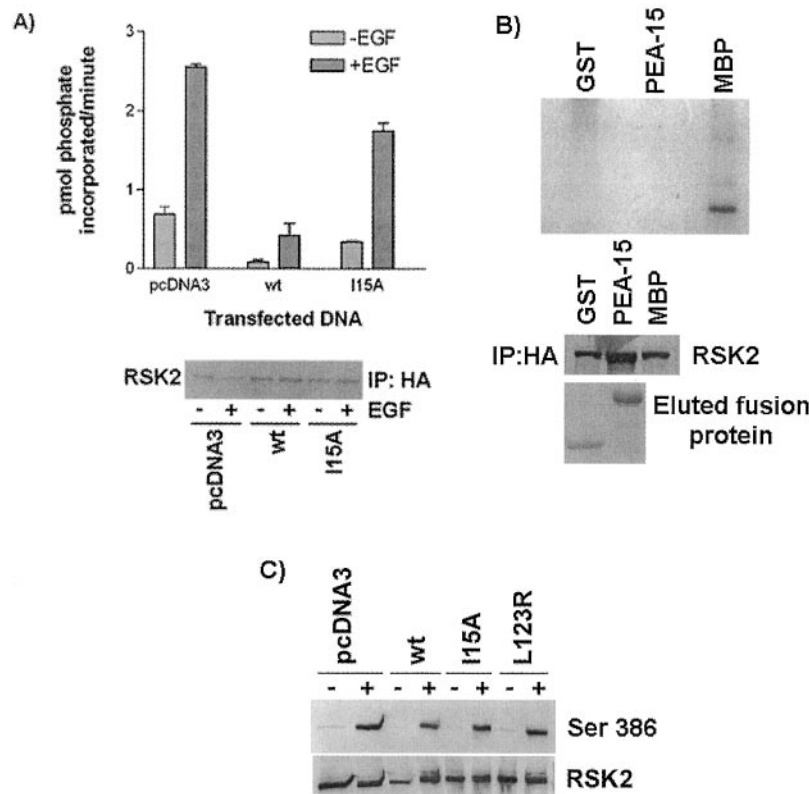


FIG. 6. **A**, PEA-15 inhibits RSK2 kinase activity. COS-7 cells were co-transfected with pMT2-HARSK2 and PEA-15 (wt or mutants). Cells were serum-starved for 16–20 h prior to stimulation with 10 ng/ml EGF for 10 min or left untreated with EGF. HA-RSK2 was immunoprecipitated with 12CA5 monoclonal antibody and subjected to an S6 peptide-based kinase assay. The figure is representative of at least two independent experiments performed in triplicate. Control immunoblots were run on the immunoprecipitates (IP) to verify protein precipitation for RSK2. **B**, PEA-15 is not a substrate for RSK2. COS-7 cells were transfected with pMT2-HARSK2. Cells were serum-starved for 16–20 h prior to stimulation with 10 ng/ml EGF for 10 min. Cells were lysed, and RSK2 was immunoprecipitated. Immune complexes were washed, and the kinase reaction was carried out using 10 μ g of GST, GST-PEA-15, and myelin basic protein (MBP) in the presence of [32 P]ATP. The reaction was terminated by the addition of sample buffer, run on an 8% SDS gel, and autoradiographed. Control immunoblots were run on the immunoprecipitates to verify protein precipitation for RSK2. GST fusion proteins are also shown. **C**, PEA-15 does not affect RSK2 phosphorylation. COS-7 cells were co-transfected with RSK2 and PEA-15. Cells were serum-starved for 16–20 h prior to stimulation with EGF for 10 min. Phospho-epitope-specific antibodies were used to detect phosphorylation of RSK2 at Ser-386. Protein loading was verified by immunoblotting for RSK2.

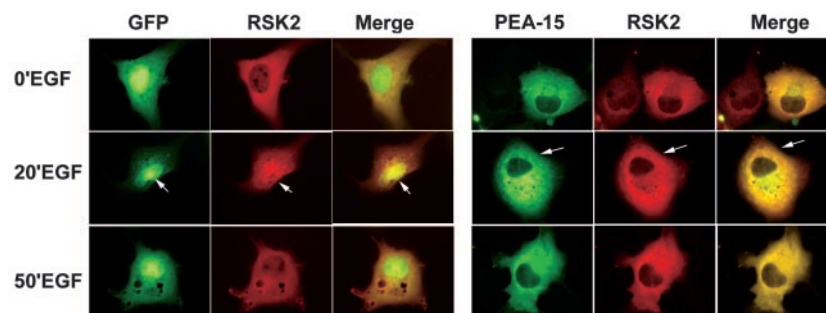


FIG. 7. **PEA-15 inhibits the nuclear translocation of RSK2**. COS-7 cells were co-transfected with pMT2-HA RSK2 and PEA-15-GFP or GFP. Cells were serum-starved for 16–20 h and then stimulated with 50 ng/ml EGF for 0, 20, or 50 min. The cells were fixed in paraformaldehyde, stained with anti-RSK2 antibody, and detected using a Texas Red-conjugated secondary antibody. The cells were imaged using a Zeiss Axiovert 100M microscope and a 100X oil immersion objective.

nase domain. To determine whether PEA-15 expression alters phosphorylation at this site, cells stimulated with EGF in the presence or absence of over-expressed PEA-15 were immunoblotted with a Ser-386 phospho-epitope antibody. PEA-15 did not affect phosphorylation at Ser-386 (Fig. 6C). Therefore PEA-15 does not affect RSK2 activity by altering its activation state.

PEA-15 Alters RSK2 Localization—A mutant of PEA-15 that accumulates in the nucleus (I15A) did not impair the kinase activity of RSK2 (Fig. 6A). In addition, PEA-15 has been shown to bind ERK and sequester it in the cytoplasm (23). This suggests that PEA-15 might regulate RSK2 similarly by altering

its subcellular localization. To test this hypothesis, we investigated whether PEA-15 regulates the translocation of RSK2 after EGF stimulation. Serum-starved cells co-transfected with RSK2 and either GFP or wt PEA-15 fused to GFP showed RSK2 predominantly localized to the cytoplasm (Fig. 7). On stimulation with EGF for 20 min, nuclear translocation of RSK2 was observed in control cells transfected with GFP alone. However, in cells transfected with the PEA-15 construct, RSK2 fails to undergo nuclear translocation. At 50 min post-EGF stimulation RSK2 re-entered the cytoplasm in the control cells, whereas no change was observed in cells transfected with PEA-15. RSK1 has been suggested to undergo transient membrane

localization as an immediate response to EGF stimulation (26). However, we were unable to detect this in our system for RSK2 even at very early time points post-EGF stimulation (data not shown). Our results therefore suggest that PEA-15 changes the subcellular localization of RSK2. This may contribute to the effect of PEA-15 on CREB-mediated transcription and appears to be a requirement for PEA-15 inhibition of RSK2 kinase activity.

DISCUSSION

We have shown that PEA-15 binds directly to RSK2 and alters both its subcellular localization and its kinase activity. In particular PEA-15 expression prevents RSK2 translocation into the nucleus and results in decreased RSK2 kinase activity. This has the functional outcome of inhibiting RSK2 activation of the transcription factor CREB and RSK2 phosphorylation of histone H3. Both PEA-15 and RSK2 are reportedly expressed in many of the same cell types including astrocytes (17, 27). Hence we have identified a novel RSK2 binding partner that may play a significant role in regulating its signaling activity.

PEA-15 is reported to affect ERK MAP kinase signaling and apoptosis (23, 28–30). Perhaps some of these effects are due to the interaction of PEA-15 with RSK2. For example, *c-fos* activation in PEA-15-null mice was shown to be twice that of wild type mice. RSK2 has been shown to be required for activation of *c-fos* (31). Hence, PEA-15 binding to RSK2 may normally play a role in limiting the ability of RSK2 to access nuclear targets such as *c-fos*, CREB, and histone H3. Cells lacking PEA-15 may then have abnormally high levels of these activities of RSK2 leading to effects on the cell cycle. Interestingly PEA-15-null cells are also reported to sustain an increased rate of proliferation (23).

We have also shown that PEA-15 binds ERK1/2 (23, 24). Here we show that RSK2 binds PEA-15 in a manner that is distinct from that of ERK. Specifically, RSK2 binds to a mutant PEA-15 that cannot bind ERK (D74A). This suggests that the two binding sites are different and that PEA-15 may be able to bind to both ERK and RSK2 at the same time, perhaps preventing or promoting their interaction. However the fact that neither RSK2 nor ERK could bind the PEA-15 tail mutant (L123R) indicates that the binding sites on PEA-15, although distinct, may overlap. The precise nature of these interactions remains to be determined.

RSK2 phosphorylates histone H3 in response to EGF stimulation (16). The phosphorylation of histone H3 by RSK2 was hypothesized by Sassone-Corsi and colleagues (16) to be important in MAP kinase regulation of transcription. Here we show that expression of PEA-15 inhibits RSK2 phosphorylation of histone H3. This is likely because of the perturbation of RSK2 translocation to the nucleus. Rapid phosphorylation of histone H3 correlates with EGF-induced expression of immediate-early response genes such as *c-fos* (32). We previously reported that cells lacking PEA-15 have a significant increase in *c-fos* expression (23). This is in agreement with the hypothesis that PEA-15 negatively regulates RSK2 activity.

It is interesting that PEA-15 alters both the subcellular localization of RSK2 and its activity. We have found that a mutant of PEA-15 that binds RSK2 but is localized to the nucleus does not block RSK2-mediated activation of transcription (Fig. 4, I15A). Moreover, the same mutant does not block RSK2 kinase activity (Fig. 6A). We propose that this is evidence that effects on transcription and RSK2 activity in the cell are predominantly due to the altered localization of RSK2. However it remains possible that binding of the I15A mutant of

PEA-15 is physically different from that of wild type and therefore is incapable of blocking RSK2 kinase activity. Alternatively, retention of RSK2 in the cytoplasm may decrease RSK2 activity by an as yet undetermined mechanism.

PEA-15 is one of the first RSK-binding proteins suggested to regulate RSK function negatively. It is the first that binds specifically to RSK2. Recently the adapter protein 14-3-3 β was reported to bind specifically to RSK1 and to regulate it negatively by interfering with RSK1 phosphorylation (33). Together these data suggest that there may be several RSK binding partners that can maintain signal specificity and modulate the growth factor response by different mechanisms. As such, this work provides important new insight for unraveling the complex regulation of RSKs.

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