Previously we showed that interactions between p90RSK1 (RSK1) and the subunits of type I protein kinase A (PKA) regulate the activity of PKA and cellular distribution of active RSK1 (Chaturvedi, D., Poppleton, H. M., Stringfield, T., Barbier, A., and Patel, T. B. (2006) Mol. Cell Biol. 26, 4586–4600). Here we examined the role of the PKARIα subunit of PKA in regulating RSK1 activation and cell survival. In mouse lung fibroblasts, silencing of the PKARIα increased the phosphorylation and activation of RSK1, but not of RSK2 and RSK3, in the absence of any stimulation. Silencing of PKARIα also decreased the nuclear accumulation of active RSK1 and increased its cytoplasmic content. The increased activation of RSK1 in the absence of any agonist and changes in its subcellular redistribution resulted in increased phosphorylation of its cytoplasmic substrate BAD and increased cell survival. The activity of PKA and phosphorylation of BAD (Ser-155) were also enhanced when PKARIα was silenced, and this, in part, contributed to increased cell survival in unstimulated cells.

Furthermore, we show that RSK1, PKA subunits, D-AKAP1, and protein phosphatase 2A catalytic subunit (PP2Ac) exist in a complex, and dissociation of RSK1 from D-AKAP1 by either silencing of PKA, depletion of D-AKAP1, or by using a peptide that competes with PKARIα for binding to AKAPs, decreased the amount of PP2Ac in the RSK1 complex. We also demonstrate that PP2Ac is one of the phosphatases that dephosphorylates RSK, but not ERK1/2. Thus, in unstimulated cells, the increased phosphorylation and activation of RSK1 after silencing of PKARIα or depletion of D-AKAP1 are due to decreased association of PP2Ac in the RSK1 complex.

Cyclic AMP-dependent protein kinase (PKA) plays a pivotal role in manifesting an array of biological actions ranging from cell proliferation and tumorigenesis to increased inotropic and chronotropic effects in the heart as well as regulation of long term potentiation and memory. The PKA holoenzyme is a heterotetramer and consists of two catalytic (PKAc) subunits bound to a dimer of regulatory subunits. To date, four isoforms of the PKAc (PKAcα, PKAcβ, PKAcγ, and PKAcδ) and four isoforms of the regulatory subunits (RIα, RIβ, RIIα, and RIIβ) have been described (1). The various isoforms of PKA subunits are expressed differently in a tissue- and cell-specific manner (2). In addition to binding and inhibiting the activity of PKAc via their pseudo substrate region (3–6), the R subunits also interact with PKA-anchoring proteins (AKAPs) and facilitate the localization of PKA in specific subcellular compartments (7, 8). More than 50 AKAP family members have been described, and although most of these have a higher affinity for the RII subunits (9), certain AKAPs such as D-AKAP1 and D-AKAP2 preferentially bind the PKARIα subunit (10–12). Because the AKAPs also bind other signaling molecules such as phosphatases (PP2B) and kinases (protein kinase C), they act as scaffolds to organize and integrate specific signaling events within specific compartments in the cells (7, 8, 13, 14).

We have shown that the PKARIα and PKAα subunits of PKA interact with the inactive and active forms of p90RSK1 (RSK1), respectively (15). Binding of inactive RSK1 to PKARIα decreases the interactions between PKARIα and PKAc, whereas the association of active RSK1 with PKAc increases interactions between PKARIα and PKAc such that larger amounts of cAMP are required to activate PKAc in the presence of active RSK1 (15). Moreover, the indirect (via subunits of PKA) interaction of RSK1 with AKAPs is required for the nuclear localization of active RSK1 (15), and disruption of the interactions of RSK1-PKA complex from AKAPs results in increased cytoplasmic distribution of active RSK1 with a concomitant increase in phosphorylation of its cytosolic substrates such as BAD and reduced cellular apoptosis (15). These findings show the functional and biological significance of RSK1-PKA-AKAP interactions.

Besides inhibiting PKAc activity, the physiological role of PKARIα is underscored by the findings that mutations in the PKARIα gene that result in haploinsufficiency of PKARIα are
the underlying cause of Carney complex (CNC) (16, 17). CNC is an autosomal dominant multiple neoplasia syndrome in which myxomas of the skin, heart, and/or viscera are recurrent and also associated with high incidence of endocrine and ovarian tumors as well as Schwannomas (18–20). The majority of patients with the multiple neoplasia CNC syndrome harbor mutations in the PKAR1A gene (21) that result in PKAR1α haploinsufficiency. Importantly, however, loss of heterozygosity or alterations in PKA activity may not contribute toward the tumorigenicity in either CNC patients or mouse model of CNC (21). This suggests that loss of function(s) of PKAR1α other than inhibition of PKA activity is(are) involved in the enhanced tumorigenicity in CNC patients and in the murine CNC model.

Because RSK1 regulates cell growth, survival, and tumorigenesis (22–27), and because its subcellular localization and activity and/or subcellular localization of RSK1. Therefore, herein we have investigated whether PKAR1α regulates the activation of RSK1 and its biological functions. Decreasing expression of PKAR1α by small interfering RNA (siRNA) enhanced the activation of RSK1, but not RSK2 or RSK3, in the absence of an agonist such as EGF. This was accompanied by an increase in the cytoplasmic localization of the active RSK1 and enhanced cell survival in the absence of any growth factor. Silencing of PKAR1α also increased PKA activity and while part of the anti-apoptotic response could be attributed to an increase in PKA activity, activation of RSK1 under basal conditions contributed significantly to cell survival. The elevation in RSK1 activity upon PKAR1α silencing was not due to increased PKA activity. Rather the activation of RSK1 in the absence of PKAR1α was due to a decrease in PP2A in the RSK1 complex. These findings demonstrate a novel role for PKAR1α in the regulation of RSK1 activation, a key enzyme that mediates the downstream actions of the ERK1/2 cascade.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Mouse lung fibroblasts (B82L) overexpressing EGF receptor were from Dr. Paul J. Bertics, University of Wisconsin, Madison, WI. Anti-phospho BAD (Ser-112 and Ser-117), anti-BAD, anti-phospho-PKA substrate antibody, anti-phospho-RSK (Thr-573), anti-phospho-RSK (Ser-380), and anti-cleaved PARP antibodies were purchased from Cell Signaling (Beverly, MA). Anti-phospho-RSK1 (Ser-380) from Epitomics (Burlingame, CA), anti-phospho-RSK1/2 (Ser-221) from R&D Systems (Minneapolis, MN), monoclonal anti-actin antibody (MP Biomedicals, Aurora, OH), anti-Erk1/2 and anti-PP2Ac antibodies (Upstate Cell Signaling Solutions, Temecula, CA), anti-RSK1 and anti-PKA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were also used. The anti-PKAR1α and monoclonal D-AKAP (catalog no. 611573) antibodies were from BD Biosciences (Palo Alto, CA). The polyclonal D-AKAP1 antibody was provided by Dr. Susan Taylor (University of California at San Diego). Control and shRNA-expressing D-AKAP1 (Mouse pLKO.1 lentiviral target gene shRNA set, catalog no. RMM4534) constructs were from OpenBiosystems.

**Cells and Culture Conditions**—Control and shRNA expressing D-AKAP1-stable B82L cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and maintained in 4 μg/ml puromycin.

**PKARIα, PP2Ac, and BAD Silencing Using siRNA**—B82L cells were plated at 50–70% confluency in serum containing media. After 24 h, and just prior to siRNA transfections, the culture medium was replaced with serum free media. Control or PKARIα (20 nm)-specific or PP2Ac (40 nm)-specific siRNAs were transfected using transkit TKO (Mirus) as described by the manufacturer. Cells were incubated for 72 h before experimentation. The 27 ± 2 ribonucleotide long PKARIα-specific siRNA (sequence: 5’-GGA GGA GGC AAG ACA GAU UCA GUC UC AC-3’ (sense)/5’-AGA CAC UGA AUC UGU CUU GCC UCC UCU UC-3’ (antisense), PP2Ac siRNA (sequence: 5’-CGU UCA AGA GGU UCG AUC AGU CAC TG-3’ (sense)/5’-GUG ACU GGA CAU CGA ACC UCU UGA AGC TT-3’ (antisense)) and control siRNA (sequence: 5’-GGA GCU GCG UUC ACU GUA AGA GAC UGU AC-3’ (sense)/5’-ACA GUC UCU UAC AGU GAA CGC AGC UCC UU-3’ (antisense)) were synthesized commercially (Integrated DNA Technologies, Coralville, IA). BAD siRNA was purchased from Ambion (catalog no. 4390771). B82L cells were transfected with 40 nm BAD siRNA, and the experiments were performed after 72 h of transfections.

**D-AKAP1 Silencing**—B82L cells were plated on 60-mm dishes at 50–70% confluency in serum-containing media. After 24 h 2 μg of DNA of shRNA expressing D-AKAP1 constructs (catalog nos. TRCN000088538, TRCN000088539, and TRCN000088541) and control were transfected using transkit TKO (Mirus) as described by the manufacturer. After 24 h the culture medium was replaced with fresh medium. After 48 h of transfection 2 μg/ml puromycin was added to the culture medium for 1 week. After 1 week of 2 μg/ml puromycin, concentration of puromycin was raised to 4 μg/ml, and the stable D-AKAP1 cell lines were maintained at this concentration for at least 2 weeks prior to experimentation. These selected cell lines are represented in Fig. 6 (D and E) as 538, 539, and 541.

**Semi-quantitative PCR**—Total RNA was isolated from semi-confluent control, and shRNA-expressing D-AKAP1 B82L cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Equal amounts of RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) as per the manufacturer’s instructions. For semi-quantitative PCR, equal amounts of reverse-transcribed RNA were used with the following D-AKAP1 primers: forward primer, 5’-TGG CTG GTG GTG GTT TTT-3’ and reverse primer, 5’-CTC TCT CGC TTT CTC TCC-3’. D-AKAP1 primers were designed using mouse D-AKAP1 sequence (accession no. NM_009648.2) nucleotides 50–77 for forward and nucleotides 544–561 for reverse primer. Prolylhydroxylase 2 mRNA (using forward primer, 5’-GAT ACA AGC TTA TGG CCA GTG ACA GC-3’ and reverse primer, 5’-GTT TAT CTA GAC TAG ACG TAT TTG CTG ACT GAA TTG GCC TGG A-3’) was monitored as internal control. Semi-quantitative PCR was performed on RoboCycler Gradient 40 (Stratagene). The reaction mixture consisted of 0.1 μg/μl cDNA template, 100 ng of each primers, 2 μl of DMSO, 50 units of Fast Start TaqDNA poly-
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merase in a 50-μl reaction volume. The PCR protocol consisted of one 4-min denaturation cycle at 95 °C followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 90 s. Final extension cycle was 10 min at 72 °C. Equal volumes of PCR products were analyzed on 1.5% agarose gel.

*Immunoprecipitations—*B2L cells that had been serum-starved were transfected with 20 nm each of either PKARI or control siRNA or 40 nm of PP2Ac siRNA, 72 or 48 h after transfection with PKARIα siRNA or PP2Ac siRNA, respectively, cells were subjected to the various treatments as indicated. RSK1 IPs were performed using 250 μg of total cell lysate protein and 700 ng of anti-RSK1 antibody for 2 h at 4 °C. BAD IPs from cells treated with either vehicle, PK1 (10 μM, overnight), or 8CPT-cAMP (100 μM for 30 min) at 37 °C were performed at 4 °C overnight using 1 mg of total cell lysate protein and 2 μg anti-BAD antibody. To monitor PP2Ac in complex with RSK1, cells were treated with 20 μM of Ht31 or Ht31P for 15 min at 37 °C. PP2Ac IPs were performed using 1 mg of cell lysate protein and 2 μg of anti-PP2Ac antibody incubated at 4 °C overnight. D-ACK1 IPs were performed using 20 μl of D-ACK1 polyclonal antibody (gift from Dr. Susan Taylor, UCSD) and 500 μg of cell lysate at 4 °C overnight. For all IPs, cells were lysed in a buffer containing 50 mM Hepes, pH 7.5, 1% Triton X-100, 0.5% CHAPS (ICN Biomedicals Inc., Aurora, OH), 150 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 100 μM phenylmethylsulfonyl fluoride, 1 μM microcystin, and 1 μg/ml each pepstatin A, aprotinin, and leupeptin. The immune complexes were precipitated by incubation with 30 μl of protein G-agarose beads (Roche Applied Science) at 4 °C and washed three times with lysis buffer. Proteins were separated on 10% polyacrylamide gels for immunoblotting.

**RSK1 Activity in IPs—**Triplicate IPs of RSK1, performed as described above, were resuspended in 20 mM Hepes, pH 7.5, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM dithiothreitol, and 25 μM β-glycerophosphate, 5 mM MgSO4, 200 μM Kemptide, 125 μM ATP, and 10 μCi of [γ-32P]ATP (final volume, 120 μl) for 10 min at room temperature. The RSK1/2 inhibitor, SL-0101 (1 μM), was added to the kinase activity reaction buffer as indicated in Fig. 1B. Reactions were terminated by the addition of equal volume of 20% trichloroacetic acid and following centrifugation (16,000 × g, 5 min), aliquots (100 μl) of each supernatant were spotted onto P81 paper (Whatman), air-dried, and washed three times with 0.5% phosphoric acid. The filters were then dried and counted in a liquid scintillation counter. One parallel set of immunoprecipitated proteins were denatured in Laemml sample buffer, separated on 10% polyacrylamide gels, and analyzed by immunoblotting to ensure that equal amount of RSK1 was immunoprecipitated under different conditions.

**Immunocytochemistry and Cell Fractionation—**B2L cells were transfected with 20 nm each of PKARIα or control siRNA under serum-free conditions. 72 h after transfection cells were stimulated with 50 nm EGF for 10 min and fixed with 100% methanol for 10 min at −20 °C, followed by 1:1 methanol: acetone for 10 min at −20 °C. After permeabilizing the cells with 0.3% Triton X-100 in phosphate-buffered saline for 5 min, rabbit anti-phospho-Thr-573 RSK (1:250 dilution), and monoclonal anti-PKARIα (1:250 dilution) antibodies were added. The secondary antibodies were goat-anti-rabbit-conjugated with Alexa Fluor 488 or goat-anti-mouse-conjugated to 594 (1:500 dilution). Images were captured as described before (15). For quantification, the fluorescence density in at least 10 cells from different fields was quantified using NIH ImageJ software. For PKAR1α whole cell fluorescence was quantified. For phospho-Thr-573 RSK1, the nuclear fluorescence intensity was subtracted from the whole cell fluorescence to obtain a value for the cytoplasmic RSK1. The mean ± S.D. values are shown. For cytosolic and nuclear fractions, cells transfected with siRNAs were lysed using a hypotonic buffer from the nuclear extraction kit (Active Motif) and incubated on ice for 15 min. Cell lysates obtained were centrifuged at 200 × g to remove intact cells and cell debris. The supernatant from this step was centrifuged at 1,000 × g for 10 min at 4 °C to pellet cell nuclei. The nuclear pellet was washed and centrifuged at the same speed. Supernatants obtained were centrifuged at 10,000 × g for 10 min at 4 °C to pellet mitochondria, etc., and the resulting supernatant was centrifuged at 100,000 × g to generate the cytosolic fraction. The proteins from the cytosolic fractions were precipitated using 4 volumes of acetone followed by 2 washes with acetone: water (4:1). The pellets obtained were air-dried and resuspended in Laemmli sample buffer. The nuclear and cytosolic proteins were analyzed on 10% polyacrylamide gels followed by Western blotting.

**Cellular Apoptosis—**B2L cells (50,000 cells/well) in a 24-well dish were transfected with control and PKARIα siRNAs in serum-free media as described above. After 72 h of siRNA treatment, cells were stimulated with 50 nm EGF for 10 min. When indicated, the RSK inhibitor (fmk, 3 μM) was added 15 min before EGF treatment. Similarly, when indicated, cells were preincubated with PK1 (10 μM) or 8CPT-cAMP (100 μM) at 37 °C for 16 h and 30 min, respectively, and then treated with or without 20 ng/ml TNF-α plus 25 μg/ml cycloheximide (CHX) to induce apoptosis. One hour after TNF/CHX treatment, DNA fragmentation was measured using the Cell Death detection kit (Roche Applied Science) as described before (15, 28). As an alternate method to monitor apoptosis, cell lysates were immunoblotted for cleaved PARP.

**RESULTS AND DISCUSSION**

RSK family members represent proteins with two kinase domains, the N-terminal kinase and C-terminal kinase, joined together by a linker region. Activation of RSK1 is absolutely dependent upon the docking of ERK to the C terminus (29, 30), which phosphorylates Thr-573, Ser-359, and Ser-363 and activates the C-terminal kinase. The C-terminal kinase autophosphorylates RSK1 on Ser-380 (31), which forms a phosphoinositide-dependent protein kinase 1 binding site (32, 33). Finally, phosphoinositide-dependent protein kinase 1 phosphorylates RSK1 on Ser-221 in the activation loop of the N-terminal kinase (34, 35) and fully activates RSK1 (31).

Previously, we showed that inactive RSK1 associates with PKAR1α while the active RSK1 interacts with PKAc (15). This indirect interaction of RSK1 with AKAPs such as D-ACK1 determines the cellular localization of the active RSK1 (15).
However, whether the interaction of RSK1 with PKA-AKAP complex regulates activation of RSK1 remains unknown. Therefore, we determined the role of the interactions between RSK1, PKARI, and D-AKAP1 on the activation of RSK1 and its biological function.

Silencing of PKARI Increases p90RSK1 Phosphorylation and Activation—In B82L, mouse lung fibroblast silencing of PKARI increased phosphorylation of RSK1 on Ser-380, Ser-221, and Thr-573 under basal, unstimulated, conditions while the phosphorylation of these sites in the presence of EGF was the same (Figs. 1, A and B). In vitro kinase activity assays of immunoprecipitated RSK1 confirmed that silencing of PKARI increased the basal activity of RSK1 in unstimulated cells and that exposure of cells to EGF elevated RSK1 activity that was not further augmented by down-regulation of PKARI (Fig. 1B). That the amount of RSK in the IPs was the same is shown by the Western blot of a portion of the IPs used in the in vitro kinase assays (Fig. 1B, top right panel). Moreover, it should be noted that the IPs of RSK1 do not contain any RSK2 or RSK3 (supplemental Fig. S1A) and that the RSK1 inhibitor, SL-0101 (23), obliterated the increase in kinase activity due to either PKARI silencing or EGF treatment (Fig. 1B). These data show that the in vitro kinase activities in the RSK1 IPs are indeed due to RSK1 and not one or more other associated kinases. Additionally, these data demonstrate that, under basal conditions, PKARI attenuates the activation of RSK1 and that the site-specific anti-phospho-RSK antibodies faithfully reflect the activation state of the immunoprecipitated RSK1. The silencing of PKARI did not alter the amount of either PKAc (Fig. 1B, top left panel) or PKARI, which is not expressed in B82L cells (supplemental Fig. S1B). To check for specificity, the activation of RSK isoforms (RSK2 and RSK3) that do not interact with PKA

**FIGURE 1.** Silencing of PKARI increases p90RSK1 phosphorylation and activation. A, B82L cells were transfected with 20 nm each of control or PKARI siRNA. After 72 h of transfection, cells were stimulated with or without EGF (50 nm, 10 min) followed by immunoprecipitation of RSK1. Immune complexes were blotted for phospho- and total RSK1 forms. Immunoblot of total cell lysates (20 μg of protein) show PKARI knockdown. Total Erk1/2 shows equal loading. A representative of three similar experiments is shown. The bottom left panel shows the quantitative analysis of phospho-RSK Ser-380 as a ratio of total RSK1 in the IPs of RSK1 from three independent experiments. Quantification of PKARI/Erk1/2 ratios showed that PKARI was silenced by 72 ± 5.5%. B, RSK1 IPs similar to those in A was used for in vitro RSK1 activity assays as described under “Experimental Procedures.” The bar graph shows the RSK1 activity from a representative of two experiments performed in triplicates. Immunoblots of total cell lysate show the levels of PKARI knockdown and PKAc (loading control). An aliquot of IPs used in the RSK1 activity assays was blotted to monitor the levels of phospho-RSK1 (Thr-573) and to ascertain equal amounts of RSK1 in the IPs. For the in vitro kinase assays, equal aliquots from the same IPs were assayed in the presence and absence of SL-0101 (1 μM). Statistical significance was assessed by Student’s unpaired t test (n = 3). Quantification of PKARI/PKAc ratios showed that PKARI was silenced by 90 ± 3.9%. C, B82L cells transfected with PKARI or control siRNAs were used to IP RSK2 or RSK3. Immune complexes were blotted using phospho-Ser-380 RSK and respective RSK antibodies. A representative of three experiments is shown. D, lysates of B82L cells treated as described in A were analyzed by immunoblotting for PKARI and phospho-Erk1/2 content. Tubulin was used as loading control. The panel on the right shows quantitative analysis of the levels of phospho-Erk1/2 as a ratio of tubulin from three different experiments. Quantification of PKARI/tubulin ratios showed that PKARI was silenced by 82 ± 6.8%.
subunits (15) was examined. Silencing of PKARIα did not alter activation of either RSK2 or RSK3 (Fig. 1C) demonstrating that disruption of the PKARIα/RSK1 interactions specifically increased activation of RSK1. Moreover, the silencing of PKARIα did not alter the activation state of ERK1/2, the upstream kinases that activate RSK1 (Fig. 1D).

Silencing of PKARIα Alters the Cellular Distribution of Active RSK and Increases Cell Survival—Previously we showed that the indirect (via PKA subunits) association of RSK1 with AKAPs is necessary for the nuclear localization of the active RSK1 (15). Therefore, we determined whether silencing of PKARIα, which would decrease the association of active RSK1 with AKAPs, also alters its cellular localization. In control cells, 10 min after EGF treatment the majority of the phospho-Thr-573 RSK1 was detected in the nucleus as puncta (Fig. 2A). However, when PKARIα was silenced, the active (phospho-Thr-573) RSK1 puncta were distributed in the cytoplasm as well as in the nucleus (Fig. 2A).

Quantification of PKARIα knockdown as well as nuclear and cytosolic distribution of phospho-Thr-573 RSK1 are shown in the lower panels of FIGURE 2. Silencing of PKARIα alters cellular distribution of RSK. A, PKARIα was silenced in B82L cells before stimulation with or without EGF (50 nM, 10 min) and fixing. The active, phospho-RSK was detected using anti-phospho-Thr-573 RSK antibody and goat anti-rabbit antibody conjugated to Alexa Fluor 488. PKARIα was detected using anti-PKARIα antibody (BD Bioscience) and goat anti-mouse antibody conjugated to Alexa Fluor 594. The bottom left panel shows the quantification of the fluorescence intensity of the PKARIα staining (red) from 10 cells using NIH ImageJ software. The bottom right panel shows quantification (mean ± S.E.) of fluorescence intensity of nuclear and cytoplasmic phospho-Thr-573 RSK1 (green) from 10 cells using NIH ImageJ software. Statistical significance was assessed by Student’s unpaired t test (n = 10). *, p < 0.01 as compared with control siRNA. B, B82L cells transfected with siRNAs as described in panel A, were subjected to cell fractionation as described under “Experimental Procedures.” The cytosolic and nuclear fractions were analyzed for the presence of phospho-Ser-380 RSK1 and total RSK1. Phospholipase C-γ and histone H1 were used as loading controls for the cytoplasmic and nuclear fractions, respectively. The panel on the top right shows the amount of phospho-Ser-380 RSK1 as a ratio of the loading controls (mean ± S.E.) from three independent experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.01; **, p < 0.001 as compared with control siRNA. Quantification of PKARIα/respective loading controls ratios showed that PKARIα was silenced by 95 ± 2.5%. C, lysates (30 μg of protein) from B82L cells treated as described in panel A were immunoblotted for PKARIα and phospho-Ser-112 BAD. Erk1/2 was used as loading control. Quantification (mean ± S.E.) of phospho-Ser-112 BAD as a ratio of Erk1/2 is shown in the right middle panel from three different experiments. Statistical significance was assessed by Student’s unpaired t test; *, p < 0.02 as compared with control siRNA. Quantification of PKARIα/Erk1/2 ratios showed that PKARIα was silenced by 72 ± 8.8%. The bottom left panel shows Western analyses of lysates from B82L cells that had been pre-treated with or without SL-0101 (20 μM) for 4 h. The cells were then exposed to vehicle or EGF (50 nM for 10 min) and lysates analyzed for phospho-Ser-112 BAD and RSK1. Quantification of the phospho-Ser-112 BAD as a ratio of RSK1 from two independent experiments is shown in the bottom right panel.
of Fig. 2A and demonstrate that silencing of PKARIα by ~50% resulted in an increase in cytoplasmic active, phospho-Thr-573 RSK1 under basal (unstimulated) conditions. Additionally, after silencing of PKARIα, more of the EGF-activated RSK1 was present in the cytosol, and its amount in the nucleus was decreased as compared with cells treated with control siRNA (bottom right panel in Fig. 2A). Although the anti-phospho-Thr-573 antibody recognized RSK1, RSK2, and RSK3, because PKARIα silencing did not activate RSK2 and RSK3 (Fig. 1C), we infer that when PKARIα was silenced the increase in cytoplasmic phospho-RSK (Fig. 2A) was due to active RSK1. As a second approach, after treating cells with control or PKARIα-specific siRNA, we fractionated the cells into nuclear and cytosolic fractions. The nuclear proteins and acetone-precipitated proteins from the cytosolic fraction were then analyzed for their total as well as phospho-RSK1 content. As shown in Fig. 2B, in the absence of any agonist, the amount of phospho-Ser-380 RSK1 in the nucleus was increased along with the amount of total RSK1. On the other hand, the amount of total and phospho RSK1 in the nuclear fractions was decreased when PKARIα was silenced; loading controls for the cytosolic (phospholipase Cγ) and nuclear fractions (histone H1) were the same (Fig. 2B). The increased cytoplasmic localization of active RSK1 upon PKARIα silencing in EGF-treated cells (Fig. 2, A and B) is akin to what we have observed when the RSK1-AKAP interaction via PKARIα is disrupted by a peptide that dissociates PKARIα from AKAPs (15). Thus, by silencing PKARIα, and thereby decreasing its interactions with RSK1 and AKAPs, the distribution of active RSK1 in the cytoplasm is increased. These findings suggest that the indirect (via PKARIα) association of RSK1 with AKAPs is necessary for the nuclear localization of active RSK1.

Because increased amounts of active RSK1 in the cytoplasm (Fig. 2, A and B) would be expected to increase phosphorylation of its substrates, we also examined the phosphorylation of BAD on Ser-112, the RSK phosphorylation site (24). As shown in Fig. 2C, silencing of PKARIα increased the amount of BAD that was phosphorylated on Ser-112 both in the absence and presence of EGF. This increase in BAD phosphorylation was markedly attenuated by SL-301, the RSK1 inhibitor, demonstrating that the augmented Ser-112 phosphorylation when PKARIα was silenced was due to increased cytoplasmic content of active RSK1. Thus, collectively, the data in Fig. 2 show that silencing of PKARIα results in increased distribution of the active RSK1 in the cytoplasm with a resultant increase in phosphorylation of its cytoplasmic substrate BAD.

Next, we investigated whether PKARIα-induced increase in phosphorylation of BAD by RSK1 affected cellular apoptosis. Serum-deprived control and PKARIα siRNA-transfected B82L cells were treated with vehicle or CHX plus tumor necrosis factor-α (TNF-α), and DNA fragmentation was monitored. Fig. 3A shows that, in control cells, TNF/CHX treatment induced apoptosis and EGF partially protected against this. When PKARIα was silenced, the ability of TNF/CHX to induce apoptosis both in the absence and presence of EGF was markedly decreased (Fig. 3A). Similar results were also observed when cleaved PARP was used to monitor apoptosis in B82L cells (Fig. 3B). These findings are consistent with an increase in RSK1 activation and an increase in BAD phosphorylation on the Ser-112 (RSK1 site) under basal (unstimulated) conditions when PKARIα is silenced.

PKARIα Silencing-induced Increase in PKAc Activity Did Not Contribute to RSK1 Activation but Partially Contributed toward Augmented Cell Survival—Silencing of PKARIα would be expected to increase PKAc activity. Indeed, as shown in supplemental Fig. S2A), silencing of PKARIα increased the phosphorylation of PKA substrates (proteins with molecular masses of ~130 kDa, 67 kDa, and 35 kDa). Moreover, preincubation of cells with the cell-permeable PKA inhibitor peptide, PKI, decreased the phosphorylation of PKA substrates when PKARIα was silenced (supplemental Fig. S2B). Preincubation of cells with PKI also inhibited the ability of cAMP to phosphorylate the PKA substrates (supplemental Fig. S2E). However, PKI did not modulate the activation of RSK1 when PKARIα was silenced (supplemental Fig. S2C). Thus, although PKAc activity is augmented upon silencing of
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PKARIα, PKAc did not contribute toward the activation of RSK1 under basal conditions. Phosphorylation of "PKA substrates" by other basophilic AGC family of protein kinases such as RSK1 could also augment the immunoreactivity of the proteins when PKARIα is silenced. Therefore, to determine whether or not RSK1 that is activated upon PKARIα silencing contributes to the increased phosphorylation of PKA substrates observed with the anti-phospho-PKA substrate antibody (FIGURE 4).

**FIGURE 4.** Decreased apoptosis after PKARIα silencing mediated protection against apoptosis. A, B82L cells were co-transfected with 40 nm each of control, PKARIα, BAD, or scrambled siRNAs. After 72 h of transfection, the cells were treated with TNF-α (20 ng/ml) plus cycloheximide (25 μg/ml) (TNF-α/CHX) for 1 h, and DNA fragmentation was monitored as described previously. Data are mean ± S.E. of A405 nm per microgram of protein (n = 3). B, immunoblots of siRNA-treated cell lysates for phospho-Ser-380 RSK, PKARIα, and BAD content. Total RSK1 was used as loading control. Quantification of PKARIα/RSK1 and BAD/RSK1 ratios showed that PKARIα and BAD were silenced by 78 ± 8.5% and 76 ± 11.2%, respectively.

PKARIα, PKAc did not contribute toward the activation of RSK1 under basal conditions. Phosphorylation of "PKA substrates" by other basophilic AGC family of protein kinases such as RSK1 could also augment the immunoreactivity of the proteins when PKARIα is silenced. Therefore, to determine whether or not RSK1 that is activated upon PKARIα silencing contributes to the increased phosphorylation of PKA substrates observed with the anti-phospho-PKA substrate antibody (FIGURE 4).

**FIGURE 5.** BAD is required for PKARIα silencing mediated protection against apoptosis. A, B82L cells were co-transfected with 40 nm each of control, PKARIα, BAD, or scrambled siRNAs. After 72 h of transfection, the cells were treated with TNF-α (20 ng/ml) plus cycloheximide (25 μg/ml) (TNF-α/CHX) for 1 h, and DNA fragmentation was monitored as described previously. Data are mean ± S.E. of A405 nm per microgram of protein (n = 3). B, immunoblots of siRNA-treated cell lysates for phospho-Ser-380 RSK, PKARIα, and BAD content. Total RSK1 was used as loading control. Quantification of PKARIα/RSK1 and BAD/RSK1 ratios showed that PKARIα and BAD were silenced by 78 ± 8.5% and 76 ± 11.2%, respectively.

**FIGURE 4.** Decreased apoptosis after PKARIα silencing is due to increased PKAc and RSK1 activities. A, after transfection of control or PKARIα siRNAs, cells were treated with or without 10 μM PKI (16 h) or 100 μM 8CPT-cAMP (30 min). Following IP of BAD, the amounts of phospho-Ser-155 BAD and total BAD were monitored by Western analyses. The bottom panel shows the quantification (mean ± S.E.) of the ratios of phospho-Ser-155 BAD/BAD in the IPs of BAD from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). B, B82L cells (50,000 cells/well) were transfected as described in panel A, followed by treatment with 10 μM of PKI for 16 h or 3 μM of fmk for 15 min. TNF-α (20 ng/ml) plus cycloheximide (25 μg/ml) (TNF-α/CHX) were then added for 1 h and DNA fragmentation was monitored. Data are mean ± S.E. of A405 nm per microgram of protein (n = 3). Statistical significance was assessed by Student’s unpaired t test (n = 3). C, lysates of cells treated as described in panel B were immunoblotted for phospho-Ser-112 BAD, phospho-Ser-155 BAD, and total Erk1/2 (loading control) content. The bottom panels show quantification (mean ± S.E.) of the ratios of phospho-Ser-155 BAD/Erk1/2 (left) and phospho-Ser-112 BAD/Erk1/2 (right) in the cell lysates from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.03 as compared with control siRNA.
strate antibody in supplemental Figs. S2A and S2B, after transfecting the cells with control or PKARI-α-specific siRNAs, we treated the cells with two RSK1 inhibitors, fmk (38) and SL-0101. As shown in supplemental Fig. S2D neither fmk nor SL-0101 diminished the increase in immuno-reactivity of cellular proteins to the anti-phospho-PKA substrate antibody when PKARI-α was knocked down. That fmk and SL-0101 effectively inhibit RSK1 is shown by data in Figs. 1, 2C, and 4C. Overall, therefore, the supplemental data provided (supplemental Fig. S2) demonstrate that the PKARI-α silencing increases PKAc activity, but this increase in PKAc activity did not contribute toward activation of RSK1 and that PKI specifically inhibited PKAc. Moreover, the data in supplemental Fig. S2D demonstrate that activation of RSK1 upon PKARI-α silencing did not contribute to the increase in anti-phospho-PKA substrate antibody reactivity.

Because PKARI-α silencing augments PKAc activity (supplemental Figs. S2A and S2B), we investigated whether the increase in PKAc activity also enhanced phosphorylation of BAD on Ser-155, the PKAc site (36, 37), and altered cell survival. As shown in Fig. 4A, like cAMP (positive control), silencing of PKARI-α increased the phosphorylation of BAD on Ser-155. That the enhanced phosphorylation of BAD on Ser-155 in PKARI-α-silenced cells is due to elevated PKAc activity is demonstrated by the ability of PKI to decrease this phosphorylation (Fig. 4A, cf. lanes 4 and 5).

Because PKARI-α silencing augments BAD phosphorylation on both the PKAc (Ser-155) and RSK1 (Ser-112) sites (Figs. 2C and 4A), using PKA- and RSK1/2-selective inhibitors PKI and fmk (38), respectively, we investigated the contribution of the two kinases in enhanced cell survival when PKARI-α is silenced. As shown before (Fig. 3A), TNF-α plus CHX increased cellular apoptosis, and silencing of PKARI-α protected the cells from

FIGURE 6. PP2Ac exists in the RSK-PKA-D-AKAP1 complex. B82L cells were treated with 20 μM each of Ht31 and Ht31P peptides for 15 min or transfected with control or PKARI-α-specific siRNAs (20 nM) for 72 h. A Cell lysates were analyzed for PKARI-α, phospho-Ser-380 RSK, and total Erk1/2 (loading control). B and C, following treatments as in panel A, RSK1 or PP2Ac, or D-AKAP1 were immunoprecipitated, and the presence of PP2Ac, PKAc, RSK1, and D-AKAP1 in the immune complexes was monitored by Western analyses. That neither Ht31 nor PKARI-α silencing altered the amounts of PKARI-α, D-AKAP1, PKAc, PP2A is shown in supplemental Fig. S3C. Quantification of the data in B and C are presented as supplemental Fig. S4. D, total RNA was isolated from clonal cells expressing control shRNA or shRNA against D-AKAP1, and equal amounts of RNA were reverse-transcribed using specific primers for D-AKAP1 and prolylhydroxylase 2 as described under "Experimental Procedures." Equal volumes of PCR products were analyzed on 1.5% agarose gel. D-AKAP1/prolylhydroxylase 2 ratios are presented at the bottom to indicate the extent of D-AKAP1 silencing. E, RSK1 was immunoprecipitated from the lysates of control or D-AKAP1 shRNA-expressing clones. Immune complexes were analyzed by Western analyses using specific antibodies.
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![Figure 7. Schematic depicting the interactions of RSK1 with subunits of PKA, D-AKAP1, and PP2Ac. As demonstrated previously (15), inactive RSK1 is bound to PKAR\(\alpha\). Activation of RSK1 results in its interactions with PKAc and PKARII subunits of PKA from AKAPs (8, 41), we showed that Ht31, but not the control peptide Ht31P, dissociates RSK1 from AKAPs such as D-AKAP1 (15). As shown in the supplemental data (Fig. S3) and Fig. 6A, Ht31, but not Ht31P, treatment of unstimulated cells increased phosphorylation and kinase activity of RSK1. These data suggest that dissociation of RSK1 from AKAP complexes results in activation of RSK1. Further, because neither Ht31 nor PKAR\(\alpha\) silencing altered the activity of the immediate upstream kinase of RSK1, ERK1/2 (Fig. 1D and data not shown), we reasoned that the influence of a phosphatase that normally dephosphorylates RSK1 is removed upon dissociation of RSK1 from AKAPs. To test this hypothesis and to identify the AKAP in this tetra-complex, we immunoprecipitated RSK1 from cells treated with \(a\) control or PKAR\(\alpha\)-specific siRNAs, \(b\) Ht31 and Ht31P, and \(c\) control or shRNA-expressing clones against D-AKAP1. The immunocomplexes were analyzed for presence of phosphatases and changes in phosphorylation of RSK1. Fig. 6A confirms that, in these experiments, PKAR\(\alpha\) silencing or Ht31 effectively increased the phosphorylation of RSK1 in unstimulated cells. Moreover, as shown in Fig. 6B, RSK1 IPs from control siRNA-treated or Ht31P-treated B82L cells contain significant amount of protein phosphatase 2A catalytic subunit (PP2Ac) when compared with PKAR\(\alpha\) siRNA- or Ht31P-treated cells. Conversely, in IPs of PP2Ac, the amount of RSK1 present was increased when the cells had been treated with either PKAR\(\alpha\)-specific siRNA or Ht31, but not Ht31P (Fig. 6B). These results suggest that treatment of cells with PKAR\(\alpha\)-specific siRNA or Ht31 dissociates RSK1 from AKAP complexes with a resultant decrease in the amount of PP2Ac in RSK1 IPs. Consistent with this notion, in IPs of RSK1 the amount of D-AKAP1 was decreased when the cells had been treated with either PKAR\(\alpha\)-specific siRNA or Ht31, but not Ht31P (Fig. 6B). Of note, BAD silencing did not alter the ability of PKAR\(\alpha\) silencing to augment RSK1 activation or affect the total amount of RSK1 (Fig. 5B). These data (Fig. 5), clearly demonstrate that the anti-apoptotic actions of PKAR\(\alpha\) are mediated by BAD.

PP2A Exists in a Complex with RSK1-PKA-D-AKAP1 and Ht31 Peptide or PKAR\(\alpha\) siRNA Dissociates PP2A from RSK1—PKAR\(\alpha\) permits the indirect association of RSK1 with AKAP(s) (15). Thus, it is likely that when PKAR\(\alpha\) is depleted RSK1 is activated due to its dissociation from the AKAP(s). In this case, dissociation of RSK1 from AKAP(s) by alternate means should also result in activation of RSK1 without the need for growth factors. Using the cell-permeable peptide Ht31 that dissociates both PKAR\(\alpha\) and PKARII subunits of PKA from AKAPs (8, 41), we showed that Ht31, but not the control peptide Ht31P, dissociates RSK1 from AKAPs such as D-AKAP1 (15). As shown in the supplemental data (Fig. S3) and Fig. 6A, Ht31, but not Ht31P, treatment of unstimulated cells increased phosphorylation and in vitro kinase activity of RSK1. These data suggest that dissociation of RSK1 from AKAP complexes results in activation of RSK1. Further, because neither Ht31 nor PKAR\(\alpha\) silencing altered the activity of the immediate upstream kinase of RSK1, ERK1/2 (Fig. 1D and data not shown), we reasoned that the influence of a phosphatase that normally dephosphorylates RSK1 is removed upon dissociation of RSK1 from AKAPs. To test this hypothesis and to identify the AKAP in this tetra-complex, we immunoprecipitated RSK1 from cells treated with \(a\) control or PKAR\(\alpha\)-specific siRNAs, \(b\) Ht31 and Ht31P, and \(c\) control or shRNA-expressing clones against D-AKAP1. The immunocomplexes were analyzed for presence of phosphatases and changes in phosphorylation of RSK1. Fig. 6A confirms that, in these experiments, PKAR\(\alpha\) silencing or Ht31 effectively increased the phosphorylation of RSK1 in unstimulated cells. Moreover, as shown in Fig. 6B, RSK1 IPs from control siRNA-treated or Ht31P-treated B82L cells contain significant amount of protein phosphatase 2A catalytic subunit (PP2Ac) when compared with PKAR\(\alpha\) siRNA- or Ht31P-treated cells. Conversely, in IPs of PP2Ac, the amount of RSK1 present was increased when the cells had been treated with either PKAR\(\alpha\) specific siRNA or Ht31, but not Ht31P (Fig. 6B). These results suggest that treatment of cells with PKAR\(\alpha\)-specific siRNA or Ht31 dissociates RSK1 from AKAP complexes with a resultant decrease in the amount of PP2Ac in RSK1 IPs. Consistent with this notion, in IPs of RSK1 the amount of D-AKAP1 was decreased when the cells had been treated with either PKAR\(\alpha\)-specific siRNA or Ht31, but not Ht31P (Fig. 6B). Of note, BAD silencing did not alter the ability of PKAR\(\alpha\) silencing to augment RSK1 activation or affect the total amount of RSK1 (Fig. 5B). These data (Fig. 5), clearly demonstrate that the anti-apoptotic actions of PKAR\(\alpha\) are mediated by BAD.

against BAD, as observed before (Figs. 3A and 4B), silencing of PKAR\(\alpha\) protected against TNF-\(\alpha\)/CHX-mediated apoptosis. However, when the expression of BAD was silenced, knockdown of PKAR\(\alpha\) did not protect against TNF-\(\alpha\)/CHX-induced apoptosis (Fig. 5A). To conclusively demonstrate that the anti-apoptotic actions of PKAR\(\alpha\) are mediated by BAD.

as shown in Fig. 5A, in cells treated with scrambled siRNA apoptosis (Fig. 4B). Treatment of cells with PKI did not modulate apoptosis in the absence of TNF/CHX. However, PKI partially reversed the PKAR\(\alpha\) silencing-induced cell survival in the presence of TNF/CHX (Fig. 4B). On the other hand, the RSK1/2-selective inhibitor, fmk, increased apoptosis in the absence of TNF/CHX and completely abolished anti-apoptotic actions of PKAR\(\alpha\) silencing (Fig. 4B). To facilitate data interpretation, we monitored the phosphorylation of BAD on the PKAc and RSK1 sites in the presence and absence of PKI and fmk. As shown in Fig. 4C, PKI inhibited the phosphorylation of BAD on Ser-155, the PKAc site, without affecting its phosphorylation on Ser-112, the RSK1 site. These data show that PKI specifically inhibited the anti-apoptotic pathway downstream of PKAc and that the partial reversal of cell survival by PKI in PKAR\(\alpha\) silenced cells observed in Fig. 4B is due to the elevated PKAc activity. Consistent with previous reports that phosphorylation of Ser-112 precedes phosphorylation of Ser-155 (39) and that binding of 14-3-3 to BAD phosphorylated on Ser-112 and Ser-136 exposes Ser-155 for phosphorylation (40), fmk inhibited phosphorylation of BAD on Ser-112, the RSK1 site, as well as phosphorylation of Ser-155 (Fig. 4C). Collectively, the data in Fig. 4 permit the following inferences. First, the elevated PKAc activity in PKAR\(\alpha\)-silenced cells, in part, contributed toward cell survival. Second, fmk by inhibiting basal phosphorylation of BAD on the RSK1 and PKAc sites (Fig. 4C), increased cellular apoptosis in the absence of TNF-\(\alpha\) and CHX (Fig. 4A). Third, fmk, by abrogating phosphorylation of BAD on both PKAc and RSK1 sites completely reversed the pro-survival actions of PKAR\(\alpha\) silencing. Overall, therefore, both kinases (PKAc and RSK1) that are activated by PKAR\(\alpha\) silencing contribute toward enhanced cell survival.

To conclusively demonstrate that the anti-apoptotic actions of PKAR\(\alpha\) silencing are mediated by changes in BAD phosphorylation, we performed experiments in which BAD was silenced in cells treated with either control or PKAR\(\alpha\)-specific siRNA. As shown in Fig. 5A, in cells treated with scrambled siRNA
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Although the data in Fig. 6 (A–C) demonstrate that D-AKAP1, RSK1, PKARια, and PP2Ac exist in a complex, they do not demonstrate which of the one or more proteins in this complex the PP2Ac is associated with, nor do they provide a role for D-AKAP1 in the activation of RSK1. Therefore, using three different shRNAs, the amount of D-AKAP1 in B82L cells was depleted. Fig. 6D shows that, as monitored by semi-quantitative PCR, the expression of D-AKAP1 was significantly inhibited by all three shRNAs used; the amount of the control mRNA (prolylhydroxylase 2) was not altered by the shRNAs against D-AKAP1. Moreover, immunoprecipitates of RSK1 from control and shRNA-treated cells showed that silencing of D-AKAP1 increased the phosphorylation of RSK1 under basal conditions and that the co-immunoprecipitation of PP2A was significantly reduced after depletion of D-AKAP1 (Fig. 6E); note that depletion of D-AKAP1 did not alter the expression of PP2A or PKARια (Fig. 6E). Collectively, the data in Fig. 6 show that, in the complex comprising D-AKAP1, RSK1, PKARια, PKAc, and PP2Ac, the PP2Ac is tethered to D-AKAP1 (see model in Fig. 7). This would explain why the amount of PP2Ac decreased in IPs of RSK1 after PKARια was silenced and why, despite a decrease in RSK1 in the IPs of D-AKAP1, the amount of PP2Ac did not change (Fig. 6C). Additionally, this model (Fig. 7) also explains why either D-AKAP1 depletion or competing the interactions between PKARια and D-AKAP1 with Ht31 peptide decreased the amount of PP2Ac in complex with RSK1. The data in Fig. 6 also strongly suggest that increased phosphorylation of RSK1 under basal conditions when RSK1 was dissociated from D-AKAP1, by silencing of PKARια or depleting D-AKAP1 or by Ht31, was due to a decrease in PP2Ac content in the proximity of RSK1 (see Fig. 7 for model).

PP2Ac Dephosphorylates RSK—That PP2Ac is one of the phosphatases that dephosphorylates RSK is shown by data in Fig. 8 (A and B). B82L cells that had been pre-treated with vehicle or okadaic acid (Fig. 8A) or siRNA against PP2Ac (Fig. 8B) were treated with EGF for 5 min. Immediately thereafter, the MEK inhibitor U0126 was added to inhibit ERK1/2-elicted phosphorylation of RSK, and the dephosphorylation of RSK on Ser-380 was followed over 2 h. In control (DMSO or sham-treated) cells, 5 min after the addition of EGF, phosphorylation of ERK1/2 and RSK was markedly enhanced and the addition of MEK inhibitor, U0126, at time point depicted by arrowheads resulted in inhibition of both ERK1/2 and RSK phosphorylation (Fig. 8, A and B). These results demonstrate that, upon MEK inhibition, there is very rapid dephosphorylation of ERK1/2 and RSK. On the other hand, when cells were decreased when PKARια was silenced (Fig. 6C, left panel), and, conversely, in IPs of D-AKAP1 the amount of RSK1 was decreased by knockdown of PKARια (Fig. 6C, right panel). Moreover, D-AKAP1 IPs also contained PP2Ac, whose amount remained unchanged when PKARια was silenced (Fig. 6C, right panel). Notably, neither silencing of PKARια nor Ht31 altered the cellular amounts of D-AKAP1, PKAc, or PP2A (supplemental Fig. S3C).

PP2Ac dephosphorylates RSK. A, serum-starved B82L cells were treated with 1 μM okadaic acid for 60 min before EGF (50 nM) addition. After 5 min of EGF treatment, U0126 (20 μM) was added (depicted by vertical arrows). Cell lysates obtained at the indicated times were immunoblotted using anti-phospho-Ser-380 RSK1, anti-phospho-Erk1/2 antibodies, and total Erk1/2 (loading control). A representative of three similar, independent experiments is shown. The lower panel represents the quantification (mean ± S.E.) of the ratios of pSer-380 RSK/total RSK1 from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.01; **, p < 0.05 as compared with DMSO control. B, same as panel A, except that 48 h before EGF treatment, B82L cells were sham transfected or transfected with 40 nm siRNA against PP2Ac (quantification of PP2A/Erk1/2 ratios showed that PP2A was silenced by 76 ± 7.8%). A representative of three similar, independent, experiments is shown. The lower panel represents the quantification (mean ± S.E.) of the ratios of phospho-Ser-380 RSK/total RSK1 from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.02 as compared with sham control.

FIGURE 8. PP2Ac dephosphorylates RSK. A, serum-starved B82L cells were treated with 1 μM okadaic acid for 60 min before EGF (50 nM) addition. After 5 min of EGF treatment, U0126 (20 μM) was added (depicted by vertical arrows). Cell lysates obtained at the indicated times were immunoblotted using anti-phospho-Ser-380 RSK1, anti-phospho-Erk1/2 antibodies, and total Erk1/2 (loading control). A representative of three similar, independent experiments is shown. The lower panel represents the quantification (mean ± S.E.) of the ratios of pSer-380 RSK/total RSK1 from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.01; **, p < 0.05 as compared with DMSO control. B, same as panel A, except that 48 h before EGF treatment, B82L cells were sham transfected or transfected with 40 nm siRNA against PP2Ac (quantification of PP2A/Erk1/2 ratios showed that PP2A was silenced by 76 ± 7.8%). A representative of three similar, independent, experiments is shown. The lower panel represents the quantification (mean ± S.E.) of the ratios of phospho-Ser-380 RSK/total RSK1 from three different experiments. Statistical significance was assessed by Student’s unpaired t test (n = 3). *, p < 0.02 as compared with sham control.
pre-treated with okadaic acid (Fig. 8A) or siRNA against PP2Ac (Fig. 8B), the basal phosphorylation of RSK, but not ERK1/2, was enhanced mimicking the actions of PKAR1α silencing, D-AKAP1 depletion, or Ht31 treatment (Figs. 1D, 6E, and supplemental Fig. S3). Addition of EGF to these cells further augmented phosphorylation of both ERK1/2 and RSK (Fig. 8, A and B). Notably, however, when PP2Ac was either inhibited or depleted, the addition of U0126 rapidly abrogated ERK1/2 phosphorylation, and the dephosphorylation of RSK took more than 2 h. These data (Fig. 8, A and B) demonstrated that inhibition of PP2Ac by two different approaches increased the basal (unstimulated) activation of RSK without any significant increase in ERK1/2 activity. Second, when PP2Ac was silenced or inhibited by okadaic acid, the dephosphorylation of RSK occurred over a longer time supporting the notion that PP2Ac is one of the phosphatases that dephosphorylates RSK. The inability of okadaic acid or depletion of PP2Ac to alter the rapid ERK1/2 dephosphorylation also demonstrates that PP2Ac does not participate in the dephosphorylation of ERK1/2. Together, the data in Figs. 6 and 8 support the model that is proposed in Fig. 7 and demonstrate that the existence of RSK1 in proximity to PP2Ac within the complex, which also includes PKA subunits and D-AKAP1, is necessary for decreasing its activity and that, when PP2Ac is no longer in proximity to RSK1, the phosphorylation of RSK1 is augmented. The latter would require that ERK1/2 be active in unstimulated cells. Indeed, as shown by longer exposures (Fig. 8B), significant amounts of ERK1/2 are phosphorylated and active in unstimulated cells so that when the PP2Ac is inhibited by either siRNA or okadaic acid, RSK1 phosphorylation is augmented without the need for growth factors (Fig. 8, A and B).

Although, PP2Cδ has been shown to associate with RSK1-4 presumably via the N-terminal kinase of RSKs (42), its role in dephosphorylation of RSKs in intact cells has not been demonstrated. In this context, our findings identify PP2A as the first phosphatase that dephosphorylates RSK1. Moreover, using at least three different approaches, our studies validate the necessity for maintaining PP2Ac in close proximity of RSK1 so that the latter is maintained inactive in resting cells under basal conditions. Disruptions of interactions among the proteins within the complex that RSK1 resides in result in the removal of PP2Ac from the proximity to RSK1 and, therefore, activation of RSK1 even in the absence of any agonists with the resultant increase in processes such as cell survival. Because D-AKAP1 contains mitochondrial targeting sequence (11), an interesting implication of our findings that PP2A, RSK1, and PKA subunits and D-AKAP1 exist in a complex is that D-AKAP1 ensures the localization of RSK1 and PKA in the proximity to BAD/Bcl complexes such that alterations in activity of either kinase can regulate BAD phosphorylation and apoptosis.

The underlying genetic defect in Carney complex (CNC) is haploinsufficiency of PKAR1α due to a number of different mutations, including large deletions in the PKAR1A gene (16–20, 43). Although PKAc activity is elevated in this condition, its role in the increased tumorigenesis in CNC has been questioned (21). Nevertheless, a recent study has suggested that elevated PKAc activity in the adrenal tissue of CNC patients, via activation of B-Raf, may augment the activity of MEK and Erk1/2 (44). While we observed that PKAR1α silencing increased the activity of PKAc, we did not observe any activation of Erk1/2. Instead, PKAR1α silencing increased activation of RSK1 downstream of Erk1/2 by decreasing the association via D-AKAP1 of RSK1 with PP2Ac. Because RSK1 augments cell survival (24, 25), and because RSK1/2 inhibitors decrease the proliferation of certain forms of tumors (22, 23), it is possible that, in CNC, decreases in PKAR1α amount lead to tumorigenesis via activation of RSK1. This would be particularly relevant in cell types and tissues in which PKA-regulated Raf forms (e.g. B-Raf) are not expressed.

Besides regulating activation of RSK1, PKAR1α also regulates the cellular localization of active RSK1 and its function. Unlike RSK3 (45), RSK1 does not have a clear nuclear localization sequence. As shown previously (15), and here, the cytoplasmic and nuclear distribution of active RSK1 depends upon its interactions, via PKAR1α, with AKAPs. Thus, in CNC when PKAR1α amounts are decreased, it is possible that, besides augmenting the activation of RSK1 and increasing its cytoplasmic content, phosphorylation of cytoplasmic RSK1 substrates as well as signaling via certain pathways may be enhanced. One of the major pathways regulated by RSK1 is the cell survival pathway, and as shown here, silencing of PKAR1α markedly augments cell survival. Whether increased cytoplasmic amounts of active RSK1 enhance the survival of tumor cells in CNC remains to be determined.

Although PKAR1α may act as a “tumor suppressor” in CNC, previous studies have also reported that PKAR1α facilitates the cell proliferative signaling of hormones and growth factors in certain types of cancer cells (46) and depletion of PKAR1α effectively inhibits transcription of certain genes and growth of tumor cells (47). Thus, PKAR1α may augment or inhibit cellular proliferation in a cell type-specific manner. Given the role of PKAR1α in ensuring the nuclear localization of active RSK1 and, thereby, augmenting phosphorylation of its nuclear substrates, it is tempting to speculate that in some cell types increases in PKAR1α facilitate the phosphorylation of nuclear substrates of RSK1 to regulate transcription of certain genes and thereby augment cell proliferation.

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REFERENCES
1. Døskeland, S. O., Maronde, E., and Gjertsen, B. T. (1993) Biochim. Biophys. Acta 1178, 249–258.
2. Skålhegg, B. S., and Taskén, K. (1997) Front Biosci. 2, d331–d342.
3. Buechel, Y. I., and Taylor, S. S. (1991) J. Biol. Chem. 266, 3491–3497.
4. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 407–414.
5. Taylor, S. S., Kim, C., Vigil, D., Haste, N. M., Yang, J., Wu, J., and Anand, G. S. (2005) Biochim. Biophys. Acta 1754, 25–37.
6. Kim, C., Xuong, N. H., and Taylor, S. S. (2005) Science 307, 690–696.
7. Wong, W., and Scott, J. D. (2004) Nat. Rev. Mol. Cell Biol. 5, 959–970.
8. Michel, J. I., and Scott, J. D. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 235–257.
9. Alto, N. M., and Scott, J. D. (2004) Cell Biochem. Biophys. 40, Suppl. 3, 201–208.
10. Huang, L. J., Durick, K., Weiner, J. A., Chun, J., and Taylor, S. S. (1997)
