Previously, we reported that the catalytic subunit of cAMP-dependent protein kinase (PKAc) binds to the active p90 ribosomal S6 kinase 1 (RSK1) (Chaturvedi, D., Poppleton, H. M., Stringfield, T., Barbier, A., and Patel, T. B. (2006) Mol. Cell. Biol. 26, 4586–4600). Herein, by overexpressing hemagglutinin-tagged RSK1 fragments in HeLa cells we have identified the region of RSK1 that is responsible for the interaction with PKAc. PKAc bound to the last 13 amino acids of RSK1, which overlaps the Erk1/2 docking site. This interaction between PKAc and RSK1 required the phosphorylation of Ser-732 in the C terminus of RSK1. Depending upon its phosphorylation status, RSK1 switched interactions between Erk1/2 and PKAc. In addition, a peptide corresponding to the last 13 amino acids of RSK1 with substitution of Ser-732 with Glu (peptide E), but not Ala (peptide A), decreased interactions between endogenous active RSK1 and PKAc. RSK1 attenuated the ability of cAMP to activate PKA in vitro and this modulation was abrogated by peptide E, but not by peptide A. Similarly, in intact cells, cAMP-mediated phosphorylation of Bcl-xL/Bcl-2-associated death promoter on Ser-115, the PKA site, was reduced when RSK1 was activated by epidermal growth factor, and this effect was blocked by peptide E, but not by peptide A. These findings demonstrate that interactions between endogenous RSK1 and PKAc in intact cells regulate the ability of cAMP to activate PKA and identify a novel mechanism by which PKA activity is regulated by the Erk1/2 pathway.

cAMP-dependent protein kinase (PKA)\(^2\) is ubiquitously distributed in a variety of tissues and cell types and has been shown to regulate a large number of biological functions ranging from regulation of inotropic and chronotropic actions in the heart to regulation of tumorigenesis as well as modulation of long term potentiation and, therefore, memory. PKA is a heterotetramer composed of two catalytic subunits (PKAc) bound to a dimer of regulatory subunits. To date four catalytic (PKAc-\(\alpha\) to PKAc-\(\delta\)) and four regulatory subunit isoforms (PKAR\(\alpha\)a, PKAR\(\alpha\)b, PKAR\(\beta\)a, and PKAR\(\beta\)b) have been described (1). Depending upon the type of regulatory subunit (PKARI or PKARII) that the catalytic subunits are bound to, the PKA holoenzyme is referred to either as type I or type II (1). The different isoforms are differentially expressed in a cell- and tissue-specific manner (2). Besides binding to the regulatory subunits, PKAc has also been shown to bind a number of proteins. For instance, akin to the binding with regulatory subunits, binding of PKAc to the inhibitory protein of nuclear factor kappa-B (NF\(\kappa\)B), IkB-\(\alpha\), inhibits PKAc catalytic activity (3). Upon degradation of IkB-\(\alpha\) during NF\(\kappa\)B activation, PKAc is released and activated in a cAMP-independent manner (3). Likewise, the small GTP-binding protein Rab13 binds with and inhibits PKAc activity during tight junction formation (4). Thus, proteins other than the regulatory subunits of PKA can modulate the activity of the catalytic subunits of this enzyme.

The p90 ribosomal S6 kinases, a family of proteins with two kinase domains, are immediately downstream of the extracellular signal-regulated protein kinases 1/2 (Erk1/2). Among the four members in this family, RSK1, RSK2, and RSK3 share considerable sequence homology with RSK4 being larger and also functionally distinct (5–7). Although RSK1, -2, and -3 are similar in their primary structure, these enzymes have non-redundant functions. Thus, despite the normal overlapping expression patterns of RSK1 and RSK3 in brains of patients with Coffin-Lowry syndrome where RSK2 is mutated, the functions of the mutant RSK2 are not taken over by the other isoforms (8). Likewise, RSK1, but not RSK2, causes differentiation of PC12 cells (9). Previously, we reported that the inactive form of RSK1 interacts with the PKAR\(\alpha\)a subunit of PKA, whereas the activated RSK1 interacts with PKAc (10). The indirect association of RSK1 with PKA anchoring proteins (AKAPs), via PKA subunits, determines the cellular localization of activated RSK1 and its ability to increase cell survival (10). Notably, RSK1, but not RSK2 or RSK3, interacts with the PKA subunits (10). Moreover, the interactions of RSK1 with PKA subunits and PKA anchoring proteins bring the RSK1 in proximity to PP2Ac, which then regulates the activation of RSK1 (11). However, the role of the interactions of RSK1 with the subunits of PKA on regulation of PKA activity has not been extensively studied. Likewise, regions on RSK1 involved in the interactions with PKAc or PKAR\(\alpha\)a are unknown.
In this report, we demonstrate that the extreme C-terminal 13 residues of active RSK1 form the PKAc binding site. PKAc binds this region on RSK1 when the Ser-732 is phosphorylated. The extracellular signal-regulated kinases Erk1/2, which initiate the activation of RSK1, bind to the same region when Ser-732 is not phosphorylated. Thus, the phosphorylation state of Ser-732 on RSK1 serves as a switch for binding either Erk1/2 or PKAc. We also show that the binding of RSK1 to PKAc decreases the ability of cAMP to stimulate PKA activity both in vitro and in intact cells. These findings provide a new mechanism for regulation of PKA activity.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Anti-RSK1, anti-PKAc polyclonal, and anti-GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PKARIα and anti-PKAc monoclonal antibodies were from BD Biosciences (Palo Alto, CA). The anti-Erk1/2 antibody was from Upstate Biotechnology (Lake Placid, NY). The anti-phospho-RSK1 (S380) was from Epitomics (Burlingame, CA). Anti-phospho-RSK (T573), anti-phospho-BAD (S112), and anti-phospho-BAD (S155) were from Cell Signaling (Beverly, MA). The anti-HA-horseradish peroxidase was from Roche Applied Science (Indianapolis, IN), and the anti-HA-phosphorylase antibody was from Covance (Berkley, CA). Purified PKAc and PKARIα proteins were provided by Dr. Susan Taylor (University of California, San Diego). PKA inhibitor peptide (PKI) was from Biomol International (Plymouth Meeting, PA). Peptides corresponding to RSK1 amino acid residues 723–735 with or without substitution of Ser were synthesized by New England Peptide (Gardner, MA). These RSK1 peptides were palmitoylated at the N terminus to facilitate cell membrane permeation. Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) was purchased from Sigma-Aldrich.

**Cell Culture and Transfection**—Both B82L cells (mouse lung fibroblasts) and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and penicillin/streptomycin. Methotrexate was added to the above medium at the concentration of 5 μg/ml for maintenance of B82L cells. HeLa cells were plated in 60-mm dishes at 4 x 10⁵/dish. On the next day, cells were transfected with plasmids using a TransIT-HeLaMONSTER® Transfection kit (Mirus Bio Corp., Madison, WI) and by following the manufacturer’s protocol.

**Plasmid Constructs**—cDNA sequences corresponding to the different regions of RSK1 were PCR-synthesized using the plasmid containing full-length rat RSK1 as template (provided by Dr. Warner Greene, University of California, San Francisco) and primers with EcoRI or NotI restriction enzyme sequences. The PCR products were digested with EcoRI and NotI and then inserted in vector pHM6 to express the HA-tagged RSK1 polypeptides.

**Silencing of RSK1**—B82L cells (1.5 x 10⁵/dish) were plated on 35-mm dishes. After 24 h, cells were transfected with control siRNA or two siRNAs against mouse RSK1. The first siRNA sequence was: sense, GGA CCA AGA UGG AGA GAG ACA UCC T; antisense, AGG AUG UCU CUC UCC AUC UUG GUC CGA. The second siRNA sequence was: sense, CCU CUA UGU GGA UGA GUC UGG GAA C; antisense, GUU CCC AGA CUC AUC CAC AUA GAG GAU. Transit TKO reagent (Mirus) was used for siRNA transfection as described in the manufacturer’s instructions. Briefly, 8 μl of transit TKO reagent was incubated with 200 μl of Opti-MEM for 12 min followed by addition of 25 or 50 pmol of siRNA and another 12-min incubation. The mixture was added to the cells cultured in 1 ml of Dulbecco’s modified Eagle’s medium supplemented with serum. After 56 h, cells were deprived of serum overnight and then used for experiments.

**Immunoprecipitation**—HeLa cells were serum-starved overnight and then were treated with or without 100 nM EGF for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline and scraped into lysis buffer containing 50 mM Hepes, pH 8.0, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 1% Triton X-100, 4 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin, leupeptin, and pepstatin. Cell lysates were cleared by centrifugation at 20,000 x g for 15 min. The supernatant was incubated for 2 h at 4 °C with 2 μg of anti-PKAc or 0.4 μg of anti-RSK1 antibody (both from Santa Cruz Biotechnology) together with 15 μl of protein G-conjugated agarose beads to immunoprecipitate PKAc, PKARIα, RSK1, HA-RSK1, or fragments of RSK1. After three thorough washes with lysis buffer, the immunoprecipitates were eluted with Laemmli sample buffer and subjected to polyacrylamide gel electrophoresis for Western analyses.

**In Vitro Kinase Assays**—Purified PKAc (0.5 pmol) and PKARIα (10 pmol) were incubated in an assay solution containing 20 mM Hepes, pH 7.4, 20 mM MgCl₂, 8 mM MnCl₂, 4 mM dithiothreitol, 20 mM NaF, 0.1 mg/ml bovine serum albumin, and 0.125 mM ATP at room temperature for 3 h. The mixture was then incubated with or without 20 μM RSK1 peptides for 5 min at room temperature followed by addition of 1 pmol of purified active RSK1 or buffer, and incubated for another 5 min. Kinase activity reactions (50-μl final volume) were started by addition of 0.2 mM Kemptide, 2.5 μCi of [γ-32P]ATP, and different concentrations of cAMP to the mixture described above. Wherever indicated, PKI (20 μM) was also added to the assay mixture. Reactions were terminated after 15-min incubation at room temperature by the addition of equal volume of 20% trichloroacetic acid. Following centrifugation (20,000 x g for 5 min), aliquots of the supernatant were applied to P81 Whatman filters that were air-dried, washed extensively in 0.5% phosphoric acid, and counted in scintillation counter.

**RESULTS**

C-terminal Region (aa 418–735) of RSK1 Interacts with PKAc—With the overall objective to determine how interactions between active RSK1 and PKAc regulate the activity of PKA, the initial goal of our investigations was to identify the site on RSK1 that interacts with PKAc. Toward this end, we utilized HA-tagged constructs of full-length RSK1 as well as its different domains shown in Fig. 1A to transfect HeLa cells. Lysates from cells treated with or without EGF were then supplemented with cAMP to dissociate the PKA subunits and, following immunoprecipitation of PKAc, the presence of full-length and deleted versions of HA-tagged RSK1 in the immunoprecipitates were monitored. As shown before for endogenous RSK1 (10), the
full-length HA-RSK1 co-immunoprecipitated with PKAc in an EGF-dependent manner (Fig. 1B); note that because cAMP was added to the cell lysates to dissociate the PKA subunits, the PKARIα subunit was not present in the immunocomplex (Fig. 1B). Having ascertained the EGF-dependent interaction between PKAc and HA-tagged full-length RSK1, similar experiments with lysates from EGF-treated cells were performed using HA-tagged N-terminal regions of RSK1. As shown in Fig. 1C, none of the N-terminal constructs of RSK1 containing the N-terminal kinase with (aa 1–418) or without (aa 1–317) the linker region were present in the immunocomplexes of PKAc. On the other hand, RSK1 constructs comprising the linker plus the C-terminal kinase domain (CTK) plus the C-terminal tail (aa 317–735) or the CTK plus C terminus (aa 418–735) were present in the PKAc immunoprecipitates (Fig. 1D). These findings suggested that the PKAc binding site was located in the C-terminal half of RSK1. That the interaction of the C-terminal region of RSK1 (aa 418–735) with PKAc was dependent upon activation of cells by EGF is shown in Fig. 2A. Substitution of Thr-573 with either Ala that cannot be phosphorylated or Asp to mimic phospho-Thr (13) did not alter the EGF-dependent interactions between PKAc and the C terminus of RSK1. Thus, the phosphorylation status of Thr-573 on RSK1 does not appear to determine binding to PKAc. To further narrow down the region on the C-terminal of RSK1 that interacts with PKAc, a GFP fusion of the C-terminal tail (aa 672–735) of RSK1 was used. GFP-RSK1 (672–735) co-immunoprecipitated with PKAc in an EGF-dependent manner (Fig. 2B). Additionally, a construct in which the last 14 amino acids of RSK1 were deleted (aa 418–721) did not co-immunoprecipitate with PKAc (Fig. 2C, compare aa 418–735 and 418–721). Furthermore, when the last 14 residues of RSK1 were substituted with the last 14 amino acids of RSK2, the C-terminal construct (418–735 RSK1/RSK2) did not co-immunoprecipitate with PKAc (Fig. 2C). Together, these data demonstrate that PKAc binds to the extreme C terminus of RSK1 and that the last 14 aa of RSK1 contribute to its binding with PKAc. The latter finding is consistent with our previous demonstration that RSK1 but not RSK2 or RSK3 interacts with PKAc (10).

Phosphorylation of Ser-732 of RSK1 Is Crucial for Its Interaction with PKAc—The last 14 residues of RSK1 contain Ser-732. This Ser residue is autophosphorylated by the NTK of RSK1 when the kinase is fully active (14). Because active RSK1 binds...
to PKAc, we reasoned that the phosphorylation of Ser-732 may determine whether RSK1 binds PKAc or not. Therefore, next, we investigated the ability of PKAc to interact with the C-terminal of RSK1 in which Ser-732 had been substituted with Ala, Asp, or Glu. The Asp and Glu substitutions were included to mimic the phospho-Ser at this position. As shown in Fig. 3A, although the wild-type sequence of the C-terminal region (418–735) of RSK1 bound to PKAc in an EGF-dependent manner, the S732A and S732D substitutions in this region markedly decreased association with PKAc, and whatever little interaction that was observed was not EGF-dependent. On the other hand, substitution of Ser-732 with Glu increased the association of the C-terminal region of RSK1 even in the absence of growth factor and EGF could not further enhance this association (Fig. 3A). Note that the expression of the different C-terminal regions of RSK1 was the same. These findings suggest that the binding of RSK1 to PKAc is dependent upon phosphorylation of Ser-732 and that the substitution of this residue with Glu, but not Asp, mimics phosphorylation at this position. This explains the constitutive (EGF-independent) association of PKAc with RSK1 C-terminal harboring the Glu substitution of Ser-732.
**RSK1 Regulates PKA Activity**

A Short Peptide Corresponding to the Extreme C Terminus of RSK1 Competes for Interactions between Endogenous RSK1 and PKAc—Having mapped the PKAc binding site to the C-terminal 14 residues (aa 721–735) on RSK1 and having identified Glu, but not Asp, as a phospho-mimic residue that can substitute phospho-Ser-732, we next investigated whether peptides corresponding to the last 13 amino acids of RSK1 would compete for association between endogenous RSK1 and PKAc. HeLa cells were incubated for 15 min with cell-permeable, palmitoylated peptides corresponding to the last 13 residues of RSK1 that contained either the wild-type (peptide wt) sequence or in which the equivalent of Ser-732 had been substituted by either Ala (peptide A) or Glu (peptide E). Cells were then treated with EGF for 10 min, and PKAc was immunoprecipitated. The association between PKAc and endogenous RSK1 was slightly decreased by the peptide wt, but the interactions between RSK1 and PKAc were not affected by peptide A (Fig. 3B). On the other hand, peptide E, in which the Ser-732 equivalent residue had been substituted with Glu, competed for the interaction of endogenous RSK1 with PKAc. These findings lend further credence to the notion that PKAc binds to the extreme C-terminal 13 amino acid region of RSK1 and that this binding is dependent upon phosphorylation of Ser-732, the NTK phosphorylation site (14).

Phosphorylation Status of RSK1 Ser-732 Determines the Interactions of RSK1 with Erk1/2 or PKAc—The C-terminal 14 residues of RSK1 also form the Erk1/2 binding site (14, 15). Erk1/2 bind to this region of the inactive RSK1, and the initial step in the activation of RSK1 is Erk1/2-mediated phosphorylation of Thr-573, Ser-359, and Ser-363 (12, 15). Following complete activation of RSK1 that also involves CTK-mediated autophosphorylation of Ser-380, docking of PDK1 to this phospho-Ser-380 region and phosphorylation by PDK1 of Ser-221, the NTK phosphorylates RSK1 on Ser-732 (14). This autophosphorylation on Ser-732 results in the dissociation of Erk1/2 from the active RSK1 (14). In light of these previous findings, our data would suggest that the phosphorylation of Ser-732 acts as a switch whereby RSK1 dissociates from Erk1/2 but now associates with PKAc. To verify this, HeLa cells expressing HA-tagged RSK1 were treated with or without EGF, and either the PKAc or RSK1 were immunoprecipitated and the presence of Erk1/2, PKAc, and RSK1 in the immunocomplex was monitored. As shown in Fig. 3C, immunoprecipitates of PKAc contained RSK1 when EGF was present but did not contain Erk1/2. On the other hand, in the absence of EGF, immunoprecipitates of RSK1 contained Erk1/2 but no PKAc. When activated by EGF, RSK1 immunoprecipitates contained PKAc, but not Erk1/2; the activation of RSK1 by EGF is evident by the slower migration of RSK1 on gels. Given that the binding sites for Erk1/2 and PKAc on RSK1 are essentially the same and phosphorylation-dependent (Ref. 14 and this report), the data in Fig. 3C show that, upon phosphorylation of Ser-732, RSK1 dissociates from Erk1/2 and binds PKAc.

**RSK1 Inhibits the Ability of cAMP to Stimulate PKA, and This Effect Is Abrogated by Peptide E That Competes for RSK1-PKA Interactions**—Next, we investigated the functional significance of the interactions between RSK1 and PKAc. Our strategy in these experiments was to utilize the 13-amino acid-long peptides corresponding to the C-terminal of RSK1 that either do (peptide E) or do not (peptide A) compete for the interactions between RSK1 and PKAc. As a prelude to using these peptides, first, we investigated whether peptides A or E altered the activities of purified PKAc or RSK1. As shown in Fig. 4A none of the peptides altered the activities of either PKAc or RSK1. Moreover, none of the peptides altered the ability of cAMP to activate the PKA holoenzyme reconstituted in vitro from purified PKAc and PKAR1α (supplemental Fig. S1). Previously, we reported that the binding of active RSK1 to PKAc increases the binding of PKAc with PKAR1α (10). Indeed as shown in Fig. 4B, the dose-response curve for cAMP-mediated stimulation of PKA holoenzyme, reconstituted with purified PKAc and PKAR1α, was shifted to the right in the presence of active RSK1. These findings are consistent with our previously reported data (10) and support the notion that PKAc/PKAR1α interactions are elevated by RSK1 and, therefore, the need for higher cAMP concentrations to elevate PKA activity. Notably, using purified proteins we have not observed phosphorylation of RSK1 by PKAc or phosphorylation of PKAc by RSK1.2 Therefore, next we determined if the association of PKAc with RSK1 is necessary for the decreased sensitivity of PKA to cAMP. In these experiments, PKA was stimulated with a submaximal concentration (30 μM) of cAMP that showed the biggest difference in PKA activation with and without RSK1 (Fig. 4B), and the ability of peptides A and E to reverse the actions of RSK1 was determined. As expected, RSK1 decreased the activation of PKA holoenzyme by 30 μM cAMP, and peptide A did not affect the ability of RSK1 to attenuate the ability of cAMP to activate PKA (Fig. 4C). In contrast, peptide E, which effectively competes for the association of RSK1 with PKAc (Fig. 3B), obliterated the ability of RSK1 to inhibit the ability of cAMP to activate PKA (Fig. 4C). Thus, binding of RSK1 via its C-terminal 13 residues to PKAc is necessary for the observed decreased ability of cAMP to activate PKA.

**RSK1 Inhibits cAMP-elicited Activation of PKA in Intact Cells, and Peptide E Reverses This Modulation**—To determine the functional significance of the interactions between active RSK1 and PKAc in intact cells, the experiments depicted in Fig. 5 were performed in B82L cells. These cells express PKAR1α and RSK1, and the interactions among these proteins are similar to those in HeLa cells (10). PKA activity in these experiments was monitored by 8CPT-cAMP (the non-hydrolyzable analog of cAMP)-mediated phosphorylation of BAD on Ser-155, the PKA site (16, 17). The activation of RSK1 in the same experiments was monitored by EGF-elicited phosphorylation of BAD on Ser-112, the RSK1 site (18). The initial experiments determined whether activation of PKA in B82L cells was modulated by activation of RSK1. The addition of different 8CPT-cAMP concentrations for 10 min did not affect RSK1 activation as monitored by phospho-Ser-380 RSK1 antibody and also did not alter phosphorylation of BAD on Ser-112, the RSK1 site (Fig. 5A). As expected, 8CPT-cAMP, in a concentration-dependent manner, increased the phosphorylation of Ser-155 on BAD (Fig. 5A). The addition of EGF in the absence of 8CPT-cAMP

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1 H. M. Poppleton and T. B. Patel, unpublished data.
increased RSK1 activation and phosphorylation of BAD on Ser-112, but not on Ser-155 (Fig. 5A). In cells that were pretreated with EGF, the addition of different concentrations of cAMP for 10 min did not alter RSK1 activation or phosphorylation of Ser-112, but the ability of 8CPT-cAMP to phosphorylate BAD on Ser-155, the PKA site, was markedly attenuated. Quantification of PKA activation as monitored by the phosphorylation of BAD on Ser-155 as a ratio of total BAD in the absence and presence of EGF (Fig. 5B) showed results similar to the activation of purified PKA holoenzyme in the presence and absence of active RSK1 (compare Figs. 4B and 5B). In subsequent experiments with intact cells, the submaximal concentration of 8CPT-cAMP (30 μM) where the difference in BAD phosphorylation with and without EGF pre-treatment was maximal (Fig. 5A) was used. That the EGF-elicited attenuation of BAD phosphorylation on Ser-155 by 8CPT-cAMP was mediated by RSK1 is shown by data in Fig. 5C. Essentially, cells were treated with control or RSK1-specific siRNAs, and the ability of submaximal concentration (30 μM) of 8CPT-cAMP to phosphorylate BAD on Ser-155, the PKA site, was monitored. In control siRNA-treated cells, EGF attenuated the ability of 8CPT-cAMP to phosphorylate BAD on Ser-155 was monitored. In control siRNA-treated cells, EGF attenuated the ability of 8CPT-cAMP to phosphorylate BAD on Ser-155. However, the EGF-mediated attenuation of BAD Ser-155 phosphorylation by 8CPT-cAMP was obliterated when RSK1 was silenced (Fig. 5C). Similar data were obtained with another RSK1-specific siRNA that targeted a different region on the mRNA (supplemental Fig. S2). These findings demonstrate that RSK1 mediates the attenuation of Ser-155 phosphorylation on BAD by 8CPT-cAMP. Note that, because the phospho-Ser-380-RSK1 antibody detects all three RSK isoforms, silencing of RSK1 decreases the amount of phospho-Ser-380-RSK to a lesser extent than the decrease in RSK1. Likewise, because RSK2 and RSK3 also phosphorylate BAD on Ser-112 (19), silencing of RSK1 did not obliterate BAD phosphorylation on Ser-112 (Fig. 5C).

Next, using the cell-permeable myristoylated peptides A and E, we investigated whether the interaction between endogenous RSK1 and PKAc is necessary for EGF-elicited attenuation of Ser-155 phosphorylation on BAD by 8CPT-cAMP. As controls, the experiments in the supplemental data (Fig. S3) were performed. Essentially, neither of the peptides altered the ability of EGF to phosphorylate RSK on Ser-380 or phosphorylate BAD on Ser-112 (19), silencing of RSK1 did not obliterate BAD phosphorylation on Ser-112 (Fig. 5C).

FIGURE 4. Purified RSK1 inhibits the ability of cAMP to activate purified PKA holoenzyme, and this effect is reversed by the peptide that competes for the interactions between RSK1 and PKAc. A, peptides A and E do not affect PKAc or RSK1 activities. Purified PKAc or RSK1 were preincubated with or without 20 μM each of peptides A or E for 10 min. In vitro kinase assays were performed in triplicates as described under “Experimental Procedures” using Kemptide and [γ-32P]ATP as substrates. B, RSK1 inhibits the activation of PKA by cAMP. Purified PKAc (0.5 pmol) was preincubated with PKARI (10 pmol) for 3 h to form the PKA holoenzyme. Active RSK1 (1 pmol) or buffer was added 5 min prior to in vitro kinase activity assays in the presence of different concentrations of cAMP. All assays were performed in triplicates in the presence and absence of PKAc inhibitor PKI (20 μM). The PKI-sensitive activity is represented as a function of cAMP concentrations. C, same as B, except that 20 μM each of the peptides A or E was added to the PKA holoenzyme just before RSK1 addition, and in vitro kinase activities were monitored in the absence or presence of 30 μM cAMP with and without RSK1. For all panels, data shown are mean ± S.E. Statistical significance was assessed by Student’s t test analyses. *, p < 0.05; **, p < 0.01 as compared with respective control.
RSK1 Regulates PKA Activity

A

| 8CPT-cAMP (mM) | none | EGF |
|----------------|------|-----|
| pRSK-S380      | 0    | 0   |
| pBAD-S155      | 0    | 0   |
| pBAD-S112      | 0    | 0   |
| BAD            | 0    | 0   |

B

**Figure 5.** Activated RSK1 regulates PKA activity in intact cells and peptide E reverses this modulation. A, B82L cells were serum-starved (16 h) and then exposed to different concentrations of 8CPT-cAMP for 10 min with or without prior treatment of cells with EGF (100 nM for 5 min). Cell lysates were analyzed for phospho-Ser-380 RSK, phospho-Ser-112 BAD, phospho-Ser-155 BAD, and total BAD. B shows quantification of the density of phospho-Ser-155 as a ratio of actin. For panel A, B82L cells were transfected with 20 nM each of control or the first RSK1-specific siRNA described under "Experimental Procedures." 56 h later, cells were serum-starved (16 h) and then exposed to 30 µM 8CPT-cAMP for 10 min with or without prior treatment of cells with EGF (100 nM) for 5 min as described for panel A. Cell lysates were analyzed for RSK1, phospho-Ser-380 RSK, phospho-Ser-155 BAD, phospho-Ser-112 BAD, and actin (loading control). D shows quantification of phospho-Ser-155 BAD as a ratio of actin. E, peptide E, but not peptide A, reverses the ability of EGF to attenuate 8CPT-cAMP-mediated phosphorylation of BAD on Ser-155. B82L cells were treated with or without 20 µM each of peptides A or E for 10 min prior to exposure to EGF or vehicle for 5 min followed by incubation with and without 8CPT-cAMP (30 µM) for 10 min. Cell lysates were analyzed for phospho-Ser-155 or Ser-112 BAD and actin (loading control). F shows quantification of the density of phospho-Ser-155 as a ratio of actin. For cumulative presentation of results from different experiments, the density values of phospho-Ser-155 BAD or loading controls were first converted to relative values by setting their highest density values in each experiment as 1. The ratios of phospho-Ser-155 BAD to loading control were then calculated by dividing the relative values of phospho-Ser-155 BAD to those of loading control. All Western blots are representative of at least three similar experiments. B, D, and F are the mean ± S.E. Statistical significance was determined by Student’s t test analyses. *, p < 0.05; **, p < 0.01 versus control.

B

**Discussion**

Besides the classic regulation of PKA by cAMP, other mechanisms also contribute to the regulation of the ubiquitously expressed PKA. Included among these is the ability of the inhibitory IκBα protein of NFκB to bind directly with PKAc and inhibit its activity much like the regulatory subunits of PKA (3). During the activation of NFκB, phosphorylation, ubiquitylation, and degradation of IκBα results in release and activation of PKAc in a cAMP-independent mechanism (3). Similarly, the small GTP binding protein Rab13 binds to and inhibits PKAc during tight junction formation (4) representing another mechanism that is independent of cAMP in terms of regulating the activity of PKA. Herein, we present yet another novel mechanism whereby cross-talk between the Erk1/2 pathway and the PKAc regulates the activity of type I PKA. Erk1 and -2 initiate the phosphorylation of their immediate downstream p90 ribosomal S6 kinases (RSKs) that belong to a family of proteins with two kinase domains (12, 20). Phosphorylation of RSK1 by Erk1/2 on Thr-573 in the C-terminal kinase domain activates this kinase to autophosphorylate RSK1 on Ser-380 (12). The phosho-Ser-380 site on RSK1 then acts as a phosphoinositide-dependent protein kinase 1 (PDK1) binding site and permits PDK1 to phosphorylate the N-terminal kinase of RSK1 on Ser-221 and, thereby, fully activates PKAc. Peptide A, which does not affect this interaction, reverses the EGF-elicited (via RSK1) attenuation of phosphorylation of BAD on Ser-155 by cAMP.

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Besides the classic regulation of PKA by cAMP, other mechanisms also contribute to the regulation of the ubiquitously expressed PKA. Included among these is the ability of the inhibitory IκBα protein of NFκB to bind directly with PKAc and inhibit its activity much like the regulatory subunits of PKA (3). During the activation of NFκB, phosphorylation, ubiquitylation, and degradation of IκBα results in release and activation of PKAc in a cAMP-independent mechanism (3). Similarly, the small GTP binding protein Rab13 binds to and inhibits PKAc during tight junction formation (4) representing another mechanism that is independent of cAMP in terms of regulating the activity of PKA. Herein, we present yet another novel mechanism whereby cross-talk between the Erk1/2 pathway and the PKAc regulates the activity of type I PKA. Erk1 and -2 initiate the phosphorylation of their immediate downstream p90 ribosomal S6 kinases (RSKs) that belong to a family of proteins with two kinase domains (12, 20). Phosphorylation of RSK1 by Erk1/2 on Thr-573 in the C-terminal kinase domain activates this kinase to autophosphorylate RSK1 on Ser-380 (12). The phosho-Ser-380 site on RSK1 then acts as a phosphoinositide-dependent protein kinase 1 (PDK1) binding site and permits PDK1 to phosphorylate the N-terminal kinase of RSK1 on Ser-221 and, thereby, fully activates PKAc. Peptide A, which does not affect this interaction, reverses the EGF-elicited (via RSK1) attenuation of phosphorylation of BAD on Ser-155 by cAMP.

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**Discussion**

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RSK1. In this context, our findings with deletion of the last 14 amino acids and, more convincingly, the studies with a 13-amino acid peptide (peptide E) that competes for interactions between endogenous RSK1 and PKAc identify the PKAc binding site to the last 13 residues in a phosphorylation-dependent manner. Moreover, when the last 14 amino acids of RSK1 were substituted with the cognate region of RSK2, the association between RSK1 and PKAc was obliterated. These latter data are consistent with our previous findings that RSK1, but not RSK2 or RSK3, interact with PKAc (10). The C-terminal sequences of the human, mouse, and rat RSK1 are identical, and although the equivalent of Ser-732 in RSK1 is conserved in RSK2 and RSK3, the residues surrounding this Ser in the extreme C-terminal regions of the three RSK isoforms are different. Thus, besides the phospho-Ser-732 other surrounding residues must play a critical role in association with PKAc. Although the structure of the C-terminal kinase domain of RSK2 was recently reported (22), the extreme C terminus remains unresolved. Moreover, the structure of the C terminus of RSK1 is not known. Thus, predictions on how, on a structural basis, the C terminus of RSK1 interacts with PKAc are presently difficult. An interesting aspect of the extreme C terminus of RSKs is that all of them have a PDZ domain binding region. It has been suggested that, because these regions are different, the different RSK isoforms may interact with different PDZ domain-containing proteins (23). In this context, because RSK1, but not the other RSK isoforms, also interacts with PKAc via its extreme C terminus that contains the PDZ binding residues (732STTL-COO−), it is possible that interaction with PKAc also modulates association of RSK1 with proteins containing PDZ domains.

An interesting observation in our studies is that the short C-terminal regions of RSK1 that contain S732 but are devoid of the NTK also interact with PKAc in an EGF-dependent manner (Figs. 1D and 2A). Because Ser-732 on RSK1 is auto-phosphorylated by the NTK of RSK1 and because phosphorylation of S732 is necessary for interactions with PKAc, it would appear that these shorter RSK1 constructs are phosphorylated by the NTK of the endogenous RSK1 that is activated by EGF.

Although the structural details of the interactions between PKAc and RSK1 remain to be determined, the functional aspects of this interaction are clearly delineated in our study. Hence, the interactions between PKAc and RSK1 do not interfere with the interactions between PKAc and PKARIα. In fact, as demonstrated in our previous report (10), RSK1 increases the interactions between PKAc and PKARIα. This is further confirmed by the evidence presented in this report that RSK1 decreases the ability of cAMP to activate PKA both in vitro assays using purified proteins as well as in intact cells. Hence, activation of the Erk1/2 and, therefore, RSK1 can modulate cAMP-elicited activation of PKA and its biological functions. This regulation of PKA activity may have implications in Carney complex, which is associated with recurring endocrine tumors, cardiac myxomas and schwannomas and is caused by familial autosomal inherited mutations in the PKARIα gene and, therefore, haploinsufficiency of PKARIα (24–26). Studies have shown that the Erk1/2 pathway, and presumably, therefore, RSK1, are more active in tumor tissues derived from patients with Carney complex (27). This is accompanied by increased PKA activity due to decreased PKARIα (27). Thus, it is possible that the active RSK1 may decrease the ability of cAMP to activate the remaining type I PKA in this condition, thereby preventing further activation of PKA. On the other hand, the PKAc that is not bound to PKARIα in cells of patients with Carney complex may also associate with the active RSK1 to then phosphorylate their substrates and perhaps augment cell survival and tumorigenesis. In this latter respect, it should be noted that PKAc and RSK1 share a number of substrates, including BAD, ETS transcription factor (ER81), GSK-3β, Nur77, and cAMP-response element-binding protein (28–32). Thus, the complex formed between these two kinases may be more efficient at phosphorylating their respective sites on these substrates. In this case, based on the sequence of peptide E, the development of small molecules that dissociate these two kinases may decrease the phosphorylation of some of these substrates that promote cell survival and proliferation and, thus, ameliorate the recurrence of tumors in Carney complex patients.

In summary, our studies presented here show that the PKAc interacts with the extreme C-terminal 13 amino acids of activated RSK1 in a phosphorylation (of Ser-732)-dependent manner. This interaction between PKAc and RSK1 decreases the sensitivity of PKA toward activation by cAMP both in vitro and in intact cells and, therefore, regulates the biological actions of PKA. These findings represent an important cross-talk between the Erk1/2 and PKA pathways with implications in diseases such as Carney complex in which the activities of these kinases are elevated.

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